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Introduction and Previous Focus of Study

Endocrine disruptors in an era of industry are under the scrutiny of epidemiologic studies. Chemicals such as phenols and phthalates often used in manufacturing have been shown to interfere with human health. Among these chemicals, BPA is a key player. Many studies have shown that exposure to BPA, a compound found in many household products and plastics, leads to a wide range of adverse health effects [1]. Considering the amount of plastic incorporated into everyday products and contamination in the environment, exposure to BPA is chronic and a cause for concern. Canada, the European Union, and major US manufacturers have already banned the use of BPA as well as other endocrine disrupting chemicals due to evidence of their interference with normal biological processes [2].

Among the concerns of the general public is the fear for women's breast health. BPA contains structural similarities to estradiol, the most abundant form of estrogen. While estrogenic activity of BPA is quite weak compared to endogenous estrogens, there are some findings that associate BPA exposure with increased breast cancer risk [3]. There is evidence that BPA may alter cytokine levels in the breast, resulting in a pro-inflammatory immune profile associated with breast density [4]. Breast density is a known risk factor for breast cancer, connecting BPA exposure with breast cancer risk. Therefore, determining the correlation between BPA and cytokines in breastmilk is a first step towards understanding the extent to which exposure to BPA might increase the risk of developing breast cancer by inducing an inflammatory profile in the breast, and thereby addressing the concern of the public.

The main aim of this honors thesis is to analyze the association between BPA levels and inflammatory cytokine levels found in breast milk. Previous studies on BPA exposure in the breast led the Arcaro lab to perform an analysis of inflammatory cytokines in 21 breast milk

samples for which BPA levels were already assessed. As shown in Figure 1, there was an association between high levels of BPA and the inflammatory cytokines leptin, IL-6, and C-reactive protein (CRP). Although the association is interesting, it is not significant. There are several limitations in this study design that could have led to the lack of a significant relationship between BPA and cytokine levels. The primary concern is that the 21 breast milk samples were collected from women nursing babies of vastly different ages (14-780 days), and cytokine levels may vary over the duration of the lactation period.

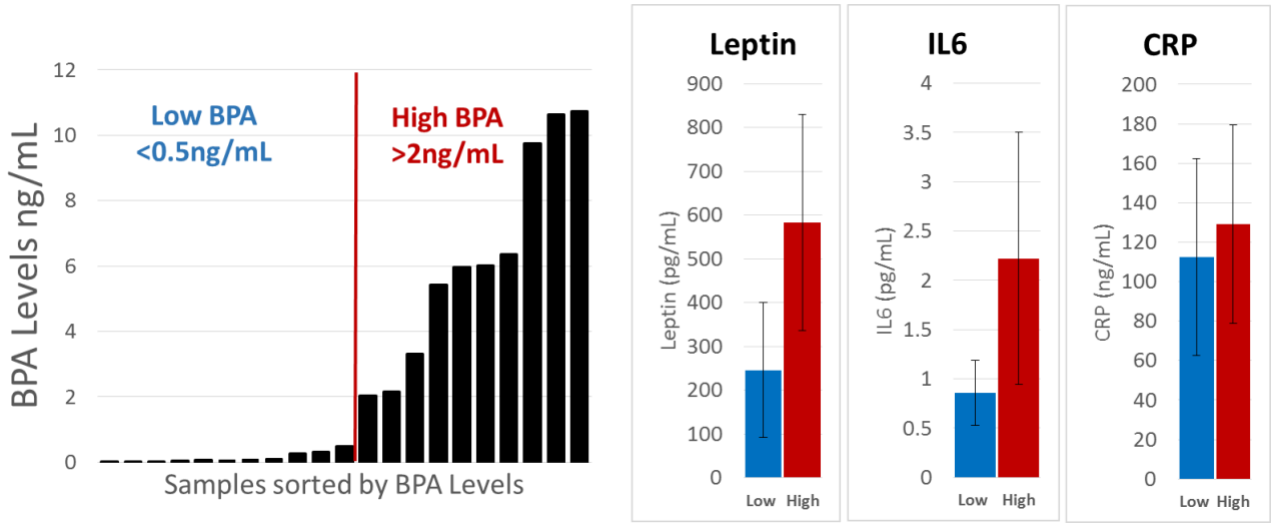


Figure 1. Relationship between BPA levels and cytokines in breast milk from 21 women. Women with higher BPA levels showed higher levels of leptin, IL-6, and CRP.

