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CUREing Exposure to Environmental Chemicals from Personal Care Products

Erika L. Doctor and Cassandra S. Korte

Lynn University, College of Arts and Sciences, 3601 N Military Trail, Boca Raton, FL 33431, USA
(edoctor@lynn.edu; ckorte@lynn.edu)

This series of laboratory modules presents students the opportunity to perform in-classroom research while building their working knowledge of the scientific process and growing their skills in analytical methods. In the laboratory, students analyze samples of urine collected as part of an intervention study focused on reducing exposure to phthalates, potentially harmful compounds found in personal care products. Students propose methods of extraction and detection, analyze samples from the intervention study, analyze data, present results, and propose future iterations of the intervention-study. In this workshop, participants experience the online student experience through the steps of extracting phthalates from urine samples, analyzing HPLC data, and proposing future research directions based on their HPLC data-derived conclusions.

Keywords: course based undergraduate research, organic chemistry, biochemistry, traditional instruction, remote instruction

Link To Supplemental Materials: https://doi.org/10.37590/able.v42.sup5

Introduction

Exposure to phthalate esters is common. Phthalates are mainly used as plasticizers, i.e., substances added to plastics to increase their flexibility, transparency, durability, and longevity (Wang et al., 2019). These compounds are commonly found in personal care products, such as liquid soap, conditioner, and deodorant (Harley et al., 2016). Our in-class research study is focused on exposures resulting from nail polish use. Phthalates are added to these products to provide flexibility of the coating and increase adherence to the nail (Craig et al., 2019). We also focus specifically on nail polish because the Environmental Working Group Skin Deep Database lists nail polish as a major source of phthalates, such as polyethylene terephthalate (Lunsford et al., 2018). Furthermore, nail salon workers tend to have higher levels of phthalate exposure after work shifts (Craig et al., 2019) and nail polish can act as a source for environmental chemical exposures in humans (Mendelsohn et al., 2016).

Thus, phthalate exposures are of public health interest—which is relatable to biology majors interested in the health sciences and the broader group of biology students, generally. Among other health effects (Radke et al., 2019, Dutta et al., 2020), previous research indicates that phthalates likely act as endocrine disruptors. These types of compounds interfere with normal physiological hormone action (La Merrill et al., 2020). This is important because the susceptible population of people who can bear children use personal care products like nail polish at higher rates than the general population, and their resultant exposures are thus fairly high (Harley et al., 2016). In our exposure assessment research study, nail polish-wearing participants collected their first urine void, removed nail polish and collected first voids on days 3, 5, and 7 of the intervention. The aim of this work is to measure how phthalate levels change over time after the removal of nail polish. This article provides student handouts and example protocols for our exposure assessment course-based undergraduate research experience (CURE) (Auchincloss et al., 2014) which is run in the second-

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year organic chemistry II lab housed within our biology major. This lab series was tested in fully face-to-face format in Spring 2019. During Spring 2020, the course transitioned to emergency remote teaching; this transition occurred between skill-building introductory cookbook-style laboratories and participation in the exposure assessment CURE. In Spring 2021, the course was offered in a blended-hybrid format, with half the students attending in-person and the other attending remotely via Zoom. Students switched attendance mode each class period. Other students in Spring 2021 attended the laboratory class fully remote. Instructor guidance is provided for both modalities: in-person and remote/online instruction. The course enrollment across each of these years was approximately 40 students, and the course was offered in two sections. This CURE, or an adaptation of it, would be appropriate for students in organic chemistry, biochemistry, environmental health, or public health courses with some modification based on course context, student learning outcomes, and student background knowledge.

The handouts and protocols provided below give students the opportunity to propose their own sample extraction method, based on their prior experience with the cookbook-style labs and searches of the primary literature. Students then extract human urine samples based on the most feasible proposed method (we have provided an example student-proposed method based on protocols from Genuis et al., 2012 and Cai et al., 2007), hydrolyze their phthalate-containing samples of glucuronide, and detect monomethyl phthalate and monoethyl phthalate via manual injection with 254 nm detection by high-performance liquid chromatography (HPLC).

