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Research Article

Targeted and Nontargeted Detection and Characterization of Trace Organic Chemicals in Human Serum and Plasma Using QuEChERS Extraction

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ABSTRACT

Humans are exposed to a broad range of organic chemicals. Although targeted gas chromatography mass spectrometry techniques are used to quantify a limited number of persistent organic pollutants and trace organic contaminants in biological samples, nontargeted, high-resolution mass spectrometry (HRMS) methods assess the human exposome more extensively. We present a QuEChERS extraction for targeted and nontargeted analysis of trace organic contaminants using HRMS and compare this method to a traditional, cartridge-based solid-phase extraction (SPE). Following validation using reference and spiked serum samples, the method was applied to plasma samples ($n = 75$) from the Prospective investigation of Obesity, Energy, and Metabolism (POEM) study. We quantified 44 analytes using targeted analysis and 6247 peaks were detected using the nontargeted approach. Over 90% of targeted analytes were at least 90% recovered using the QuEChERS method in spiked serum samples. In nontargeted analysis, 84% of the peaks were above the method detection limit with area counts up to 3.0×10^5 times greater using the QuEChERS method. Of the targeted compounds, 88% were also identified in the nontargeted analysis. We categorized the 4212 chemicals assigned an identity in using EPA's CompTox Dashboard and 1076 chemicals were found in at least one list. The category with the highest number of chemicals was "androgen or estrogen receptor activity." The findings demonstrate that a QuEChERS technique is suitable for both targeted and nontargeted analysis of trace organic contaminants in biological samples.

Key words: QuEChERS, nontargeted analysis, GC-Orbitrap, exposome, persistent organic pollutants.

The human exposome, which is intended to represent the totality of chemical exposure that individuals experience over their lives, is extremely complex (Cui *et al.* 2016; Miller and Jones 2014; Wild 2005, 2012). The exposome encompasses a wide variety of chemicals, some of which are known to cause adverse health effects in humans, including metals, small and large organic molecules, and reactive electrophile species (Patel *et al.* 2010; Rappaport 2011). Unbiased, nontargeted discovery-based analytical methods must be developed to detect and identify as many xenobiotics in the exposome as possible. Coupling nontargeted analytical strategies with traditional targeted quantification methods is one exposome surveillance approach that could address the need to simultaneously quantify specific chemicals and detect unknown toxicants in human samples.HRMS paired with chromatographic separation provides a platform for detecting both the chemical and biological markers of exposure due to its high mass precision, accuracy, and sensitivity. Liquid chromatography (LC)-HRMS is routinely used for metabolomics and is a convenient tool for exposure assessment; however, LC-HRMS alone cannot fully capture the

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Impact Statement

This study presents a QuEChERS extraction method for detecting persistent organic pollutants (POPs) and trace organic chemicals in the human exposome using targeted and nontargeted gas chromatography high-resolution mass spectrometry (GC-HRMS). Identified chemicals are classified using EPA's CompTox Dashboard illustrating the broad range of chemicals that can be detected using nontargeted GC-HRMS.

exposome. Recent advances in HRMS, namely the Orbitrap mass analyzer, have been integrated into gas chromatography (GC) systems to facilitate nontargeted analysis of small, volatile organic molecules in biological and environmental samples (Gómez-Ramos *et al.* 2019; Peterson *et al.* 2014; Sapozhnikova 2021).

Many nontargeted GC studies employ time-of-flight mass analyzers and often require two-dimensional (GC × GC) chromatography to distinguish compounds due to the relatively low resolving power (>40 000 full width at half maximum [FWHM]) (Cordero *et al.* 2010; Kujawinski 2018; Pelander *et al.* 2011; Sapozhnikova 2021; Yang *et al.* 2020). The Orbitrap is a high-resolution mass analyzer that provides 120 000 FWHM resolving power (Scheltema *et al.* 2014) and enables separation of *m*/z differing by <1 ppm (*m*/z with accuracy of 200.0000 ± 0.0002) at large mass ranges (total range 30–3000 *m*/z), mitigating the need for GC × GC (Perry *et al.* 2008). Fourier transform ion cyclotron resonance (FT-ICR) MS also provides high resolving power and mass accuracy (>1 000 000 FWHM, <1 ppb) (Bowman *et al.* 2020; Marshall *et al.* 2002); however, the size and cost of the GC-FT-ICR-MS limits accessibility (Zubarev and Makarov 2013). The high mass accuracy renders the GC-Orbitrap ideally suited for both targeted and nontargeted analysis of POPs.

POPs which include organic compounds characterized by their environmental persistence, bioaccumulative behavior, ability to undergo long-range transport, and adverse human health and environmental effects represent an important component of the human exposome (Breivik *et al.* 2016; Muir and Howard 2006). Prominent POPs, such as polychlorinated biphenyls (PCBs), pesticides, polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polybrominated diphenyl ethers (PBDEs) (Jones and de Voogt 1999), have been analyzed using targeted GC-MS or GC-electron capture detectors (Brevik 1978; Yu *et al.* 2011). Sample preparation techniques for POPs analysis in human samples (eg, serum, plasma) is time consuming and labor intensive, and includes methods such as solid-phase extraction (SPE), Soxhlet extraction, and liquid-liquid extraction (Johnson *et al.* 2021; Loconto *et al.* 2008; Salihovic *et al.* 2013; Yu *et al.* 2020). Alternative methods, such as QuEChERS (quick, easy, cheap, efficient, rugged, and safe), can improve sample throughput, reduce solvent, and provide sufficient recovery for a broad range of analytes (Anastassiades *et al.* 2003). QuEChERS methods (Anastassiades *et al.* 2003; González-Curbelo *et al.* 2015) include a liquid-solid extraction using buffering salts and sample clean-up using dispersive solid-phase extraction. Various QuEChERS extractions have been applied to human serum, whole blood, and human plasma for targeted analysis of tetrabromobisphenol A, hexabromocyclododecane isomers, and PBDEs (Li *et al.* 2017; Plassmann *et al.* 2015; Srivastava *et al.* 2017).

In this work, we present a novel QuEChERS-based extraction method that is applicable to both targeted and nontargeted analyses of POPs and trace organic contaminants, and is benchmarked against compared a traditional SPE sample preparation method. We directly compared the QueEChERS and SPE procedures using standards spiked into fetal bovine serum (FBS), NIST 1958 SRM, and 75 plasma samples collected from the Prospective investigation of Obesity, Energy, and Metabolism (POEM) cohort (Lind *et al.* 2020). The 2 sets of plasma extracts were analyzed using a Thermo GC-Orbitrap MS operated in full-scan mode to allow for targeted analysis of 44 trace organic contaminants and nontargeted detection of unknown chemicals. We then compared target analytes recovery in the 2 methods, performed a pair-wise comparison of the 2 extraction methods on a subset of samples, and assessed the method efficacy for nontargeted analysis.

