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Development of a Chemical Separation Framework for Effects-Directed Analysis of Water and Wastewater

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DEVELOPMENT OF A CHEMICAL SEPARATION FRAMEWORK FOR EFFECTS-DIRECTED ANALYSIS OF WATER AND WASTEWATER

by

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Abstract

Pochiraju, Sesha Sai Susheera (M.S., Department of Civil, Environmental and Architectural Engineering)
Development of a chemical separation framework for effects-directed analysis of water and wastewater
Thesis directed by Prof. Karl Linden

Water scarcity is a global issue related to population growth, climate change, and industrial or agricultural water uses. Therefore, assessing, treating, and reusing wastewater has become more important than ever. Waters and wastewaters may contain a complex mixture of compounds with a wide variation in physical and chemical properties. Chemical and toxicological assessment methods have improved over the years, but traditional methods using *in vivo* techniques are both costly and time intensive to identify a compound or compounds, from a highly complex sample matrix, that result in a toxicological response. These issues demonstrate the need to develop more rapid and cost-effective methods for fractionating and identifying compounds responsible for a water sample's toxic response. Therefore, the aim of this study was to develop a simplified chemical separation framework and divide a water/wastewater matrix into different fractions based on its polarity or adsorptive capacity; and then validate this separation framework through an effects-directed approach (EDA) using an *in-vitro* bioassay.

Six estrogenic compounds, Ethylparaben (EPB), Bisphenol A, 17ß-estradiol (E2), 2,4,6-trichlorobiphenyl (TCBP), Octylphenol (OP) and Bis-(2-ethylhexyl)-phthalate

(DEHP), were chosen to create a synthetic water mixture that was used to develop and test a separation framework consisting of two-steps: liquid-liquid extraction (LLE) followed by solid phase extraction (SPE). Based on the polarity of these compounds and their adsorption onto the SPE phases, the procedure generated six fractions from the simulated mixture: a concentrate and a flow-thru fraction for each of the polar, mid-polar and non-polar phases. The individual fractions were then assessed for cytotoxicity and estrogenic activity using yeast estrogen screening (YES), an *in vitro* bioassay conducted with a recombinant yeast strain, *Saccharomyces cerevisiae*. All the fractions were concurrently analyzed by Liquid Chromatography Mass Spectrometry (Ion Trap) and Gas Chromatography Flame Ionization Detection for the identification and quantification of the target compounds. The compounds were distributed among four of the six fractions namely polar concentrate, mid-polar concentrate and flow-thru, along with the nonpolar concentrate. This was validated by YES results of the synthetic water mixture and select fractions exhibiting a relative estrogenic activity (% REA) ranging between 80%-95% while the fractions devoid of estrogenic compounds showed non-detectable levels of REA. The developed framework coupled with an EDA approach could be more cost-effective and time saving, compared to TIE, while also providing insights into the chemical nature of contaminants and subsequent identification, that could be used to inform its removal through targeted treatments. Overall, this generalized method could be applied to environmental water samples, in an effort to improve the identification and evaluation of toxic chemicals.

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CHAPTER 1
INTRODUCTION

Water scarcity is a global issue related to population growth, climate change, and industrial or agricultural water uses. Therefore, assessing, treating, and reusing wastewater has become more important than ever. Water reuse has become the most important research focus in areas which have the economy to support reuse infrastructure and with populations high enough to generate sufficient volume of wastewater that can be treated and reused. The treated wastewater, also called reclaimed water (or recycled water) can then be further treated to achieve drinking water quality standards. However, in most treatment plants, even after a secondary treatment, the wastewater effluents constitute a complex mixture of chemical compounds from anthropogenic sources with a wide variation in physical and chemical properties (Kolpin, Skopec, Meyer, Furlong, & Zaugg, 2004; Roberts & Thomas, 2006).

Understanding the composition of these mixtures is critical to optimize treatment plants to meet reuse criteria set by federal and state regulatory agencies (Levine, Tchobanoglous, & Asano, 1985; Ramona, Green, Semiat, & Dosoretz, 2004). One way to improvise analysis of environmental waters is by separating the compounds present in a sample into different fractions based on their physicochemical properties such as molecular weight, polarity, adsorption, etc. (Klinkow et al., 1998). Because, more often than not, the complexity of an environmental sample leads to discrepancies between predicted and observed effects, thus posing a challenge in indicating the compound or compounds that are responsible for the sample's toxicity (Brack et al., 2016; Escher, van Daele, Dutt, Tang, & Altenburger, 2013). These discrepancies arise due to various factors under complex exposure conditions such as a) masking effects, i.e. dominance of one

compound over another in stimulating a response in a microorganism(Weiss et al., 2009), and b) synergistic effects, where the effects of more than one compound are combined in generating a response in a bioassay(Hecker & Hollert, 2009). Such complications in interpreting assay results lead to inconsistency in compound identification, thus affecting overall characterization of the water sample (Lahr et al., 2003).

The current study aims to solve such problems in characterization of water as described above by developing chemical processes that can separate different classes of compounds in the water into fractions which will be individually characterized using a bioassay. Based on the response generated by each fraction, they are subjected to chemical analysis using liquid-chromatography and gas chromatography detection techniques to identify compounds that would have caused the response in the bioassay. This method is called effects-directed analysis (EDA), which is a US EPA approved tool for toxicological assessment of water samples. The hypothesis of the study is that EDA based separation framework will make characterization of complex water samples simpler and more informative by showing the nature of toxic compounds besides being easier for compound identification.

CHAPTER 2
REVIEW OF LITERATURE

Effects-directed Analysis (EDA)

The process of toxic compound identification and quantification has improved over the years, but traditional methods such as EPA's Toxic Identification Evaluation (TIE) are both costly and time intensive, involving *in-vivo* assays that evaluate the response of the whole organism (EPA Methods for Toxicity Identification Evaluation, 1991). In contrast, effects-directed analysis (EDA) uses *in-vitro* biotesting tools with toxicological endpoints such as estrogenicity, mutagenicity, acute toxicity, and others (Hug et al., 2015; You & Li, 2017).

Although both TIE and EDA share the same goal of classifying and identifying the contaminants, TIE suffers from the disadvantage of a lengthy three-phase methodology (characterization-identification-confirmation) in which target analysis is limited to Phase II (You & Li, 2017). On the other hand, EDA promises quicker diagnosis of key organic toxicants by combining using sophisticated chemical separation techniques and advanced analytical tools for target, suspect and non-target analyses as seen in previous studies (Klinkow et al., 1998; la Farré et al., 2001; Nakada et al., 2004).

Chemical Separation Framework:

There is currently a paucity of literature investigating separation techniques based on a sample's chemical nature (e.g. chemicals present), towards an EDA. Recently, Muschket et al. (2017) have linked the detection of anti-androgenic compounds in surface waters with a parallel fractionation approach. Similar work involving techniques such as silica gel chromatography (Nakada et al., 2004), SPE-HPLC fractionation (Thomas, Hurst, Matthiessen, & Waldock, 2001), gel permeation chromatography-HPLC (Fetter et al., 2014) showed toxicity-directed separation as an

effective approach in contrast to traditional TIE. Review studies done on integrating TIE with EDA carried out with fractionation (Li, Zhang, & You, 2017) indicate potential research scope in application of biotesting and chemical analysis on environmental waters (Brack et al., 2016). Single step separations using liquid-liquid extraction coupled with GC-MS and a whole cell-based assay (using MCF-7) were proposed for monitoring drinking water and wastewater samples (Shrivastava et al., 2017).

However, the need to have a more generalized yet simple procedure to carry out EDA as the first step to understand the water/wastewater composition prior to designing treatment technologies inspired us towards the current study. This work considers the scope and potential of EDA, as demonstrated in research by above studies, and explores the flexibility EDA provides in choosing fractionation techniques based on research needs. Fig. 1 shows the relation between the chemical separation processes and toxicological assessment which is the crux of EDA.

The aim of this study is to develop a basic separation framework with techniques that are applicable to a wide variety of compounds and can be done in a short duration of time in most labs at a low cost. One such technique we used is liquid-liquid extraction (LLE) which involves separation of compounds based on the differences in their solubility and partition coefficients. Another technique that has been frequently used in the research studies with EDA and is coupled with LLE in the current work is solid phase extraction (SPE). SPE separates compounds based on adsorption and is used for extracting semi-volatile and non-volatile analytes besides solids that are pre-extracted into solvents. It has advantages such as lower solvent consumption, enormous saving of time, increased extraction efficiency, decreased evaporation volumes, higher selectivity, cleaner extracts, greater reproducibility,

avoidance of emulsion formation, and easier automation (Hennion, 1999, Juhascik & Jenkins, 2009).

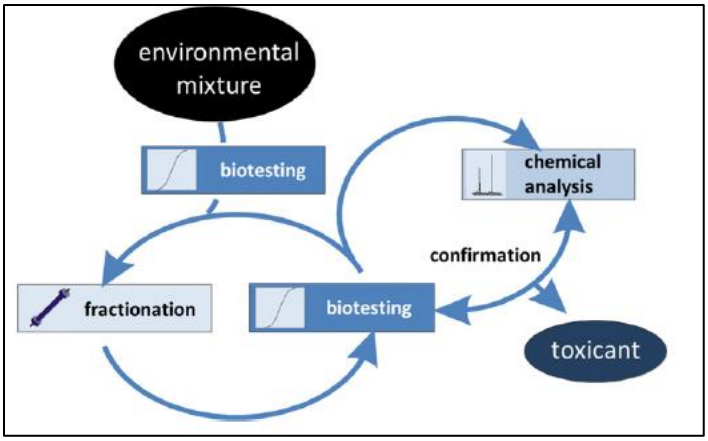


Fig. 1: Schematic illustration of effects-directed analysis (EDA) using biotesting and chemical analysis along with fractionation (from Brack et al., 2016).