As originally planned for this thesis, I began analyzing 32 breast milk samples obtained from 16 primiparous women at 6 and 8 weeks postpartum, as shown in Figure 2. Two time points were selected because this should allow a more accurate assessment of the relationship between BPA exposure and cytokine levels, as BPA is metabolized and cleared from the body. For example, if a participant ate a lot of canned food the day prior to providing a milk sample their BPA measurement might be high and I would expect an increased inflammatory profile. On the other hand, if BPA exposure was very low (as estimated by answers to questionnaires), we

would expect their BPA level to be low and to see a reduced inflammatory profile. Through liquid chromatography and tandem mass spectrometry, I will extract and analyze BPA levels found in breast milk. Using an electrochemiluminescence assay, inflammatory cytokine levels will be assessed in each sample of breast milk. Determining this correlation and the extent to which BPA increases inflammation in the breast will provide insight into a possible mechanism behind environmental exposures affecting breast health.

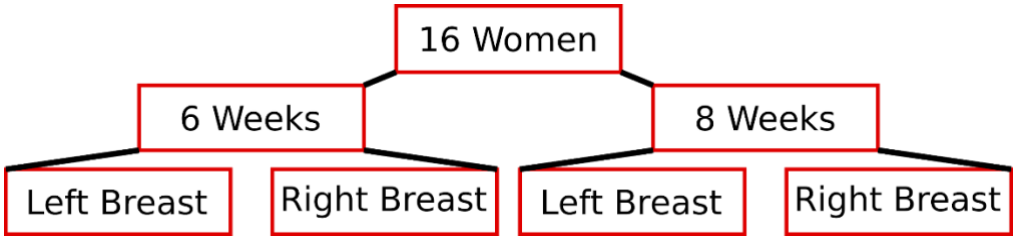


Figure 2. The samples collected from 16 women followed this study design. At 6 and 8 weeks, each woman provided left and right breast milk samples. For the current analyses, we combined an aliquot from the left and right breast and are analyzing this combined sample.

Due to the current COVID-19 pandemic and suspension of laboratory access as of March 23rd, 2020, sample analysis has been delayed until further notice. In terms of this thesis project, I will focus on the practice data collected from August 2019 through early March 2020 that was used to optimize our methods and test the performance of the UPLC instrument. Although I did not complete the cytokine assays or analyze the actual study samples, this practice data is just as important to my analysis as the actual study sample data and has provided an important learning experience. In order to proceed through this project with consistency and reliability, practice trial results acted as method diagnostics to point out areas of weakness or instrumental dysfunctions. Without perfected practice results, I would not have been able to move forward in this project.

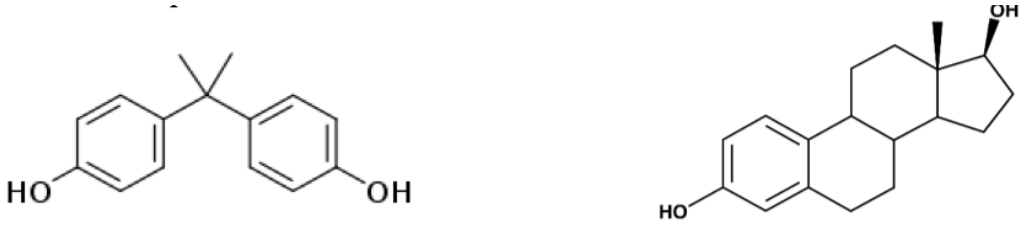
The logic behind my original intentions along with my newly revised thesis plan will be outlined in the following literature review.