MATERIALS AND METHODS

Chemicals and materials. Certified reference standards (purity ≥97%) were obtained from AccuStandard (New Haven, Connecticut) and are listed in the Supplementary Data. Organic solvents, including acetonitrile (≥99%), *n*-hexane (≥99%), acetone (99.8%, HPLC grade), dichloromethane (99.8%, HPLC grade), and methyl tertiary-butyl ether (MtBE, 99.9% extra pure) were purchased from Fisher Scientific. NIST 1958 Standard reference material (SRM) and charcoal-stripped FBS were purchased from Millipore Sigma (St Louis, Missouri). QuEChERS tubes were purchased from United Chemical Technologies (UCT, Bristol, Pennsylvania). Three extraction formulations were tested: (1) 15 ml tubes containing 150 mg dispersive C18 powder and 900 mg anhydrous $MgSO_4$, (2) 15 ml tubes containing 150 mg dispersive PSA powder and 900 mg anhydrous $MgSO_4$, and (3) 15 ml tubes containing 150 mg dispersive PSA powder, 45 mg graphitized carbon black (GCB), and 900 mg anhydrous $MgSO_4$. SPE cartridges containing 5 g Florisil were purchased from Sigma Millipore (Supelclean LC, Darmstadt, Germany), Thermo Scientific (Hypersep, Waltham, Massachusetts), UCT (Enviroclean, Bristol, Pennsylvania), and Waters (Sep-pak, Milford, Massachusetts). The following certified reference standards were purchased from AccuStandard: Furan Mix, Dioxin Mix, PBDE Congeners of Primary Interest Calibration Mix, Pesticide Mix 1, Pesticide Mix 2, AccuGrand 8270 Semi-Volatile Standard (AG01), Method 525.2 Organochlorine Pesticides, Triphenyl phosphate, WHO/NIST/NOAA Congener List, tris(2-Chloroethyl) phosphate (TCEP), PCB Congeners Mix 2, and Pesticide/Herbicide Mix. Surrogate standards (PCB-65 and 166), internal standard mix (Phenanthrene-d10 and Chrysene-d12), and Carbon Distribution Marker (retention time marker) were also purchased from AccuStandard.

Plasma sample study population. A total of 75 human plasma samples from the POEM study were extracted (Lind *et al.* 2020). Blood was drawn from participants in the morning (8–10 AM) after an overnight fast in EDTA-plasma tubes that were kept cool during spinning. Plasma was thereafter put in a −80°C freezer. POEM is a human cohort of inhabitants of Uppsala, Sweden aged 50 years (50% female) collected from October 2010 to October

2016 (Lind *et al.* 2020). The primary aim was to explore the links between obesity and cardiovascular disease (CVD).

Sample preparation. All samples were stored at −80°C, defrosted in a 4°C refrigerator, and then brought to room temperature prior to extraction. The samples were thoroughly vortexed (Thermo LP Vortex Mixer) to ensure uniformity and homogeneity for at least 30 s. The plasma samples were homogenized by vortex mixing (Thermo LP Vortex Mixer) and then split into equal volumes (400 µl plasma) prior to SPE and QuEChERS extractions. Each sample aliquot was transferred to an amber glass 4-dram vial with a pipette and spiked with 10 µl of a standard solution containing PCB congeners 65 and 166 in hexane so that the final concentration was 5 µg/l. These two congeners were used because they were never commercially produced and are not a part of the exposome. To each 4-dram vial containing sample, 5 ml of a 1:1:1 mixture of hexane: acetone: dichloromethane was added and the vials were vortexed for 30 s, placed in a sonication batch (Fisherbrand, CPX2800, 2.8 l) for 60 min, and placed on an orbital shaker (Fisherbrand 3D Platform Rotator) for at least 15 h. For both extraction methods tested in this study (SPE and QuEChERS), NIST 1958 SRM, FBS blanks, and FBS spiked with the certified reference standards purchased from Accustandard and POEM plasma samples were extracted by 2 different people so that extractor (human operator) biases and reproducibility could be assessed. Reference materials used to validate the methods, such as NIST, are only provided in serum. Serum samples from the POEM study were not available. Although serum samples are commonly used for analysis, the extraction and cleanup procedures minimizes issues related to protein interference and conjugated species; thus, the procedure can be applied to both plasma and serum. Previous studies have applied similar extraction methods to both plasma and serum samples when not enough of one material was available (Stubleski *et al.* 2018; Wolf *et al.* 2019).

SPE extraction. The SPE procedure (Supplementary Figure 1) was based on methods described previously (Caudle *et al.* 2007; Hatcher *et al.* 2007; Johnson *et al.* 2021). SPE cartridges containing 5 g Florisil were purchased from 4 different vendors: Sigma Millipore (Supelclean LC, Darmstadt, Germany), Thermo Scientific (Hypersep), UCT (Enviro-clean), and Waters (Sep-pak). The extractions were performed by 2 people to assess the SPE method reproducibility. The SPE cartridges were placed on a vacuum manifold, prepped by adding 1 g anhydrous sodium sulfate, and conditioned with 10 ml MtBE. The entire sample in the 4-dram vial and two 3-ml rinses of hexane: acetone: dichloromethane were transferred to the SPE cartridge. Vacuum was applied and the SPE cartridge was then rinsed with 10 ml MtBE, 5 ml hexane: acetone, and 5 ml dichloromethane. All fractions were collected in a clear glass test tube. The final eluent was evaporated down to 0.5–1 ml using an Organomation 30 position Multivap Nitrogen Evaporator (Organomation Associates Inc.) operated at 40°C, transferred to a glass autosampler vial, and reduced to a final volume of 150 µl. The final extract was transferred to an amber autosampler vial containing a 250-µl glass insert, spiked with 10 µl of an internal standard solution containing 62.5 µg/l of phenanthrene D-10 and chrysene D-12 and with 10 µl of the retention time marker, and sealed with a cap.

QuEChERS extraction. The following describes the steps that were taken to extract samples using the QuEChERS extraction procedure (Supplementary Figure 1):

1. The entire sample (solvent and serum or plasma) in each 4-dram vial was transferred to a 15-ml QuEChERS tube containing 150 mg dispersive C18 powder and 900 mg $MgSO_4$ (UCT, Bristol, Pennsylvania). We also tested the following QuEChERS tubes: 150 mg PSA powder with 900 mg anhydrous $MgSO_4$ and 150 mg PSA powder with 45 mg GCB and 900 mg anhydrous $MgSO_4$.
2. The QuEChERS tube containing the sample and solvent mixture was vortexed for 30 s (Thermo LP Vortex Mixer), hand-shaken for 30 s, and mixed on an end-over-end shaker (Thermo Scientific, Waltham, Massachusetts) for 15 min.
3. The QuEChERS tubes were centrifuged at 5000 rpm for 5 min.
4. The supernatant from the QuEChERS tube was transferred to a 35-ml clear glass centrifuge tubes and capped.
5. Steps 1–4 were repeated 2 additional times with 2 ml hexane: acetone: dichloromethane (9 ml hexane: acetone: dichloromethane total). We also tested the following extraction solvent mixtures, but these were not used in the final procedure: MtBE, 1:1 hexane: acetone, dichloromethane, and acetonitrile.
6. The final extract in the glass centrifuge tube was evaporated down to 150 µl as described previously for SPE and spiked with 10 µl of the internal standard and retention time marker solutions.

