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Analyzing a broader spectrum of endocrine active organic contaminants in sewage sludge with high resolution LC-QTOF-MS suspect screening and QSAR toxicity prediction†

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Endocrine active contaminants (EACs) in environmental samples can pose a range of toxicological threats to ecosystems, especially through their impacts on reproductive pathways mediated by the estrogen receptor. The physicochemical properties of known organic EACs vary greatly and typically require different sample preparation techniques to identify different classes of compounds. EAC sources are similarly diverse, including both endogenous compounds and anthropogenic chemicals found in personal care products, pharmaceuticals, and their transformation products, which are often disposed of to sewers at their end of use. Looking for EACs in sewage sludge proposes a bottom-up, or end-of-use and treatment approach to discover environmentally relevant EACs, since many EACs accumulate in sludges even after application of robust wastewater treatment processes. This study demonstrates an extraction and analytical method capable of detecting a broad spectrum of known and suspected EACs *via* High Resolution Liquid Chromatography Quadropole Time-of-Flight Mass Spectrometry (LC-QTOF-MS) suspect screening of fourteen California sewage sludge samples. Spike-recovery experiments were performed using twelve carefully selected surrogates to assess different extraction solvents, sample weights, extraction pH values, procedures for combining extracts with different extraction pH's, and solid phase extraction cartridges. Using LC-QTOF-MS, identifications of several other organic compounds in the samples were made, a goal unachievable with unit resolution mass spectrometry. Suspect screening of California sludge samples discovered 118 compounds including hormones, pharmaceuticals, phosphate flame retardants, recreational drugs, antimicrobials, and pesticides. Additionally, 22 of these identified compounds are predicted to interfere with estrogen receptors or other reproductive/developmental pathways based on the VEGA QSAR toxicity prediction model.

Environmental significance

Many chemicals used in consumer products interfere with reproductive and developmental success in exposed organisms. Many of these chemicals are robustly engineered and persist their intended use and reach wastewater treatment plants (WWTPs). It is impossible to design treatment processes that eliminate each of these chemicals as they are engineered with vastly different physiochemical properties, but by looking at sewage sludges we can gain find those that are exceptionally persistent. In this study, we identified 118 anthropogenic using LC-QTOF-MS suspect screening, 22 of which were predicted to interfere with reproductive and/or developmental signalling. Confirming the endocrine activity of these 22 compounds in subsequent studies would provide the evidence necessary to lobby for removal of such compounds from consumer products altogether.

1. Introduction

Endocrine disrupting compounds (EDCs) have been implicated as potential contributors to diabetes, cancer, fertility decline, and a host of other environmental and public health issues.[1] Suspected EDCs are found in many classes of consumer products including pharmaceuticals, plastics, cosmetics, clothing dyes, and food packaging. In addition to the link between EDCs in commerce and adverse health outcomes,[2–4] many of these compounds persist beyond their intended use and through wastewater treatment techniques.[5–9] The potential for reproductive failure caused by EDCs in aquatic ecosystems (*e.g.*, fathead minnows, zebra fish and white sucker fish) has been extensively documented.[10–13] The exposure, toxicity, and bioaccumulation of EDCs in terrestrial organisms has been less

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studied, and although evidence has suggested potential risks similar to those observed in aquatic species, the findings from these studies have been disputed. Kinney *et al.* (2008) reported the ability of anthropogenic compounds, like triclosan and galaxolide to bioaccumulate in earthworms, but this study was quickly challenged in 2009 for lack of data validation with standard methods and overstatement of results.[14,15] In 2006, Hayes *et al.* reported the increased effects of pesticide mixtures over individual exposures on developmental toxicity in leopard frogs, but this too led to a series of editorial responses from the scientific community.[16,17] Although exposure of EDC's to terrestrial organisms is not thoroughly or unobjectively documented, there are many studies reporting evidence suggesting this concern is not one that should be ignored.

Ultimate disposal of many consumer products to the environment occurs *via* wastewater treatment, so discharges from wastewater treatment facilities are obvious places to search for endocrine active compounds (EACs). Endocrine active compounds, in contrast to endocrine disrupting compounds are those that are predicted or shown to interact with hormone receptor binding mechanisms and cause cellular responses, but evidence of resulting adverse health outcomes are unclear due to the lack of exhaustive *in vivo* studies required to label compounds as endocrine "disrupting". Contaminants in their original form, or products of metabolism or other forms of transformation, originate from pharmaceuticals, personal care products, pesticides, cleaning materials, manufacturing by-products, roadside runoff, and other sources. Many of these contaminants persist beyond their intended use, are transported to and treated within a wastewater treatment facility, and ultimately disposed of into the environment *via* effluent discharge or land application of sewage sludge. The rapid development of new organic compounds used in consumer products has made it difficult to keep up with associated fate, transport and toxicity issues. This paper presents an analytical method developed in support of a bottom-up search for EACs in commerce by studying their presence after use and treatment rather than the more traditional top-down approach of predicting their environmental fate prior to use. This work also seeks to identify any transformation products that may have formed after their intended use that are recalcitrant in the sludge, suggesting that these products may be persistent once formed. This is a direct complement to US EPA efforts to screen chemicals and evaluate their endocrine activity as part of the EPA Endocrine Disruptor-Screening Program (EDSP). By looking in sewage sludge at the end of robust treatment trains, we aim to identify highly persistent, endocrine active contaminants.

A large majority of semi-polar and nonpolar organic compounds are known to partition into the sludge fraction during wastewater treatment. Synthetic hormones like ethinyl estradiol,[23–25] estrone,[26,27] norgestrel;[28] sulfonamide antibiotics like sulfamethoxazole, sulfamethazine, and sulfathiazole;[29] polychlorinated biphenyls,[30,31] polyaromatic hydrocarbons,[5,6,32] and phthalates[33] have been ubiquitously detected at ng g^{-1} to μg g^{-1} ranges.[34] These contaminants, among others, are suspected to be endocrine active with possible endocrine disrupting effects.[35] Several published methods are available to extract pharmaceuticals and other personal care products from sewage sludge, however most are optimized for a handful of compounds. Accelerated Solvent Extraction (ASE) is commonly used for the extraction of hormones from soils, sediments and sludges,[36–41] but requires a large upfront cost and sample throughput is limited by the number of ASE cells available. Sonication, shaking and other forms of liquid–liquid extraction followed by Solid Phase Extraction (SPE) are used as ASE replacements.[8,23,42–46] Solvents, pH's, SPE cartridges, SPE elution buffers and evaporation techniques vary widely among these methods but in each case are optimized for 2 to 25 compounds. There are currently no published techniques outlining a sonication-based extraction for suspect and/or non-targeted analysis of sewage sludge capable of encompassing a broad spectrum of analytes in a single extraction method.

The majority of trace contaminant analyses of sewage sludge are performed in a targeted fashion, but through the incorporation of suspect screening (screening against spectral libraries), we can significantly broaden the spectrum of compound identifications. Suspect screening workflows have been used extensively for a wide range of environmental samples,[47–51] and although sensitivity is moderately compromised, the breadth of identifications is far greater than conventional targeted methods, thus allowing a more comprehensive chemical profile analysis.

Quantitative Structure–Activity Relationship (QSAR) modelling is used frequently to predict structural similarity and biological activity for compounds where experimental data is unavailable. QSARs have been used to estimate toxicity of compounds in a variety of environmental samples, such as the estrogenicity prediction of pharmaceuticals and their metabolites in urine,[18] acute toxicity estimates of polycyclic aromatic hydrocarbons exposure to *Daphnia magnia* and *Hyalella azteca*,[19] and toxicity of aromatic pollutants and photooxidative intermediates in water.[20] QSARs have also been used to assess the ability of a compound to bind to estrogen receptor ligands,[21] and their ability to sorb to sewage sludge during waste treatment.[22] Using *in silico* techniques to predict endocrine activity of consumer product chemicals is a suitable initial screen for evaluating a compounds biological/toxicological activity.

This work presents a sample preparation and analytical method capable of identifying a wide range of compounds within a physicochemical space created by carefully selected surrogate compounds. The utility and significance of the method is demonstrated by conducting suspect screening of fourteen California sewage sludge samples and using QSAR modelling to identify biologically relevant identifications through the prediction of each compounds' estrogenic activity and/or reproductive and developmental toxicity. The results suggest that the method is capable of extracting many diverse contaminants and transformation products that can be identified using qualitative analytical approaches.