Student Outline

Objectives

Gain research experience while contributing to the scientific body of knowledge
Explain sources of environmental exposure
Distinguish and properly conduct methods of liquid-liquid extraction and HPLC detection
Quantitatively assess collected data
Propose a feasible method of analysis for assigned sample
Design a future research experiment

Activity 1 - Extraction of Phthalates from Urine

Objectives

1. Explain metabolic processes that result in conjugated metabolites
2. Explain and perform procedures of enzymatic hydrolysis
3. Explain chemical principles underlying liquid extraction
4. Perform extraction of phthalates from participant urine samples

Background

As previously discussed, this exposure assessment study will focus on examining exposure of phthalates from nail polish. Participants were asked to provide their first morning void while still wearing nail polish (Day 1), then they removed their polish. On Day 3, Day 5, and Day 7, participants also provided a sample of their first morning void. In order to determine the types and quantities of phthalates that were present, these phthalates first need to be extracted from the urine samples.

This procedure focuses on the two most common phthalates found in nail polish – dibutyl phthalate and polyethylene terephthalate. Phthalates are all polar due to the phenyl ester base structure. However, they have varying degrees of polarity based on the species that make of the R groups (see Figure 1). For example, dibutyl phthalate is more polar than polyethylene terephthalate due to the length of the carbon chains as part of the R groups.

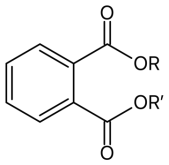

Figure 1: Phthalate base structure

After absorption in the body, these phthalates undergo metabolism to produce glucuronide-conjugates (see Figure 2). The addition of glucuronide increases water-solubility, thereby favoring elimination of phthalates through urinary excretion. In order to increase the sensitivity of the extraction and detection techniques, the glucuronic acid part of the phthalate glucuronide must be removed through acid hydrolysis.

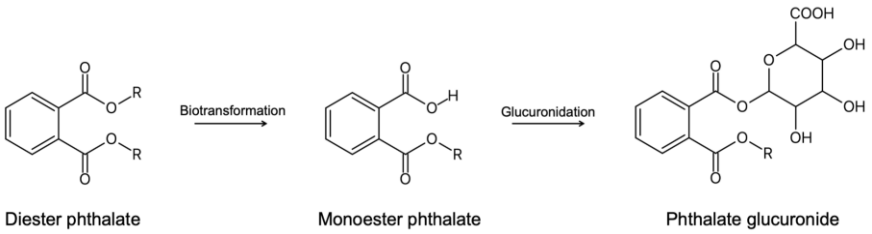

Figure 2: Metabolism of Phthalates

Timing

This activity will take 180 minutes total.

Remote/Online students

You have been provided with a video demonstration of this process. In order to receive credit on completion you must watch the video in its entirety and answer the quiz questions embedded in the video. Review the below procedure prior to viewing the video.

Materials for each group

- 6 x 2 Dram clear glass vials
- 6 x 1 Dram amber vials
- Two urine samples
- Acetate buffer (pH = 6)
- 2-propanol (HPLC grade)
- $MgSO_4$
- 1000-µL pipette
- 1000-µL pipette tips
- β-glucuronidase

PRECAUTIONS

This lab requires using human biological samples; therefore, lab safety is of utmost importance. All personal protective equipment must be worn at all times. When handling samples, gloves must be worn. Remove gloves if you handle anything else. All disposables must be placed into the biohazard waste container (gloves, tips, vials, etc.). Glassware will be collected to be autoclaved.

Procedures

Each group will be given two urine samples from a participant (another group will have the other two samples). Each group member will extract one aliquot from each of the samples. Make sure to label ALL vials with permanent marker. **For all measurements disposable pipette tips will be used.**

Enzymatic hydrolysis (Genuis et al., 2012)

1. Pipette 2 mL of urine into an 8 mL clear glass vial
2. Add 1 mL of acetate buffer (pH = 6) and 20 µL of β-glucuronidase
3. Cap the vial with a small piece of aluminum foil and place in water bath at 37˚C for 90 minutes
4. Allow to cool to room temperature before starting liquid-liquid extraction

Liquid-Liquid extraction (Cai et al., 2007)

1. To the vial, add 1.5 mL of 2-propanol, cap with the lid and shake to make a homogenous mixture
2. Add 1.3 g of $MgSO_4 \cdot 7H_2O$ or 0.63 g of $MgSO_4$ anhydrous salt to the vial and shake until dissolved. Two distinct layers should begin to form
3. The top layer (2-propanol), will be removed carefully (Do not take any of the aqueous phase) using a pipette set at 1000 µL and placed into a labeled amber vial for future analysis

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Activity 2 - Separation and Detection of Phthalates Extracted from Urine

Objectives

1. Describe the principle of HPLC as it relates to phthalates
2. Using principles of polarity and partitioning, predict elution order of phthalates from a mixture

Background

After extraction, separation of extracted analytes from other compounds that may have coextracted is an important step before quantitation. In high performance liquid chromatography (HPLC), compounds are separated based on their partitioning between the mobile phase and the stationary phase. Here a reversed phase system is used, where the stationary phase is non-polar and the mobile phase is polar. The less polar an analyte is, the longer it will take to elute and be detected by the UV-visible detector.

