### Title

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### Journal

Environmental Science Processes & Impacts, 21(7)

### ISSN

2050-7887



### Authors

Black, Gabrielle P
Anumol, Tarun
Young, Thomas M



### Publication Date

2019-07-17

### DOI

10.1039/c9em00144a

Peer reviewed

HHS Public Access

Author manuscript

*Environ Sci Process Impacts*. Author manuscript; available in PMC 2020 July 17.

Published in final edited form as:

*Environ Sci Process Impacts*. 2019 July 17; 21(7): 1099–1114. doi:10.1039/c9em00144a.

# Analyzing a broader spectrum of endocrine active organic contaminants in sewage sludge with High Resolution LC-QTOF-MS suspect screening and QSAR toxicity prediction

**Gabrielle P. Black**[a], **Tarun Anumol**[b], **Thomas M. Young**[a]

[a.]Civil & Environmental Engineering and Agricultural and Environmental Chemistry Graduate Group, University of California Davis

[b.]Agilent Technologies, Inc.

## Abstract

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 endocrine receptor. The physicochemical properties of known organic EACs vary greatly and typically require targeted analytical platforms and workflows for accurate identification. 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 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 on twelve carefully selected surrogates to assess different extraction solvents, sample weights, extraction pH values, procedures for combining extracts with different extraction pH, 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.

## 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.

Conflicts of interest

The authors declare no conflicts of interest.

Electronic Supplementary Information (ESI) available: See DOI: 10.1039/x0xx00000x

[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 effects [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 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 using 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; compounds able to interact with endocrine systems where adverse health effects are not yet confirmed, as is the case with EDCs). Contaminants in their original form, or products of metabolism or other forms of transformation, originate from pharmaceuticals, personal care products, pesticides, cleaning materials, manufacturing byproducts, roadside runoff, and other sources. Many of these contaminants persist beyond their intended use, are transported to and treated within a wastewater treatment facility, with ultimate disposal to 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, in addition to any transformation products that may have formed after their intended use. 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[18–20], estrone[21,22], norgestrel[23]; sulfonamide antibiotics like sulfamethoxazole, sulfamethazine, and sulfathiazole[24]; polychlorinated biphenyls[25,26], polyaromatic hydrocarbons[5,6,27], and phthalates[28] have been ubiquitously detected[29] at ng/g to ug/g ranges. These contaminants, among others, are suspected to be endocrine active with possible endocrine disruptive characteristics[30]. 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, [31–36] 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.[8,18,37–41] Solvents, pH's, SPE cartridges, SPE elution buffers and evaporation techniques vary widely among these methods but in each case they 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.

The majority of trace contaminant analysis 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, [42–46] 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. This work presents both a sample preparation and analytical method capable of identifying a wide range of compounds within a physicochemical space created by carefully selected surrogate compounds for which this method was optimized. The utility and significance of the method is demonstrated by conducting suspect screening of fourteen California sewage sludge samples. 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

### 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 (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, ammonium hydroxide and sodium phosphate were purchased from Fisher Scientific (Hampton, NH). Double-deionized (DDI, 18.2MOhm*cm) low TOC (<50ppb) water was produced by a Milli-Q® Integral 5 Water Purification System. Solid Phase Extraction cartridges were purchased from Waters (Oasis HLB, 60 um particle size, 500 mg, 6 cc, Milford, MA) and Agilent Technologies (Bond Elut Plexa (45 um particle size, 500 mg, 6 cc, Santa Clara, CA), Bond Elut ENV (45 um particle size, 500 mg, 6 cc, Santa Clara, CA)). PTFE syringe filters were purchased from Agilent Technologies (Captiva, 15mm diameter, 0.2um 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. The plants represent various geographical locations and feature diverse influent characteristics and treatment processes. 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 for each sample can be found in Table S1.

### 2.3 Sample Preparation

Prior to extraction, the dry weight is 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 1,000 ng $g^{-1}$. 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 an sonication bath. Samples were centrifuged for 5 minutes at 3,000 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 water. Gravity elution was performed with 12 mL 5% MTBE in 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 labeled internal standards were added to all extracts (SI-1).

### 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. 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. Isotopically labelled compounds were paired with analytes based upon similar matrix interference factors. LC-QTOF-MS parameters are summarized in the Supporting Information. 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, (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:[47] 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) and log($K_{OW}$) and MW were obtained from the PubChem database (pubchem.ncbi.nim.nih.gov). The distribution of each physicochemical property of these 50 compounds is presented in the Supporting Information (S2). 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 (Figure 1).

The 12 surrogates chosen were 2-phenylphenol, 4-tertoctylphenol, 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 (after sample preparation, before injection) and non-spiked samples (used as a matrix blank) (n=3). Recoveries ranged from 32-104% and are summarized in Figure 2.