2. Experimental methods

2.1 Materials

All analytical standards used in this study were >97% purity and purchased from Sigma Aldrich (St. Louis, MO), or AccuStandard

(New Haven, CT). Isotopically labelled internal standards were purchased from Cambridge Isotope Laboratories (Andover, MA) or Toronto Research Chemicals (North York, ON) and were >98% purity. Acetonitrile (ACN; LCMS grade) and methanol (MeOH; LCMS grade) were acquired from Honeywell – Burdick & Jackson (Muskegon, MI). *tert*-Butyl methyl ether (MTBE, HPLC grade) was obtained from ACROS organics (Morris Plains, NJ). Phosphoric acid (H_3PO_4, 85% w/w%) solution (99.99% trace metals basis), formic acid (ACS grade), and ammonium fluoride (HPLC grade) were purchased from Sigma Aldrich (St. Louis, MO). Ethylenediaminetetraacetic acid (EDTA), hydrochloric acid (HCl), ammonium hydroxide (NH_4OH) and sodium phosphate ($NaH_2PO_4 \cdot H_2O$) were purchased from Fisher Scientific (Hampton, NH). Double-deionized (DDI, 18.2 MΩ cm) low TOC (<50 ppb) water was produced by a Milli-Q® Integral 5 Water Purification System. Solid Phase Extraction cartridges were purchased from Waters (Oasis HLB, 60 μm particle size, 500 mg, 6 cm^3, Milford, MA) and Agilent Technologies (Bond Elut Plexa (45 μm particle size, 500 mg, 6 cm^3, Santa Clara, CA), Bond Elut ENV (45 μm particle size, 500 mg, 6 cm^3, Santa Clara, CA)). PTFE syringe filters were purchased from Agilent Technologies (Captiva, 15 mm diameter, 0.2 μm pore size).

2.2 Sample collection and treatment

Milwaukee Milorganite (heat treated and pelleted sewage sludge) was purchased from a local nursery and stored in an airtight glass container in a dark room at 4 °C. In addition, twelve California wastewater treatment plants provided sludge samples for this study. Two treatment plants each contributed two samples that represent before-and-after additional sludge treatment processes employed during wet *versus* dry months. All 14 samples fall under either Class A or Class B sewage sludge as defined by the EPA and are appropriate for disposal *via* land application, regardless of the multiple treatment techniques employed at two of the WWTPs. The majority of the plants that participated in this study employ advanced secondary wastewater treatment processes. The plants represent various geographical locations and feature diverse influent characteristics. All facilities participated anonymously and have been assigned randomized identifiers. Grab samples were collected in a clean 1-gallon glass jar and shipped overnight in ice-filled coolers. Samples were immediately transferred into a dark room at 4 °C until extraction. Dry weights and sample descriptors for each sample can be found in Table S4.†

2.3 Sample preparation

Prior to extraction, the dry weight was determined by drying a 100 g sample at 100 °C for ~24 h to attain a constant weight. Dry weights were calculated and starting samples were weighed so that a sample mass equivalent to 0.5 g (dry weight solids) was achieved. The 0.5 g was split into two-0.25 g fractions. Extracts used for calculating recovery were fortified at 1000 ng g^{-1} in acetonitrile and allowed to sit overnight prior to sample extraction. DDI water (5 mL) was added to one fraction and the pH was adjusted to 6–8 using HCl and NH_4OH (neutral fraction). In the other fraction, 5 mL phosphate buffer (pH 2, 99 mL DDI water, 1.93 g $NaH_2PO_4 \cdot H_2O$, 1 mL 85% H_3PO_4) was added and acidified to pH 2 using HCl (acidic fraction). MeOH : ACN (5 mL, 1 : 1 v/v%) was added to both the acidic and neutral fractions and vortexed for 5 minutes, followed by 30 minutes in a sonication bath. Samples were centrifuged for 5 minutes at 3000 rpm. Supernatants were removed and combined from the two fractions, diluted with water to 200 mL and 200 mg Na·EDTA was added to promote metal chelation. Agilent BondElut Plexa SPE cartridges were preconditioned with 20 mL MeOH followed by two-6 mL volumes of DDI water. Diluted extracts were run over the SPE cartridge under vacuum at 5–10 mL min^{-1}. Prior to drying, the cartridges were washed with 10 mL DDI water, followed by 10 mL 10% MeOH in DDI water. Gravity elution was performed with 12 mL 9 : 1 H_2O : MeOH and evaporated to 2 mL, filtered through a 0.2 μm PTFE syringe filter, evaporated to 0.2 mL and brought up to 1.0 mL with DDI water. Post-spiked samples were fortified prior to the final evaporation step. 400 ng g^{-1} of isotopically labelled internal standards were added to all extracts (Table S1†).

2.4 Analytical

An Agilent 1260 Infinity HPLC pump equipped with a 100 μL sample loop was used for all analyses. Chromatographic separation was performed using an Agilent Zorbax Eclipse Plus C18 column (2.1 × 100 mm, 1.8 μM). DDI water with 0.1% (v/v%) formic acid (A) and ACN with 0.1% formic acid (v/v%) (B) were used as mobile phases for positive electrospray ionization (ESI^+), and DDI water with 1 mM ammonium fluoride (A) and ACN (B) were used for negative electrospray ionization (ESI^-). The initial gradient was held at 2% B for 1.5 min, followed by a linear increase to 100% B at 16.5 min and held for 4 min. A post-run column equilibration time of 3.0 min was implemented resulting in a total sample run time of 23.5 min. An injection volume of 5 μL was used, the mobile phase flowrate was 350 μL min^{-1} and the column temperature was maintained at 30 °C for the duration of the run.

Mass spectra were acquired using an Agilent 6530 quadrupole time-of-flight (QTOF) mass spectrometer. Fragmentation voltage, collision cell voltage, gas temperature and sheath gas temperature were tuned to achieve maximum sensitivity and abundance of surrogate compounds (Table S5†). The Agilent All-Ions, data-independent acquisition mode was used for this analysis where collision cell voltages were repeatedly cycled among 0 eV, 10 eV and, 40 eV over the course of each run. Static electrospray ionization was performed in negative and positive modes in separate instrument runs. LC-QTOF-MS parameters are summarized in the ESI.† Data analysis and processing used MassHunter Quantitative Analysis (B.08), and Qualitative Analysis Workflows (B.08) software (Agilent Technologies).

2.5 Target quantification

Fifty chemicals were identified in the literature as (1) frequently detected in sewage sludge, and (2) suspected to have endocrine activity, or (3) are structurally analogous to known or suspected endocrine active compounds. To assess the range of physicochemical properties encompassed by these 50 compounds,

their Abraham solvation parameters:[52] hydrogen bonding acidity (A), hydrogen bonding basicity (B), polarizability/polarity (S), partitioning coefficient between gas phase and hexadecane (L), McGowan volume (V) and excess molar refraction (E) were used along with their molecular weight (MW) and octanol–water partitioning coefficient (K_{OW}) as descriptors. Abraham parameters were estimated using ACD labs Percepta software (2012, build 2254). log(K_{OW}) and MW were obtained from the PubChem database (https://pubchem.ncbi.nim.nih.gov). The distribution of each physicochemical property of these 50 compounds is presented in the ESI (Tables S2-1–S2-3†). A subset of 12 compounds was selected from the 50 literature compounds to serve as representatives of the minima, second and third quartile values, and maxima of all 50 compounds (Fig. 1).