The UV-visible detector for this experiment as a set wavelength of 254 nm. Due to the benzene ring present in all phthalates, they absorb light at this wavelength producing high peak areas at lower concentrations than what we observed for caffeine.

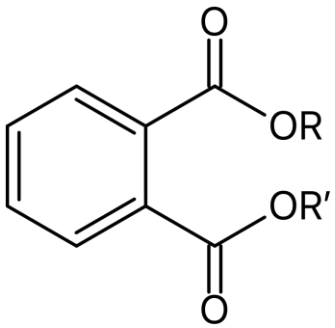


Figure 1: Phthalate base structure

In order to identify the phthalates extracted from urine and separated by HPLC, a standard mixture of 6 phthalates (benzyl butyl phthalate (BBP), bis(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP), di-n-octyl phthalate (DOP), diethyl phthalate (DEP), and dimethyl phthalate (DMP)) was analyzed under the same conditions as your extractions will be. The peak area observed is proportional to the concentration of each phthalate. Calibration curve data will be provided during data analysis.

Timing

Each sample run and cleanup takes 45 total minutes.

Remote students

You have been provided a video on Canvas of this injection process. Make sure you review the procedure and watch the video at the beginning of the class period.

Materials

- Buck Scientific BLC Series isocratic HPLC with UV detector of 254 nm
- 100-µL HPLC gas tight syringe

- Pinnacle DB AQ C18 5 µm column, column length 250 mm, inside diameter 4.6 mm
- Mobile phase (made with 35% HPLC-grade water, 20% HPLC-grade propanol, 40% HPLC-grade acetonitrile, and 5% HPLC-grade methanol)
- Extracted urine samples from Activity 1

Procedure

1. A mobile phase was prepared containing by volume: 35% water, 20% propanol, 40% acetonitrile, and 5% methanol
2. The HPLC instrument was previously set up to have flow rate of 1 mL/min of the isocratic mobile phase
3. The Peak Simple software will be open with parameters set to record data for 20 minutes
4. Press “zero” on the front of the HPLC to remove the background absorption of the mobile phase
5. To run your unknown sample:
    a. Pull 50 µL of your urine extract into a needle syringe
    b. The injection port should be turned to “Load”
    c. Push the needle into the injection port and inject your sample
    d. Turn the injection port to “Inject” and press the spacebar on the keyboard to start recording data (this must be done immediately with no delay between these steps.
6. Once the 20 minutes are done, continue running the pump for 20 minutes
7. Save you file in the folder labeled “SCI 391 X” with the following file name:
    a. Initials_SCI391_Participant number_day number
    b. You will also save a screen shot of your chromatogram and your data table (contains peak area) – This will be emailed to you
8. This procedure will be repeated over the next 3 weeks in order to analyze the urine samples that were extracted

Post-Lab Worksheet

1. How does the mobile phase compare chemically to the mobile phase used for caffeine? (Do not just tell me the chemicals, but what do they do? E.g. Polarity, pH, etc.)