Toxicological assessment

Effects-directed analysis (EDA) relies on a toxicological bioassay to diagnose compounds responsible for toxicity in a sample and its fractions. Out of numerous endpoints and assays available, a yeast estrogen screening assay (YES) was chosen for this study to assess estrogenic response induced by fractions of an environmental sample and the sample itself. YES uses a genetically engineered strain of *Saccharomyces cerevisiae* and has been shown to be highly sensitive and robust (Jarque et al., 2016).

Estrogen is the primary female sex hormone that is responsible for the development of female reproductive system and sex characteristics. Estrogenic compounds or synthetic estrogens are a class of compounds which are structurally similar to natural estrogen hormone and thus mimic the hormone or block its activity, causing reproductive interferences (Bistan et al., 2012). The main sources of estrogenic compounds in water come from birth control pills (Aris, Shamsuddin, &

Praveena, 2014), natural estrogens produced by plants (phytoestrogens), therapeutic drugs such as anti-depressants (Chèvre, 2014), and other synthetic compounds (xenoestrogens) used in pesticides (Kolok et al., 2014), and skin and hair care products to list a few (Myers et al., 2014).

Challenges in EDA directed fractionation are often related to the selection of relevant separation techniques, availability of user-friendly identification software tools, authentic standards and an up-to-date database (Simon et al., 2015). As mentioned before, this study attempts to focus on developing a combination of commonly applicable chemical separation techniques that will form a simple framework which can be implemented on any type of complex environmental water sample. The study hypothesizes that this framework will make toxicity assessment of a complex water sample easier through separation of its constituents. The toxicity data can then be used to address the missing gaps between compound identification and potential treatment selection.

CHAPTER 3
METHODOLOGY

3.1 Chemical Methods

3.1.1 Selection of Target Compounds:

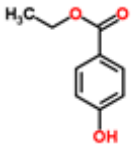
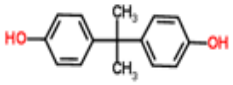
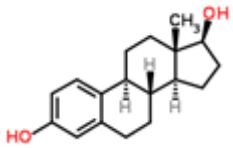
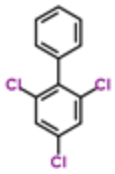
The objective of this study was to develop and demonstrate the separation framework based on EDA approach based on the toxicological endpoint as estrogenic induction in yeast. To accomplish this, a synthetic water sample was prepared in the lab using targeted compounds that could mimic the complexity of an environmental sample. A total of six compounds were chosen for this purpose which were reported to be estrogenic in previous studies: Ethylparaben (EPB, Engeli et al., 2017), Bisphenol A (BPA, Elliott, Ettinger, Leaderer, Bracken, & Deziel, 2017), 17ß-estradiol (E2, Routledge & Sumpter, 1996), 2,4,6-trichlorobiphenyl (TCBP, Harris et al., 2014; Layton et al., 2002), Octylphenol (OP, Puy-Azurmendi et al., 2014; Routledge & Sumpter, 1996) and Bis (2-ethylhexyl) phthalate (DEHP, Elliott et al., 2017; Jin, Sun, & Li, 2008).

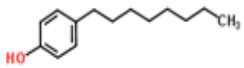
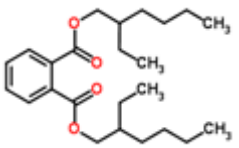
The compounds were screened based on research-supported knowledge of their estrogen induction in recombinant yeast assay in the cases of BPA, OP, TCBP and E2 (Elliott et al., 2017; Layton et al., 2002; Puy-Azurmendi et al., 2014; Routledge & Sumpter, 1996) or a cell-based reporter assay in the case of EPB (Engeli et al., 2017). The chemical properties of the compounds were also considered such as size, polarity, adsorptivity, which would play a key role in the fractionation process. The compounds varied mainly in size, with molecular weights spread between 166.5 g/mol and 390 g/mol, and in polarity, with octanol-water coefficients ranging from 2.47 - 7.60.

Preparation of Synthetic Water Sample: Prior to making our synthetic water sample, each estrogenic compound was tested at numerous concentration levels

ranging from 0.1 ng/L to 1000 mg/L to evaluate the minimum effective concentration that could generate an estrogenic response. All the estrogenic assays with target compounds were carried out with 17ß-estradiol internal standard as positive control and ultrapure DI water for negative control as described in Section 2.2. The target estrogen compounds with their corresponding concentrations used in the simulated mixture are shown in Table 1. All the compounds were acquired from Sigma-Aldrich (MO, USA), and were of at least 98% purity.

Table 1: Estrogenic compounds used for preparation of synthetic water mixture

Chemical Name	**Chemical formula**	**log k_{ow}**	**Structure**	**Molecular weight (g/mol)**	**Solubility in water at 25°C**	**Conc. in synthetic water sample (mg/L)**
Ethyl 4-hydroxybenzoate (EPB, 99%)	$C_9H_{10}O_3$	2.47[a]		166.06	885 mg/L [b]	50
Bisphenol A (BPA, >99%)	$C_{15}H_{16}O_2$	3.32[a]		228.28	120 mg/L [c]	10
17beta-estradiol (E2)	$C_{18}H_{24}O_2$	4.01[a]		272.177	3.9 mg/L [b]	0.0027
2,4,6-Trichlorobiphenyl (TCBP)	$C_{12}H_7Cl_3$	5.47[a]		255.96	0.25 mg/L [b]	0.005

Octylphenol (OP)	$C_{14}H_{22}O$	5.50[a]		206.16	3.11 mg/L [d]	5*
Bis(2-ethylhexyl) phthalate (DEHP)	$C_{24}H_{38}O_4$	7.60[a]		390.27	0.27 mg/L [e]	73.87*

Note: Compounds that were not soluble or sparingly soluble in water (at the concentration level used) were dissolved in acetonitrile (>99.93%, HPLC grade) and then added to the synthetic water mixture.
a) Martin, 1996 b) Myrdal, Ward, Dannenfelser, Mishra, & Yalkowsky, 1992; c) Dorn, Chou, & Gentempo, 1987; d) Estimated using US EPA EPISuite™ e) USEPA Water Quality Standards (https://www.epa.gov/sites/production/files/2015-06/documents/il_hh_157_06202006.pdf)

3.1.2 Separation Framework:

Prefiltration: Prior to the separation framework, the mixture to be fractionated was filtered using Corning® 250mL sterile vacuum filter system containing cellulose acetate membrane filters of 0.22µm pore size (Product *#430767*) to remove the native microorganisms and particulate matter that might interfere with subsequent extraction steps and toxicity tests. For each fractionation, a volume of at least 550mL of synthetic water was filtered under vacuum suction and stored in a sterile glass container at 20°C.

Separation of compounds based on polarity:

Solvent selection is a major part of a liquid-liquid extraction (LLE) experiment design. Solvents used for extraction should be as immiscible as possible with aqueous phases for better separation of the compounds in the sample (Table 2). One of the equations that illustrate these processes is the Nernst distribution law (Equation 1). It states that any neutral species will distribute between two immiscible solvents so that the ratio of the concentration remains constant.

Equation 1: Nernst distribution law

$$K_D = \frac{C_o}{C_{aq}}$$

Where K_D is the distribution constant; C_o is the concentration of the analyte in the organic phase; and C_{aq} is the concentration of the analyte in the aqueous phase.

As K_D value increases, the solubility of the compound in the organic phase increases with respect to the aqueous phase. For non-ionizable compounds, K_D is equal to K_P or K_{ow} (partition coefficient).

Table 2: Log K_{ow} of some common organic solvents

Compound	**Log K_{ow}**
Methanol	-0.77
Acetonitrile	-0.34
Ethyl Acetate	0.73
Dichloromethane	1.25
Chloroform	1.97
Pentane	3.39
Heptane	4.66

Solvents with higher log K_{ow} are immiscible enough with water to form two separate layers. As log K_{ow} decreases, miscibility with water increases and hence the ability to solubilize organic compounds decreases. For example, ethyl acetate is 8.1% water soluble compared to dichloromethane (1.3%) and chloroform (0.79%) which makes it less efficient in extracting mid-polar and nonpolar compounds from water.

LLE Procedure

To separate nonpolar compounds from the aqueous matrix, pentane was used as a solvent with low polarity. In this step, 500 mL of the simulated mixture was decanted into a Kimax 500 mL glass separatory funnel and extracted using 50 mL of pentane (HPLC grade, ≥99.7%) to separate the nonpolar compounds from the synthetic mixture water. After gently introducing the extraction solvent into separatory funnel, mixing of the two phases was done for about 2 min by manually vortexing the funnel at an angle of 45°. The funnel was then held stationary for about 5-10 min supported by a stand with a clamp. After the separation of layers becomes visibly clear, the aqueous and pentane fractions are collected into separate sterile glass containers for further processing. If emulsions were present after shaking and settling, the samples were sonicated for 30 minutes (Branson 1200 Ultrasonic Bath) prior to decanting.