The 12 surrogates chosen were 2-phenylphenol, 4-*tert*-octylphenol, carbamazepine, estriol, estrone, ethinyl estradiol, metoprolol, miconazole, norgestrel, sulfamethoxazole, triclocarban and trimethoprim. These compounds were used for method development and validation through a series of spike-recovery experiments. Additional validation was performed with diclofenac, efavirenz, flunixin, fluoxetine, fluvoxamine, gemfibrozil, lamotrigine, mefenamic acid, methyl dihydrojasmonate, *N*,*N*-diethyl-*meta*-toluamide (DEET), and triclosan. Spike recoveries of 23 compounds (12 surrogates and 11 compounds used for supplemental validation) were calculated using MassHunter Quantitative Analysis (B.08). Quantifier ions were $[M + H]^+$ for positive mode and $[M - H]^-$ for negative mode with a mass accuracy window of 10 ppm. The two most abundant MS/MS fragments that were identified from library spectra were used as qualifying ions. Spike recovery calculations and quality control used pre-spiked samples (before sample preparation), post-spiked samples (spiked before the final evaporation step) and non-spiked samples (used as a matrix blank) (n = 3). Recoveries ranged from 32–104% and are summarized in Fig. 2. Isotopically labelled compounds were paired with analytes based upon similar matrix factors measured in post-spiked samples (eqn (1)), where a matrix factor (MF) equal to 1 represents negligible signal suppression or enhancement, MF > 1 represents signal enhancement, and MF < 1 signifies signal suppression.

$$\text{Matrix factor} = \frac{\text{area STD } 500\,\frac{\text{ng}}{\text{mL}}}{\text{area STD}_{\text{postspike}}\left(500\,\frac{\text{ng}}{\text{mL}}\right) - \text{area STD}_{\text{nonspike}}} \quad (1)$$

2.6 Suspect screening using All-Ions find by formula workflow

Suspect screening was conducted using MassHunter Qualitative Analysis (B.08) and the Find by Formula search against the Agilent Pesticide PCDL (Personal Compound Database Library) containing 1684 compounds (770 with MS/MS spectra), the Forensic Toxicants PCDL containing 8998 compounds (3497 with MS/MS spectra) and the Water Contaminants PCDL containing 1451 compounds (1083 with MS/MS spectra). Compounds present in these PCDL's contain not only parent compounds, but a variety of metabolic and transformation products as well. The MS/MS spectra for each compound in the PCDLs were catalogued using a similar All-Ions acquisition method (0 eV, 10 eV, 20 eV, and 40 eV collision energies) as was used in this study. Positive identifications required less than a 10 ppm mass error, intensities greater than 1000 counts, confirmation with at least one coeluting fragment ion (with a coelution score > 85%), an overall match score of >70% (weighted score of accurate mass, isotopic spacing and isotopic abundance), abundance greater than five times that in a blank,

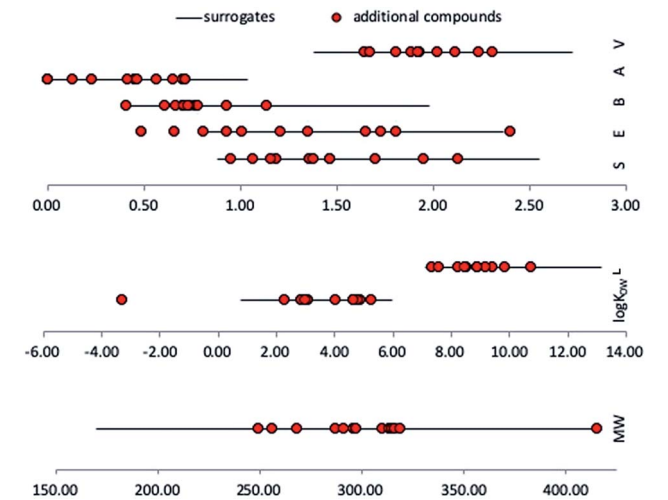

Fig. 1 Distribution of physiochemical properties of surrogate compounds. The black line represents the range of each physiochemical property covered by the 12 surrogate compounds. The red circles represent the value of each parameter represented by the 11 additional compounds used for supplemental validation. MW = molecular weight, log K_{ow} = octanol water partitioning coefficient, (Abraham solvation parameters) L = partitioning coefficient between gas phase and hexadecane, S = polarizability/polarity, E = excess molar fraction, B = hydrogen bonding basicity, A = hydrogen bonding acidity, V = McGowan volume.

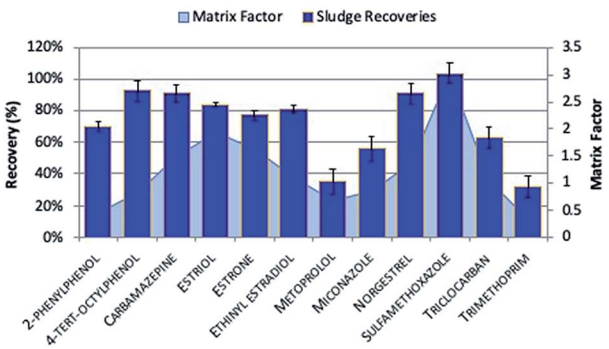

Fig. 2 Spike-recovery and matrix factors of surrogates using the combined extraction workflow with milorganite (n = 4). Matrix factor (MF; see eqn (2)) is represented by the light blue area on the secondary y-axis where a MF = 1 is equivalent to no signal suppression or enhancement, MF < 1 is equivalent to a matrix suppression, and MF > 1 is equivalent to matrix enhancement.

and presence across all technical replicates. Additional Find by Formula parameters used are reported in Table S6.† In positive mode, $[M + H]^+$, $[M + Na]^+$, and $[M + NH_4]^+$ adducts were searched, and in negative mode, $[M - H]^-$, $[M + HCOO]^-$, and $[M + CH3COO]^-$ were searched. Identified compounds complying with all of the mentioned parameters were designated as "qualified". Compounds that were not qualified due to missing MS/MS fragments, high mass error or any of the other identification parameters were unmet, were not investigated further (this included all library entries that did not contain MS/MS spectra). All qualified compounds were manually inspected for peak shape, signal-to-noise ratio, fragment-to-precursor abundance ratio, and the plausibility of found fragment ions. The qualified compounds that complied with all of the parameters mentioned and were subsequently confirmed with manual inspection, were accepted as Level 2a (probable structure by library spectrum match) identifications. Where reference standards were available and retention times and MS/MS fragments were confirmed, identifications were classified as Level 1 (confirmed structure by reference standard). When two or more structural isomers were unable to be deciphered between, identifications were labelled as Level 3 (unequivocal molecular formula).[53]

2.7 QSAR toxicity prediction

To better evaluate the potential biological relevance of the compounds identified in the suspect screen, a toxicity prediction model was used to evaluate the toxicity of each compound on three biological endpoints, the Estrogen Receptor Mediated Effect (ERME, EPA-CERAPP model), Estrogen Receptor Binding Affinity (ERBA, IRFMN model) and, Developmental/Reproductive Toxicity library (Dev/Rep, PG model). The VEGA-QSAR model (version 1.2.4, downloaded from https://www.vegahub.eu), a Quantitative-Structure Activity Relationship model with read-across was used for this study.[54] The advantage of VEGA over other QSAR models is the ability to predict toxicity based on functional groups of structural analogues within the VEGA dataset. SMILES (Simplified Molecular Input Line Entry System) codes were imported into the VEGA-QSAR software and predicted for both estrogen endpoints. The reliability of each prediction is measured in the Applicability Domain Index (ADI) which sums the statistical values, elements of case-based reasoning and, possible presence of active substructures to a score between 0 and 1 (1 being the most reliable score, 0 the least). Results with an ADI value >0.7 were evaluated, meaning the results were based either on experimental data (ADI = 1) or they had moderate to high reliability based on the level of analogous structures present in the database.

