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A high-throughput analytical method for complex contaminant mixtures in biosolids

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Abstract

Biosolids are rich in organic matter and other nutrients that contribute to environmental and agricultural sustainability by improving soil textural and biological properties and enhancing plant growth when applied to agricultural crops. Land application of biosolids encourages resource recovery and circumvents drawbacks associated with landfilling or incineration. However, biosolids contain numerous chemicals at trace levels, and quantitative analysis of such mixtures in this complex matrix is crucial for understanding and managing application risks. There are currently few analytical methods available that are capable of extracting and quantifying a large range of the emerging contaminants found in biosolids. In this study, a simplified, rapid, and robust method of analysis was developed and validated for a high-priority organic contaminant mixture of 44 endocrine disrupting compounds known to occur in biosolids. Analytes consisted of chemicals from many classes with a wide range of physiochemical properties (e.g., log K_{ow} values from -1.4 to 8.9). The biosolids extraction and cleanup protocol was validated for 42 of the targeted compounds. The UPLC-MS^2 parameters were validated for all 44 organic contaminants targeted for study. From the two batches of biosolids tested using this analytical method, most of the targeted contaminants (86%) were detected with 100% frequency at concentrations ranging from 0.036 to 10,226 µg/kg dw. Performance results highlighted that internal standards alone could not negate biosolids matrix effects; thus, internal standards and the standard addition method were used for residue quantification. This was the first study to detect and quantify 6PPD-q in biosolids, and the first to quantify lidocaine and 11 other chemicals in biosolids using a single analytical method. This method may be expanded for analysis of additional chemicals in biosolids and comparable matrices.

Keywords: Chemical mixtures; Complex matrices; Extraction methods; UPLC-MS^2 analysis; Endocrine disrupting compounds analysis; Multi-class, multi-residue analysis

1. Introduction

When wastewater is collected and treated, large quantities of biosolids (i.e., treated sewage sludge) are generated as a byproduct (Seiple et al., 2017; Beecher et al., 2022). Biosolids are rich in organic matter and other nutrients that contribute to environmental and agricultural sustainability by improving soil textural and biological properties and enhancing plant growth when applied to agricultural crops (Kumar et al., 2017; Sumner, 2000; Eid et al., 2020). The United States produces between 5.65 and 6.71 Mt of dried biosolids annually (Seiple et al., 2017; Rauch-Williams et al., 2019; Beecher et al., 2022), of which 2.08 to 2.3 Mt was reportedly land-applied (Rauch-Williams et al., 2019; Beecher et al., 2022). Applying biosolids to land allows for significant resource recovery while circumventing the disadvantages associated with landfilling and incineration (Hope, 1986; Ødegaard et al., 2002; Peccia and Westerhoff, 2015). However, biosolids contain numerous trace contaminants that may pose a threat to human and environmental health if released into agricultural systems. The Chemicals in Biosolids List (Richman et al., 2022) on the US EPA CompTox Chemistry Dashboard (v2.2.1: Williams et al., 2017) revealed the presence of 726 chemicals in U.S. biosolids and a global literature search uncovered an additional 13, totaling 739 chemicals in biosolids. Concerns regarding the occurrence, fate, and risks of such chemicals in agricultural systems hinder the expansion of beneficial land applications of biosolids (Hiemstra and de Kok, 2007; Vulliet et al., 2008; Cincinelli et al., 2012). It is not feasible to investigate all contaminants in biosolids, and it is likely that not all of them are a health concern due to their low concentrations, short half-lives, or low biological activity. Therefore, chemical prioritization for a short list of “high priority” organic contaminants and development of reliable and rigorous methods for identifying and

quantitatively measuring such contaminants in complex matrices such as biosolids are urgently needed.

Technological advances in analytical instrumentation, data processing software, and chemical extraction techniques (Christensen et al., 2005; Wiest et al., 2011; Danezis et al., 2016) allow for a better understanding of the occurrence, fate, and risks of biosolids-borne contaminants in agricultural systems. For example, the U.S. EPA Method 1694 was standardized for HPLC-MS^2 analysis of 74 pharmaceuticals and personal care products (PPCPs) in biosolids (U.S. EPA, 2007) and other methods have been developed for analysis of complex mixtures in complex biological and environmental matrices (Kruve et al., 2008; Barrek et al., 2009; Wiest et al., 2011; Wu et al., 2012; Berlioz-Barbier et al., 2014; Gago-Ferro et al., 2015; Petrie et al., 2016; Zhao et al., 2018; Lopardo et al., 2019; Melekhin et al., 2022). However, environmental samples contain numerous co-extractives that cause ionization enhancement or suppression in mass spectroscopy-based analyses which can falsely influence quantification results (Kebarle and Tang, 1993; Hajšlová and Zrostlıkova, 2003; Kruve et al., 2008; Kebarle and Verkerk, 2009; Trufelli et al., 2011). Therefore, analysis of complex chemical mixtures in complex matrices remains a significant challenge (Feron et al., 1998; Groten et al., 2001; Gibson et al., 2019).