### 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 1,684 compounds (770 with MS/MS spectra), the Forensic Toxicants PCDL containing 8,998 compounds (3,497 with MS/MS spectra) and the Water Contaminants PCDL containing 1,451 compounds (1,083 with MS/MS spectra). 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 1,000 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, and presence across all technical replicates. In positive mode, [M+H]+, [M+Na]+, and [M+NH4]+ 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. 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.[48] Where reference standards were available anc 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).

## 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[49] and method 1698: Steroids and Hormones in Water, Soil, Sediment, and Biosolids by HRGC/HRMS[50] 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-ESI-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.

Starting sample masses of 0.25 g and 1.0 g were analyzed for extraction recovery and matrix effects. The former was chosen due to its reduction in matrix effects 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. 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. 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 (500mg, 6cc), Agilent Bond Elut Plexa (500mg, 6cc), and Waters Oasis HLB (500mg, 6cc), Solid Phase Extraction (SPE) cartridges were analysed for greatest reduction in matrix interferences while maintaining adequate recoveries. 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 effects from each tested method are reported in the Supporting Information.

## 3.2 Method validation

**3.2.1 Targeted Analysis—**To calculate extraction recoveries, eight-0.25 g samples were fortified at 2,000 ng/g (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) directly prior to LC-QTOF-MS analysis (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:

$$\mathrm{R} = \frac{C_{PRE} - C_{NON}}{C_{POST} - C_{NON}} \times 100 \qquad \text{Equation 1:}$$

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 samples. Isotopically labelled standards were spiked in all samples (200 ng $mL^{-1}$) for internal calibration. Recoveries for the twelve surrogates are shown in Figure 2. Recoveries were calculated using an 8 point linear calibration curve ranging from 0.25 – 1,000 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 effects were calculated using Equation 2.

$$Matrix\ Factor = \frac{Area\ STD\ 500\frac{ng}{mL}}{Area\ Postspike\left(500\frac{ng}{mL}\right) - Area\ Nonspike} \qquad \text{Equation 2:}$$

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 >50% with <20% RSD (Figure 3).

To further assess the robustness of this method in non-heat treated and pelleted sludges, the method was tested using a sludge sample (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 Figure 4 and the 11 additional compounds in Figure 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, salt concentration and salt composition have been shown to affect sulfamethoxazole recoveries in previous studies.[51] The starting salt content of each sludge sample was not determined and could be a contributing factor in this variation between samples. The variation in miconazole recoveries may be due to the close proximity between the compound's pKa (6.77) and the pH of the neutral extraction (pH 6-8). Slight underestimation of the extraction pH could result in miconazole being fully protonated and interacting with negative surface charge on the sludge particles thus hindering its extractability. The formation of miconazole nitrate salts and their interaction with matrix components is another potential explanation for the observed variation. To further examine the appropriateness of this method for the compounds that had >30% variability between extraction recoveries, we performed a secondary spike-recovery study in two additional California sludges. 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 a broad spectrum of compounds from all sludges for suspect screening and other qualitative analysis and believe that salt content variation between sludge samples plays 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.

#### 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 in the three 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 extraction method described. Starting sample weights were calculated based on percent solids and are reported

in the Supporting Information. Suspect screening was performed using the *Find by Formula* workflow discussed in Section 2.6. 118 compounds were tentatively identified as Level 2a identifications (80 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[52]. 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, in pesticide formulation and in personal care products[53]. 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, a common ingredient in the street drug “bath salts”. 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-propinoate, gestonorone, boldione, hydrocortisone buteprate and progesterone, were found in 9 or fewer samples. The pesticides detected were fludonixil, dichloroprop, fenoprop, fipronil, methoprene, dicamba, difenzoquat, and novaluron. A complete list of the 83 compounds determined to be Level 2a (probable structure by library spectrum match) identifications are reported in Table 1.

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 medroxyprogestrone was unable to be determined in the acquired data. Similarly, the hydroxylation site between 3-hydroxyphenylacetic aicd and 4-hydroxyphenylacetic acid was unable to be deciphered without reference standards available. Table 2 summarizes the list of Level 3 identified compounds.

Reference standards for 31 of the 118 Level 2 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. Upon confirmation with reference standards, these 28

compounds qualified as Level 1 (confirmed structure by reference standard) identifications (Table 3).

The false positive rate observed through reference standard validation was 9.7%, similar to that reported by Moschet et. al. (2017) who observed a 9% false positive rate after investigating 70 compounds.[54] It was also concluded here that false negatives were generally due to low molecular ion abundances and not because of algorithmic errors. This efficiency and false detection rate is low considering the high occurrence of coeluting ions in *All Ions* acquisition.

### 3.4 Toxicity prediction of suspect identifications

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 www.vegahub.eu), a Qualitative-Structural Activity Relationship model with read-across was used for this study.[55] 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. 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.