3. Results and discussion

3.1 Method development

Solid matrix extraction techniques from the EPA standard method 1694: [the extraction of] Pharmaceuticals and Personal Care Products in Water, Soil, Sediment, and Biosolids by HPLC/MS/MS[55] and method 1698: Steroids and Hormones in Water, Soil, Sediment, and Biosolids by HRGC/HRMS[56] were used as starting points to develop this method capable of extracting pharmaceuticals, personal care products, steroids, hormones, and other ingredients originating from consumer products in a single extraction for analysis by LC-QTOF-MS. Using the twelve surrogate compounds described in Section 2.5, a series of spike-recovery experiments were conducted to optimize starting sample weight, extraction pH, extraction solvents, solvent ratio (mL solvent : g starting material), and solid phase extraction cartridges. All method development, unless otherwise stated, was performed using milorganite as a sample matrix, and later confirmed using California sludge samples. This method aimed at achieving extraction recoveries >30% with minimal matrix effects, an obstacle prevalent in electrospray ionization and complex matrices like sewage sludge that has reportedly high concentrations of humic substances, lipids, bacterial biomass, and lignin residues.[57] Ion suppression or enhancement can be caused by coeluting compounds, undetected matrix components, and "cross-talk" effects and generally varies between compounds, internal standards, and with sample type.[58]

Starting sample masses of 0.25 g and 1.0 g were analyzed for extraction recovery and matrix factors (Table S3-1†). The former was chosen due to its reduction in matrix factors compared to the larger starting weight. ACN : water (1 : 1 v/v) and MeOH : ACN (1 : 1 v/v) were tested as extraction solvents, ultimately the MeOH : ACN resulted in higher extraction recoveries (Table S3-2†). Five methods for adjusting the pH in the extractions were examined: (1) one-0.5 g sample was extracted under acidic conditions at pH 2, (2) one-0.5 g sample was extracted under basic conditions at pH 10, (3) one-0.5 g sample was extracted under neutral conditions at pH 7, (4) two-0.25 g fractions of a sample were extracted alongside each other, one at acidic pH (pH 2) and one at a neutral pH (pH 7) and combined prior to SPE, and (5) one-0.5 g sample was extracted first under neutral conditions (pH 7) then again under acidic conditions (pH 2) and both supernatants were combined prior to SPE (Fig. S3-3, Table S3-4†). Combining the neutral and acidic fractions prior to SPE was chosen due to its recovery efficiency and convenience of injecting only one extract. Agilent Bond Elut ENV (500 mg, 6 cm^3), Agilent Bond Elut Plexa (500 mg, 6 cm^3), and Waters Oasis HLB (500 mg, 6 cm^3), Solid Phase Extraction (SPE) cartridges were analyzed for greatest reduction in matrix factors while maintaining adequate recoveries (Table S3-5†). The BondElut ENV and Oasis HLB performed similarly, but the BondElut Plexa has the most significant reduction in matrix interferences and was chosen for this method. Extraction recoveries and matrix factors from each tested method are reported in the ESI.†

3.2 Method validation

3.2.1 Targeted analysis. To calculate extraction recoveries, eight-0.25 g samples were fortified at 1000 ng g^{-1} (with the 12 surrogates and 11 additional compounds) and allowed to dry overnight prior to sample preparation (pre-spikes, $n = 4$). Post-extraction fortifications were made in a subsequent 4 replicates

(neutral and acidic fractions were combined) before the last evaporation step (post-spikes, $n = 4$). The remaining replicates were left unfortified as a matrix blank (non-spikes, $n = 4$). Absolute recoveries (R) were calculated as follows:

$$R = \frac{C_{PRE} - C_{NON}}{C_{POST} - C_{NON}} \times 100\% \quad (2)$$

where C_{PRE} is the measured concentration of the analyte in the pre-spiked samples, C_{NON} is the measured concentration in the non-spiked samples, and C_{POST} is the measured concentration in the post-spiked extracts. Isotopically labelled standards were spiked in all extracts (400 ng g^{-1}) for internal calibration. This approach to calculating absolute recovery is necessary to correctly account for matrix factors in complex samples. Using more conventional recovery calculations (*e.g.* recovery = C_{PRE}/amount added × 100%) where matrix factors are not incorporated would result in inaccurate evaluations of concentration. Recoveries for the twelve surrogates are shown in Fig. 2. Recoveries were calculated using an 8 point linear calibration curve ranging from 0.25–1000 ng mL^{-1} with $R^2 > 0.99$ for all compounds with 1/× weighting. Recoveries for ten of the twelve surrogates were over 50%, while metoprolol and trimethoprim were recovered at 35% and 32%, respectively. Relative Standard Deviations (RSD) across all replicates were <20% for all compounds. Matrix factors were calculated using eqn (1), and matrix factors below 10 were considered acceptable for this work.

In addition to the 12 surrogates, 11 additional compounds were analyzed to further validate the method for compounds within the physicochemical bounds of this method. Recoveries for each of these compounds were >30% with <20% RSD (Fig. 3).

To further assess the robustness of this method in non-heat treated and pelleted sludges, the method was tested using a sludge sample (Sample #9, 23% solids w/w%) collected from a wastewater treatment plant in California. Absolute recoveries of a spike experiment with the 12 surrogates are shown in Fig. 4 and the 11 additional compounds in Fig. 3. Recoveries for 10 of the 12 surrogates have <30% variation between the CA sludge and the milorganite recoveries reported previously. Sulfamethoxazole and miconazole had >30% variation in recoveries between the two samples. Sulfamethoxazole is an ampholytic compound and slight differences in sample composition could affect its extractability. Salt content and composition have been shown to affect sulfamethoxazole recoveries in previous studies.[59] Sulfamethoxazole is sensitive to photodegradation and no sample preparation actions were taken to protect fortified samples from light exposure, so variations in light exposure between extraction days may also affect extraction recoveries.[60] Additionally, the most probable hypothesis is that the most acidic proton's pK_a is 6.16 for sulfamethoxazole, and 6.77 for miconazole, which are both very close to the desired pH's of the neutral extraction method (pH 6–8). Any slight underestimation of the neutral fraction extraction pH could result in either of these compounds being fully protonated and interacting with negative surface charge on the sludge particles, thus effecting its extractability. This is likely the main source of extraction variability observed and it is hypothesized that more tightly controlling the extraction pH may drastically reduce this variation. Subsequently, the formation of miconazole nitrate salts and their interaction with matrix components is another potential explanation for the observed variation with miconazole. Hörsing *et al.* (2011) reported the inability to obtain a sorption isotherm for miconazole in sewage sludge due to its affinity to sorb to glass surfaces and/or water surfaces, so it is possible that extractability is hindered by sorption onto sample preparation materials.[61] To further examine the appropriateness of this method for these two compounds, we performed a secondary spike-recovery study in two additional California sludges (Samples 10 and 11). For these additional samples, sulfamethoxazole was recovered at 16% and 88%, and miconazole at 24% and 60%. Although there is variation between samples, we believe this method is still capable of extracting

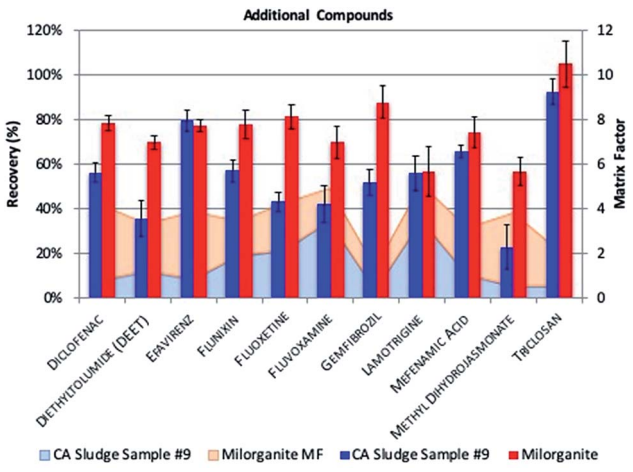

Fig. 3 Absolute recoveries of additional compounds in milorganite (86% w/w%) and a California sewage sludge sample (Sample #9, 23% solids, w/w%). Matrix Factor (MF) is represented by the light red and light blue area on the secondary axis. MF = 1 is equivalent to no signal suppression or enhancement, MF < 1 is equivalent to a matrix suppression, and MF > 1 is equivalent to matrix enhancement.