The aqueous extract following the first step was subjected to second extraction using 50 mL of dichloromethane (≥99.9%, Optima™, Fisher Chemical) to extract moderately polar compounds from water, which were not soluble in pentane. Following this extraction, which occurred as described for pentane, both the DCM (~50ml) and aqueous phases (~500ml) were collected into sterile containers for the subsequent steps in the separation framework. Similar to that of pentane, if emulsions were observed, the DCM/aqueous mixture was sonicated for 30 min prior to decanting.

After homogenization, the pentane and DCM fractions were dried using sodium sulfate (technical, anhydrous, granular, ≥99%, 10-60 Mesh). The bed was made by carefully adding 3 grams of Na_2SO_4 into a glass pipette that formed a packed drying column. Each fraction, DCM or pentane, were then passed through a packed drying column to remove any excess water that might have been present.

The aqueous fraction obtained after the two extractions was then concentrated to 100mL using a Buchi Rotovapor (RE 111 and Water Bath B 461, Flawil, Switzerland). This was done at 25°C at 15 psi. The concentrate was collected into a burned glass bottle and stored at 20°C until further processing. The methods described above are detailed in Fig. 2.

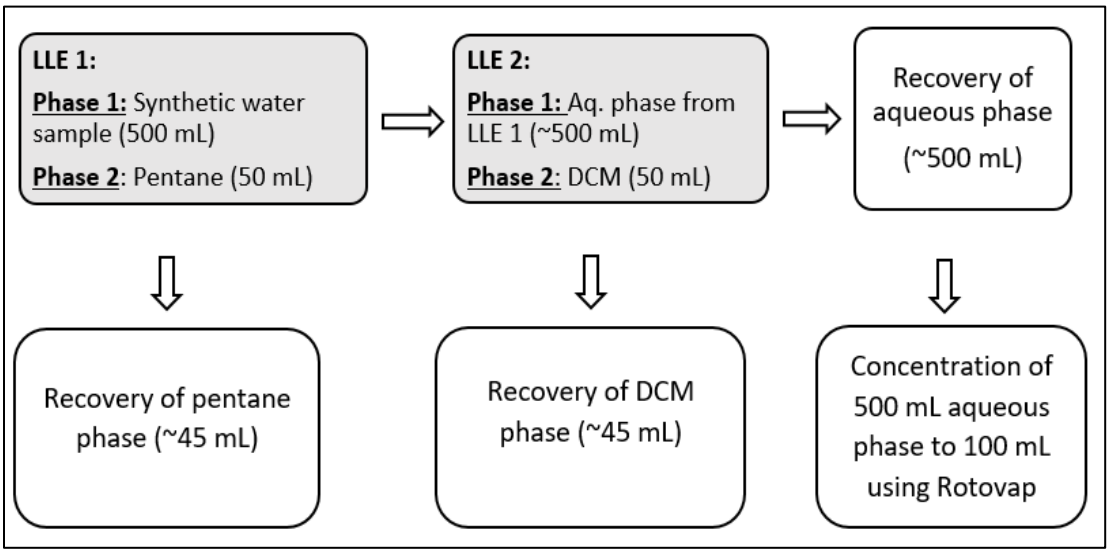


Fig. 2: Flowchart showing procedure of liquid-liquid extraction step in separation framework

Separation based on adsorption

Also known as "Liquid-solid phase extraction", SPE has become increasingly popular among adsorption or affinity-based extraction techniques due to many advantages, most important one being its flexibility to switch phases that are more suitable for subsequent chromatographic analyses.

As explained in Appendix II, SPE follows a simple procedure with four main steps: conditioning, sample addition, washing and elution (3 and 4 are often considered as one step). Based on the stationary phase used, an SPE cartridge is classified into many categories. Some of the most important types which are of

significance for this study are listed below (refer to Appendix II for detailed descriptions):

1. Normal phase SPE
2. Reversed-phase SPE

SPE Procedure:

After separating the water sample into polar (aqueous), mid-polar and nonpolar phases, they were subjected to solid phase extraction (SPE) for further separation of compounds based on their adsorption onto stationary phases such as graphitized carbon and magnesium silicate. For each phase, the extraction generated an eluent fraction with compounds that could adsorb strongly (higher log K_{oc}) onto a nonpolar stationary phase (in the case of aqueous phase) or a polar stationary phase (in the case of DCM and pentane phases), while the flow-thru (or the runoff) fractions were supposed to retain the unadsorbed organic compounds with low log K_{oc}. Thus, the three fractions obtained from LLE generated two fractions each: aqueous SPE concentrate, aqueous flow-thru, DCM SPE concentrate, DCM flow-thru, pentane SPE concentrate and pentane flow-thru as shown in the diagram below (Fig. 3).

The 100mL aqueous concentrate was passed through SPE cartridges made of graphitized non-porous carbon to separate organic polar and nonpolar compounds with the help of adsorption. Supelclean™ ENVI-Carb™ cartridges with a carbon surface area of 100m^2/g and a bed weight of 500 mg (sample volume of 6mL) have been used for this purpose. The columns were pre-treated with 6mL of 99% methanol and then equilibrated with 6mL of ultrapure water (Sartorius atrium® 611VF). The mobile phases were passed through the columns under vacuum suction and flow through was collected into sterile glass tubes (1 mL/min). Elution of adsorbed phase was done using 6 ml of a mixture of 20% methanol in acetonitrile.

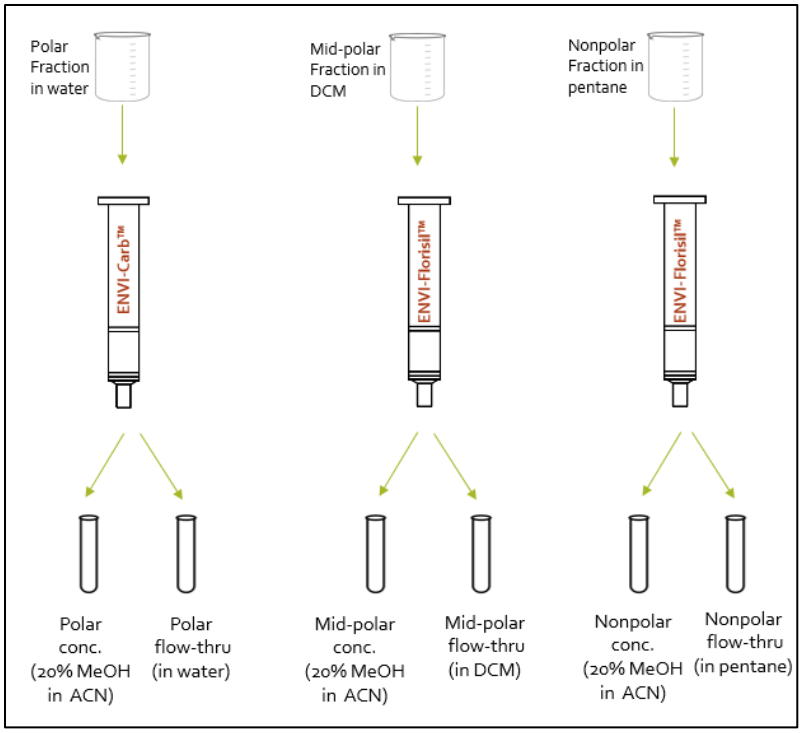


Fig. 3: Diagram showing procedure of solid-phase extraction of phases obtained from LLE.

The eluate fractions are concentrated under nitrogen gas using a TurboVap® LV (Biotage, Charlotte, NC) evaporator system to about 50 µL, with a small aliquot (5 µL) being taken for analytical methods, with the remaining 45 µL being re-suspended in 5mL of ultrapure water (HPLC Grade, Optima™, Fisher Chemical). Meanwhile the flow through fraction, owing to its large volume, was concentrated to 5mL using Buchi Rotovapor under vacuum suction of about 15psi.

The LLE mid-polar and nonpolar phases suspended in DCM and pentane respectively, are passed through columns loaded with magnesium silicate (housed in PTFE frits) to separate polar organic compounds from both the phases. Supelclean™ ENVI-Florisil™ cartridges with a bed weight of 500mg (sample volume of 3mL) have been used for this purpose. The cartridges were pretreated with 3mL of appropriate solvent (100% DCM or 100% pentane depending on the fraction being passed). The samples were passed under vacuum suction and flow through was collected. The

columns were then eluted with 6 mL of 20% methanol in acetonitrile to extract the compounds adsorbed onto magnesium silicate. The overall schema for chemical separation framework with LLE and SPE as described above is presented in Fig. 4.