Compounds were ranked by assigning a value of 1 to each endpoint that returned a toxic result (maximum of 3). Bisphenol A, a plasticizer, was detected in one sample and was the only compound with positive results on each biological endpoint. Other plasticizers bis(2-ethylhexyl)phthalate (DEHP) and diethyl phthalate (DEP) were also predicted to be developmental/reproductive toxins. Other toxins positively identified on this pathway include the pesticides, dichloroprop and fenoprop and, the pharmaceuticals adenosine, amphetamine, ciprofloxacin, dinoprostone, diphenhydramine, flumequine and gestonorone. Benzophenone-1, a UV filter used in personal care products, and ethyl 4-hydroxybenzoate, an antifungal preservative in packaged food, returned active results from both of the 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 toxicity prediction results can be found in Table 2.

## 4. Conclusions

The 22 widespread, persistent compounds identified in this study that were predicted to interfere 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 unavoidable with the sophisticated medical system in California, alternatives for DEHP-, and BPA-containing

plastics, cosmetics containing benzophenone-1, pesticides, and preservatives are widely available to consumers. These concerns are 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 binding mechanisms, so while only 22 compounds were highlighted in this work, the remaining 96 compounds identified in the suspect screen could exhibit toxicity on other endocrine endpoints. Better understanding the toxicity mechanisms for compounds that persist through wastewater treatment is a thorough environmental approach for identifying consumer-product chemicals of high concern. Future work is needed to investigate additional compounds and transformation products that have estrogenic characteristics 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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

Research reported in this publication was supported by the UC Davis Superfund Research Center, National Institutes of Health, NIEHS award (P42ES004699), the National Science Foundation Graduate Research Fellowship Program, the Jena and Michael King Foundation, John Wick and Peggy Rathmann. The authors would like to thank Agilent Technologies for instrumental support and assistance in sample preparation, instrument operation, and analytical workflows. We would also like to thank the participating wastewater treatment plants for their cooperation in providing sewage sludge samples, as well as Dr. Christoph Moschet for his insight and assistance in experimental design and analytical methods and Dr. Peter Green for his analytical assistance.

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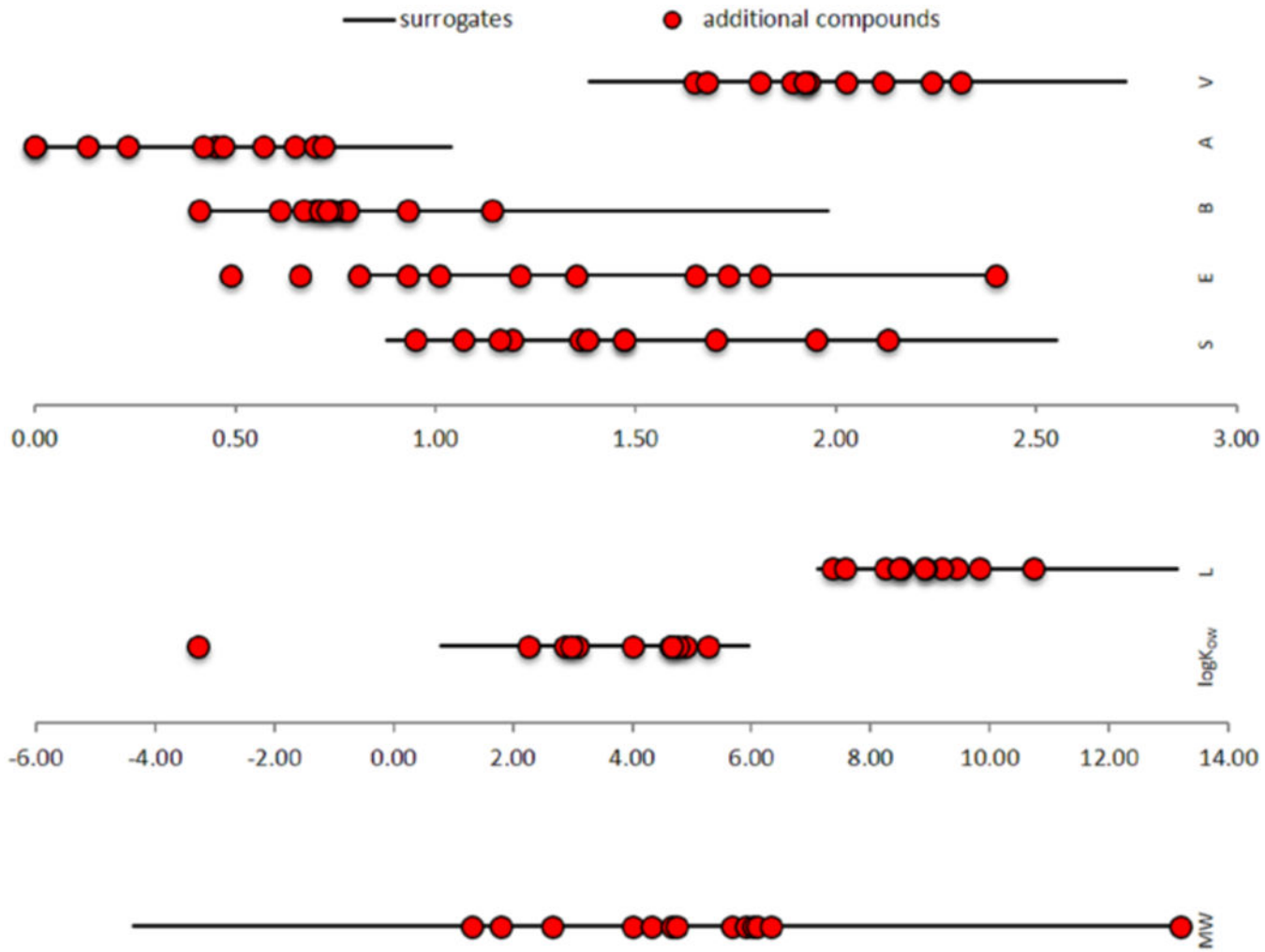