Targeted analysis. Sample extracts were analyzed for POP concentrations using a high-resolution Thermo Q Exactive Orbitrap MS equipped with a Thermo Trace 1300 GC and a TriPlus RSH Autosampler. Helium (99.9999% purity) and nitrogen (99.999% purity) were used as the carrier and c-trap gases, respectively. The samples were analyzed for POP concentration on 2 analytical columns: one column was used for furans, dioxins, and BDEs and the second column was used to analyze PCBs, pesticides, and other nonbrominated flame retardants. The details of the GC methods are described in the Supplementary Data. For both methods, the instrument was operated in electron ionization (EI) mode (70 eV). Data were collected in full-scan mode (ranges listed below) with 60 000 resolution and 1×10^6 automatic gain control. The extracted ion chromatogram (XIC) was used for quantification using the most abundant peak in the mass spectrum (Supplementary Table 1). Analyte identity was confirmed using the 2confirming ions ratios and retention time (Supplementary Table 1). Quantification for both methods was performed using 8-point calibration curve prepared by serial dilution of calibration standards in hexane (0.025–15 µg/l). The limit of detection (LOD) for each target analyte is displayed in Supplementary Table 1 was determined from 7 injections of calibration standards extracted from FBS (400 µl) and calculated using:

$$LOD = \frac{t*s}{m} \quad (1)$$

where t is the Student's t value for a 99% confidence level with $n - 1$ degrees freedom ($t = 3.14$), s is the standard deviation of the mean, and m is the slope of the calibration curve (Armbruster and Pry 2008; Long and Winefordner 1983).

Nontargeted analysis. Nontargeted analysis was performed on a subset of POEM plasma samples ($n = 29$) for which SPE was performed with UCT brand cartridges (UCT cartridges achieved the highest recovery in targeted analysis). Peak, or "feature," detection and deconvolution and library matching and scoring were performed in Thermo Compound Discoverer Version 3.2 using the GC EI Workflow with Statistics. The GC EI workflow in Compound Discoverer deconvolves the spectra, detects chromatographic peaks, aligns peaks across input files, and performs peak identification by running a library search. In this process,

Compound Discoverer aggregates all *m/z* features corresponding to a single peak. Peaks were detected with 5 ppm mass tolerance, 10 S/N threshold, and a 98% allowable ion overlap window. Each chromatogram retention time was aligned using the carbon distribution marker spiked into each sample and retention indices (RIs) were calculated for each peak detected. The RI of each peak was used to limit suspects during identification; the allowed maximum RI difference was 150. Compounds were identified by searching their mass spectra in the NIST Mass Spectra Library (NIST/EPA/NIH EI and NIST Tandem Mass Spectral Library Version 2.3) and a high-resolution library developed in-house using certified standards containing 354 unique compounds. A minimum Match Factor (SI) and Reverse Match Factor (RSI) score of 500 was used for assigning library matches. The high-resolution filtering (HRF) score was also used to score mass spectra matched to the library. Peaks with scores less than 500 were not assigned the identification. The HRF score ranges from 0% to 100% and is used to help reduce the number of compounds matched and to filter good matches (Kwiecien *et al.* 2015; Yang *et al.* 2019). Peak areas detected in blank samples were excluded from the experimental samples if the peak areas were less than the method detection limit (MDL). The MDL was calculated using:

$$\mathrm{MDL} = \bar{X} + t_{(n-1,\ 1-\alpha=0.99)} S_b \quad (2)$$

where X is the method blank peak areas mean, $t_{(n\ -\ 1,1\ -\ \alpha\ =\ 0.99)}$ is the Student's t value for the single-tailed 99th percentile t-statistic with $n - 1$ degrees freedom, and S_b is the sample standard deviation of replicate blanks peak area (EPA 2016).

Chemical categorization. The EPA CompTox Dashboard (Williams *et al.* 2017) to determine which chemicals identified in the nontargeted analysis have known uses or have been detected previously. The batch search routine was used to search all of chemicals detected in nontargeted analysis based on the QuEChERS extraction method. All currently available lists (253) were used to categorize the identified chemicals into groups (Supplementary Data, Excel file, "Assigned Categories"). Lists that did not include a chemical purpose or a chemical class (eg, targeted MS lists contributed by other labs) were classified as "Other Lists" and were not used in subsequent analysis.

Statistics. Statistical analysis was performed using R (version 4.0.3) and MATLAB (version 9.11 [R2021b]). One-way analysis of variance (ANOVA) paired with Dunn's post hoc test was used to determine if there was a statistical difference in the means recovery for the 4 SPE cartridge brands used to extract (α=0.05). To compare the mean recovery between 2 different people performing the SPE and QuEChERS extractions, a t test (α = 0.05) was used to determine if there was a significant difference in the means. For nontargeted analysis, a paired samples t test was used to determine the significance of the peaks detected in both the SPE and QuEChERS extraction. The p values were adjusted using Benjamini-Hochberg for the false-discovery rate. To determine the correlation between groupings of nontargeted annotations assigned to EPA CompTox lists, pairwise Pearson correlation coefficients were calculated in MATLAB (α = 0.05).

RESULTS

Gas Chromatograms

Total ion chromatograms (TIC) for a 10-μg/l standard extracted from FBS and reconstituted in hexane on the 15 and 30 m columns are shown in Supplementary Figures 2A and 3A, respectively. Two columns were used because PBDEs are thermally labile and break down in columns longer than 15 m due to the higher temperatures required for separation (Björklund *et al.* 2004). Each analyte was initially identified from the full-scan mode chromatogram using the NIST mass spectra library, and subsequently quantified using the *m/z* feature with the highest abundance, while and the two next highest were used as confirmation ions (Supplementary Table 1). When applicable, isomers were differentiated first using the Kovats Retention Index data in the NIST library and then confirmed using certified reference standards. The quantification ion was extracted from the TIC, as displayed in the XICs in Supplementary Figures 1B and 2B and 2C. The XICs validate that sufficient separation of each isomer was achieved. The XICs also demonstrate the advantage of using HRMS for targeted analysis. In the full-scan TIC, the peaks for each analyte are barely visible; however, the high resolving power, mass accuracy, and precision of the GC-Orbitrap allow the exact masses to be separated from the other ions in the TIC. Calibration curves were determined using the quantification ion and linearity was assessed by calculating the square of correlation coefficient, or the coefficient of determination (R^2), which was greater than 0.95 for all analytes.