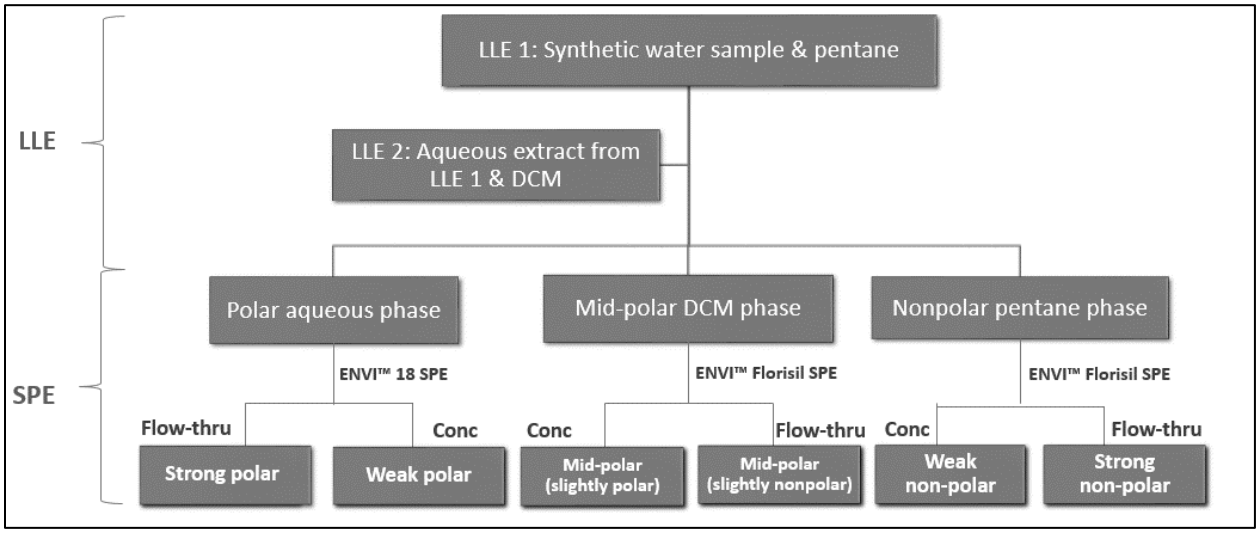


Fig 4: Schema of two-step chemical separation framework with LLE followed by SPE generating six fractions from synthetic water sample.

Concentration by evaporation: Both the eluent and flow through fractions were subjected to evaporation under nitrogen gas using TurboVap® LV (Biotage, Charlotte, NC) and concentrated down to about 50 µL. This was reconstituted with 100 µL of the same solvent in which the solution is originally suspended in. An aliquot of 20-40 µL is transferred to a sterile glass chromatography vial with a glass insert and stored at -20°C for LC-MS and GC-MS analyses. The rest of the solution is re-suspended in 5 mL ultrapure DI water (Sartorius atrium® 611VF) and stored at -20°C to conduct bioassays.

Control extraction: A control or blank extraction was carried out with 500 mL of ultrapure DI water (Sartorius atrium® 611VF) by executing every step of the process to generate six control fractions. These were tested for estrogenicity and then

assessed using LC-MS and GC-FID to ensure that toxicity was not being induced by materials used during the procedure.

3.2 Analytical Methods

3.2.1 Liquid Chromatography-mass spectrometry (LC-MS)

High performance liquid chromatography (HPLC) with ion trap mass spectrometry (MS) was used for the analysis of the synthetic water and its fractions. The instruments used were an Agilent 1100 series HPLC equipped with a reverse phase C8 analytical column of 150 mm × 4.6 mm and a 3.5 µm particle size (Zorbax Eclipse XDB-C8) that led to a XCT Plus ion trap (Agilent, Santa Clara, CA). Electrospray ionization was used, in positive-ion mode, for analysis of samples. The mobile phase consisted of HPLC grade water (Honeywell Burdick & Jackson, Morristown, NJ) with 0.1% formic acid (Fluka, St. Louis, MO) and HPLC grade acetonitrile (Honeywell Burdick & Jackson). The gradient elution started at 10% acetonitrile and 90% water and increased to 100% acetonitrile over 24 min, after which it continued at 100% acetonitrile for an additional 2 min.

Following each run, a post-run of 10 min was used (Thurman et al., 2014). Agilent ChemStation was used to load method parameters, data collection and analysis for both LC and MS. Three concentrations of internal standards between the range of 1 ppm and 100 ppm were prepared for each compound based on their concentration used in simulated mixture to obtain calibration standard curves. All the standards and subsequent dilutions were prepared using acetonitrile ($\geq$99.93%, HPLC grade, Sigma-Aldrich, MO, USA).

The fractions that were suspended in water or 20% methanol in acetonitrile solution (SPE concentrates) were analyzed on the LC along with internal standards

and the synthetic water sample itself. Two control vials, one with ultrapure DI water and the other with 20% methanol in acetonitrile mixture were run through the column before injecting the samples. All the samples and standards were run in positive ion mode except for Bisphenol A which was run in negative ion mode.

3.2.2 Gas Chromatography with Flame Ionization Detection (GC-FID)

For compound identification in fractions and standards that were not amenable to LC-MS analysis, an Agilent 7890a purge and trap gas chromatograph with a flame ionization detector (Agilent Technologies, Santa Clara, California) was used. A Resteck Rxi-1ms column (20m, 0.18 mm ID, 0.18 μm) was used with front inlet operating in splitless mode. The injection volume was 10 μL with Helium as carrier gas at a flow rate of 10 mL/min and 14.505 psi pressure. The ramp program was run at 40^{o}C for 2 min followed by 20^{o}C min^{-1} to 330^{o}C and held for 20 min. The front detector (FID) temperature was at 350^{o}C and injector temperature was 275^{o}C (Rosenblum, Sitterley, Thurman, Ferrer, & Linden, 2016). Agilent GC ChemStation was used to load method parameters, data collection and analysis.

As described above with LC-MS, internal standards were prepared at three different concentration levels between 1 ppm and 100 ppm to obtain calibration curves. All the standards and subsequent dilutions were prepared using dichloromethane (≥99.9%, Optima™, Fisher Chemical).

3.3 Toxicological assessment of the separated fractions

After separation of the synthetic mixture into six fractions based on polarity and adsorption, the next step in our effects-directed analysis was to screen the fractions for estrogenic induction using the recombinant yeast assay. The assay

duration is 5 days including the incubation time for the yeast and an exposure time of 72 hours.

3.3.1 Yeast Estrogen Assay (YES):

The recombinant strains of yeast *Saccharomyces cerevisiae* were initially acquired from J.P. Sumpter stored at -20°C in 250 µl of culture aliquots. Assay was carried out in a controlled environment inside a biosafety hood at constant room temperature of 21°C for every run.

Procedure for YES:

Day 1: On the first day of the assay, a 24-hour culture of yeast was initiated by inoculating an aliquot of cryogenic stock of the organism into the growth medium. The yeast growth medium consisted of 45 mL of minimal medium (Routledge & Sumpter, 1996), 5 mL of glucose solution, 1.25 mL of L-aspartic acid, 0.5 mL of vitamin solution and 0.5 mL of L-threonine. All the media were prepared in the lab, autoclaved (or filter sterilized in case of heat sensitive compounds) and stored at room temperature, expect for L-threonine, vitamin solution, CuSO4, CPRG (indicator compound) and estradiol, which were stored at 4°C. The chemicals used for preparing culture media were acquired from Sigma-Aldrich (MO, USA). In a class III biosafety cabinet, growth medium was added to a sterile 250 mL Erlenmeyer flask into which 125 µl of yeast was inoculated. The flask is sealed with aluminum foil and incubated in a controlled heating shaker cabinet at 31°C and 125 rpm for 24 hours.

Day 2: The yeast culture from 24 hours was passed into fresh growth medium to achieve better growth. 2 mL of 24-hour culture is inoculated into 50 mL of sterile growth medium and incubated at the same temperature and rotation as Day 1.

Day 3: 17ß-estradiol was used as the assay positive control to generate a standard curve and was diluted serially using absolute ethanol (mol. bio. grade, 200 proof, Sigma Aldrich E7023) to get 7 different concentrations. All the concentrations were plated in triplicates on a 96-well microtiter plate (Greiner CELLSTAR®, sterile, flat-bottomed with lid) by transferring 10 microliters aliquots into each well and allowed to dry. Once the ethanol evaporated, 100 microliters of ultrapure water was added to the positive control wells and an entire row (the bottom most row) of empty wells to test for negative control.

The fractions that were resuspended in water were used for testing the estrogenic activity. Each fraction, along with the raw water, was serially diluted with ultrapure water to obtain 9 different concentration: 1x (original sample), 0.5x, 0.1x, 0.05x, 0.01x, 0.005x, 0.001x, 0.0005x, 0.0001x. Each concentration was plated in triplicates by transferring aliquots of 100 microliters into each of three consecutive wells in a row.

The 24-hour yeast culture from Day-2 was removed from the incubator. To enumerate the cell growth, a $1/10^{th}$ dilution was made by adding 0.3 mL of yeast culture to 2.7mL of sterile minimal medium. A 40 microliters aliquot of this dilution was added onto the counting chamber (Hausser Scientific Bright-Line™ Counting Chamber, Sigma-Aldrisch, MO, USA) under a glass cover slide (20 microliters per side). This was viewed under 40x magnification to count the number of yeast cells in each of the 16-square grids. An average was calculated and used to determine the volume of cell culture to be added to the final assay medium. Usually this volume ranged between 1-2 mL depending on the density of the 24-hour culture.

Once the volume was determined, the cells were added to assay medium which contained 45 mL of 2x minimal medium, 10 mL of glucose solution, 2.5 mL of L-

aspartic acid, 1 mL of vitamin solution, 0.8 mL of L-threonine, 0.25 mL of CuSO4 and 0.5 mL of CPRG (chromogenic substrate). This seeded medium was added in 100 microliters aliquots to each of the wells that contained positive and negative controls, and dilutions of fractions. After adding yeast, the plates were sealed as shown in Fig and were shaken at 125 rpm for 2 minutes before placing them in a ventilated heating cabinet at 31°C.