**Figure 1.**

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, logKow= 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.

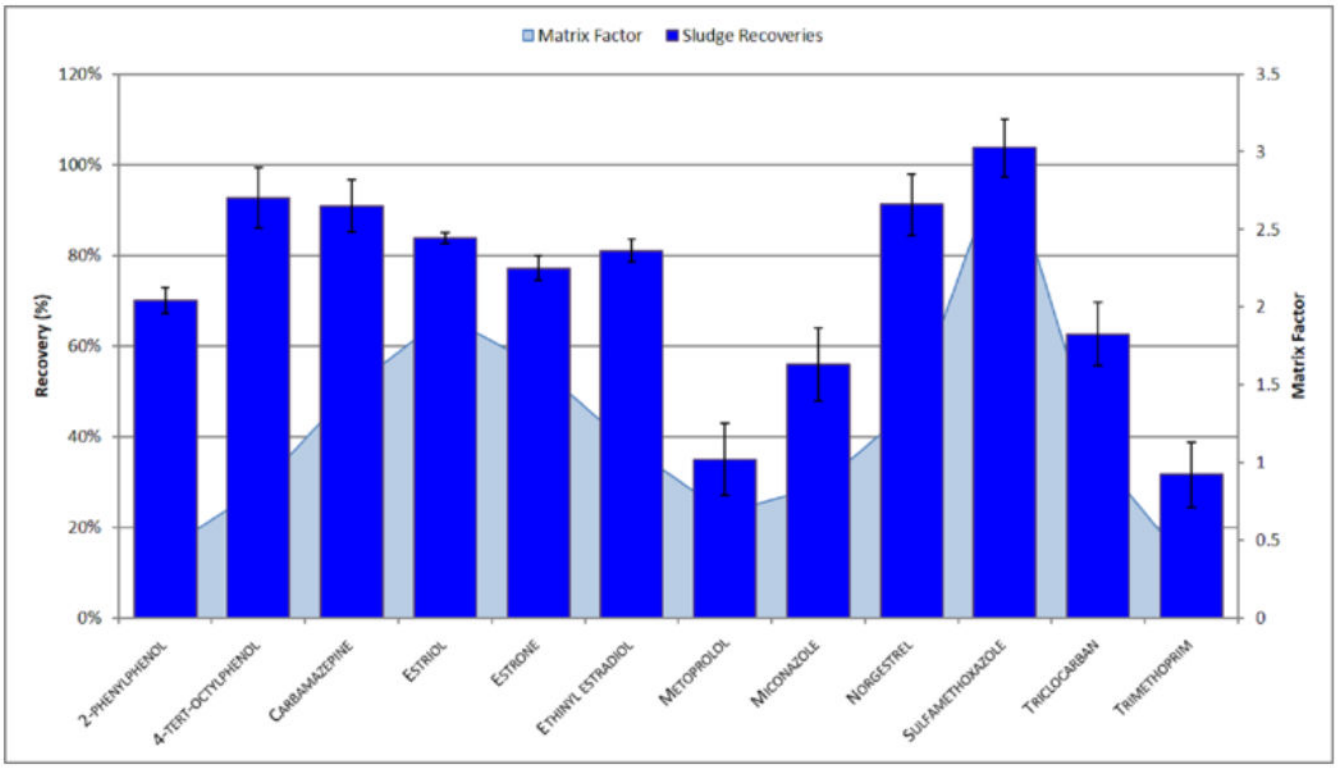


**Figure 2.**

Spike-recovery and Matrix Factors of surrogates using the combined extraction workflow (n=4). Matrix Factor= area of standard (500 ng/g)/(area of matrix spiked (500 ng/g) − area of unspiked matrix)