Methods currently available for analysis of chemicals in biosolids show room for improvement. ~~This is because biosolids extractions~~Existing methods often require repeated sample extractions or clean up steps, consume large volumes of solvents, produce separate extracts, contain time-intensive steps, or are limited to a single chemical class (e.g., U.S. EPA, 2007; Sepulvado et al., 2011; Petrie et al., 2016; Choi et al., 2019; Lazcano et al., 2019; Lopardo et al., 2019; Proctor et al., 2019; Madrid et al., 2020). Biosolids extraction, cleanup, and chemical residue analysis also

continues to pose a challenge due to the complexity of the matrix itself (Kruve et al., 2008; Trufelli et al., 2011; Petrie et al., 2016; Proctor et al., 2019). This challenge is compounded by the number of chemicals ~~sequestered in biosolids~~ having ~~unknown and~~ potential~~ly numerous~~ interactions among them when introduced into solution (Feron et al., 1998; Groten et al., 2001). Therefore, the purpose of this study was to develop a ~~validated,~~ simplified, rapid, robust, and comprehensive analytical method for high throughput screening of a high-priority, complex contaminant mixture known to occur in biosolids~~ that comprised more than three major chemical classes~~. The method was validated against Class A biosolids samples to detect a wide range of biosolids-borne chemicals quickly and reliably.

2. Materials and Methods

2.1 Chemicals

From the 739 chemicals that occur in biosolids, 44 were selected as high priority for research based on several criteria (**Figure S1**), i.e., regulatory status in food and water, ability to permeate biological membranes and enter the food chain, endocrine disrupting potential, prevalence in source materials, and reported environmental or human health toxicity (**Table S1**). This complex chemical mixture collectively comprised more than three major chemical classes including fluorinated, brominated, and phosphonated flame retardants; anti-microbial, hormonal, and fragrant personal care products; antibiotic, anti-anxiety, and pain-relieving pharmaceuticals; plastic leachates; food additives; a tire-wear product, and others. Specifically, the 44 chemicals prioritized and selected for method development were: 4-chloraniline, 4-n-nonylphenol (4-NP), 4-octylphenol, 2-anilino-5-[(4-methylpentan-2-yl)amino]cyclohexa-2,5-diene-1,4-dione (6PPD-q), acetaminophen, atenolol, bisphenol A, caffeine, cannabidiol, cannabinol, carbamazepine,

chlortetracycline, diazepam, diisodecyl phthalate (DIDP), diisononyl phthalate (DINP), estrone (E1), 17β-estradiol (E2), fluoxetine, galaxolide, hexabromocyclododecane (HBCD), ibuprofen, indomethacin, lidocaine, metformin, methamphetamine, methylparaben, miconazole, naproxen, norethindrone, oxybenzone, perfluorobutane sulfonate (PFBS), perfluorohexanoic acid (PFHxA), perfluorohexane sulfonate (PFHxS), sulfathiazole, tetrabromobisphenol A (TBBPA), tris-(2-butoxyethyl) phosphate (TBEP), tributyl phosphate (TBP), tris(2-chloroethyl) phosphate (TCEP), tonalide, triphenyl phosphate (TPhP), triclocarban, triclosan, triphenyl tin, and Δ9-tetrahydrocannabinol (Δ9-THC). Chemical details and toxicological reference values (sourced from PubChem (Kim et al., 2023) and CompTox Chemical Dashboard (v2.2.1: Williams et al., 2017)), environmental concentrations, and source literature are given in **Table S1**.