Day 4: After 24 hours of incubation, the plates were shaken vigorously for 2 min to mix and disperse the growing cells and were returned to the incubator.

Day 6: After 3 days of incubation, the plates were taken out of the incubator and shaken again for 2 min and left for 1 hour for the yeast to settle down. Then the plates were read for absorbance at 540 nm (OD_{540}) for color and at 620 nm (OD_{620}) for turbidity. Readings were taken using microplate reader (BioTek® Epoch, VT, USA) with Gen5 software for data collection and analysis. The corrected value for absorbance was calculated by plugging the equation shown below (Eq. 2) into the Gen5 software protocol.

Equation 2: Calculation of corrected absorbance at 540 nm (Routledge & Sumpter, 1996)

$$Corrected\ value\ (Corr\ 540) = sample\ abs.at\ 540\ nm - (sample\ abs.at\ 620\ nm - blank\ abs.at\ 620\ nm$$

Deep red color in the wells indicate positive activity while light orange or yellow indicates negative response. Turbidity in the wells suggest growth of yeast, and clear wells suggest inhibition of the organism.

Relative estrogenic activity (REA): The estrogenic induction of positive control, synthetic water fractions, original synthetic water sample and negative control was

estimated in terms of % relative estrogenic activity which was calculated using the formula shown below (Eq. 3).

Equation 3: Relative estrogenic activity (% REA) in yeast (Bistan et al., 2012)

$$\%\, REA\ = \frac{EA_{sample} - EA_{blank}}{EA_{E2} - EA_{blank}} * 100$$

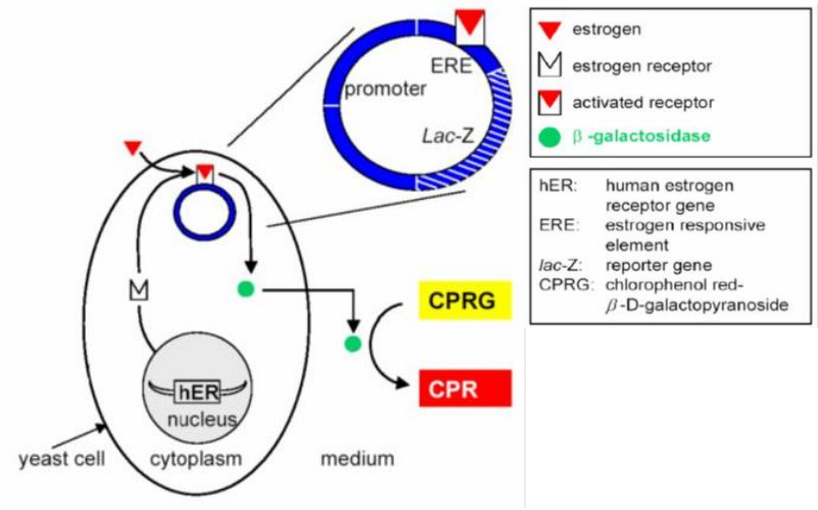


Fig. 5: Figure showing mechanism of estrogen-inducible expression in yeast (Routledge & Sumpter, 1996).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Chemical analysis of separated fractions

The fractions analyzed on LC-MS and GC-FID showed a distribution of estrogenic compounds across four of the fractions: aqueous SPE concentrate, DCM phase SPE concentrate, DCM phase SPE flow-thru, and pentane phase SPE concentrate (Table 3). The fractions aqueous SPE flow-thru and pentane SPE flow-thru did not show any peaks or ions that align with the six estrogenic compounds studied, when analyzed by LC-MS or GC-FID.

Most of the compounds were found to be present in more than one fraction, suggesting a distribution of compounds between water and the organic solvents, pentane and DCM. The fact that each chemical compound had its own pattern of distribution helped to understand the influence of various chemical properties such as polar surface area (PSA), adsorption coefficient (k_{oc}) and octanol-water partition coefficient (k_{ow}) on the fate of each compound.

The mass spectra of the SPE concentrate fractions suspended in 20% MeOH in acetonitrile were analyzed using ion trap LC-MS and the results were compared with those of the internal standards. The polar (aqueous) SPE concentrate showed signal peaks at a retention time of 20.8 min which matched with the standard retention time of ethylparaben (EPB). Evidently, the presence of the compound in the concentrate fraction indicated that EPB showed good adsorption onto activated carbon during SPE of aqueous phase although EPB had a relatively low log K_{oc} (2.365).

Table 3: Distribution of estrogenic compounds into six fractions separated from synthetic water sample

Estrogenic compound	Log K_{ow}	Log k_{oc}	Aqueous (polar) conc	Aqueous (polar) flow-thru	DCM (mid-polar) conc	DCM (mid-polar) flow-thru	Pentane (non-polar) conc	Pentane (non-polar) flow-thru
Ethylparaben	2.47	2.3	+	–	+	+	+	–
Bisphenol A	3.32	4.8	+	–	–	–	+	–
17β-Estradiol	4.01	3.3	–	–	+	+	+	–
2,4,6-Trichlorobiphenyl	5.47	4.4	–	–	+	–	–	–
Octylphenol	5.5*	4.5	–	–	+	–	–	–
Bis (2-ethylhexyl) phthalate	7.5	5.6	+	–	+	–	+	–

* Estimated value using US EPA's EPISuite™

The GC-FID spectra of aqueous concentrate suggested the presence of bisphenol A and DEHP with peaks at retention times of 11.82 min and 13.574 min respectively. The internal standard peaks for BPA had a retention time of about 11.8 min and DEHP had about 13.58 min. The detection of DEHP in aqueous phase was also supported by LC-MS spectra which showed molecular ion peaks for the compound (m/z 391.3) along with the stable ion peaks (m/z 413.3). This was assumed to be due to possible formation of $[M+Na]^+$.

One possible reason for DEHP's detection in aqueous phase might be the polar surface area (PSA) of the molecule which is 53 A^2 units, a relatively high amount for a non-polar compound. The presence of 4 -H bond acceptors in its structure could explain the possibility of its mobile nature in polar and mid-polar phases. Studies on plasticizer usage in food and water products show leaching of phthalates which can be used to support our above assumption (Erythropel et al., 2014; Jin et al., 2008; Khedr, 2013). After a proportion of the compound entering aqueous phase during LLE, the subsequent solid phase extraction using activated carbon (AC) would have

enabled adsorption of DEHP, given its nonpolar nature and a high log K_{oc} (5.6). Similarly, EPB with a log K_{oc} of 4.8 was able to be adsorbed onto AC, illustrated by its presence in the aqueous concentrate fraction. This assumption was in agreement with the absence of signal peaks for these compounds in the aqueous flow-thru fraction.

The concentrate fraction of DCM contained five out of the six target compounds added in simulated mixture: EPB, E2, TCBP, OP and DEHP. EPB has a log K_{ow} of 2.47, while the compounds E2, TCBP and OP fall within the range of log values at 4.01, 5.47 and 5.5 respectively. DEHP has the highest at 7.5. Although EPB has a higher water solubility of 885 mg/L (Myrdal et al., 1992), its log K_{ow} 2.47 suggests that it is neither too polar to entirely stay in aqueous phase nor too non-polar to restrict itself to organic phases. That could explain its wide distribution among all the three phases: polar, mid-polar and a small percentage in non-polar. The compound peaks for EPB (m/z = 166.5) were seen on the mass spectra of both standards and fractions at a retention time of 20.8 min. The compound mass spectrum along with chromatograms of aqueous, DCM and pentane concentrates with EPB peaks are shown in Fig. 6. Standard curve extrapolation shows as estimated concentration of 65.54 mg/L, 60.24 mg/L and 47.83 mg/L in aqueous, DCM and pentane concentrates respectively.

17ß-estradiol (E2) is sparingly soluble in water, while TCBP and OP are mid-polar compounds. Hence it was not surprising when they were detected in the DCM phase. Nevertheless, EPB and E2 detection was low compared to other compounds indicating weak adsorption onto polar stationary phase such as magnesium silicate when present in a strong mid-polar mobile phase like DCM. This leaves reason to expect them in higher concentrations in the flow-thru fraction of DCM compared to

the SPE concentrate. E2 was identified with stable ion peaks (m/z = 254.9) at a retention time of 21.8 min with its molecular ion peak.