SPE Extraction

SPE was used to extract FBS blanks, FBS spiked with a known about of standard solution, NIST SRM, and POEM serum samples. Recovery efficiencies for each type of sample was assessed by spiking the samples with PCB 65 and 166 and averaging the recovery for these two compounds. Supplementary Figure 4A displays the distribution of the recoveries in 75 total samples extracted by 2 people and 4 different SPE cartridge brands (Waters Sep-Pak, n = 12; Thermo Scientific HyperSep Florisil, n = 17; Sigma Millipore Supelclean LC-Florisil, n = 17; and UCT Enviro-clean Florisil, n = 29). The average recovery for all samples was 61.7%. Supplementary Figure 4B displays the distribution of the averaged PCB 65 and 166 recoveries of the samples by the SPE brand used to extract. One-way ANOVA with Dunn's Multiple Comparison post hoc test was performed to evaluate the difference in the mean among the 4 SPE cartridge brands, which were Waters Sep-Pak (n = 12, $\bar{x}$ = 0.557), Thermo Scientific HyperSep Florisil (n = 17, $\bar{x}$ = 0.451), Sigma Millipore Supelclean LC-Florisil (n = 17, $\bar{x}$ = 0.561), and UCT Enviro-clean Florisil (n = 29, $\bar{x}$ = 0.773). The results from the statistical test determined that the differences in the mean values among the cartridge brands are greater than would be expected by chance and that there is a statistically significant difference (H = 26.237, df = 3, p = <.001). Thus, the SPE brand impacted recovery and the variation could compromise the concentration results for targeted POP analysis. Extraction bias due to person performing the extraction was also tested (Supplementary Figure 4C) (extractor 1: n = 37, $\bar{x}$ = 0.557; extractor 2: n = 39, $\bar{x}$ = 0.677). To compare the means of the 2 people extracting, a t test with a 95% 2-tailed confidence interval was performed. The results of the statistical test indicated that the mean of samples extracted by Extractor 2 exceeds the sample mean of those extracted by Extractor 1 by an amount that is greater than would be expected by chance, rejecting the hypothesis that the population mean of samples extracted by Extractor 1 is greater than or equal to the population mean of those extracted by Extractor 2 (t = −2.401, df = 73, p = .009).

To assess target analyte recovery using SPE, standards were spiked into FBS and extracted using UCT brand cartridges, which had the highest internal standard recovery. Supplementary

Figure 5 shows the recovery for each analyte from 4 replicate FBS samples spiked with a known concentration of each compound. For compounds recoverable using this method, recovery ranged between 3.75% and 106.4%. OCDF, OCDD, BDE-180, BDE-183, BDE-209, Endrin, and PCB-206 were not recovered from the spiked FBS. For 58% of the analytes, the 4 replicates varied more than 5% relative standard deviation, indicating poor method precision. The reason for the variation between replicate extractions could potentially arise from matrix interference but is more likely due to inconsistencies in the SPE method that can lead to poor reproducibility. For example, imprecise conditioning of SPE media, such as incomplete bed wetting or drying out after solvation, leads to variation in the capacity of the sorbent. This compromises the retention of undesirable matrix components and, thus, the degree of extraction matrix interference. Additionally, sample loading and analyte elution flow rate, which is controlled by vacuum pressure in SPE, can also impact recovery. For example, if the elution flow rate is too fast, analytes may not sufficiently desorb from the SPE cartridge.

QuEChERS Extraction Optimization

A mixture containing 64 analytes was first used for optimizing and developing the QuEChERS extraction method. Similar to SPE, QuEChERS techniques may employ a variety of solvents or solvent combinations and materials. The most common solvent used in QuEChERS is acetonitrile (Anastassiades *et al.* 2003; Rejczak and Tuzimski 2015). Therefore, acetonitrile and the solvents used in the SPE, hexane, acetone, dichloromethane, and MtBE, were tested as the extraction solvent to recover the target analytes from FBS (C18 and $MgSO_4$ QuEChERS tubes used) (Supplementary Figure 6). Despite the commonality of using acetonitrile in QuEChERS techniques, 1 wash of acetonitrile did not recover the highest amounts of the target analytes. The 1:1:1 hexane: acetone: dichloromethane mixture exhibited the highest recovery of all target analytes than any of the other extraction solvents tested, and therefore 1:1:1 hexane: acetone: dichloromethane was used in further optimization of the QuEChERS extraction.

Three common dispersive powders used in QuEChERS techniques to remove lipids, fatty acids, and matrix interference are primary secondary amine (PSA), C18, and GCB. Supplementary Figure 7 displays recovery of PCDFs, PCDDs, and BDEs using Florisil SPE and PSA with GCB PSA without GCB, and C18 QuEChERS extraction (all QuEChERS extractions were performed with a single wash of 1:1:1 hexane: acetone: dichloromethane and contain 900 mg $MgSO_4$). In comparison to the SPE, more analytes were recovered and in similar or greater percentages in the at least one of the QuEChERS materials tested. Of all the materials tested, C18 recovered the greatest percentage of all analytes, with the least amount of variability between replicate samples. Although recovery using PSA powder alone was not as high as the C18 powder, the addition of GCB negatively impacted recovery of the target analytes. GCB has been shown to retain planar pesticides in SPE (Shimelis *et al.* 2007). Most of the planar compounds extracted in this analysis were retained by the GCB, except for 2,3,78-TCDF, 1,2,3,4,7,8-HCDF, 1,2,3,4,6,7,8-HCDD, and OCDD. Nonplanar compounds were also retained by the GCB, including BDE 28, BDE 47, BDE 153, and BDE 154. C18 was chosen as the QuEChERS material most suitable for recovering the target analytes; thus QuEChERS tubes containing C18 and $MgSO_4$ were used in solvent optimization testing.

To increase extraction efficiency, consecutive solvent washes can be performed on the sample in the QuEChERS tube. Supplementary Figure 8 shows the effects of subsequent solvent washings on the final recovery. Excluding the cases where analytes were nearly fully recovered after the first wash, subsequent washes resulted in a higher final recovery. After a total of 3 washings, final recoveries were in the range of 84%–114%, except for BDE 209, of which 64% of the initial mass was recovered.

Evaluation of QuEChERS Extraction Method

The final QuEChERS extraction method used 15 ml QuEChERS tubes with 150 mg dispersive C18 powder and 900 mg anhydrous $MgSO_4$ and 3 washes of 1:1:1 hexane: acetone: dichloromethane. All 75 serum samples were extracted using the QuEChERS method. The average PCB-65 and 166 recovery for all of the samples (Supplementary Figure 9A) was 95.2%, which is 33.5% higher than in the SPE extracted samples. The QuEChERS extraction method also exhibited improvement in biases due to the person performing the extraction (Supplementary Figure 9B). The mean recoveries for each person performing the extractions were 96.7% and 94.6%. The variances in the recovery for the two sets of samples extracted by different people were not normally distributed (n for extractor 1 was <30), so a Mann-Whitney-Wilcoxon test was performed to determine if the two sets of samples extracted by different lab personnel had the same distribution. The results of the statistical test concluded that there is not a statistically significant difference ($p = .719$) in the distribution between the extractors.

The QuEChERS extraction procedure was then compared with the SPE using UCT brand cartridges by extracting FBC spiked with a standard solution containing all 44 target analytes. Figure 1 shows the results of the finalized QuEChERS method using C18 powder and 1:1:1 hexane: acetone: dichloromethane for 3 washes compared with the SPE (also summarized in Supplementary Table 2). The finalized QuEChERS method exhibited higher recovery than the SPE across all target analytes except for *o,p′*-DDT, for which full recovery was achieved using both methods.

Comparisons to NIST 1958 SRM

To validate concentration data, measurements were compared against certified NIST 1958 SRM. Supplementary Figure 10 displays the recovery of each analyte that NIST 1958 SRM contained that had an expected concentration greater than 0.010 ng/l in the SPE and QuEChERS extraction methods ($n = 3$ for both). Although some of the compounds with expected concentrations less than 0.01 ng/l in NIST 1958 SRM were detected, the recovery was not displayed because the concentration was below the LOD. The QuEChERS method exhibited greater recovery and less variability for the 24 compounds evaluated (Supplementary Figure 10).