The targeted chemicals and isotopically labeled analogs were purchased from several sources, including C/D/N Isotopes (Pointe-Claire, Quebec, Canada), Cambridge Isotope Laboratories (Tewksbury, MA), HPC Standards (Atlanta, GA), Santa Cruz Biotechnology (Dallas, TX), Sigma-Aldrich (Saint Louis, MO), Tokyo Chemical Industry (Portland, OR), and Wellington Laboratories (Guelph, Canada). Purchasing details are listed in **Table S2**. All chemicals were purchased either neat or as dissolved solutions and were further dissolved or diluted in 100% methanol (MeOH) or 100% acetonitrile (ACN) and stored at -20°C as individual stock solutions ranging from 10 to 1000 mg/mL before use. From the individual stocks, 3 separate solutions were prepared at 1 mg/mL (except E2 (5 mg/mL) and d2-E2 (10 mg/mL)) in 100% MeOH. One solution contained only the 44 native test chemicals, another solution contained only the 23 labeled surrogate internal standards, and the third solution contained only the 2 labeled quantitative internal standards. Reagents used for matrix extraction (HPLC grade MeOH, ACN,

acetone, and methyl tert-butyl ether (MTBE)) and instrument analysis (Optima® LC/MS grade water (H2O), MeOH, ACN, formic acid, ammonium acetate, acetic acid, ammonium formate) were purchased from Fisher Scientific (Fair Lawn, NJ). A Barnstead E-Pure water purification system (Thermo Scientific, Dubuque, IA) produced the Mili-Q H2O that was used for sample extraction.

2.2 Biosolids Sampling and Extraction

The goal here was to produce a single sample extract for analysis from a one-step extraction and cleanup process with a high sample throughput. The detailed extraction and cleanup protocol are described in **Text S1**. Dried and pelleted Class A biosolids **(Text S3)** were provided by the Irvine Ranch Water District (IRWD, Irvine, CA) in two separate batches (9/22, 1/23). A sample was collected from each batch in a large container, transported to the laboratory, and stored at 4°C. Upon arrival to the lab, each biosolids sample was tested for moisture content, pH, and contents of nitrogen, phosphorus, and total carbon (**Table S3**). To simplify chemical extractions from complex solid samples, QuEChERS salts are widely used and can be applied in conjunction with different cleanup materials to address matrix effects (Anastassiades et al., 2003; Kruve et al., 2008; Wiest et al., 2011). Therefore, the biosolids samples were extracted using original QuEChERS salts (4 g MgSO4 and 1 g NaCl, Thermo Scientific, Rockwood, TN) with 1.5 g added sodium citrate dihydrate buffer (Fisher Scientific) to both decrease the solution pH to ~ about pH 4 and to increase the extraction efficiency. The extracts were cleaned up using Supelclean™ ENVI-Carb II/PSA SPE cartridges (900 mg, 6 mL; MilliporeSigma, Burlington, MA). Nine composite samples of the two batches were used for method development and validation, and 5 samples from each batch were used to verify the validated method.

Briefly, for method development and validation, 4 g of the lyophilized biosolids mix was weighed into 50-mL polypropylene centrifuge tubes. Procedural sample blanks (n=2) were spiked with 400 µL of the surrogate internal standard mixture prior to extraction and the replicate samples (n=7) were spiked with 400 µL of both the native test chemical mixture and the surrogate internal standard mixture prior to extraction. All samples were reconstituted with 100 µL of the quantitative internal standard mixture. The labeled surrogate internal standard mixture was used to monitor for analyte loss during extraction and to compensate for matrix effects during analyte quantification. The quantitative internal standards were used to monitor for instrument signal variation and to compensate for chemical activity during analyte quantification. A series of extraction solvents (2 mL Milli-Q H2O, 3 mL MeOH, 10 mL ACN, 2 mL Acetone, 1 mL MTBE) was added to the samples with vortexing (15 s) between additions. Buffered QuEChERS salts were added to each sample and the samples were shaken and vortexed (20 s), sonicated (15 min), and centrifuged (4000 rmp, 5 min). ENVI-Carb cartridges were preconditioned with 1 mL each MeOH, ACN, and MTBE. Supernatant (3 mL) from the centrifuged sample was passed through the cartridge (at ~ 2 mL/min) and eluted with 1 mL each MTBE, ACN, and MeOH without allowing the cartridge to run dry. Eluants were nitrogen evaporated (N-EVAP 111- Organomation Associates, Inc. Berlin, MA) to dryness (< 2 h), and the dried extracts were reconstituted with 900 µL of MeOH and 100 µL of the quantitative standard mixture. To assess matrix effects and calculate chemical recovery, procedural sample blanks were prepared using composite biosolids samples (n=5); these samples were not spiked with any chemical solution prior to extraction but instead were spiked during reconstitution with 75 µL of the native test chemical mixture, 75 µL of the labeled surrogate internal standard mixture, 100 µL of the labeled quantitative standard mixture, and 750 µL of MeOH. All cleaned and reconstituted biosolids extracts were transferred

into 2-mL glass LC vials without filtering and immediately analyzed or stored at -20°C for up to 24 h prior to analysis.