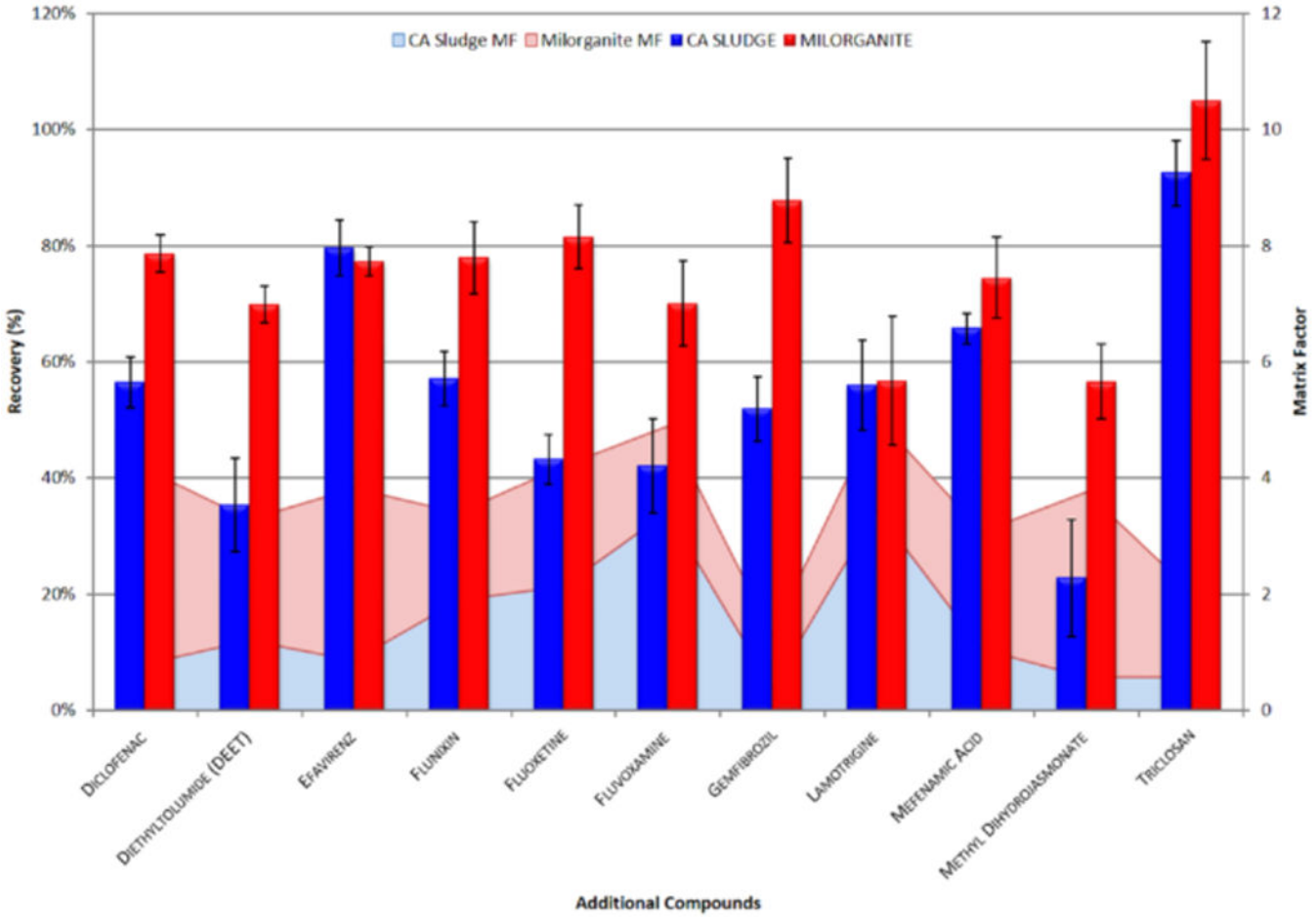


**Figure 3.**

Recoveries of additional compounds in milorganite (86% solids, w/w%) and a sewage sludge sample collected in Northern California (23% solids, w/w%)