2. The following chromatogram was produced under the same conditions as your urine extracts will be:

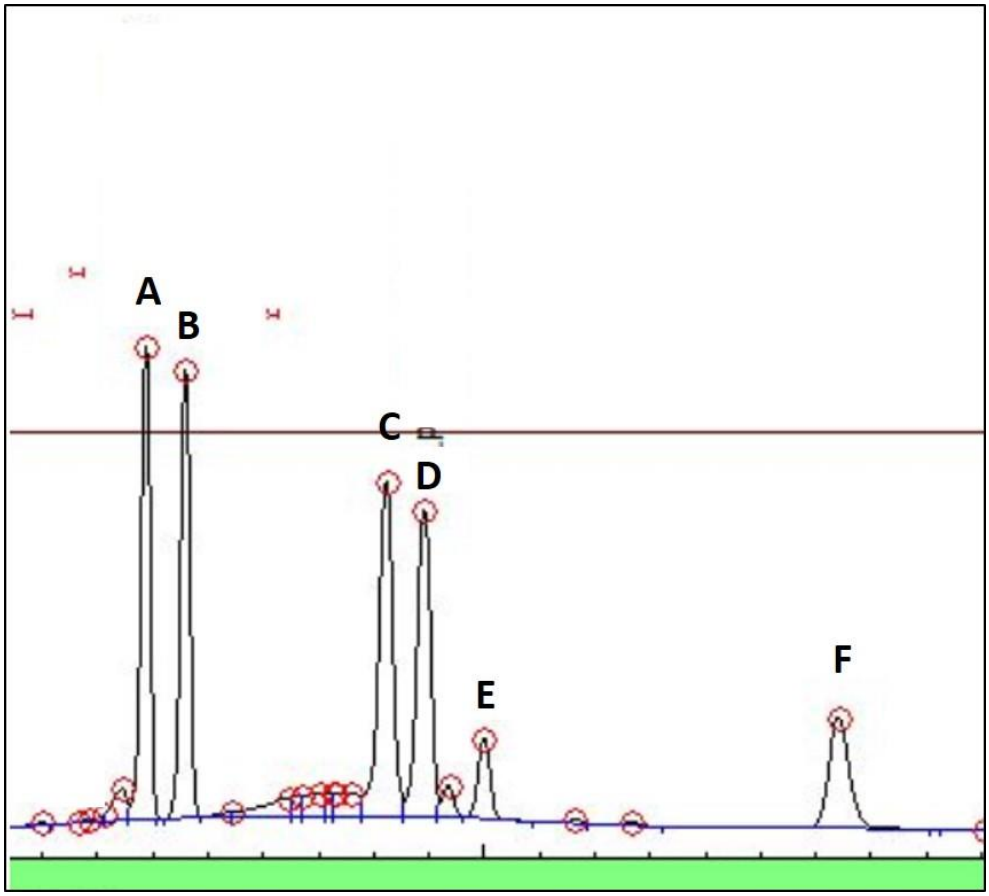


Figure 2: Chromatograph of separation of 6 phthalate standards.

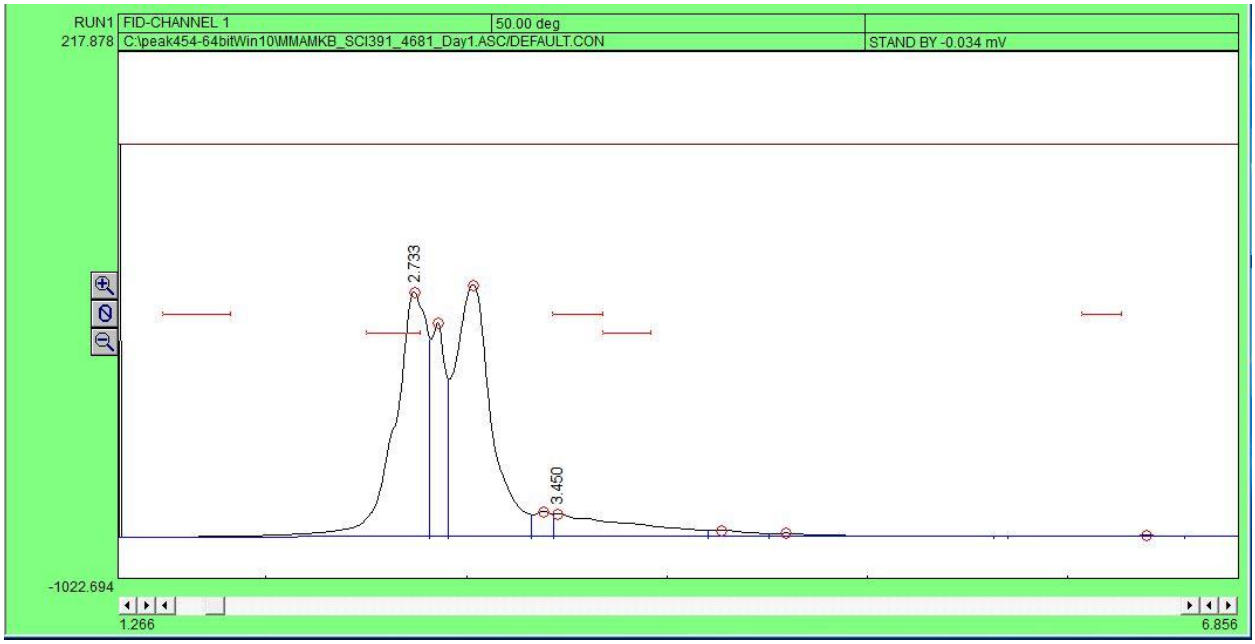
Fill out the following table including the elution order of the phthalates. Draw the structures of R and R' of each phthalate and provide the corresponding letter for the phthalate from the chromatogram (A, B, C, D, E, F).