2.3 UPLC-ESI-MS^2 Optimization

During the optimization process, the final extract solvent (100% MeOH, 100% ACN, 1:1 MeOH:H2O), organic mobile phase (100% MeOH, 9:1 MeOH:ACN, 1:1 MeOH: ACN, 1:9 MeOH:ACN) with and without buffers (formic acid, ammonium acetate, acetic acid, ammonium formate), aqueous mobile phase with and without buffers, solvent gradient, and flow rate (0.25 and 0.3 mL/min) were evaluated to determine the conditions producing the most favorable separation, resolution, selectivity, sensitivity, and calibration range for the majority of the chemicals in the mixture.

A parent and two product ions, wherever possible, were monitored for identification and quantitation of each chemical using multiple reaction monitoring (MRM) in both positive and negative modes. Preferred electrospray ionization (ESI) polarities, retention times, monitoring ions, cone voltages, and collision energies are listed in **Table S4**. Mass spectrometer conditions were optimized for each chemical using the instrument software (Intellistart®, Waters) and individual stock solutions diluted to 1 mg/mL in pure MeOH. For the chromatographic separation, solutions were prepared containing a test chemical, surrogate internal standard, and quantitative standard mixture at 1 mg/mL (except E2 (5 mg/mL) and d2-E2 (10 mg/mL)) in 100% MeOH. Separation of the mixture was performed using a Waters ACQUITY UPLC BEH C18 column (2.1 x 100 mm, 1.7 um i.d.). A Waters ACQUITY ultra performance liquid chromatograph (UPLC) coupled with a Waters Micromass triple quadrupole (TQD) tandem mass

spectrometer (MS^2) was used to separate analytes and acquire analytical data (Waters, Milford, MA).

2.4 Method Validation

Method verification for the extraction and instrumental analysis of 44 biosolids-borne contaminants was accomplished following several internationally recognized guidelines (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), 2005; United Nations Office on Drugs and Crime (UNODC), 2009; Eurachem, 2014). Method performance data were acquired during a single batch acquisition and processed using v4.1 of the manufacturer provided software (Waters, Milford, MA: MassLynx® – Acquisition, TargetLynx® – Processing). At the acquisition start, end, and after every 10 sample injections, 2 solvent blanks, a continuing calibration check standard of known concentration, and 2 additional solvent blanks were sequentially injected to monitor for chemical carryover and ensure calibrated results. Internal standards were assigned to each analyte for signal normalization during instrument calibration, assessment of instrument and extraction performance metrics, and quantification of environmental grab samples to address matrix effects. The surrogate internal standards were similarly linearly calibrated with and without signal normalization using a quantitative standard (as was appropriate for the specific analysis) to further account for instrument variation and/or chemical activity.

Extraction performance metrics included an examination of extraction efficiency, matrix effects, and limits of detection and quantification. Extraction efficiency or accuracy was determined from a traditional spike and recovery test:

$$\%R = (C_A - C_B)/C_C * 100 \quad (1)$$

where $\%R$ was the percent of the chemical recovered, C_A was the concentration in the replicate samples spiked pre-extraction, C_B was the concentration in the unspiked sample blanks, and C_C was the concentration in replicate samples spiked post-extraction (i.e., during reconstitution). Extraction precision was represented by the standard error of the mean percent recovery for each chemical. Absolute recovery was calculated by replacing C_C with the nominal concentration spiked (**Table S5**).

The validation guidelines ~~used~~ recommended using 3 concentrations (low, middle, and high) and 3 samples for each concentration for the spike and recovery test. The 70 chemicals purchased for this analysis in total were very expensive and data processing for targeted analysis of 70 chemicals is extremely time consuming. Therefore, a more appropriate spike and recovery test for this complex mixture and matrix was to use additional replicates and 1 middle and environmentally relevant spiking concentration. Additionally, biosolids produce large matrix effects that would likely cause variable results at low spiking concentrations, especially using only 3 replicates. High spiking concentrations could render the method environmentally irrelevant. Therefore, because biosolids samples often contain trace amounts of chemicals, we chose to validate the method using 2 procedural blanks (to ensure concentrations in the blanks did not interfere with the recovery calculation) and 5 spiked replicates in addition to 5 procedural blanks spiked immediately prior to injection to assess matric effects and calculate recovery.

Absolute matrix effects for the extraction process were calculated and reported as a percent absolute effect where the mean C_C in **Eqn 1** was divided by the nominal concentration spiked and the resultant multiplied by 100 (**Table S5**). However, in order to visualize the effect direction

(e.g., ionization suppression or enhancement), an actual percent effect, i.e., the percent difference from a null effect, or 100%, was also calculated:

$$\%ME = (\%A_{ME} - 100)/100 * 100 \qquad (2)$$

where $\%ME$ was the percent actual matrix effect and $\%A_{ME}$ was the percent absolute effect.