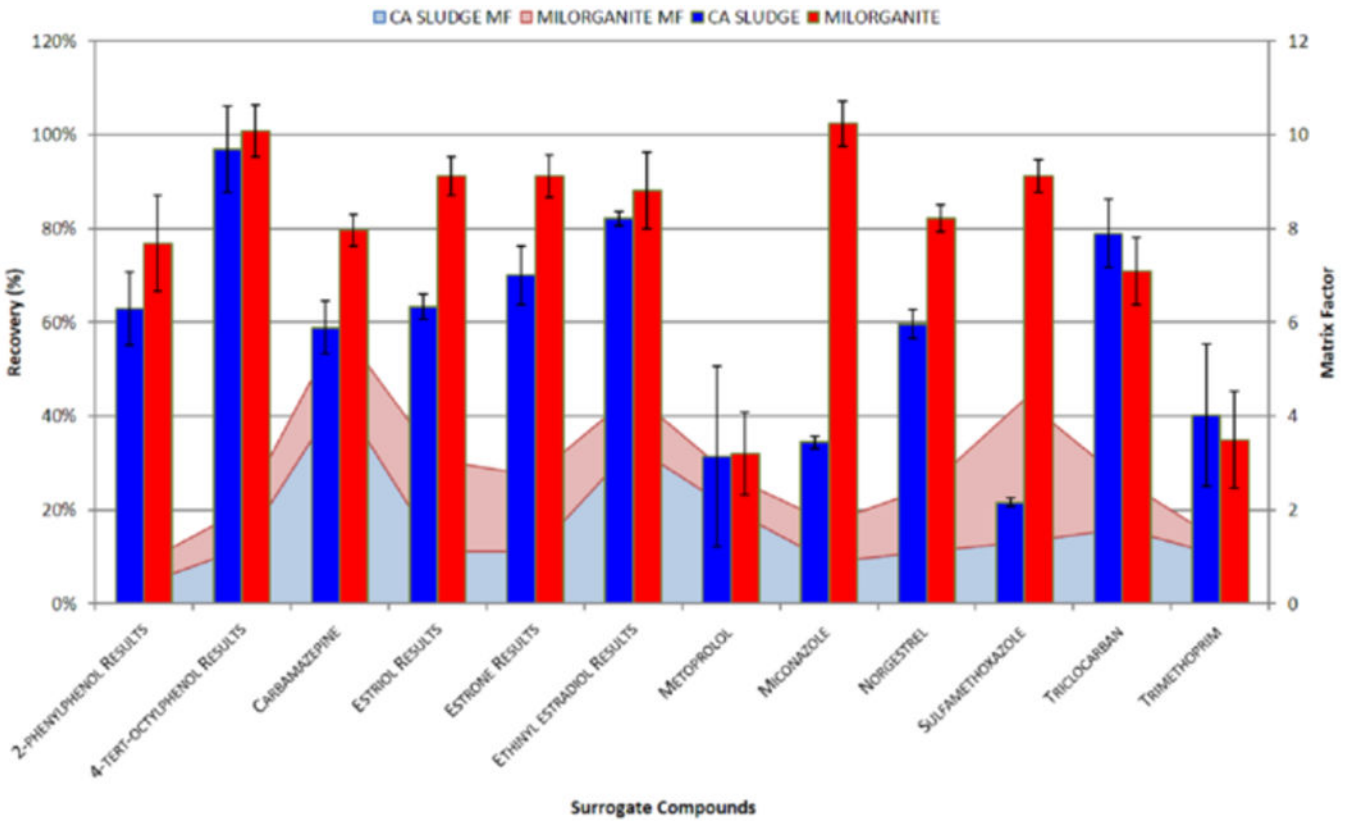


**Figure 4.**

Recoveries of surrogate compounds in milorganite (86% solids, w/w%) and a sewage sludge sample collected in Northern California (23% solids, w/w%)

**Table 1**

Level 2b suspect screening identifications

| Detection Frequency | Compound | CAS-ID | Molecular Formula |
|---|---|---|---|
| 13 | Benzoic acid | 65-85-0 | C7 H6 O2 |
| 11 | Azelaic acid | 123-99-9 | C9 H16 O4 |
| | Methyl nicotinate | 93-60-7 | C7 H7 N O2 |
| 10 | 8-Hydroxyefavirenz | 205754-33-2 | C14 H9 Cl F3 N O3 |
| | Sertraline | 79617-96-2 | C17 H17 Cl2 N |
| 9 | Caffeic acid | 331-39-5 | C9 H8 O4 |
| | Curcumin | 458-37-7 | C21 H20 O6 |
| | Inositol nicotinate | 6556-11-2 | C42 H30 N6 O12 |
| | T-Tryptamine | 61-54-1 | C10 H12 N2 |
| | Testosterone-17-propionate | 57-85-2 | C22 H32 O3 |
| 6 | Ethyl 4-hydroxybenzoate | 120-478 | C9 H10 O3 |
| | Phenylalanine | 63-91-2 | C9 H11 N O2 |
| 7 | Perfluorononanoic acid | 375-95-1 | C9 H F17 O2 |
| | Phenylacetic acid | 103-82-2 | C8 H8 O2 |
| 6 | Arachidonic acid | 506-32-1 | C20 H32 O2 |
| | Cannabidiol | 13956-29-1 | C21 H30 O2 |
| | Cannabinol | 521-35-7 | C21 H26 O2 |
| | Indole-3-acetic acid | 87-51-4 | C10 H9 N O2 |
| | Ipriflavone | 35212-22-7 | C18 H16 O3 |
| | Para-aminobenzoic acid | 150-13-0 | C7 H7 N O2 |
| 5 | Berberine | 2086-83-1 | C20 H18 N O4 |
| | Dichloroprop | 120-36-5 | C9 H8 Cl2 O3 |
| | Ezetimibe | 163222-33-1 | C24 H21 F2 N O3 |
| | Palmidrol | 544-31-0 | C18 H37 N O2 |
| | Perfluorodecanoic acid | 335-76-2 | C10 H F19 O2 |