Phthalate	**R**	**R'**	**Elution Order (Letter from Chromatogram)**
benzyl butyl phthalate (BBP)			
bis(2-ethylhexyl) phthalate (DEHP)			
dibutyl phthalate (DBP)			
di-n-octyl phthalate (DOP)			
diethyl phthalate (DEP)			
dimethyl phthalate (DMP)			

3. Explain how you arrived at this elution order.

Activity 3 - Phthalate Peak Area Data Analysis

In the attached Excel spreadsheet you will find all the data collected for the semester between both classes. There are two tables presented, one for the dimethyl phthalate (DMP) metabolite monomethyl phthalate (MMP) and one for diethyl phthalate (DEP) metabolite monoethyl phthalate (MEP), with peak heights obtained over the 7 days of participant collection. Feel free to use the spreadsheet to perform your calculations. Peak areas were obtained from your chromatograms like the one pictured below.



To analyze these samples, you will be calculating the percent change for each participant compared to their Day 1 samples. For example:

$$\frac{(peak\ area\ Day\ 3 - peak\ area\ Day\ 1)}{peak\ area\ Day\ 1} \times\ 100\% = percent\ change\ in\ phthalate\ on\ Day\ 3$$

If % change is negative, that shows a decrease in the amount of phthalate compared to Day 1. Whereas if % change is positive, phthalate levels have increased compared to Day 1. You will be preparing a plot of percent change for each participant over the 7 day study period for each phthalate. Day 1 will be plotted as 0%. You can do this with two graphs, one for each phthalate, with five curves on the same graph, one for each participant. You will be including these graphs in your posters.

Post Lab

In your notebook include a table that contains all these calculations (you can tape in a printout with your Excel calculations or do them by hand) and answer the following post lab questions.

1. Why do you think that we need to look at the percent change in the amount of phthalates and not the average peak area for each day? [HINT: Think about the differences in the Day 1 and where those may come from.]
2. Based on the collected class data, did the intervention work? Did we see a decrease in the amount of phthalate after the participant stopped using the nail polish? Consider what happened at Day 3, Day 5, and Day 7. Remember Day 1 was right before the nail polish was removed. If there was an increase in the % change of phthalate, what does that mean?
3. Each data point is a single HPLC run of one sample. Considering random errors, what would need to be done to improve this set of data?

Materials

This section contains a general summary of materials for class of 25 and equipment needed to carry out the activities outlined in the previous section. If you are running this lab remotely, the amount of materials will depend on how much data you want to provide the students. All the data from these modules can be collected ahead of time. It is helpful to have teaching assistants or research assistants who can do the HPLC runs independently.

For All Activities

- Gloves
- Goggles
- 1000-µL pipette tips
- 1000-µL micropipetters (10 total)
- Computers
- Balances (do not need to be analytical, need to measure to one decimal place)

Activity 1 Setup

For each group of students

- Water bath at 37˚C (for enzymatic hydrolysis)
- 6 x 2 Dram clear glass vials (Fisher)
- 6 x 1 Dram amber vials (Fisher)
- Two urine samples (previously collected through proper IRB)
- Acetate buffer (pH = 6)
- 2-propanol (HPLC grade)
- $MgSO_4 \cdot 7H_2O$ or $MgSO_4$ anhydrous salt
- 1000-µL pipette
- 1000-µL pipette tips
- β-glucuronidase (Fisher)