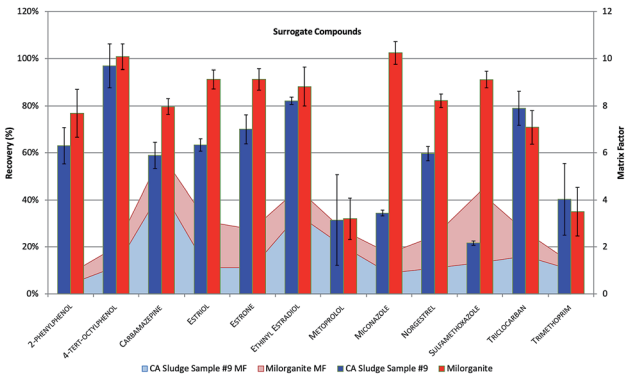

Fig. 4 Absolute recoveries of surrogate compounds in milorganite (86% w/w%) and a California sewage sludge sample (Sample #9, 23% solids, w/w%). Matrix Factor (MF) is represented by the light red and light blue area on the secondary axis. MF = 1 is equivalent to no signal suppression or enhancement, MF < 1 is equivalent to a matrix suppression, and MF > 1 is equivalent to matrix enhancement.

and detecting a broad spectrum of compounds from all sludges for suspect screening and other qualitative analysis but that salt content, the variation of pH's during extraction, and the ability of different sludges to act as better buffering mediums than others likely play a role in these compounds' extractability.

The Limits of Detection (LOD) for the method were determined as the concentrations at which the signal-to-noise ratio was greater than 3 for the quantifying ions. The Limits of Quantification (LOQ) were determined as the concentration at which all transitions had a signal-to-noise ratio greater than 10. The LOQ for all surrogates and additional compounds were between 1-50 ppb with the exception of 2-phenylphenol for which the LOQ was 100 ppb (Table S1†). While the LOQ's for many compounds are within range of similarly reported limits in the literature,[5,62] others reported here are slightly higher due to the multiple transitions we required for qualification, and likely due to the compromised sensitivity related to Data Independent Acquisition methods, like the All-Ions method used here.

3.2.2 Suspect screening using All-Ions workflow. Using the Suspect Screening workflow described in Section 2.6, we positively identified all 23 compounds fortified in pre-spiked sludge samples ($n = 3$) at concentrations over 5 times the concentration in the non-spiked extracts, with mass accuracies <10 ppm, match scores >70%, coelution score >85%, and presence across all technical replicates.

3.3 Suspect screening of fourteen California sewage sludge samples

Fourteen sewage sludge samples were prepared and analyzed using the method described. Starting sample weights were calculated based on percent solids and are reported in the ESI.† Suspect screening was performed using the Find by Formula workflow discussed in Section 2.6. 121 compounds were tentatively identified as Level 2a identifications (78 in ESI^+, 37 in ESI^-, and 6 that were identified in both ESI^+ and ESI^-). Two compounds were found in all sludge samples, bis(2-ethylhexyl) phthalate and tributylphosphate, both of which are abundant in manufacturing and in consumer products. Tributylphosphate is used in industry as a solvent, plasticizer, heat-exchange unit and anti-foaming agent in products like hydraulic fluid, brake fluid, resins, adhesives, pesticides, detergents, and paints.[63] Bis(2-ethylhexyl) phthalate, or DEHP, is the most common phthalate and is mainly used as a plasticizer, but also as an industrial solvent, lubricant, an additive in textiles, pesticide formulations, and personal care products.[64] Three compounds were identified in 13 of the 14 sludges: benzoic acid, a precursor to food preservatives and plasticizers, but also used in some topical ointments; diphenhydramine, an antihistamine; and miconazole, the antifungal component in athletes foot cream and vaginal yeast infection treatments.

Other compounds identified included the following perfluorinated compounds: perfluoro -hexanoic, -heptanoic, -octanoic, -nonanoic, -decanoic acids and perfluorooctanesulfonic acid. Cannabinol, cannabidiol, and delta9-tetrahydrocannabinol, primary compounds found in cannabis, were each found in 6, 6, and 1 sample(s), respectively. In addition, the two main metabolites formed after cannabis consumption, 11-nor-9-carboxy-THC and 11-hydroxy-TCH were found in 2 and 3 samples, respectively. Other recreational drugs identified were etryptamine, a psychoactive stimulant, and methedrone, an ingredient in the street drug "bath salts".[65] Tryptamine was also identified in 9 samples, a compound that by itself closely mimics the amino acid tryptophan, but is a common functional group on psychoactive recreational drugs due to its serotonin-agonistic behavior. Phosphate flame retardants tris(2-butoxyethyl) phosphate, and tri(2-chloroisopropyl)phosphate were detected in 3 and 9 samples, respectively. Antibiotics azithromycin, ciprofloaxacin, doxycycline, flumequine, ofloxacin and sulfapyridine were each found in one or two samples. A variety of hormones including dinoprostone, nandrolone, testosterone isocapronate, testosterone-17-propionate, gestonorone, boldione, hydrocortisone buteprate and progesterone, were found in 9 or fewer samples. Pesticides detected included fludonixil, dichloroprop, fenoprop, fipronil, methoprene, dicamba, difenzoquat, and novaluron.

83 compounds were identified as Level 2a (probable structure by library spectrum match) identifications. In seven instances, an unequivocal structure was unable to be identified among two isomers, classifying these identifications as Level 3, or tentative candidates. For example, the site of hydroxylation between the structures of hormones medrysone and medroxyprogesterone was unable to be determined in the acquired data. Similarly, the hydroxylation site between 3-hydroxyphenylacetic acid and 4-hydroxyphenylacetic acid was unable to be deciphered without reference standards available.

Reference standards for 31 of the 121 level 2a or level 3 identified compounds were available in our lab. Retention times and MS/MS fragments were confirmed for 28 of the 31 compounds, while 3 were rejected (3-hydroxychinolin, flecainide, and tricresylphosphate). Upon confirmation with reference standards, 28 compounds qualified as Level 1 (confirmed structure by reference standard) identifications. Identifications are reported in Table 1 with their respective detection frequencies, the percentage of the samples each compound was positively identified in ((number of detects/total samples) × 100%).

The false positive rate observed through reference standard validation was 9.7%, which is similar to that reported by Moschet *et al.* (2017) who observed a 9% false positive rate after investigating 70 compounds.[66] It was also concluded here that false negatives were generally due to low molecular ion abundances and not because of algorithmic errors. In the case of flecainide ($C_{17}H_{20}F_6N_2O_3$) in this work, the molecular ion abundance was ~10 000 resulting in very low fragment ion abundances. In addition, with this relatively low molecular ion abundance, isotopic information is lost in the noise region, but in this case, the overall match score is not compromised because there are no strong isotopes (such as halogens) expected. Tricresylphosphate returned a match score of 88.73%, a mass error of 1.63 ppm, but was missing the main two fragment ions. The confirming fragment (m/z 243.0569), which ultimately may have been a coeluting ion from a different compound, was expected at only 11% relative abundance to the

Table 1 Compounds identified *via* suspect screening in California sewage sludge. Detection frequencies equate to the percentage of samples each compound was detected in (# detects/total samples × 100%, $n = 14$). Level 1 identifications were confirmed with reference standards, Level 2a identifications are considered "probable structures" and were discovered *via* MS/MS database screening, and Level 3 identifications are "tentative candidates" where a single structure was not able to be determined. For Level 3 ID's, the CAS numbers are provided for the suspected structures. Compounds that have been previously reported in sewage sludge globally are referenced in the far-right column. If evidence was not readily available of a compounds' previous detection in sewage sludge, this field was left blank, suggesting that this study was a compounds' first identification in sewage sludge