The detection and quantification limits for the extraction protocol were estimated according to Lopardo et al. (2019), with modification:

$$MDL = LOD/(\%R * C_f) \qquad (3)$$

$$MQL = LOQ/(\%R * C_f) \qquad (4)$$

where *MDL* and *MQL* were the extraction process detection and quantification limits, *LOD* and *LOQ* were the instrumental limits of detection and quantification, and C_f is the concentration conversion factor for the amount of sample extracted (e.g., 4 g biosolids into 1 mL extract).

To assess the instrument performance metrics, calibration data were acquired in triplicate with 10 data points spanning 3+ orders of magnitude using the optimized chromatographic conditions. Instrument performance metrics included an evaluation of goodness of fit for linear calibration, estimates of measurement repeatability and reproducibility, and measurement sensitivity. Goodness of fit was achieved when the linear regression coefficient of determination for each chemical calibration was > 0.99. Repeatability and reproducibility (i.e., intra- and inter-day accuracy and precision) were assessed using spiked replicates (n=7: intraday, n=3: interday) of the chemical mixtures in pure MeOH according to guideline recommendations (ICH, 2005; UNODC, 2009; Eurachem, 2014). Accuracy was determined by dividing the concentration

detected in spiked solvent by the nominal amount spiked. Precision was expressed as the coefficient of variation of the spiked replicates. Measurement sensitivity was demonstrated for each chemical by a calculated limit of detection (LOD) and quantification (LOQ) using calibration curve data:

$$LOD = 3.3\,\sigma / m \qquad (5)$$

$$LOQ = 10\,\sigma / m \qquad (6)$$

where σ was the standard deviation of the y-intercept or regression line and m was the slope of the regression line. Because the quantitative standards were used as external standards, they were not linearly calibrated; thus, their *LOD* and *LOQ* values were calculated using the relative response factor standard deviation and the mean response factor in place of σ and m, respectively.

2.5 Application to Environmental Samples

Biosolids grab samples were extracted and analyzed for a high-priority complex chemical mixture to validate the utility of this analytical method. Five samples from each batch were extracted for chemical residues (n=10 total). The grab samples were extracted without test chemical spiking but with surrogate internal standard spiking (100 ng/g) prior to extraction and were reconstituted in 900 µL methanol and 100 µL of the 1 ng/mL quantitative internal standard mixture. To compensate for trace level analysis, matrix effects, and chemical activity, residues in the grab samples were quantified using the same internal standard arrangement as was used during method development. To ensure accurate residue results, the standard addition method was also employed for analysis of the grab samples because low residue concentrations were expected (e.g., **Text S2**). For the standard addition method, residue data was acquired from

the extracts, the extracts were then spiked with 200 µuL of the test chemical mixture, data was reacquired, and the residues were quantified accordingly. Residue data acquired at lower values than the specific range of calibration for each chemical were reported as not detected. Averaged chemical residue values were reported as both uncorrected and method recovery-corrected concentrations for each biosolids batch.

3. Results and Discussion

3.1 Biosolids Analysis

Due to the complexity of the matrix, a delicate balance existed between cleaning the sample extracts well enough for introduction to the instrument and removing the analytes during cleanup. Even with advanced extraction and cleanup technologies utilized, biosolids extracts still contained many co-extractives that entered the analytical column, passed through to ionization, and fouled the ESI cone, ultimately decreasing detection sensitivity. Co-extracted residues in biosolids extracts interfered with chemical analysis by causing ionization suppression or enhancement among analytes when compared to dilution in pure solvent. This change in ionization led to shifts in analyte retention time, split or fanned chromatographic peaks, and/or failed continuing calibration check standards by the end of a longer batch acquisition, as has been previously reported (Petrie et al., 2016; Choi et al., 2019; Lazcano et al., 2019; Lopardo et al., 2019; Proctor et al., 2019; Madrid et al., 2020). These complications suggest that there is continued room for improvement with regards to analytical method development for complex mixtures extracted from complex matrices such as biosolids.