| Detection Frequency | Compound | CAS-ID | Molecular Formula |
|---|---|---|---|
| | Thymopentin | 69558-55-0 | C30 H49 N9 O9 |
| | Tryptophan | 54-12-6 | C11 H12 N2 O2 |
| 4 | 5-(4-methylphenyl)-5-Phenylhydantoin | 51169-17-6 | C16 H14 N2 O2 |
| | Aminorex | 2207-50-3 | C9 H10 N2 O |
| | Azobenzene | 103-33-3 | C12 H10 N2 |
| | Dicamba | 1918-00-9 | C8 H6 Cl2 O3 |
| | Dinoprostone | 363-24-6 | C20 H32 O5 |
| | Fenofibric acid | 42017-89-0 | C17 H15 Cl O4 |
| | Isosteviol | 27975-19-5 | C20 H30 O3 |
| | Lappaconitine | 32854-75-4 | C32 H44 N2 O8 |
| | Norharman | 244-63-3 | C11 H8 N2 |
| | Pentedrone | 8797722-57-3 | C12 H17 N O |
| | Triamterene | 396-01-0 | C12 H11 N5 |
| 3 | 11-Hydroxy-tetrahydrocannabinol | 36557-05-8 | C21 H30 O3 |
| | Amphetamine | 300-62-9 | C9 H13 N |
| | Hydroxychloroquine | 118-42-3 | C18 H26 Cl N3 O |
| | Losartan | 114798-26-4 | C22 H23 Cl N6 O |
| | Pramipexole | 104632-26-0 | C10 H17 N3 S |
| | Serotonin | 50-67-9 | C10 H12 N2 O |
| 2 | 11-Nor-9-Carboxy-tetrahydrocannabinol | 56354-06-4 | C21 H28 O4 |
| | 4-Chlorosalicylic acid | 5106-98-9 | C7 H5 Cl O3 |
| | Abietic acid | 514-10-3 | C20 H30 O2 |
| | Adenosine | 58-61-7 | C10 H13 N5 O4 |
| | Azithromycin | 83905-01-5 | C38 H72 N2 O12 |
| | Ciprofloxacin | 85721-33-1 | C17 H18 F N3 O3 |
| | Diosmetin | 520-34-3 | C16 H12 O6 |
| | Doxylamine | 469-21-6 | C17 H22 N2 O |
| | Fludioxonil | 131341-86-1 | C12 H6 F2 N2 O2 |
| | Labetalol | 36894-69-6 | C19 H24 N2 O3 |
| | Lidocaine | 137-58-6 | C14 H22 N2 O |

| Detection Frequency | Compound | CAS-ID | Molecular Formula |
|---|---|---|---|
| | Methedrone | 530-54-1 | C11 H15 N O2 |
| | Nandrolone | 434-22-0 | C18 H26 O2 |
| | Ofloxacin | 82419-36-1 | C18 H20 F N3 O4 |
| | Oxybenzone | 131-57-7 | C14 H12 O3 |
| | Sulfapyridine | 144-83-2 | C11 H11 N3 O2 S |
| | Telmisartan | 144701-48-4 | C33 H30 N4 O2 |
| | Testosterone isocapronate | 15262-86-9 | C25 H38 O3 |
| | Tyrosine | 556-03-6 | C9 H11 N O3 |
| 1 | 2-Hydroxyethyl salicylate | 87-28-5 | C9 H10 O4 |
| | Alantolactone | 546-43-0 | C15 H20 O2 |
| | Boldione | 897-06-3 | C19 H24 O2 |
| | delta9-Tetrahydrocannabinol-2-carboxylic acid | 23978-85-0 | C22 H30 O4 |
| | Difenzoquat | 49866-87-7 | C17 H17 N2 |
| | Doxycycline | 564-25-0 | C22 H24 N2 O8 |
| | Fenoprop | 93-72-1 | C9 H7 Cl3 O3 |
| | Flumequine | 42835-25-6 | C14 H12 F N O3 |
| | Gestonorone | 2137-18-0 | C20 H28 O3 |
| | Hexachlorophene | 70-30-4 | C13 H6 Cl6 O2 |
| | Hydrocortisone buteprate | 72590-77-3 | C28 H40 O7 |
| | Loperamide | 53179-11-6 | C29 H33 Cl N2 O2 |
| | Metamfepramone | 15351-09-4 | C11 H15 N O |
| | Methoprene | 40596-69-8 | C19 H34 O3 |
| | O-Desmethylvenlafaxine | 93413-62-8 | C16 H25 N O2 |
| | Phenylpyruvic acid | 156-06-9 | C9 H8 O3 |
| | Progesterone | 57-83-0 | C21 H30 O2 |
| | Raloxifene | 84449-90-1 | C28 H27 N O4 S |
| | Tioconazole | 65899-73-2 | C16 H13 Cl3 N2 O S |
| | α-Ethyltryptamine | 2235-90-7 | C12 H16 N2 |