Sample to Sample Comparison of the Extraction Methods Applied to POEM

SPE and QuEChERS were used to extract the target trace organic contaminants from 75 human plasma samples. Supplementary Table 2 summarizes the maximum amounts of sample that could be processed and the time required for extraction. Using the SPE vacuum manifold, a maximum of 12 samples could be processed in a single batch. The total extraction time for 12 samples using the SPE was between 4.5 and 5 h. With the QuEChERS extraction method, the number of samples in a batch can be increased because the extractor is not limited by the SPE vacuum manifold capacity. Using QuEChERS, 12 samples can be processed within 3 h. Therefore, the QuEChERS method provides higher throughput than the SPE. Unlike the SPE, the steps of the QuEChERS technique involve mixing and does not require the extractor to continuously monitor the extraction process.

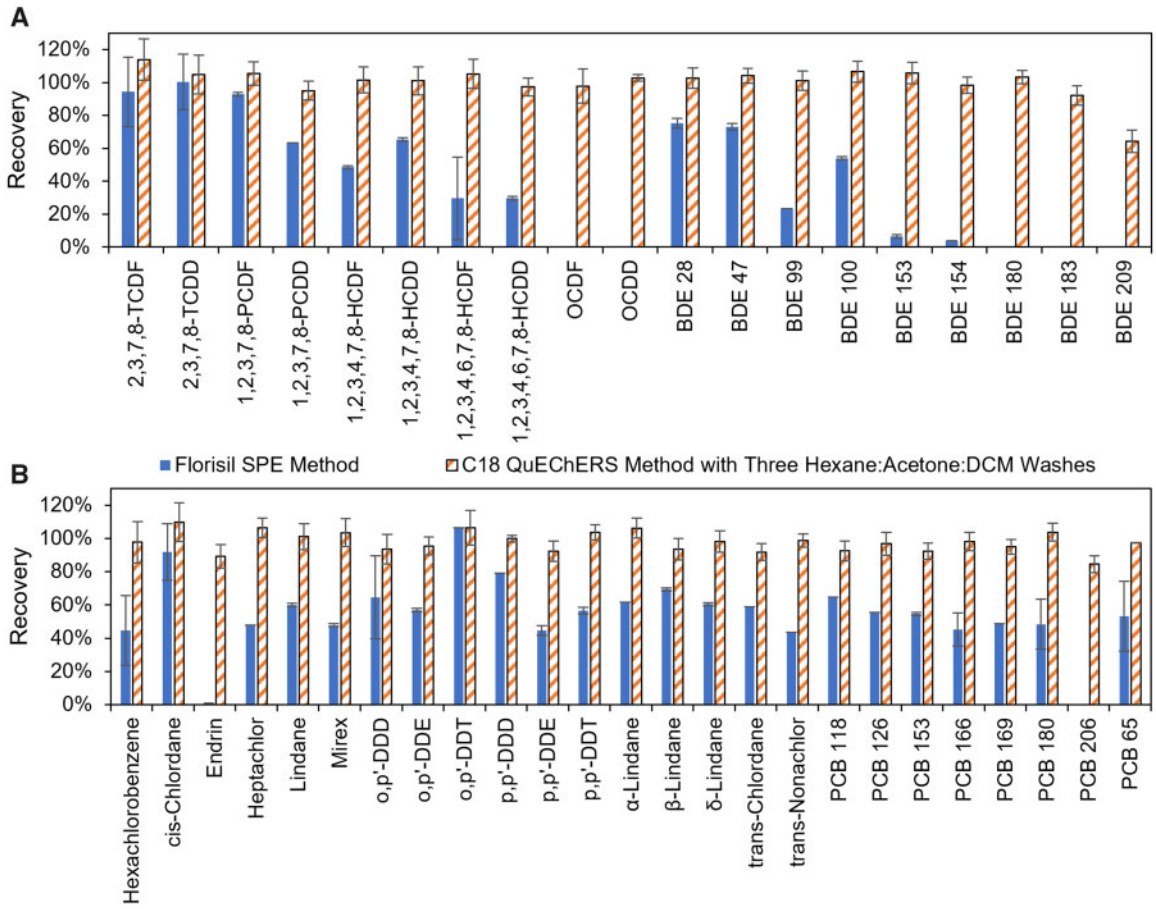


Figure 1. Comparison of SPE to QuEChERS using standards spiked in to FBS ($n = 3$).

A comparison of the concentrations of the target analytes per volume of serum extracted in the SPE and QuEChERS methods was performed (Figure 2) for a subset of the POEM plasma samples ($n = 29$). Because UCT brand SPE cartridges outperformed the other cartridges tested, only samples that were extracted using UCT brand SPE cartridges were compared. The diagonal line in Figure 2 represents a 1:1 ratio of the concentration detected in QuEChERS to SPE. The majority of the points in this figure lie above the 1:1 ratio line, indicating that the QuEChERS method extracted a greater mass of the environmental analytes from the plasma samples than was extracted using SPE. In some SPE extracted samples, analytes were not detected; but when the same sample was extracted by the QuEChERS method, up to 9 μg/l was detected.

Sample to Sample Comparison of the Extraction Methods for Nontargeted Analysis

Nontargeted screening was performed on the samples that were extracted by both SPE using UCT brand cartridges (highest recovery) and QuEChERS. In total, 6247 peaks were detected (Figure 3A) and 5268 were above the MDL. Of these peaks, 5159 were detected in both the SPE extracted samples and the QuEChERS extracted samples; however, 18 of these peaks were less than the MDL, which was calculated for each peak detected based on the peak area in extracted blank FBS (equation 2). The number of peaks detected only using SPE was 662, but 649 (98%) of these peaks were less than the MDL. The number of peaks detected in samples extracted by the QuEChERS procedure was 426, but 312 (73%) were less than the MDL. For the 5141 peaks detected in both methods and above the MDL, a volcano plot was produced to evaluate the efficiency of the extraction methods for nontargeted analysis using log2 fold change of the peak areas (Figure 3B). The p values in this plot were calculated using a paired 1-way ANOVA test to determine the significance of individual peaks within the 2 extraction methods. The x-axis represents Log2 fold change of the peak area with respect to the SPE, which indicates differences between the 2 groups. A more positive Log2 fold change value indicates that QuEChERS was the favored extraction technique for this peak; whereas a negative Log2 fold change value represents a peak that was favored in the SPE extraction method. The abundance of points on the positive side of the volcano plot (Figure 3B) indicates that more peaks were favored (had higher area counts) in the QuEChERS extraction than in the SPE. In total, 3173 peaks were favored in QuEChERS and 1968 were favored in SPE.