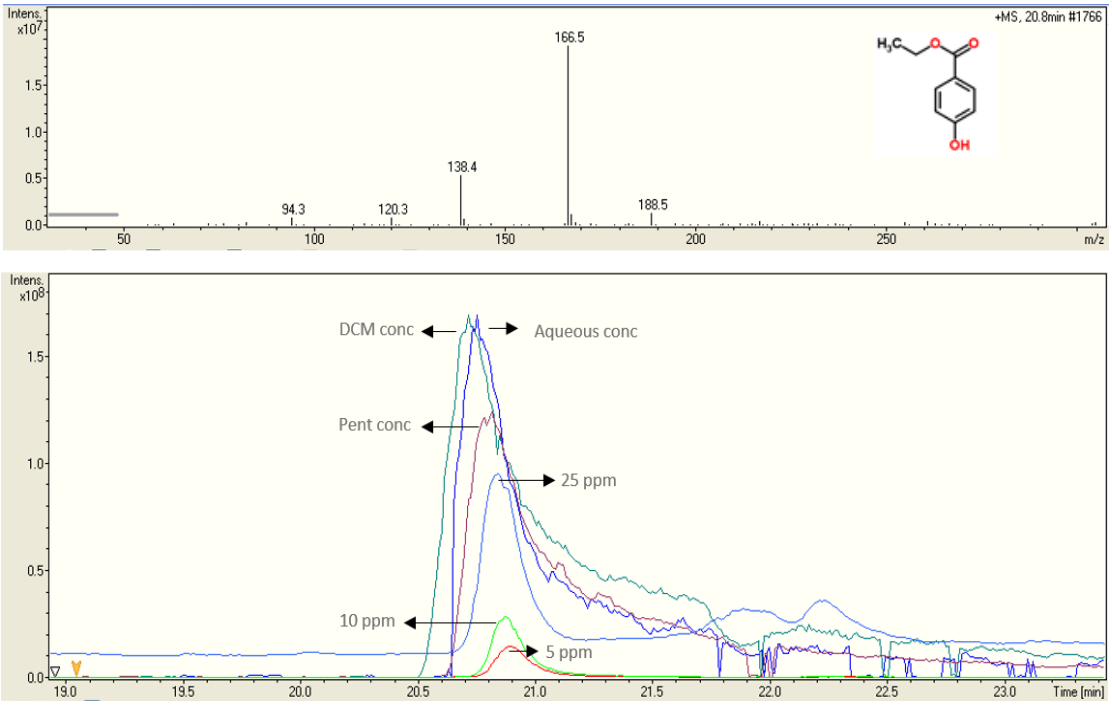


Fig 6: Mass spectrum of EPB (top) with the compound peak (m/z = 166.5) at retention time 20.8 min. LC chromatograms showing aqueous, DCM and pentane concentrates along with EPB standards.

Fig. 7 shows mass spectrum of E2 compound along with chromatograms of 5 ppm, 10 ppm and 25 ppm standards overlapped on DCM concentrate and pentane concentrate fractions showing compound peaks for estradiol. Standard curve extrapolation showed as estimated concentration of 0.00004 mg/L and 0.0003 mg/L in DCM and pentane concentrates respectively.

While the fact that the partition coefficients of these chemicals might explain their reasonable affinity with DCM to some extent, it was also important to consider other physical and chemical properties of seemingly non-polar compounds like DEHP as to what would have caused its partition into DCM. As explained above with DEHP detection in polar aqueous phase, the same can be implied here with an assumption

that the compound's high polar surface area and high log K_{oc} might have resulted in its scattering among weak polar (aqueous concentrate), strong mid-polar (DCM concentrate) and weak non-polar (pentane concentrate) fractions.

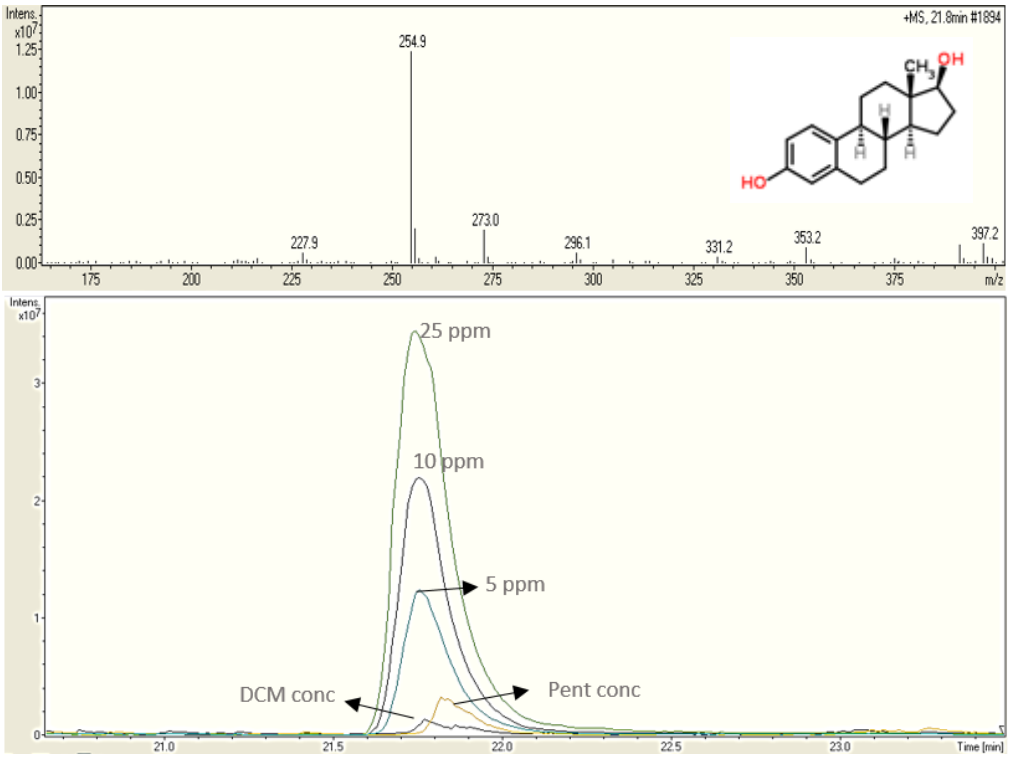


Fig 7: Mass spectrum of E2 (top) with the stable ion peak (m/z = 254.9) at retention time 21.8 min. LC chromatograms (bottom) showing EPB peaks at 21.8 min in DCM and pentane concentrates along with the standards.

The SPE flow-thru fraction of DCM was analyzed using GC-FID since it is suspended in dichloromethane and the spectra showed two signal peaks that matched the standard retention times of the compounds EPB and E2. One signal with decent peak area was observed at a retention time of 8.33 min and the other at 13.89 min. EPB internal standards suspended in DCM were injected into the GC at three concentration levels: 10 ppm, 35 ppm and 100 ppm which showed retention times of 8.318 min, 8.32 min and 8.34 min respectively. Similarly, E2 standards at 5 ppm, 10 ppm and 100 ppm showed signal peaks at 13.885 min, 13.882 min and 13.903 min

respectively. From the standard curve extrapolation, the projected concentrations for EPB and E2 were 175 mg/L and 0.031 mg/L respectively.

As explained above while discussing the mid-polar concentrate fraction, only a minor portion of these compounds that went into the DCM phase during LLE had been adsorbed onto magnesium silicate during SPE in the next step. This caused a major percentage to remain in the mobile phase thus ending up in flow-thru. This could be because EPB and E2 have adsorption coefficients of 2.3 (US EPA's EPISuite™) and 3.3 (Lee, Strock, Sarmah, & Rao, 2003) respectively, which are relatively low compared to the rest of the compounds that are in the LLE phase of DCM. Although they have polar surface area (PSA) of 47 $Å^2$ (EPB) and 40 $Å^2$ (E2), there is a possibility that other compounds with higher adsorption coefficients such as OP and DEHP would have dominated the active sites compared to EPB and E2. Besides, both the compounds exhibit strong mid-polar characteristics that would augment their affinity with DCM.

The SPE concentrate phase of pentane phase was analyzed using both LC-MS and GC-FID. A total of four compounds: EPB, BPA, E2, and DEHP were detected in the chromatograms. Bisphenol A peaks were seen in GC-FID spectra at a retention time of 11.818 min respectively. This was in line with the retention time of internal standard of BPA dissolved in DCM, that showed 11.82 min for 100 ppm concentration. Although BPA has a relatively higher water solubility of 120 mg/L (US EPA's EPISuite™), it has a log K_{ow} of 3.32 (Hansch C, 1995) and is slightly hydrophobic in nature. This would have likely resulted in it partitioning into pentane during the LLE. Yet, it is natural that one would assume an uneven distribution with a higher percentage to be in aqueous phase. This assumption was supported by a GC peak area ratio for BPA in aqueous and pentane concentrates which was found to be 12:1.

This showed BPA's affinity towards aqueous phase with effective adsorption onto activated carbon.

Although the stationary phases used for mid-polar and nonpolar fractions were the same (magnesium silicate), the pentane concentrate fraction showed adsorption of all the compounds that were in LLE pentane extract, as none of them appeared in the flow-thru fraction unlike DCM flow-thru. This might be due to compounds having relatively low concentrations in pentane eluent according to the inferences drawn from both recovery estimates and estrogenic responses.

The six fractions of control extraction performed with ultrapure DI water alongside the synthetic water extraction showed no compounds when analyzed with LC-MS and GC-FID. This showed us that there was no contamination from materials or glassware that interfered with analytical results or toxicological response.

4.2 Toxicological analysis of separation fractions:

The Yeast Estrogen Screening (YES) assay results of chemical separation fractions were analyzed for cytotoxicity and estrogen induction. Fig. 8 showed estrogenic activity observed during the assay with the simulated mixture, SPE concentrates of aqueous, DCM and pentane phases along with DCM flow-thru. The flow-thru fractions of aqueous and pentane phases showed no detectable response in comparison with the positive control. This could be explained using the dose-response data obtained from preliminary assessment (as mentioned in Section 2.1.1) which showed an inhibition of 33% with 1000 mg/L (and about 15% with 100 mg/L) of BPA and 89% with 1000 mg/L of EPB. As mentioned in Section 3.1, estimated compound concentrations suggested 230 mg/L for BPA and 60 mg/L for EPB. Such high concentrations of both the compounds could have had individual and/or synergistic effects on inhibition of yeast growth at 1x concentration.