**Table 2**

Level 3 suspect screening identifications

| Detection Frequency | Compound | CAS-ID | Molecular Formula |
|---|---|---|---|
| 7 | 3-hydroxyphenylacetic acid or 4-hydroxyphenylacetic acid | 120-47-8 | C8 H8 O3 |
| 4 | Medrysone or Medroxyprogesterone | 2668-66-8, 520-85-4 | C22 H32 O3 |
| 3 | 2,6-Xylidine or Phenethylamine | 87-62-7, 64-04-0 | C8 H11 N |
| | Adenosine or Vidarabin | 5536-17-4, 58-61-7 | C10 H13 N5 O4 |
| | Tyramine or Phenyl ethanolamine | 7568-93-6, 51-67-2 | C8 H11 N O |
| 2 | Isotretinoin or Tretinoin | 4759-48-2, 302-79-4 | C20 H28 O2 |
| 1 | Phenpromethamine or Phentermine | 93-88-9, 122-09-8 | C10 H15 N |

**Table 3**

Level 1 suspect screening identifications

| Detection Frequency | Compound | CAS-ID | Molecular Formula |
|---|---|---|---|
| 14 | Bis(2-ethylhexyl) phthalate | 117-81-7 | C24 H38 O4 |
| | Tributylphosphate | 126-73-8 | C12 H27 O4 P |
| 13 | Diphenhydramine | 58-73-1 | C17 H21 N O |
| | Miconazole | 22916-47-8 | C18 H14 Cl4 N2 O |
| 11 | Leucine | 328-39-2 | C6 H13 N O2 |
| 10 | 7-Desoxycholic acid | 10538-65-5 | C24 H40 O4 |
| | Perfluorooctanoic acid | 335-67-1 | C8 H F15 O2 |
| 9 | Tri-(2-chloroisopropyl)phosphate | 13674-84-5 | C9 H18 Cl3 O4 P |
| 7 | Octodrine | 543-82-8 | C8 H19 N |
| 6 | Fexofenadine | 83799-24-0 | C32 H39 N O4 |
| | Triclosan | 3380-34-5 | C12 H7 Cl3 O2 |
| 5 | Celecoxib | 169590-42-5 | C17 H14 F3 N3 O2 S |
| | Diethyl phthalate | 84-66-2 | C12 H14 O4 |
| | Gemfibrozil | 25812-30-0 | C15 H22 O3 |
| 4 | Piperine | 94-62-2 | C17 H19 N O3 |
| 3 | 2,4-Dihydroxybenzophenone | 131-56-6 | C13 H10 O3 |
| | Fipronil | 120068-37-3 | C12 H4 Cl2 F6 N4 O S |
| | Perfluorohexanoic acid | 307-24-4 | C6 H F11 O2 |
| | Tris(2-butoxyethyl) phosphate | 78-51-3 | C18 H39 O7 P |
| 2 | 4-Hydroxybenzoic acid | 99-96-7 | C7 H6 O3 |
| | Perfluoroheptanoic acid | 375-85-9 | C7 H F13 O2 |
| | Valsartan | 137862-53-4 | C24 H29 N5 O3 |
| 1 | 9-Octadecenamide | 3322-62-1 | C18 H35 N O |

| Detection Frequency | Compound | CAS-ID | Molecular Formula |
|---|---|---|---|
| | Bisphenol A | 80-05-7 | C15 H16 O2 |
| | Novaluron | 116714-46-6 | C17 H9 Cl F8 N2 O4 |
| | Perfluorooctanesulfonic acid | 1763-23-1 | C8 H F17 O3 S |
| | Phenylacrylic acid | 621-82-9 | C9 H8 O2 |
| | Triclocarban | 101-20-2 | C13 H9 Cl3 N2 O |