Literature Review

BPA: An Endocrine Disruptor

Bisphenol A (BPA) is a synthetic compound industrially used to induce plasticity in many household products, such as shampoo bottles, or commonly used to coat the inside surface of metal food cans [5]. BPA is known to leach, or migrate from plastics and other materials into dust, food stored in plastics, and eventually into the environment [6]. Due to its presence in the environment and incorporation into a wide range of daily used products, humans are exposed to BPA through multiple routes of contact: oral exposure, transdermal exposure, and inhalation. A recent national report showed that a majority of people in the U.S. had detectable levels of BPA in their urine, indicating that these daily exposures are pervasive [7].

The pervasive nature of BPA brings into question its biological activity when introduced to human tissues. BPA is a known endocrine disruptor, a chemical that interferes with the endocrine system to produce adverse developmental, reproductive, neurological, and immune effects. The mechanism by which many endocrine disruptors act is through mimicking natural hormones and interfering with hormone receptor activity [8]. BPA contains structural similarities to that of the hormone estradiol, as shown in Figure 3.



Bisphenol A 17β-Estradiol

Figure 3. Structural similarity between Bisphenol A and 17β-Estradiol. The estrogenic activity of BPA is between 1,000 and 10,000 times lower than the natural hormone.

Although structural similarity provides potential to interfere with endogenous receptor activity, the potency of BPA is between 1,000 and 10,000 times lower than the potency of 17β-estradiol. Weak potency and low daily exposure levels question the extent to which BPA exposure poses a health risk. As of 2004, numerous studies have demonstrated significant effects of exposure to low doses of BPA, including at or below what is considered to be a safe reference level of 50 µg/kg per day [9]. A large number of *in vitro* studies revealed an estrogenic mode of action even at low exposure levels, where xenoestrogens such as BPA triggered estrogen-response mechanisms in doses as low as 1 pM [10]. In human and animal studies, the estrogenic activity of BPA has also been observed to affect metabolism and obesity, increase diabetes risk, induce polycystic ovaries syndrome, and increase risk of hormonal cancers in response to environmental exposures [1]. Through these studies, there is strong evidence that low-dose daily exposures are still a cause for concern despite low potency.

BPA and Breast Health: Breast Cancer Risk

While many BPA-induced estrogenic effects have been studied, not many studies explicitly examine the effect of BPA on breast health and breast cancer risk. As a hormonal cancer, it is possible that BPA may initiate breast cancer formation through estrogen receptor

activity. However, the estrogenic activity of BPA is weak and findings among the few studies that do focus on BPA exposure and breast cancer risk are inconsistent. A Korean case control study found a higher median level of serum BPA in cases than controls but the difference was not significant [11]. A Canadian case-control study observed striking associations between breast cancer risk and working in plastics manufacturing and in food canning [3]. However, a recent case control study found no association between a single assessment of urinary concentrations of BPA and breast cancer risk [12]. To date, it is unclear as to whether BPA's estrogenic property is responsible for this weak association with breast cancer risk, as the biological mechanism behind this association remains uncertain.

BPA, Breast Density, and Breast Cancer Risk

Breast density is one of the strongest risk factors for breast cancer [13]. BPA exposure has been associated with breast density, although this relationship is also controversial [14]. A study by Sprague and colleagues supports the association between BPA and breast cancer after finding a positive correlation between serum BPA and mammographic breast density [15]. While breast cancer is a hormonal disease, it is unlikely that the weak estrogenic activity of BPA supports the correlation between exposure and breast cancer risk. No clear mechanism has been shown to explain this association, however, one proposed method of action is through inflammation. Inflammation of the breast tissue has been shown to play a role in breast cell proliferation, a fundamental feature of cancer [16]. One way we can assess inflammation is through the presence of cytokines, which are a class of proteins that act as inflammatory/anti-inflammatory biomarkers. This allows for the quantification of breast inflammation to associate with BPA levels in breast tissue.