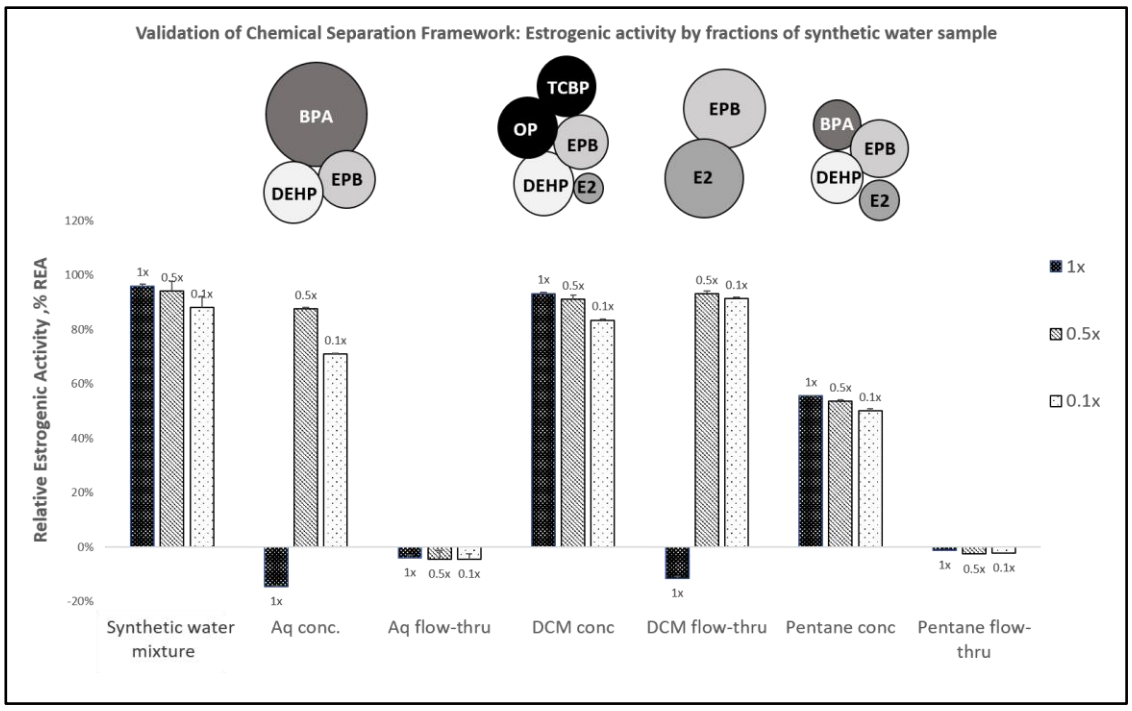


Fig. 8: Compound distribution after chemical separation framework and corresponding estrogenic response. All the fractions were tested at 1x, 0.5x and 0.1x concentrations including the parent synthetic mixture.

The aqueous SPE concentrate showed negative estrogenic response at 1x concentration (Fig. 5) due to cytotoxicity (Fig. 9) while the dilutions showed relative estrogenic activity (REA) of 88%, 71% and 63% for 0.5x, 0.1x and 0.05x respectively. As discussed in the chemical analysis in Section 3.1, the presence of EPB, BPA and DEHP likely resulted in this observed response.

The SPE concentrate of the DCM phase, which contained five compounds as per the chemical analyses, showed REA of 93%, 93% and 83% for 1x, 0.5x and 0.1x, respectively. The SPE concentrate of pentane phase which contained four of the target compounds as per our analytical results had an estrogenic induction of 56%, 54%, and 62% for 1x, 0.5x and 0.1x concentrations. The pentane fraction showed an estrogenic response that was almost half of what was seen with DCM concentrate. This suggests that the pentane phase might have had one or more compounds in a

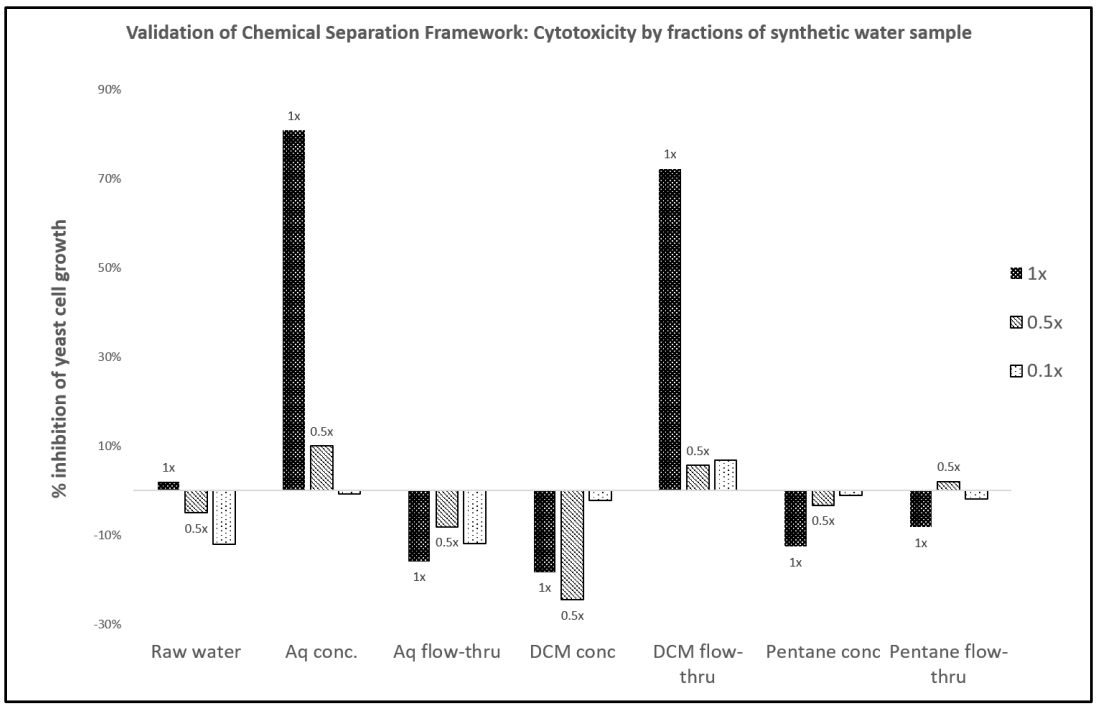


Fig. 9: Cytotoxicity (% inhibition of yeast) by separated fractions. All the fractions were tested at 1x, 0.5x and 0.1x concentrations including the parent synthetic mixture.

lower concentration compared to DCM concentrate. The concentrations calculated from LC-MS and GC-FID peak areas as discussed in Section 3.1 support the above assumption which showed EPB concentration in pentane concentrate to be 47.8 mg/L compared to DCM concentrate which contained 60.24 mg/L. Moreover, the presence of OP and TCBP in the DCM concentrate would also have caused higher activity compared to pentane concentrate.

The flow-thru fraction of DCM showed negative estrogenic induction of -9% due to a high cytotoxicity of 72% at 1x concentration, similar to what was observed from the aqueous concentrate. The fraction dilutions of 0.5x, 0.1x and 0.05x showed REAs of 92%, 91% and 71%, respectively. The DCM flow-thru chromatographic analyses showed presence of two compounds, EPB and 17β-estradiol, the peak areas observed were much higher compared to internal standards and other fractions in

which these compounds were seen. It is possible that at such high concentrations these compounds could have caused significant cytotoxicity of the yeast. As explained with aqueous concentrate cytotoxicity, the dose-response data showed cytotoxic response with EPB at concentration levels more than 100 mg/L. For E2, the same was observed at concentrations approximately above 0.013 mg/L. This strongly supports the above assumption of cytotoxic response being caused by high concentrations of EPB and E2.

As mentioned in Section 3.1, the DI water fractions screened for estrogenicity did not exhibit any estrogenic activity nor cytotoxicity in the YES assay. This provided a validation to the framework procedure and the toxic responses demonstrated by the synthetic water fractions.

The separation framework developed using synthetic water sample can be used for separating any water or wastewater sample into fractions based on the nature of the sample's constituents. High quality waters such as drinking water, groundwater, etc. could be directly subjected to the separation framework without pretreatment while more complex samples such as raw sewage, wastewater effluent, produced water samples (from oil and gas wells) must be filtered prior to the separation to prevent interference of already existing microorganisms or particulate matter.

As discussed in the beginning, the method is an attempt to make toxicity assays more reliable and informative by dividing the compounds. The results showed that by separating the fractions, it is easier to understand the nature of the fraction that generated a positive response in the toxicity assay and hence informs about the class of the toxic compounds, e.g., nonpolar adsorbing, or mid-polar non-adsorbing or highly polar salts. Lahr in 2003 and Escher *et al.* in 2013 studied that there is a huge knowledge gap in toxicity assay response data and regulated contaminant lists,

resulting in a lot of unexplained effects caused by unknown chemicals (Escher et al., 2013; Lahr et al., 2003). Brack in 2016 reported the need to have methods developed based on effects-directed analysis to understand and identify the toxic compounds (Brack et al., 2016). The method developed as a part of this thesis was intended to serve the above stated purpose of implementing an EDA based separation framework to improvise toxicological assessment of complex water samples and to aid toxic compound identification, and the corresponding results are in agreement with the hypothesis of the study. Further, the framework can be optimized according to research requirements and can be coupled with different toxicity assays based on the toxic endpoints being tested.