Although identification of the unknown peaks was not a priority for this study, as the focus was developing an extraction procedure that could be used for both targeted and nontargeted exposomics, the percentage of peaks tentatively identified using the NIST and in-house library searches was examined. The SI score is considered a forward search or direct comparison search, whereas RSI is considered a reverse comparison search. The RSI search compares the library entry to the unknown compound from the data set; a forward search compares the mass spectrum of the unknown compound from the data set to a mass spectral library entry. Compound assignment was

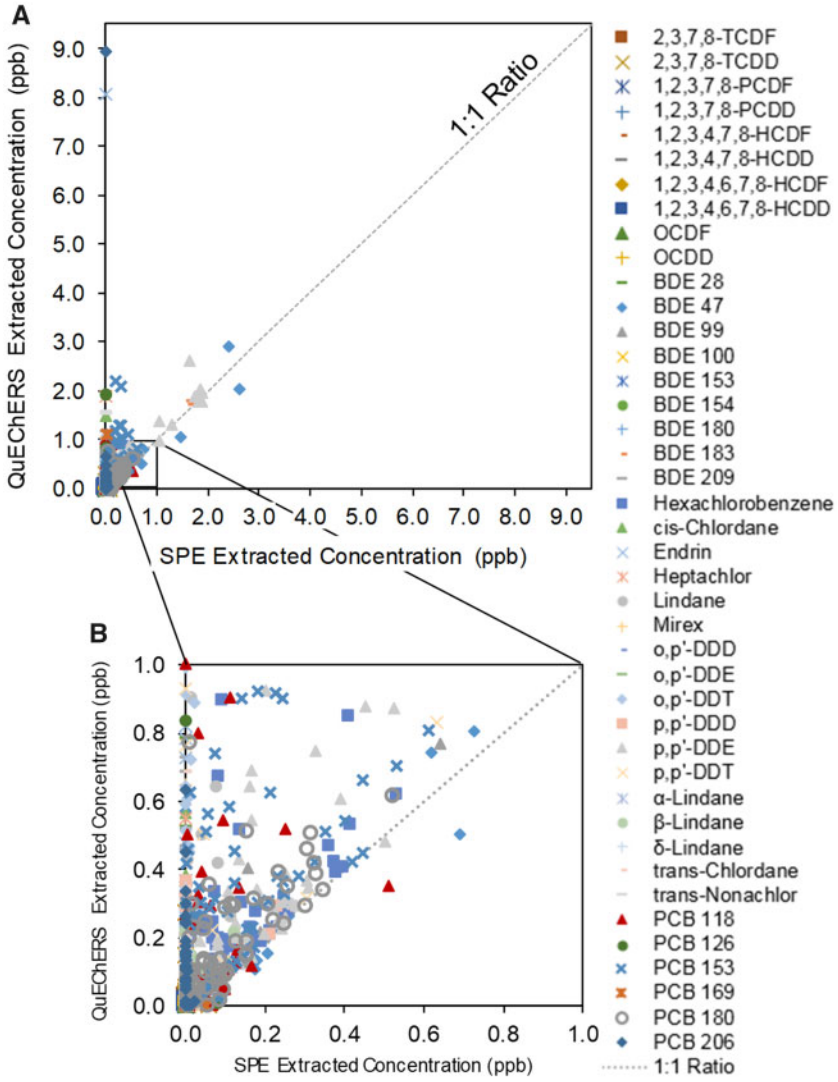
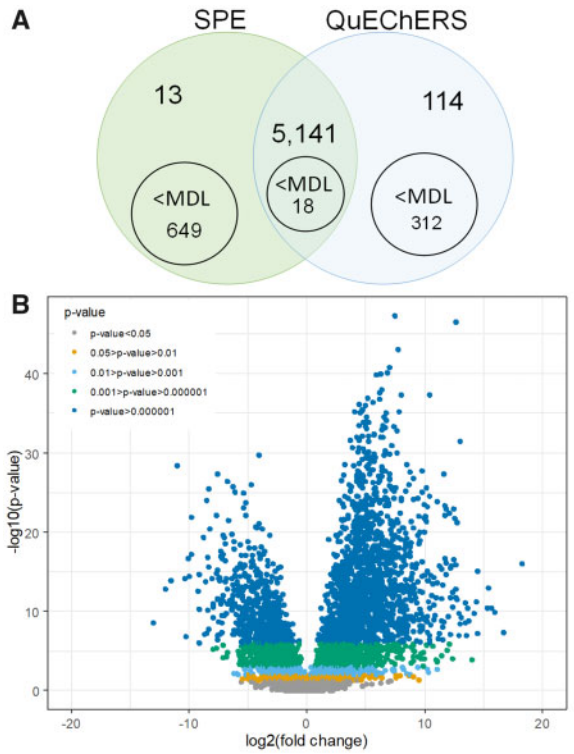


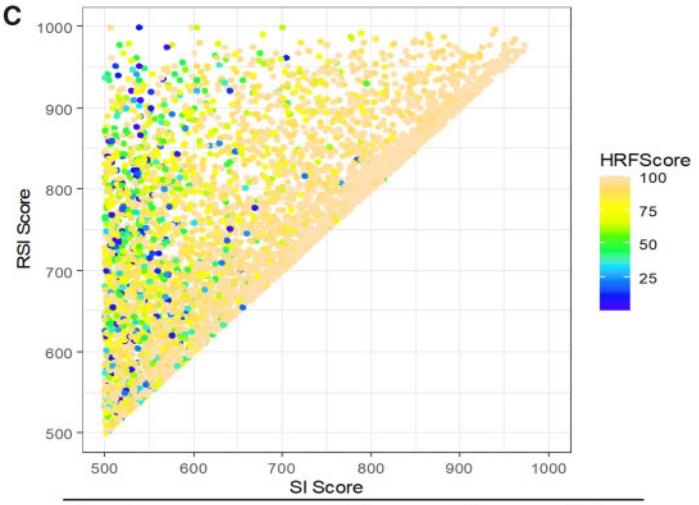
Figure 2. Comparison of the QuEChERS method to the SPE. Individual samples were vortexed to ensure that they were well mixed and then split for the extraction ($n = 29$). Concentrations are expressed in ppb (μg/l) per volume of sample extracted.

performed using a minimum RSI and SI criteria. Peaks with SI or RSI scores less than 500 were not assigned an identification. The RSI and SI scores are a direct comparison score of the experimental spectrum to the library spectrum. RSI and SI scores greater than 900 are considered an excellent match; scores between 750 and 900 are a good match; scores from 600 to 750 are a fair match; and scores less than 600 are considered a poor match. Of the 5255 peaks detected and above the MDL in the QuEChERS method, 4440 (84%) compounds were matched to the in-house high resolution or NIST spectral libraries. A total of 833 compounds did not have library matches greater than 500, and thus were not assigned an identification ("unknown-unknowns"). Of those assigned an identification, 4212 (95%) compounds were assigned unique annotations. A total of 22 compounds that were in the targeted method were also detected in the nontargeted method (88% in the targeted method using the 30-m column). The standard mixture used to create the calibration curves contained 260 compounds that were not used in the targeted method. Of these compounds, 61 were detected in the nontargeted analysis.

Figure 3C displays the RSI, SI, and HRF scores for each compound tentatively identified using the QuEChERS extraction. In all cases, the RSI score was greater than the SI score. The RSI score was evenly distributed score between the minimum and maximum (500–999 for RSI and SI); whereas the SI and HRF scoring were not. SI scoring was skewed toward the lower range (500–699) and HRF scoring was skewed toward the higher range (80%–100%). Factors that affect both scores are peaks coming from background or impurities that were not removed during deconvolution and the ratios of peaks present in the spectrum (Stein 1994). Using either RSI or the HRF score for data filtering will lead to more compounds of interest when conducting exposomics studies.