BPA and Inflammatory Cytokines

Characteristic of many inflammatory diseases, pro-inflammatory cytokines are produced and are used as biomarkers of inflammation. Cytokines are small proteins important to the cell signaling of immune system responses. Common inflammatory cytokines are interleukins (IL), tumor necrosis factors (TNF), and C reactive proteins (CRP) which acts as a global marker for inflammation and infection [17]. Animal and *in vitro* studies show that low-level BPA exposure induces inflammation [18, 19]. Of particular interest to my hypothesis are studies by Ben-Jonathan and colleagues. In one study, they found that exposure of human breast adipose tissue in cell culture to BPA led to an increase in leptin [4]. In a related study, they found that exposure of abdominal adipose tissue in cell culture to BPA led to an increase in IL-6 [20].

The alteration of inflammation biomarkers in breast tissue from the study by Ben-Jonathan and colleagues led the Arcaro lab to analyze cytokine levels in breast milk samples for which BPA levels were already assessed. As displayed in figure 1, women with higher BPA levels showed higher levels of leptin, IL-6, and CRP. These associations were not significant but the increased levels of cytokines support the role of BPA in increasing inflammation. To determine whether these preliminary findings accurately reflect the relationship between BPA and inflammation, or are a spurious result, I will use an improved study design that includes collecting two milk samples at standardized times. In this new study design, I will be determining the levels of BPA and the following cytokines: IL-6, TNFα, CRP, and leptin, all of which are pro-inflammatory cytokines.

The Importance of an Effective Procedure: BPA Extraction from Breast Milk

Concern over BPA contamination in baby formula, cow's milk, and other dairy products has led to the development of a procedure to effectively extract bisphenols from such products

for analysis. With similar properties to cow's milk, breast milk is a complex matrix enriched with lipids and other non-polar molecules that potentially provide the perfect environment for soluble endocrine disruptors. Due to a high fat content, traditional methods of bisphenol extraction used for paper goods and industrial products are not sufficient for complete separation and removal of organic molecules. Prior to a solid phase extraction, a hexane partitioning step will be added in order to remove all fatty substances following an adapted procedure from Stephanie Zimmers' methods paper [21], originating from the Kang and Kondo methods paper [22]. Extensive separation allows for a highly selective and sensitive method for analysis and contributes to a higher recovery rate of BPA in breast milk [22].

The Importance of an Effective Procedure: Formation of a BPA Derivative

Like gas chromatography, liquid chromatography may require derivatization of the target analyte to increase volatility and stability in order to enhance instrumental sensitivity. This is especially applicable to steroidal estrogens and environmental estrogens such as BPA that are weakly ionizable in the electrospray ionization (ESI) technique of LC-MS/MS, giving phenolic compounds an inherently low sensitivity [24]. BPA is a neutral compound under the pH conditions necessary for clear LC separation, the main contributor of low ESI efficiency. Although an extra layer of manipulation and error susceptibility is introduced, adding a derivatization step to this procedure with great precaution would be highly beneficial to increase the overall strength and sensitivity of the analyte signal.

Dansyl-chloride has been widely used to derivatize BPA in many different biological, environmental, and food samples [24]. While this has been a consistent choice, recent studies have shown a preference for pyridine-3-sulfonyl (PS). Dansyl-chloride MS/MS fragmentation of the parent ion results in reagent-specific daughter ions, which reduces method specificity and

increases the likelihood of mistaking matrix component interference for possible daughter analyte signals. Unlike dansyl-chloride, PS fragmentation of the parent ion yields analyte-specific daughter ions and reduces matrix interference. In this procedure, I will be using PS to derivatize BPA. Figure 4 displays the derivatized form of BPA (BPA-diPS) along with its fragmentation pattern.