CONCLUSION

A separation framework has been developed and tested using a simulated mixture of known estrogenic chemicals, and an estrogenic bioassay. The LC-MS and GC-FID analyses of simulated mixture fractions showed the presence of estrogenic compounds in four of the fractions. Neither estrogenic activity nor compound peaks were observed in the other two fractions: aqueous flow-thru and pentane flow-thru. The results of this study validated our initial assumption that the effects-directed analysis (EDA) approach with an *in vitro* bioassay coupled with simple separation techniques followed by analysis using advanced tools (LC-MS and GC-FID) might be a cost-effective and helpful technique in characterizing a complex water sample.

Efficient characterization of environmental samples would help not only in identification of unknown toxic compounds but might also inform about their physical and chemical properties which would be helpful in modifying/upgrading the removal methods in a water/wastewater treatment plant. Moreover, the non-specificity of the techniques used in the framework increases its applicability to a wide variety of compounds in water and wastewater. The method itself can suggest a direction for selection and optimization of treatment for removing identified compounds. For instance, most of the compounds in the synthetic water used in this study were seen in SPE concentrate fractions, suggesting that an adsorption process with activated carbon might be an effective removal strategy which could also be combined with tertiary treatment techniques to break down the organic compounds observed in the fractions.

Similar studies can be carried out on different types of environmental water samples to localize contaminants and apply separation techniques at lab scale. The framework provides flexibility to be customized by replacing the recombinant yeast

assay (YES) with a bioassay of desired endpoint (for e.g. bacterial mutagenicity assay such as AMES to detect genotoxic compounds), or by optimizing solvent selection and adsorption media to suit specific research needs. Moving further, this EDA based separation framework can be considered as a first step of an in-depth research study where it could be used along with chromatographic separation (e.g. fraction collector) to isolate individual compounds for subsequent toxicological assessment and determine which compound is causing the specific toxic response. This could further guide the selection of treatment approach, optimized based on the information and data acquired, rather than evaluating each possible removal technique for the whole environmental sample which can be time-consuming. Furthermore, the framework can find its applications in priority research areas such as identifying unknown compounds and understanding unregulated chemicals such as contaminants of emerging concern (CECs). This research contributes towards bridging the knowledge gap between identification of toxic compounds and development of targeted removal strategies, while attempting to keep the method cost-effective, simple, understandable, and implementable in any water quality lab.

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APPENDIX I

Liquid-liquid extraction (LLE):

Liquid-liquid extraction involves adding an extracting solvent to the sample followed by selective partitioning of analytes versus contaminants between two phases. Once the solvent is added, physical mixing is done by vortexing or shaking before the two phases are allowed to separate. The mixing step helps solute molecules to redistribute and disperse into extracting solvent phase (dispersed phase). The agitated mixture is then allowed to settle, and phases are separated once again, now with solute molecules mostly present in extracting solvent.

Separation is carried out either by pipetting or "freeze-pouring" in which the aqueous layer is allowed to freeze so that the organic layer can poured off easily. Most common solvent used in LLE for extraction are ether, ethyl acetate, methyl tertiary-butyl ether ("MTBE"), methylene chloride, hexane, chloroform, toluene, etc.

Since LLE works on the basic principle of differences in chemical structure, a mixture of chemicals which are chemically diverse in terms of functional groups, polarity, etc. which eventually affect the solubility of the constituents in a specific solvent. Usually, extraction is preferred in case of mixtures with solutes that are relatively less volatile, with solutes that are both inorganic and organic and solutes in small concentrations.

Extractions of triclosan, bisphenol, sulfamethoxazole, fluoranthene and sulfathiazole have been predominantly done by liquid-liquid extraction. While dichloromethane was used as extraction solvent for triclosan, bisphenol and fluoranthene, ethyl acetate has been used for sulfamethoxazole and sulfathiazole.

2.1.2 Drying and Reconstitution:

After collection of extraction solvent along with the solute, the solvent is dried down or reconstituted usually with a different solvent. If the samples are to be sent for a chromatographic analysis, a more compatible solvent may be used for reconstitution since most of the mobile phases in LC-MS are aqueous in nature and are thus incompatible with organic solvents. The mobile phase of LC itself is also used sometimes as reconstitution solvent.

For solutes that are highly water-soluble, separation is achieved by extracting all the non-polar and organic soluble interferences leaving only the desired solute in the aqueous phase.

APPENDIX II

Solid Phase Extraction (SPE)

Solid phase extraction (SPE), uses solid particles (stationary phase) to chemically separate different components in a liquid (mobile) phase. There are four kinds of packing used in SPE namely: Reversed phase, normal phase, ion exchange phase, adsorption packings. SPE is most commonly used for extracting semi-volatile and non-volatile analytes besides solids that are pre-extracted into solvents.

Solid phase extraction has become more popular in the last ten years owing to developments in formats, automation, introduction of new phases and optimization of cartridges. One of the major reasons behind rapid progress in SPE was increasing requirement in laboratories to adopt solvent-free techniques and use as less organics as possible. This method can be performed off-line by separating sample preparation from subsequent chromatographic analysis.

Steps Involved in SPE

SPE primarily involves four different steps: conditioning, sample addition, washing and elution.

a) Conditioning: The most suitable solid phase (sorbent) is selected based on the chemical nature of the desired analyte and then conditioned using an appropriate solvent. While conditioning the functional groups of the sorbent bed are solvated which enables them to interact with the sample.

b) Sample Addition: This is followed by passing the sample (contained in mobile phase) through the sorbent bed during which analytes and some other components in

the mixture are retained onto the solid matrix. Different components react in different ways with the sorbent bed depending on their physico-chemical properties.

c) Washing and Elution: The components present on the solid particles, analytes and interferents, can be subsequently separated by selective washing or selective extraction or selective elution. While selective extraction and washing remove interferents leaving the analytes onto sorbent bed, selective elution removes the analyte of concern leaving the interferents with the stationary phase.

In SPE, choice of stationary phase is an important choice since it influences crucial parameters such as selectivity, affinity and capacity. The physical and chemical characteristics of the analyte drive the selection of sorbent. Eluotropic strength of adsorption on silica and polarity index can provide a measure of solvent's ability to interact as proton donor or acceptor or dipole [Lucci et al., 2012]. Most common type of interactions between sorbent and analyte are hydrophobic, hydrophilic, cationic-anionic and selective antigen-antibody reactions (in the case of immunological assays).

Chemicals like Ibuprofen, kanamycin, 2,4 – Dinitrophenol, chloramphenicol and benzoquinone have been extracted using solid phase extraction cartridges. The nature of cartridge used depends on the chemical and so does the eluent (solvent used for elution).

SPE has become increasingly popular among adsorption or affinity based extraction techniques due to many advantages, most important one being its flexibility to switch phases that are more suitable for subsequent chromatographic analyses. As explained in Section 2.2, SPE follows a simple procedure with four main steps: conditioning, sample addition, washing and elution (3 and 4 are often considered as one step). Based on the stationary phase used, an SPE cartridge is

classified into many categories. Some of the most important types which are of significance for this study are:

1. Normal phase SPE
2. Reversed-phase SPE

3.2.1 Normal phase SPE:

Normal phase SPE is by far the most used technique which has a stationary phase that can retain polar compounds from organic samples using polar interactions such as hydrogen bonding, dipole-dipole and induced dipole-dipole interactions. Typical sample matrices that use normal phase SPE include hydrocarbon or fatty oils diluted in an organic solvent such as hexane, DCM ethyl acetate, etc. Analytes retained onto the stationary matrix are eluted with polar organic solvents like methanol, isopropanol or acetonitrile. Analytes eluted mostly consist of hydroxyl groups, carbonyls, amines and double bonds including functional groups with resonant properties. Normal phase SPE is mostly applied in fractionation of petroleum hydrocarbons, cleanup of organic extracts of soils and sludge and for environmental samples. Polar-functionalized bonded silicas (e.g. LC-CN, LC-NH2, and LC-Diol), and polar adsorption media (LC-Si, LC-Florisil, ENVI-Florisil, and LC-Alumina) typically are used under normal phase conditions.

3.2.2 Reverse phase SPE:

Reverse phase SPE involves a polar or moderately polar sample matrix (mobile phase) and a nonpolar stationary phase. The common interactions are nonpolar-nonpolar interactions and van der Waals or dispersion forces. The analyte of interest is typically mid- to nonpolar. Several SPE materials, such as the alkyl- or aryl-bonded silicas (LC-18, ENVI-18, LC-8, ENVI-8, LC-4, and LC-Ph) are in the reversed phase

category. Retention of organic analytes from polar solutions (e.g. water) onto these SPE materials is due primarily to the attractive forces between the carbon-hydrogen bonds in the analyte and the functional groups on the silica surface. Carbonaceous adsorption media, such as the ENVI-Carb materials, consist of graphitic, nonporous carbon that has a high attraction for organic polar and nonpolar compounds from both polar and nonpolar matrices.