EPA's CompTox Dashboard (Williams *et al.* 2017) was used to further understand the data by classifying the identified compounds into categories (Figure 4A). We searched all compounds that were assigned unique annotations (4212 compounds) and used all lists that were currently available in the CompTox Dashboard (outlined in Supplementary Data, Excel File). The number of chemicals identified in the QuEChERS method and





Score Range RSI/SI, HRF Score	RSI	SI	HRF
500-599, 0—20%	530	1,914	116
600-699, 20—40%	1,037	1,040	135
700-799, 40—60%	1,188	783	233
800-899, 60—80%	1,142	580	523
900-999, 80—100%	543	123	3,433

Figure 3. Samples extracted by SPE (UCT cartridges) and the QuEChERS method ($n = 29$) were compared in nontargeted analysis. The total number of peaks detected in each extraction method is displayed in the Venn diagram in (A), including the number of peaks less than the MDL in each technique. For the peaks detected in both methods (overlap in the Venn diagram) and above the MDL, the data were plotted as a volcano plot (B) to display which method was favored. The x-axis represents Log2(fold change) of the peak area. Points with Log2(fold change) greater than 0 favor the QuEChERS extraction, whereas peaks with Log2(foldchange) less than 0 favor the SPE. Peaks were matched to the NIST library and an in-house mass spectra library and then scored with SI, RSI, and HRF scores and are shown in (C) for those detected in the QuEChERS method. RSI and SI scores range from 500 to 999, whereas HRF scores range from 0 to 100%.

found in at least 1 list or category in the CompTox Dashboard was 1076. The remaining 3136 chemicals could not be grouped into any category. The category with the highest amount of chemicals was "Androgen or Estrogen Receptor Activity," which included 4 lists from the EPA CompTox Dashboard. The second highest category was "EPA—Toxicity Database," which included 6 lists containing chemical databases or lists from the EPA with data on chemical toxicity. The amount of the identified compounds that were previously reported in the blood exposome was 267.

Figure 4B highlights the extent of overlapping annotations between the 49 categories from Figure 4A. We grouped these categories into 4 larger classes (Exposome, Health Related, Regulatory, and Uses) based on the category descriptions provided in the CompTox Dashboard. The Exposome class included organic molecules that have previously been detected in the exposome and the metabolome and 2 sources of chemical exposure (water and dust). The Health Related class included categories related to health outcomes or are associated with toxicity. The Regulatory class includes categories that were created by the EPA or other regulatory agencies. The Uses class contains categories that were associated with a specific chemical purpose or class. Using pairwise Pearson correlation coefficients, we quantified the correlation of each annotation between categories and obtained p values ranging from −.0593 to .946. A majority of the categories within the health-related class are correlated (p value range: .425–.705, average p value: .558). Within the Exposome class, the categories are weakly correlated (p value range: .259–.546, average p value: .428). The correlations within the Regulatory class ranged from weak to strong (p value range: −.047 to .946, average p value: .369). Within the Uses class, categories were weakly correlated (p value range: −.044 to .741, average p value: .153). Between different classes, there are notable correlations between Exposome and Health Related (average p value: .438), Health Related and Regulatory (average p value: .422), and Exposome and Regulatory (average p value: .356). Less significant correlation exists between Health Related and Uses (average p value: .252), Exposome and Uses (average p value: .2081), and Regulatory and Uses (average p value: .155). The 2 categories with the greatest correlation are "EPA–CERCLA" and "EPA–Underground Storage Tanks" with a p value of .946, indicating these two lists contained almost the exact same constituent annotations.

DISCUSSION

This research developed and evaluated a QuEChERS extraction technique and compared this approach to a traditional SPE technique using targeted and nontargeted GC-HRMS analysis. The methods were tested using 44 reference standards spiked into FBS and using 24 compounds in NIST 1958 SRM. The QuEChERS method developed here outperformed SPE in reliability and analyte recovery, achieving analyte recoveries greater than 90% in at least 90% of the target analytes. The SPE data showed biases in the cartridge brand used and the person performing the extraction. UCT brand cartridges exhibited the greater recovery for a range of trace organic contaminants; therefore, this brand was used when comparing analyte recovery to the QuEChERS method. The QuEChERS technique allowed analyte recovery to overcome the limitations of SPE, including