Activity 2 Setup

For whole class

- Buck Scientific BLC Series isocratic HPLC with UV detector of 254 nm
- 100-µL HPLC gas tight syringe
- Pinnacle DB AQ C18 5 µm column, column length 250 mm, inside diameter 4.6 mm
- Mobile phase (made with 35% HPLC-grade water, 20% HPLC-grade propanol, 40% HPLC-grade acetonitrile, and 5% HPLC-grade methanol)

For each group of students

- Extracted urine samples from Activity 1

Activity 3 Setup

No additional setup

Notes for the Instructor

This research project is performed during a 3 hour long Organic Chemistry II laboratory course. It is adaptable to other courses such as biochemistry, depending on the analyte you choose to examine. The CURE itself involves biological samples, human urine, and thus requires an approved IRB from your institution, however, the methods can be adapted for other analytes such as environmental samples or personal care products themselves that will not require an IRB. Table 1 shows the overall course schedule. The sections that pertain to this research project are bolded. In this schedule, there are traditional organic chemistry laboratories interspersed. The HPLC runs only take a few minutes to inject with about 40 minutes of down time between injections, so to keep students engaged, we perform other experiments in the down time.

WEEK	EXPERIMENT
1	Introduction
2	**Lecture on Methods**
3	Extraction of Caffeine
4	HPLC of Caffeine Isolation of Chlorophyll from Spinach
5	**Introduction to Phthalate Project**
6	**Extraction Proposals**
7	**Extraction/Sample Prep**
8	**Extraction/Sample Prep**
9	**HPLC & Ethics of Human Research**
10	**HPLC** Isolation of Cinnamaldehyde
11	**HPLC** Formation of Aspirin
12	**Data Analysis**
13	**Poster Proposals**
14	**Poster Proposals and Critiques**

Table 1: Structure of the course with embedded CURE

At the start of the course (Week 2) students are given a lecture that covers the concepts of

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polarity, extraction, partitioning, and HPLC separation and detection. Where this course is offered in our biology curriculum, students have only previously been exposed to the topic of polarity, so this lecture is included in order to provide them with the necessary background information. If you implement this in a course where these topics are already discussed, you may not need to provide the lecture.

After this initial scientific background, students spend two weeks (Weeks 3-4) working on a "cookbook" style experiment where they extract and quantitate the amount of caffeine in cold brew coffee. These experiments expose them to all the techniques they will be using in the research project.

Once the cookbook experiments are completed, students are introduced to the research project involving phthalates (Week 5). During this class session we discuss the IRB (which is provided to students) for the intervention research project and the methodology of the participant sampling in that study. We also go over some basics of toxicology: how phthalates absorb, metabolize, and distribute in the body, chemical properties of phthalates, and why examining these types of compounds is important. This background information provides students with the why and the what information for the research project. An example of this background lecture can be found in the Supplemental Materials. During the next class period (Week 6), students apply this background knowledge and what they have learned in previous weeks about extraction to propose their own extraction methods to remove phthalates from urine. Students work in groups and spend 2 hours researching feasible methods and then the rest of the class period presenting their methods. The most scientifically sound, feasible method is used for the rest of the experiment by the entire class. The method provided in Activity 1 – Extraction of Phthalates from Urine, is a student proposed method.

Weeks 7 and 8 are spent performing the sample extraction (Activity 1). This usually only takes one 3-hour class period, however, if your lab period is shorter, you may need to perform part of the extraction and finish in a second period. Activity 1 can be completed in a regular laboratory classroom if there is a biological waste container for tips and used vials. Depending on the length of the laboratory, a good cutoff is after the enzymatic hydrolysis, which takes about 2 hours. In a three-hour class you may have to encourage students at the beginning to work quickly to leave enough time for the liquid-liquid extraction. During the enzymatic hydrolysis, we review the importance of this step and what it does for our samples. We want to look at the free phthalate and our peak intensities will go up. We also discuss that we will not recover the whole phthalate, but a hydrolyzed form. For example, dimethyl phthalate will convert to monomethyl phthalate after enzymatic hydrolysis in vivo. For remote students, they are still required to be in the class on Zoom so this discussion still includes them. The liquid-liquid extraction produces two very distinct layers once all the components are added to the vial. Students are reminded that is better to leave some of the organic phase behind rather than taking off some of the aqueous phase. They commonly panic about this, but an explanation that we will measure concentration, not the total amount extracted, helps with them overcome the idea of leaving something behind. This section is ended with a discussion about which phase we are going to see the phthalates during the liquid-liquid extraction.