Det. freq. (%)	Compound	CAS-ID	Molecular formula	ID level	Ionization mode	Exact mass	RT	Main MS/MS fragment	Previously reported in sewage sludge
100	Bis(2-ethylhexyl) phthalate	117-81-7	$C_{24}H_{38}O_4$	1	ESI^+	390.2766	19.16	279.1591	72 and 73
	Tributylphosphate	126-73-8	$C_{12}H_{27}O_4P$	1	ESI^+	266.1657	13.68	98.9842	73
93	Benzoic acid	65-85-0	$C_7H_6O_2$	2a	ESI^-	121.0293	2.14	77.0397	74
	Miconazole	22916-47-8	$C_{18}H_{14}Cl_4N_2O$	1	ESI^+	413.9855	11.55	158.9745	5,76
	Diphenhydramine	58-73-1	$C_{17}H_{21}NO$	1	ESI^+	255.1624	9.00	167.0855	5, 64, 73 and 79
79	Leucine	328-39-2	$C_6H_{13}NO_2$	1	ESI^+	131.0945	2.08	86.0964	19
	Azelaic acid	123-99-9	$C_9H_{16}O_4$	2a	ESI^-	187.0976	2.13	125.0972	
	Methyl nicotinate	93-60-7	$C_7H_7NO_2$	2a	ESI^+	137.0478	5.58	79.0417	
71	7-Desoxycholic acid	10538-65-5	$C_{24}H_{40}O_4$	1	ESI^-	391.2854	12.10	345.2799	
	8-Hydroxyefavirenz	205754-33-2	$C_{14}H_9ClF_3NO_3$	2a	ESI^-	330.0153	11.87	237.9883	78
	Perfluorooctanoic acid	335-67-1	$C_8HF_{15}O_2$	1	ESI^-	412.9667	9.92	368.9766	76 and 79
	Sertraline	79617-96-2	$C_{17}H_{17}Cl_2N$	2a	ESI^+	305.0742	10.42	275.0389	78
64	Caffeic acid	331-39-5	$C_9H_8O_4$	2a	ESI^-	179.0345	7.09	135.0452	
	Curcumin	458-37-7	$C_{21}H_{20}O_6$	2a	$ESI^{+/-}$	368.1267	11.75	134.0373	
	T-Tryptamine	61-54-1	$C_{10}H_{12}N_2$	2a	ESI^+	160.0982	5.67	115.0542	5
	Tri-(2-chloroisopropyl)phosphate	13674-84-5	$C_9H_{18}Cl_3O_4P$	1	ESI^+	326.0011	11.71	174.9922	80
	Testosterone-17-propionate	57-85-2	$C_{22}H_{32}O_3$	2a	ESI^+	344.2343	11.55	191.1078	
	Inositol nicotinate	6556-11-2	$C_{42}H_{30}N_6O_{12}$	2a	ESI^+	810.1910	9.27	106.0287	
57	Ethyl 4-hydroxybenzoate	120–478	$C_9H_{10}O_3$	2a	ESI^-	165.0558	2.80	93.0346	74
	Phenylalanine	63-91-2	$C_9H_{11}NO_2$	2a	ESI^+	165.0792	4.09	120.0808	81
50	Octodrine	543-82-8	$C_8H_{19}N$	1	ESI^+	129.1521	5.68	57.0699	
	3-Hydroxyphenylacetic acid or 4-hydroxyphenylacetic acid	120-47-8	$C_8H_8O_3$	3	ESI^-	151.0397	4.10	107.0502	
50	Phenylacetic acid	103-82-2	$C_8H_8O_2$	2a	ESI^+	136.0528	8.00	91.0542	
	Perfluorononanoic acid	375-95-1	$C_9HF_{17}O_2$	2a	ESI^-	462.9641	10.54	418.9734	76 and 79
43	Arachidonic acid	506-32-1	$C_{20}H_{32}O_2$	2a	ESI^-	303.2327	16.70	259.2431	
	Ipriflavone	35212-22-7	$C_{18}H_{16}O_3$	2a	ESI^+	280.1102	13.68	106.0287	
	Cannabidiol	13956-29-1	$C_{21}H_{30}O_2$	2a	ESI^-	359.2252	10.02	245.1563	82
	Cannabinol	521-35-7	$C_{21}H_{26}O_2$	2a	ESI^-	309.1877	16.25	279.1391	82
	para-Aminobenzoic acid	150-13-0	$C_7H_7NO_2$	2a	ESI^+	137.0484	6.82	120.0444	83
	Indole-3-acetic acid	87-51-4	$C_{10}H_9NO_2$	2a	ESI^+	175.0631	8.11	130.0651	73
	Fexofenadine	83799-24-0	$C_{32}H_{39}NO_4$	1	ESI^+	501.2905	9.28	466.2741	84 and 85
	Triclosan	3380-34-5	$C_{12}H_7Cl_3O_2$	1	ESI^-	286.9443	14.09	141.9827	73
36	Celecoxib	169590-42-5	$C_{17}H_{14}F_3N_3O_2S$	1	ESI^-	380.0693	12.83	276.0904	78
	Dichloroprop	120-36-5	$C_9H_8Cl_2O_3$	2a	ESI^-	252.9838	13.47	124.9801	
	Ezetimibe	163222-33-1	$C_{24}H_{21}F_2NO_3$	2a	ESI^-	408.1449	12.33	271.1151	78
	Gemfibrozil	25812-30-0	$C_{15}H_{22}O_3$	1	ESI^-	249.1500	13.07	121.0659	5, 64 and 86
	Diethyl phthalate	84-66-2	$C_{12}H_{14}O_4$	1	ESI^+	222.0902	11.43	149.0233	72
	Tryptophan	54-12-6	$C_{11}H_{12}N_2O_2$	2a	ESI^+	204.0902	5.32	188.0706	81
	Thymopentin	69558-55-0	$C_{30}H_{49}N_9O_9$	2a	ESI^+	679.3653	7.23	663.3461	87
	Perfluorodecanoic acid	335-76-2	$C_{10}HF_{19}O_2$	2a	ESI^-	512.9602	11.13	468.9702	76 and 79
	Berberine	2086-83-1	$C_{20}H_{18}NO_4$	2a	ESI^+	336.1209	8.33	320.0917	
	Palmidrol	544-31-0	$C_{18}H_{37}NO_2$	2a	ESI^+	299.2822	11.25	57.0699	88

Table 1 (*Contd.*)