3.2 UPLC-ESI-MS^2 Analysis

The optimal chromatographic conditions for analysis of this chemical mixture utilizes a different combination of instrumental parameters than similar studies (U.S. EPA, 2007; Kruve et al., 2008; Barrek et al., 2009; Wiest et al., 2011; Wu et al., 2012; Berlioz-Barbier et al., 2014; Petrie et al., 2016; Zhao et al., 2018; Choi et al., 2019; Lazcano et al., 2019; Lopardo et al., 2019; Proctor et al., 2019; Melekhin et al., 2022). Dilution of the mixture in 1:1 MeOH:H2O caused precipitation of some of the analytes, possibly due to the several analytes in the mixture that are not very soluble in water. This could raise concerns for the quantitative integrity of mixtures studies using this solvent combination for chemical dilution or reconstitution of extracts. For the dilution/reconstitution solvent, most of the analytes in this mixture gave optimal resolution, separation, and sensitivity when in MeOH as compared to ACN. For both positive and negative mode analysis, optimal sensitivity and resolution was achieved when there was at least 10% ACN in the organic mobile phase. For negative mode analysis, too much ACN in the organic mobile phase (i.e., > 60%) in combination with > 0.5% (v/v) ammonium acetate and acetic acid in the aqueous mobile phase encouraged the salting out of ammonium acetate within the column and caused unacceptable backpressure (EPA, 2007). Therefore, the amounts of ammonium acetate, acetic acid, and ACN were reduced, alleviating the occurrence of salt precipitation and system backpressure. For positive mode analysis, addition of ammonium formate in combination with formic acid in the mobile phases decreased signal intensity for many of the analytes relative to using formic acid alone. This may be due to the ammonia moiety possibly changing the solution pH and the strength of analyte ionization. For both positive and negative mode analyses, reduction of the flow rate from 0.30 to 0.25 mL/min increased signal intensity for the majority of

the analytes. ~~This may suggest that many of the analytes preferred partitioning in and out of the column lining under lower vs higher fluid pressures.~~ The optimal chromatographic conditions for instrumental analysis of the targeted chemical mixture are listed in **Table 1**.

3.3 Method Performance

The performance metrics for the biosolids extraction are detailed in **Figure 1**, showing percent chemical recovery; **Figure 2**, depicting percent matrix effect; and **Table 2**, listing the *MDL* and *MQL* values. The optimized extraction process was completed on 24 biosolids samples in < 4 h; whereas other methods may take up to 3 d for processing. For compensation of matrix effects and instrument signal variation, 3 of the negative mode analytes were quantified using a surrogate internal standard for signal normalization, 5 were quantified using the quantitative standard for normalization, and the remaining 6 were quantified without signal normalization (i.e., external calibration). For the positive mode: 12 analytes were quantified using a surrogate internal standard for signal normalization, 16 were quantified using the quantitative standard for normalization, and the remaining 2 were quantified without signal normalization. Some of the analyte/analog pairs behaved differently through the extraction and analysis process, indicating that signal normalization of analytes using a similar surrogate chemical for quantification did not negate matrix effects, but instead overly increased or decreased the quantified concentration relative to the known amount.

To fully understand what is happening such a complex and real solution or extract, we would need to account for chemical activity of all of the chemicals present in the solution for proper quantification. That is not possible because environmental samples contain many chemicals that are not known (Feron et al., 1998; Groten et al., 2001) and therefore, cannot be quantified as

such. That said, many assumptions that are made regarding how analyte/analog pairs behave in ideal conditions do not hold true for real and complex solutions (Feron et al., 1998; Groten et al., 2001), therefore all of the analyte/analog pairs are not likely to behave ideally in this real solution. Finding an appropriate labeled chemical for analyte normalization (i.e., one that behaves similarly to the analyte when in solution due to similar chemical activity) or using no normalization to ensure accurate quantification relative to a known amount seemed to be the only logical and comprehensive approach, considering both the complexity of the mixture and the matrix. For example, when triclosan was analyzed against either labeled standard, the surrogate or the quantitative, the QCs failed, and the analyte signal was overly corrected relative to the known spiking concentrations, failing recovery validation parameters. Consequently, the more accurate analysis for triclosan in biosolids extracts was quantification without signal normalization, wherein the QCs and recovery parameters passed validation criteria. This approach allowed for increased confidence in passing QCs and producing accurate residue concentration results from unspiked grab samples wherein we did not know the concentration of the analyte.