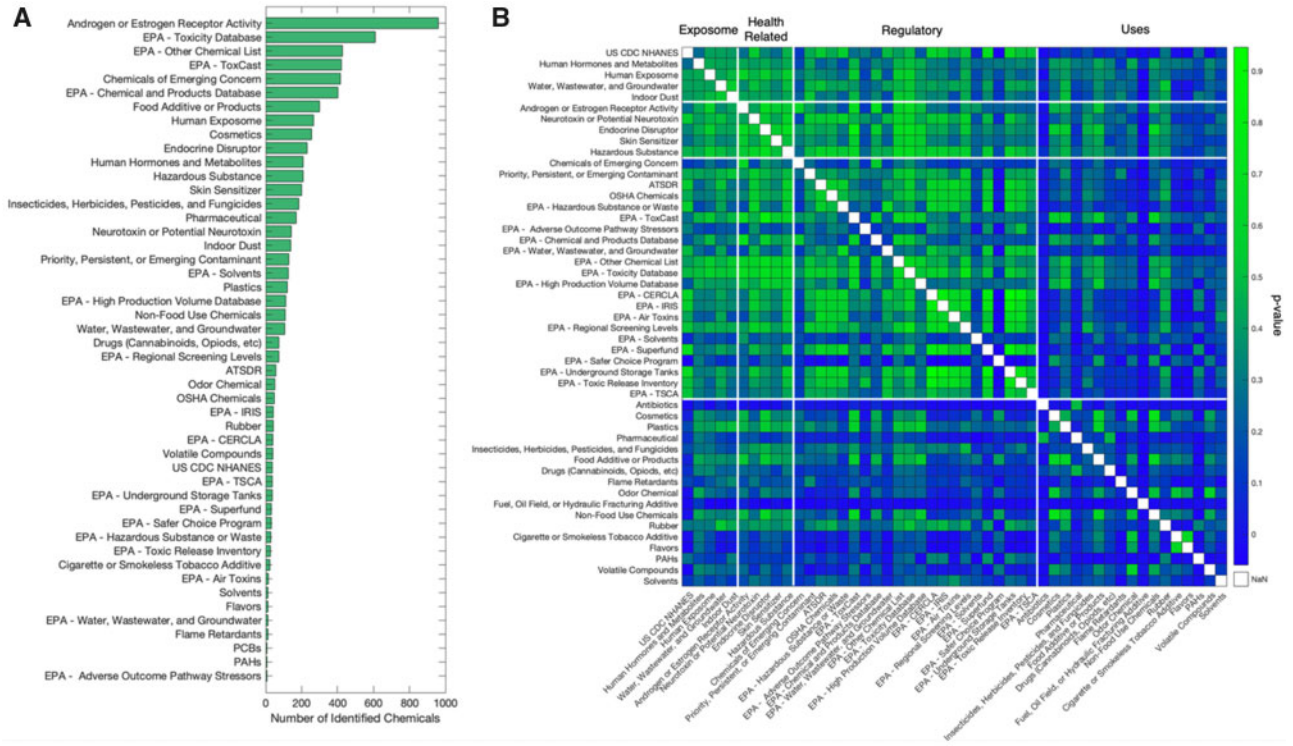


Figure 4. Chemicals assigned an annotation were evaluated using EPA's CompTox Dashboard. We used all lists that were currently available in the database and group the lists based on chemical purpose or description. Categories displayed in the histogram (A) contained at least 7 chemicals. We also analyzed the overlap of annotations within these categories using pairwise Pearson correlation, shown in the heatmap (B). p values greater than 0 indicate a positive correlation between lists and p values closer to 1 indicate a strong positive correlation. From left to right and top to bottom are the following groupings of lists: exposome, health related, regulatory, and uses. Because the overlap between equivalent lists would have p values equal to 1 and this could be misinterpreted, the overlaps are displayed as white boxes to indicate no value.

variation due to extractor or cartridges and higher sample throughput. Thus, the QuEChERS method presented here is more reliable, convenient, and effective than the SPEs evaluated.

The 2 methods were then applied and compared using plasma samples from the POEM cohort ($n = 29$). Comparing analyte concentrations in each sample showed that the ratio of analyte recovered in the QuEChERS method to the analyte recovered by SPE was greater than 1:1 in most cases, indicating QuEChERS obtained higher plasma concentrations. Data were collected in full-scan mode, which allowed retrospective nontargeted evaluation. The nontargeted data analysis showed that the QuEChERS technique extracted 114 peaks that were above the MDL and were not detected in SPE. Only 13 peaks were detected in SPE above the MDL and not detected in QuEChERS. Peaks detected in both methods had higher Log2(foldchange) peak area in the QuEChERS technique, indicating that the extraction efficiency was favored in the QuEChERS extraction. The QuEChERS extraction technique simplifies the extraction procedure and provided improved sensitivity and reproducibility in comparison to the SPE. Thus, the QuEChERS method developed herein is suitable for a larger set of target analytes and for nontargeted analysis of environmental chemicals in human serum and plasma samples. Improving sample extraction for both targeted and nontargeted methods, as demonstrated here, has the potential to advance current knowledge surrounding the human exosome. Using effective extraction techniques, like QuEChERS, aids in exposome characterization and can provide critical data on chemical exposures.

Following nontargeted analysis, we used the EPA's CompTox Dashboard to categorize the identified compounds into endogenous and exogenous categories (Williams *et al.* 2017). Although we were able to categorize more than 1000 chemicals, a large number of chemicals were not categorized. A number of the chemicals detected were categorized into groups describing adverse effects, such as neurotoxin or potential neurotoxin, estrogen receptor activity, androgen receptor chemicals, endocrine disruptor, and skin sensitizer. Chemicals were also grouped into sample types the chemicals have previously been detected in, including water, house dust, cosmetics, and pharmaceuticals. Numerous chemicals have previously been detected in the human exposome or were human hormones or metabolites (Zhao *et al.* 2021). Water and indoor dust contribute to the exposome, and these categories were correlated with the adverse effects mentioned above. Therefore, the QuEChERS technique developed here can capture a wide range of exogenous and endogenous chemicals. Future studies can utilize this approach to assess chemical exposure.

Another notable result from chemical categorization using the EPA CompTox Dashboard is that the Centers for Disease Control and Prevention (CDC) National Health and Nutrition Examination Survey (NHANES) data, which should represent substances in the human exposure space (Centers for Disease Control and Prevention 2019), had high p values for all categories within the Exposome, Health Related, and Regulatory classes. Yet, CDC NHANES has weak, if any, positive correlation with categories pertaining to Uses, which should theoretically contribute to the NHANES data. For example, CDC NHANES was more strongly correlated with water and indoor dust (Exposome

class) than any of the categories in the Use class. The weak correlations between NHANES and the Uses class may be a result of categories within the Uses class not containing regulated chemicals because they have already been mostly removed as a result of imposed regulations. Additionally, the overall trends suggest that annotated compounds are most varied between categories within the Uses class, which may be a contributing factor for the minimal correlation between Uses and the other 3 classes.

The chemicals that appeared most frequently in the EPA CompTox Dashboard are displayed in Supplementary Table 3. This table is limited to the compounds that appeared in at least 24 different lists. The compounds detected include hydrocarbons, chlorinated compounds, and phthalates. The 5 compounds that occurred most frequently were toluene (41 lists), styrene (38 lists), di(2-ethylhexyl) phthalate (DEHP) (38 lists), dibutyl phthalate (DBP) (37 lists), and ethylbenzene (36 lists). These chemicals are potentially human toxins (Camara-Lemarroy *et al.* 2015; Chen *et al.* 2020; Gibbs and Mulligan 1997; Henderson *et al.* 2007; Rowdhwal and Chen 2018). The degree of toxicity of each of these varies and depends on exposure amounts (Mackay *et al.* 2014). Toluene has a wide range of uses, including paint, nail polish, stain removes, explosive, and glues; therefore, humans are most commonly exposed to toluene through skin contact or inhalation (Greenberg 1997). Ethylbenzene is produced to make styrene, both of which can cause irritation to the skin and eyes after exposure (Santodonato *et al.* 1980). The 2 phthalates, DEHP and DBP, can be absorbed by the body through skin contact and can lead to cancer or reproductive harm (Meeker *et al.* 2009). All of these chemicals were found in 31 different categories from the classifications curated from the lists in the EPA CompTox Dashboard (Williams *et al.* 2017). Despite their common appearance in lists associated with human health (Androgen or Estrogen Receptor Activity, Neurotoxin or Potential Neurotoxin, Endocrine Disruptor, and Skin Sensitizer), they were also all found in lists associated with products that humans consume or use in their daily lives (Rubber, Plastics, Food Additives or Products, and Cosmetics).

The EPA CompTox Dashboard (Williams *et al.* 2017) is a useful tool for interpreting and understanding nontargeted data for human blood samples. However, a large portion (75%) of the chemicals identified in the nontargeted data using the QuEChERS method were not found in the database. Our results suggest that a large portion of small exogenous organic molecules have not been of concern for regulators or environmental health research. The results could also indicate that a large portion of both exogenous and endogenous small organic molecules are not represented in this database, which could be largely due to the reliance of metabolomics research on LC-MS, rather than GC. The large presence of uncategorized chemicals demonstrates the need to continually expand and strengthen the analytical methods and databases that cover chemicals in the human exposome. Additionally, small organic molecules in the human exposome can be metabolized and may transform in the human body (Brown 1994). In exposome measurements, it is important to include databases that include transformations reactions that could help to uncover the exposures. The incorporation of nontargeted methods into traditional targeted analysis, as demonstrated herein, has the potential to help environmental researchers discover new moieties in the human exposome and metabolome that cause or could indicate a threat to human health.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

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DECLARATION OF CONFLICTING INTERESTS

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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