Det. freq. (%)	Compound	CAS-ID	Molecular formula	ID level	Ionization mode	Exact mass	RT	Main MS/MS fragment	Previously reported in sewage sludge
28	Aminorex	2207-50-3	$C_9H_{10}N_2O$	2a	ESI^+	162.0795	7.98	77.0386	
	5-(4-Methylphenyl)-5-Phenylhydantoin	51169-17-6	$C_{16}H_{14}N_2O_2$	2a	ESI^+	266.1072	7.00	77.0386	
	Norharman	244-63-3	$C_{11}H_8N_2$	2a	ESI^+	168.0699	6.28	115.0542	
	Dicamba	1918-00-9	$C_8H_6Cl_2O_3$	2a	ESI^-	218.9626	7.78	174.9723	
	Pentedrone	8797722-57-3	$C_{12}H_{17}NO$	2a	ESI^+	191.1305	10.27	119.0491	
	Dinoprostone	363-24-6	$C_{20}H_{32}O_5$	2a	ESI^-	351.2211	11.05	333.2071	
	Fenofibric acid	42017-89-0	$C_{17}H_{15}ClO_4$	2a	$ESI^{+/-}$	318.0666	12.32	233.0364 (ESI^+) 231.0218 (ESI^-)	89
	Triamterene	396-01-0	$C_{12}H_{11}N_5$	2a	ESI^+	253.1087	0.00	237.0883	90
	Isosteviol	27975-19-5	$C_{20}H_{30}O_3$	2a	ESI^+	318.2225	10.16	301.2162	
	Medrysone or medroxyprogesterone	2668-66-8 or 520-85-4	$C_{22}H_{32}O_3$	3	ESI^+	344.2352	8.76	327.2319	91
	Lappaconitine	32854-75-4	$C_{32}H_{44}N_2O_8$	2a	ESI^+	584.3027	6.62	567.3065	
	Azobenzene	103-33-3	$C_{12}H_{10}N_2$	2a	ESI^+	182.0853	6.58	77.0309	92
	Piperine	94-62-2	$C_{17}H_{19}NO_3$	1	ESI^+	285.1383	11.64	201.0546	
21	2,4-Dihydroxybenzophenone	131-56-6	$C_{13}H_{10}O_3$	1	$ESI^{+/-}$	214.0639	10.85	91.0189	94
	Amphetamine	300-62-9	$C_9H_{13}N$	2a	ESI^+	135.1041	4.45	65.0386	93
	Pramipexole	104632-26-0	$C_{10}H_{17}N_3S$	2a	ESI^+	211.1132	6.58	77.0386	
	Tyramine or phenyl ethanolamine	7568-93-6 or 51-67-2	$C_8H_{11}NO$	3	ESI^+	137.0833	2.29	77.0386	
	Fipronil	120068-37-3	$C_{12}H_4Cl_2F_6N_4OS$	1	ESI^-	434.9319	13.34	329.9595	101
	Adenosine or vidarabin	5536-17-4 or 58-61-7	$C_{10}H_{13}N_5O_4$	3	ESI^+	267.0972	3.08	134.0459	95
	Serotonin	50-67-9	$C_{10}H_{12}N_2O$	2a	ESI^+	176.0925	3.97	160.0757	
	Hydroxychloroquine	118-42-3	$C_{18}H_{26}ClN_3O$	2a	ESI^+	335.1760	5.46	191.0366	
	11-Hydroxy-tetrahydrocannabinol	36557-05-8	$C_{21}H_{30}O_3$	2a	ESI^+	330.2161	9.34	191.1078	82
	Losartan	114798-26-4	$C_{22}H_{23}ClN_6O$	2a	$ESI^{+/-}$	422.1682	9.90	405.1589	78
	Perfluorohexanoic acid	307-24-4	$C_6HF_{11}O_2$	1	ESI^-	312.9746	8.51	268.9830	76
	Tris(2-butoxyethyl) phosphate	78-51-3	$C_{18}H_{39}O_7P$	1	ESI^+	398.2436	14.39	299.1618	92
	2,6-Xylidine or phenethylamine	87-62-7 or 64-04-0	$C_8H_{11}N$	3	ESI^+	121.0881	8.51	105.0699	
14	11-Nor-9-carboxy-tetrahydrocannabinol	56354-06-4	$C_{21}H_{28}O_4$	2a	ESI^-	389.1991	9.71	191.1078	96
	4-Chlorosalicylic acid	5106-98-9	$C_7H_5ClO_3$	2a	ESI^-	170.9872	6.74	126.9956	
	Adenosine	58-61-7	$C_{10}H_{13}N_5O_4$	2a	ESI^-	286.0928	4.75	134.0459	95
	Abietic acid	514-10-3	$C_{20}H_{30}O_2$	2a	ESI^+	302.2272	11.62	93.0699	
	4-Hydroxybenzoic acid	99-96-7	$C_7H_6O_3$	1	ESI^+	138.0323	5.77	95.0491	74
	Testosterone isocapronate	15262-86-9	$C_{25}H_{38}O_3$	2a	ESI^+	386.2802	16.81	109.0648	5
	Diosmetin	520-34-3	$C_{16}H_{12}O_6$	2a	$ESI^{+/-}$	300.0637	9.59	284.0326 (ESI^-) 258.0523 (ESI^+)	
	Fludioxonil	131341-86-1	$C_{12}H_6F_2N_2O_2$	2a	ESI^-	247.0328	11.75	126.0349	
	Methedrone	530-54-1	$C_{11}H_{15}NO_2$	2a	ESI^+	193.1082	6.67	146.0600	
	Sulfapyridine	144-83-2	$C_{11}H_{11}N_3O_2S$	2a	ESI^+	249.0592	6.01	156.0114	97
	Isotretinoin or tretinoin	4759-48-2 or 302-79-4	$C_{20}H_{28}O_2$	3	ESI^+	300.2089	14.38	255.2107	
	Labetalol	36894-69-6	$C_{19}H_{24}N_2O_3$	2a	ESI^+	328.1795	7.71	311.1754	98
	Telmisartan	144701-48-4	$C_{33}H_{30}N_4O_2$	2a	ESI^+	514.2402	9.35	497.2336	85
	Azithromycin	83905-01-5	$C_{38}H_{72}N_2O_{12}$	2a	ESI^+	748.5035	7.13	591.4215	76
	Perfluoroheptanoic acid	375-85-9	$C_7HF_{13}O_2$	1	ESI^-	362.9715	9.29	318.9862	76
	Ofloxacin	82419-36-1	$C_{18}H_{20}FN_3O_4$	2a	ESI^+	361.1466	6.32	261.1033	5, 64, 76 and 86
	Ciprofloxacin	85721-33-1	$C_{17}H_{18}FN_3O_3$	2a	ESI^+	331.1346	6.38	314.1299	5, 64, 76, 86 and 98
	Tyrosine	556-03-6	$C_9H_{11}NO_3$	2a	ESI^+	181.0732	2.09	91.0542	92
	Doxylamine	469-21-6	$C_{17}H_{22}N_2O$	2a	ESI^+	270.1722	5.82	182.0964	
	Lidocaine	137-58-6	$C_{14}H_{22}N_2O$	2a	ESI^+	234.1733	6.36	86.0964	98

Table 1 (*Contd.*)

Det. freq. (%)	Compound	CAS-ID	Molecular formula	ID level	Ionization mode	Exact mass	RT	Main MS/MS fragment	Previously reported in sewage sludge
	Nandrolone	434-22-0	$C_{18}H_{26}O_2$	2a	ESI^+	274.1918	9.17	257.1900	
	Oxybenzone	131-57-7	$C_{14}H_{12}O_3$	2a	ESI^+	228.0779	13.01	105.0335	94
	Valsartan	137862-53-4	$C_{24}H_{29}N_5O_3$	1	$ESI^{+/-}$	435.2283	11.27	91.0553 (ESI^-) 207.0917 (ESI^+)	87
7	2-Hydroxyethyl salicylate	87-28-5	$C_9H_{10}O_4$	2a	ESI^-	201.0568	10.09	93.0346	
	Raloxifene	84449-90-1	$C_{28}H_{27}NO_4S$	2a	ESI^+	473.1676	8.66	77.0386	78
	Alantolactone	546-43-0	$C_{15}H_{20}O_2$	2a	ESI^-	231.1394	13.27	187.1492	
	Bisphenol A	80-05-7	$C_{15}H_{16}O_2$	1	ESI^-	227.1091	10.39	77.0397	73
	Gestonorone	2137-18-0	$C_{20}H_{28}O_3$	2a	ESI^+	316.2042	11.20	109.0648	
	Boldione	897-06-3	$C_{19}H_{24}O_2$	2a	ESI^+	284.1778	10.64	121.0648	99
	Difenzoquat	49866-87-7	$C_{17}H_{17}N_2$	2a	ESI^+	249.1382	8.12	130.0651	
	Fenoprop	93-72-1	$C_9H_7Cl_3O_3$	2a	ESI^-	266.9397	8.07	194.9177	
	Tioconazole	65899-73-2	$C_{16}H_{13}Cl_3N_2OS$	2a	ESI^+	385.9815	10.23	130.9717	100
	Hexachlorophene	70-30-4	$C_{13}H_6Cl_6O_2$	2a	ESI^-	404.8395	12.84	194.9177	76 and 86
	Flumequine	42835-25-6	$C_{14}H_{12}FNO_3$	2a	ESI^+	261.0840	11.70	244.0768	5
	9-Octadecenamide	3322-62-1	$C_{18}H_{35}NO$	1	ESI^+	281.2707	16.97	265.2526	101
	Loperamide	53179-11-6	$C_{29}H_{33}ClN_2O_2$	2a	ESI^+	476.2263	10.13	266.1539	61
	Methoprene	40596-69-8	$C_{19}H_{34}O_3$	2a	ESI^+	310.2492	16.95	279.2319	
	Hydrocortisone buteprate	72590-77-3	$C_{28}H_{40}O_7$	2a	ESI^+	488.2828	10.69	401.2323	
	Doxycycline	564-25-0	$C_{22}H_{24}N_2O_8$	2a	ESI^+	444.1561	7.67	428.1340	98
	Novaluron	116714-46-6	$C_{17}H_9ClF_8N_2O_4$	1	ESI^-	491.0050	14.39	470.9988	
	Metamfepramone	15351-09-4	$C_{11}H_{15}NO$	2a	ESI^+	177.1156	8.52	105.0699	
	Perfluorooctanesulfonic acid	1763-23-1	$C_8HF_{17}O_3S$	1	ESI^-	498.9304	11.31	98.9558	76 and 79
	α-Ethyltryptamine	2235-90-7	$C_{12}H_{16}N_2$	2a	ESI^+	188.1317	5.58	130.0651	
	Phenylacrylic acid	621-82-9	$C_9H_8O_2$	1	ESI^-	147.0452	6.65	103.0553	
	Triclocarban	101-20-2	$C_{13}H_9Cl_3N_2O$	1	ESI^+	313.9782	14.03	161.9867	5 and 76
	Phenylpyruvic acid	156-06-9	$C_9H_8O_3$	2a	ESI^-	163.0402	4.94	91.0553	
	delta9-Tetrahydrocannabinol-2-carboxylic acid	23978-85-0	$C_{22}H_{30}O_4$	2a	ESI^+	358.2153	10.05	341.2111	
	O-Desmethylvenlafaxine	93413-62-8	$C_{16}H_{25}NO_2$	2a	ESI^+	263.1881	6.50	246.1852	102
	Phenpromethamine or phentermine	93-88-9 or 122-09-8	$C_{10}H_{15}N$	3	ESI^+	149.1202	5.84	91.0542	
	Progesterone	57-83-0	$C_{21}H_{30}O_2$	2a	ESI^+	314.2236	13.13	97.0648	5