Triclocarban and TBBPA could not be recovered from biosolids using this extraction protocol, however, matrix effects could be estimated (**Fig 1**). Chemical recovery was low for indomethacin (1.47 ± 0.57%), miconazole (15.9 ± 2.7%), chlortetracycline (14.2 ± 2.2%), and triclosan (9.7 ± 9.7%), likely due to the low spiking concentration (100 ng/g); however, the precision values were validated (i.e., < 25%). Precision values for the remaining analytes were validated with the exception of galaxolide, 4-octylphenol, and bisphenol A (34.7, 36.7, and 31.0%, respectively); however, because their recovery values were validated, analytical results were considered fully

quantitative. Excluding triclocarban, TBBPA, indomethacin, miconazole, chlortetracycline, and triclosan, recovery of the analytes in biosolids ranged from 54.0 to 127%. Atenolol, E1, and E2 had lower than desired recoveries (54.0, 55.4, and 55.5%, respectively); however, their precision values were validated (1.9, 7.5, and 20.7%, respectively) for a fully quantitative analysis. Matrix effects from the biosolids extracts (**Fig 2**) were ± 30% from null for 16 of 44 analytes, indicating a limited effect on quantification of concentrations for those chemicals. Signal suppression (-31.8 to -99.4) and enhancement (32.5 to 115%) ranges were large for the remaining 28 analytes. Regardless of the matrix effects observed, signal normalization and matrix-corrected recovery allowed for quantification of *MDL* and *MQL* values for all analytes and internal standards (except for triclocarban and TBBPA) from spiked replicates at values ranging from 0.046 to 31.5 ng/kg dw biosolids (**Table 2**), indicating its suitability for trace level analysis.

This validated biosolids extraction protocol is capable of simultaneously extracting trace amounts (ng/kg) of 42 of the 44 high-priority contaminants from lyophilized biosolids using one extraction and cleanup step to produce a single extract for a high throughput multi-class, multi-residue chemical analysis. This biosolids extraction protocol may be expanded to analyze other high-priority chemicals sequestered in biosolids having similar physiochemical properties to the targeted contaminants because the protocol is not chemically selective and complex environmental samples contain many chemicals of unknown composition and concentration (Feron et al., 1998; Groten et al., 2001).

The UPLC-ESI-MS^2 performance metrics for the targeted high-priority mixture are given in **Table 3**. Linearity covered nearly 3 orders of magnitude for most of the analytes and R^2 values ranged from 0.991 to 0.999. Because the quantitative standard monitors for both chemical activity

393 and instrument variation and the surrogate internal standard monitors for analyte loss and matrix
394 effects, either standard or both in combination should have been sufficient for signal
395 normalization and accurate quantification. However, the normalized signal using the surrogate
396 internal standard either unacceptably increased or decreased (greater than ± 30%) the analyte
397 quantification results relative to a known, spiked value for diazepam, miconazole, bisphenol A,
398 TBBPA, HBCD, E1, triclosan, and triclocarban; therefore, the quantitative standard was
399 alternatively used for signal normalization and analyte quantification. The standard assignment
400 arrangement used to validate the instrument performance metrics for the high-priority mixture in
401 pure solvent (**Table 3**) was markedly different from the arrangement used to validate the biosolids
402 extraction protocol (**Table 2**), demonstrating that some analyte/analog pairs do not always behave
403 the same when in solution. This suggests that signal normalization using a surrogate or
404 quantitative standard alone may not fully compensate for chemical activity and matrix effects
405 during analyte quantification.

406 Compared to most guidelines for analytical method validation (ICH, 2005; UNODC, 2009;
407 Eurachem, 2014), repeatability accuracy fell outside of the desired range (70 to 130%) for 2
408 analytes and 3 surrogate internal standards and corresponding precision values were acceptable
409 (< 20%) for all but d16-bisphenol A (21.5%). Reproducibility accuracy fell outside of the desired
410 range for only 1 analyte (65.5%) and 1 surrogate internal standard (59.4%). Although the intraday
411 accuracy values for d3-ibuprofen and d4-4-NP were lower than desired (68.8 and 51.0%,
412 respectively), they were retained for sample analysis because their precision values were
413 acceptable, and they did not adversely affect their corresponding analyte accuracy values. The
414 intraday variability observed could be due to chemical activity, stock solution instability, or

415 standard impurities. The limit of detection and quantification values ranged from 0.0003 to 0.111
416 µg/L and from 0.001 to 0.336 µg/L, respectively, which was acceptable for analyzing trace levels
417 of organic chemicals sequestered in complex environmental samples.

418 Collectively, the instrument performance metrics validated these UPLC-ESI-MS^2 conditions for
419 analysis of a high-priority complex chemical mixture (**Table S1**). These instrument conditions
420 are capable of quickly separating and quantifying 44 analytes, 21 surrogate internal standards,
421 and 2 quantitative standards contained in a single extract. These UPLC-ESI-MS^2 conditions,
422 inclusive of both a positive and negative mode analysis, are proficient for quantifying chemical
423 residues from a wide range of major chemical classes and offer shorter analysis times than similar
424 multi-class, multi-residue method development studies.