After extraction, samples are separated and detected through HPLC for the next 3-4 weeks of the course using Activity 2 – Separation and Detection of Phthalates Extracted from Urine. Each sample run and column cleanup takes 45 total minutes. The total time required for completion depends on number of total samples and may require outside class time to complete. Our research assistants complete all the class samples outside of class time. Due to our equipment limitations, only one sample can be run at a time through manual injection, however, if you have the availability of an autosampler, these samples can be run overnight, and the experiment will take much less time. If not covered in previous course material, the principles of HPLC and partitioning may need to be added to this laboratory module. While remote students are provided with a video for the injection process, this only takes about a minute, so the video is very short. Depending on the circumstances of the course, in person students may not actually do the injection either but the worksheet is very important in understanding the elution order as well as the next step of data analysis. HPLC methods are described in the Activity 2 - Separation and Detection of Phthalates from Urine, it is important to note that the method is specific for the instrument used, Buck Scientific BLC Series isocratic HPLC with UV detector of 254 nm, depending on your own instrument limitations, you will have to optimize the method, including the mobile phase. During the downtime of the HPLC runs, students are provided with worksheets to determine elution order for the potential phthalates we may encounter in our samples. They are also exposed to the ethics of human research as it applies to biological samples (Week 9). At the end of this research project, we want

students to propose their own future project, and this gives them the information they need to propose a study and to start thinking about the type of project they may want to propose.

In concert with HPLC runs, students are also performing traditional organic chemistry laboratory activities in Weeks 10-11. Once all the HPLC data is collected, a research assistant pulls the peak areas from all the data files and enters it into a SCI 391 Results Table spreadsheet. During the Data Analysis class (Week 12) students are given an example of the raw data (see Activity 3 - Data Analysis) as well as an Excel spreadsheet containing all the peak area data contained in the SCI 391 Results Tables. Students are then asked to prepare a graphical representation of the data. We discuss in class what peak areas are actually measuring. We also review resolution. Additionally, we discuss how we can make the best representation of the data for the question we are trying to answer. On Canvas students are provided with a video of how to use the Excel document. If students only have access to the application version of Excel (iPad or online) then students will not be able to create the graphs and you may have to provide a separate Excel with the graphs formulated and they just insert the data. Students are provided with a spreadsheet of the peak areas, but they must do their own calculations in the program. Students complete this in class and these graphs are reviewed before they leave. There is usually time left in the three-hour class period where students can start looking at these results and begin a discussion with their group about future iterations of the study and their own proposals.

Students then work in groups during the rest of Week 12 and Week 13 to examine the data, determine what it tells us about our study, and to create a poster presentation proposing a new direction for the research. These new studies can make any changes to the previous study but must still be an exposure assessment for potentially harmful chemicals in personal care products.

Remote/Online Students

In Spring 2020, we performed the entirety of these modules online. Those remote/online students were provided with video tutorials of the processes used to collect the data but still participated through Zoom in all classroom discussions and group activities. In Spring 2021, we had split instruction, with some students opting to be entirely remote while others are in the classroom. Students in the classroom performed the techniques so those procedures have been provided with modifications for remote/online students. Videos were provided through our learning management software, Canvas, which records whether the video is viewed and has capabilities of embedding quiz questions. The questions are based on the procedures of the activities to make sure students viewed the video.

Answers to all worksheets and post-lab questions are available upon request to the authors.

Conclusion

In conclusion, we have shown a course-based undergraduate research experience geared toward our student population interests which can be embedded into courses ranging from organic chemistry to biochemistry depending on the focus of the analyte. This project has been assessed for student learning by comparing student exam gains in a completely in-person iteration of the course and the remote semester of Covid-19 and no statistical difference was found (Doctor et al., 2020b). Additionally, the in-person iteration of the course was previously assessed showing students had learning gains and an increase in their self-efficacy (Doctor et al., 2020a). Overall, this project provides students with a real-world problem to solve through scientific inquiry.

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About the Authors

Dr. Erika L. Doctor has been a faculty member at Lynn University since 2015. She's the academic program coordinator for Lynn's forensic science programs and teaches chemistry lab within the biology major.

Dr. Cassandra S. Korte has been a faculty member at Lynn University since 2014. She's the academic program coordinator for the Masters of biological sciences program. She teaches biology lectures and labs primarily in the first year and in the environmental science and policy program.

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