most abundant fragment (*m*/*z* 91.0542), which was missing. The misidentification of 8-hydroxychinolin is not as straightforward. The match score was 99.29% with a 1.37 ppm mass error and the main fragment was identified. Because of the high match score, it is hypothesized that the assigned molecular formula is correct, but the correct structure is not in the databases used. It is also possible that because the mass of this compound (*m*/*z* 143.0602) is relatively small, and the molecular formula contains little uniqueness (C_9H_7NO), it is possible this ion and its confirming fragment are fragmentation products of larger $C_xH_xN_xO_x$-containing compounds. Overall, the efficiency and false detection rate reported here are low considering the high occurrence of coeluting ions in All Ions acquisition.

Slightly more than half of the compounds identified in the suspect screen are consistent with identifications made *via* targeted analysis in the literature, which are referenced to in Table 1. To the best of our knowledge, 51 of the 118 compounds identified here have not been detected in sewage sludge previously. Of these compounds, octodrine, a dietary supplement advertised to "burn fat" and "increase weight loss", and novaluron, an insect growth regulator, were confirmed with reference standards.

3.4 QSAR toxicity prediction of suspect identifications

All compounds identified in the suspect screen were analysed in the VEGA-QSAR toxicity prediction model, regardless of the level of identification. Estrogen Receptor Binding Affinity (ERBA), Estrogen Receptor-Mediated Effect (ERME), and Developmental/Reproductive Toxicity (DRT) were predicted.

Bisphenol A was the only compound to return positive results on all three toxicity endpoints, but was only detected in one sample. Other plasticizers bis(2-ethylhexyl)phthalate (DEHP) and diethyl phthalate (DEP) were predicted to be developmental/reproductive toxins and were detected in 100% and 36% of samples, respectively. Other developmental/reproductive toxins identified in this prediction include the pesticides, dichloroprop

Table 2 Compounds identified in California sewage sludge samples (detection frequencies equate to the percentage of samples each compound was detected in (# detects/total samples × 100%, $n = 14$)) that are predicted to be developmental/reproductive toxins or interact with estrogen receptors (VEGA-QSAR, v.1.2.4)

Compound	Developmental/reproductive toxicity library (PG) (version 1.0.0)	Estrogen receptor relative binding affinity model (IRFMN) (version 1.0.1)	Estrogen receptor-mediated effect (IRFMN/CERAPP) (version 1.0.0)	Detection frequency (%)
Bis(2-ethylhexyl) phthalate	•	•		100
Diphenhydramine	•			93
Ethyl 4-hydroxybenzoate		•	•	57
Ipriflavone			•	43
Cannabinol		•		43
Cannabidiol		•		43
Dichloroprop	•			43
DEP/diethyl phthalate	•			43
Ezetimibe			•	43
Dinoprostone	•			29
Fenofibric acid			•	29
Adenosine	•			21
Amphetamine	•			21
Benzophenone-1		•	•	21
Adenosine	•			14
Ciprofloxacin	•			14
Diosmetin			•	14
BPA/bisphenol a	•	•	•	7
Fenoprop	•			7
Flumequine	•			7
Gestonorone	•			7
Hydrocortisone buteprate			•	7

and fenoprop, and pharmaceuticals adenosine, amphetamine, ciprofloxacin, dinoprostone, diphenhydramine, flumequine and gestonorone. Benzophenone-1, a UV filter used in personal care products, and ethyl 4-hydroxybenzoate (ethyl paraben), an anti-fungal preservative added to packaged food products, were predicted to be active on both estrogen toxicity pathways. Diosmetin, ezetimibe, hydrocortisone buteprate, ipriflavone, cannabinol, cannabidiol and, fenofibric acid were predicted to exhibit activity on the estrogen mediated response element pathway. A summary of predicted developmental/reproductive toxicity and/or ER interactions results for these compounds can be found in Table 2.

The activity of Bisphenol A (BPA) on all toxicity endpoints examined here came with little surprise given the publicity and ultimate Food and Drug Administration ban of the substance in baby bottles, sippy cups, and infant formula packaging in 2012. The spotlight on BPA resulted in the examination of many BPA-replacements and led to the discovery of similar toxic effects.[67] Similarly, other plasticisers have been under regulatory scrutiny for toxic effects on development, and ultimately bis(2-ethylhexyl) phthalate (DEHP) has been banned by the European Union for use in all infant products, a process that began in 2000, and was effective in the United States as of 2009.[68] The safety of diethyl phthalate has also been investigated, but no federal bans have been implemented. Other phenolic compounds like benzophenone-1 and ethyl paraben have been investigated for placental transmission and developmental delays in infants.[69,70]

Many of the compounds identified as estrogenic or reproductive toxins are pharmaceuticals, whose presence in these samples are not as heavily scrutinized due to their importance to modern medicine. Compounds that were predicted to be estrogenic, or reproductive toxins like plasticizers (BPA and DEHP), preservatives (ethyl paraben), and pesticides (dichloroprop) are suggested for further evaluation regarding the appropriateness of their use in consumer products. If less persistent and/or non-toxic alternatives are available, it is possible that limiting their use may lessen the adverse anthropogenic load on terrestrial organisms.

Six of the 22 compounds identified as estrogen active or reproductive/developmental toxins have not previously been detected in sewage sludge to the best of our knowledge. Dichloroprop, a common ingredient in weed killers, was detected for the first time in 6 sludge samples and predicted to be a reproductive/developmental toxin. Synthetic hormones including hydrocortisone buteprate, used for topical treatment of dermatitis, gestonorone, used for the treatment of enlarged prostates, endometrial, and breast cancers, and dinoprostone, a naturally occurring and synthetic hormone used to aid in cervical dilation in pregnant women, were detected and predicted to be active on one of the three toxicity pathways investigated. Diosmetin, a naturally occurring antioxidant and antineoplastic, and ipraflavone, a pharmaceutical used to inhibit bone restoration and prevent osteoporosis, were also identified in sewage sludge for the first time and predicted to interact with Estrogen Receptor Mediated Effects.

4. Conclusions

The 22 compounds identified in this study that were predicted to interact with estrogen and reproductive pathways are cases of chemicals in consumer products that should be of exceptional

concern to both consumers and regulators alike. While pharmaceuticals, hormones, and other medications are somewhat unavoidable with the sophisticated medical system in California, alternatives for DEHP-, and BPA-containing plastics, cosmetics containing benzophenone-1, pesticides, and preservatives are generally available to consumers. Concerns about these compounds are quite appropriate from an environmental health perspective, but in addition, very little is known about chronic exposure of these chemicals on human health. This study focused on reproductive and developmental pathways, but many other homeostatic processes are controlled by hormone-receptor mechanisms. Better understanding the toxicity mechanisms for compounds that persist through wastewater treatment requires a thorough environmental approach for identifying consumer-product chemicals of high concern. In this work, we identified 51 compounds that to the best of our knowledge, have not previously been detected in sewage sludge. Six of these compounds are predicted to interact with the ER, exhibit estrogenic activity and/or predicted to be reproductive/developmental toxins, five of which originate from anthropogenic sources, thus making their evaluation in consumer products paramount. The analytical and sample preparation method developed in this work allows for future analysis investigating additional compounds and their transformation products using non-targeted chemical analysis in conjunction with effects-directed analysis to gain a more comprehensive understanding of endocrine active consumer-product chemicals that persist beyond their intended use in consumerism and enter the environment upon ultimate disposal.

Conflicts of interest

The authors declare no conflicts of interest.

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