425 *3.4 Environmental Sample Analysis*

426 This validated analytical method was used to quantify contaminant residues in biosolids grab
427 samples. Many of the chemicals sequestered in biosolids were present at low concentrations and
428 quantification was subject to matrix effects. Therefore, in order to neutralize the biosolids matrix
429 effects and provide more accurate results, both the standard addition method and signal
430 normalization were used for grab sample residue quantification. Analysis of sample residues
431 revealed the presence of 42 of the 44 high-priority chemicals in biosolids at the µg/kg level
432 (**Table 4**), demonstrating the robustness and utility of the analytical method. Due to extraction
433 limitations, triclocarban and TBBPA could not be detected. Triphenyl tin was found in one of the
434 two biosolids batches at 100% frequency, while E1, triclosan, and PFHxS were detected in one
435 batch at 80% and the other batch at 100% frequency. Confirming the reliability of the analytical

method, the remaining 38 chemicals targeted for analysis were detected in both biosolids batches with 100% frequency.

The chemical residues in both biosolids batches ranged from 0.146 to 8479 µg/kg dw, similar to that previously reported (**Table S1**). This was the first study to both confirm the presence and quantify the amount of 6PPD-q and lidocaine in biosolids (6PPD-q: 35.3 ± 2.9 and 18.8 ± 1.5 µg/kg dw, lidocaine: 7.13 ± 0.34 and 26.9 ± 1.7 µg/kg dw). This study further quantified the concentrations of oxybenzone, cannabinol, Δ9-THC, cannabidiol, triphenyl tin, DINP, DIDP, 4-octylphenol, PFBS, and HCBD in biosolids for the first time using one analytical method. All three per-and-polyfluoroalkyl substances (PFAS) investigated were detected in both biosolids batches at concentrations similar to previous studies. Both HHCB and Δ9-THC were detected at high levels, consistent with that previously reported. Methamphetamine, atenolol, cannabinol, cannabidiol, and indomethacin were found at higher concentrations and the phthalates (DINP and DIDP) were detected at lower concentrations than were previously reported.

From the chemical classes included in this study, personal care products, recreational drugs, and plasticizers were found to contribute the most (80%) to those sequestered in biosolids as shown in **Figure 3**. This suggests that there may be other personal care products, recreational drugs, and plasticizers sequestered in biosolids that are not currently accounted for. Because each of the study compounds are potential endocrine disruptors (Karthikeyan et al., 2019, 2021), environmental exposure to a mixture of them at any level could pose a health risk (Barrek et al., 2009; Gibson et al., 2019; Lopardo et al., 2019; Tanner et al., 2020), suggesting the need for additional exploratory studies involving similar chemical mixtures prior to expanding the beneficial use of biosolids. Furthermore, the confirmed presence of 6PPD-q in biosolids coupled

with its environmental mobility and acute toxicity (Tian et al., 2020, 2022; Zoroufchi Benis et al., 2023) warrants further research.

4. Conclusions

This is the first multi-class, multi-reside analytical method that is capable of simultaneously extracting, detecting, and quantifying a mixture of residues from more than three major classes that additionally includes a progestin and two estrogenic hormones. Overall, this validated method offers a more rapid, robust, and reproducible analysis than similar multi-class, multi-residue method development studies. The biosolids extraction protocol represents a robust and simple approach to quickly (< 4 h) and simultaneously extract a wide range of organic contaminants from biosolids samples. The optimized UPLC-ESI-MS^2 conditions represent a rapid (29 min/sample) and versatile technique capable of quantifying a wide range of major chemical classes contained in a single sample. Together, the verified extraction protocol and instrument conditions offer a high throughput and fully quantitative analytical method for screening a wide range of chemicals in biosolids samples. Additionally, this study highlights that not all analyte/analog pairs behave the same when in solution. Therefore, internal standards may not fully compensate for chemical activity and matrix effects during residue quantification from complex environmental samples.

This is the first study to detect and quantify 6PPD-q in biosolids, and the first to quantify lidocaine and 11 other chemicals in biosolids using a single analytical method. The list of analytes prioritized for study is exclusive of metabolites and contains few chemicals from the same class. Additionally, the extraction protocol is not chemically selective. Therefore, this analytical method may be expanded to study additional chemicals of emerging concern with

analogous physicochemical properties in comparable complex matrices. Finally, this study highlights that in order to retain realism and accuracy in analytical results, it will be imperative to establish best practices and find more flexible approaches to the way complex mixtures extracted from complex matrices are analyzed and validated.

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