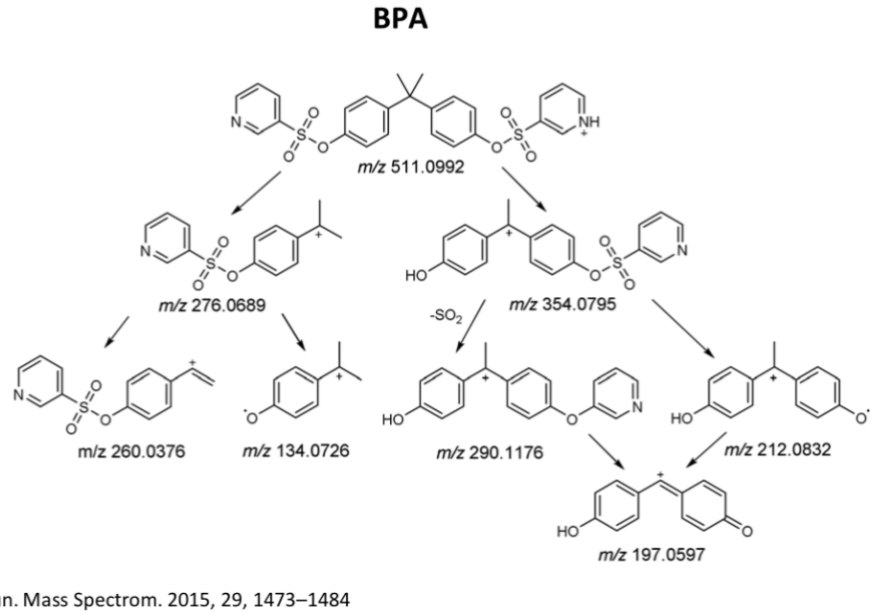


Figure 4. MS/MS fragmentation pattern of derivatized BPA (BPA-diPS) which a mass to charge (*m/z*) ration of approximately 511.0092. The daughter ion *m/z* rations are shown below the parent ion with their respective chemical structures.

The fragmentation pattern from Figure 4 depicting the *m/z* ratios of the parent and daughter ions will guide the analysis of signals detected by LC-MS/MS to separate BPA from any other noise or contamination peaks. According to Zimmers and colleagues as well as Xu et al., the retention time of BPA-diPS is just under 6 minutes, around when I should expect to see BPA-diPS detected [21, 23].

Methods

Below is the most recent version of our modified procedure, as adapted from Stephanie Zimmers' methodologies paper [21]. I was trained to perform this procedure during the summer of 2019, when I began running numerous practice samples to ensure quality control and consistency among different phases of the procedure. I was trained by Ph.D. candidate Soon-Mi Kim in Engineering Lab II, where all our resources and instruments have been made available. Since BPA is present in many plastics, the materials and supplies used in the analysis are glass. To remove all chemical residue, the glassware is baked at 500 ºC for 8 hours prior to running each sample batch.

Breast Milk Sample Composition from Study Design

In the current study design, 32 breast milk samples obtained from 16 primiparous women at 6 and at 8 weeks postpartum will be analyzed. Each sample is a combination of left and right breast milk. Three mL of each sample will be used to perform the following BPA extraction and analysis. The 32 breast milk samples will be run in triplicate (n=96) over 9 batches of 12 samples per batch including controls (run in either duplicate or singly) for a total of 108 samples. A small amount of each sample will be preserved for the electrochemiluminescence immunoassay for cytokine analysis. Practice samples were breast milk samples saved from a previous Arcaro Lab study, and any methods/instrumental standards were prepared with MilliQ water instead of breast milk.

BPA Extraction

Hexane Partitioning:

The purpose of hexane partitioning is to remove carbohydrates, lipids and other nonpolar compounds in the milk. Hexane partitioning separates compounds based on their solubility characteristics. For each sample, I will add 1 mL of milk to a 15 mL centrifuge tube, and for the controls, 1 ml of MiliQ water. Each sample and control will be spiked with 6 µL of 1 ng/µL $^{13}C_{12}$-labeled BPA in methanol. I will then vortex each tube for 30 seconds. I will add 3 mL of LC/MS acetonitrile and vortex the tubes again for 30 seconds. I will then add 6 mL of hexane and invert each tube by hand for 5 minutes. The tubes will be vortexed for 30 seconds then centrifuged at 5,500 rpm for 10 minutes. After centrifuging, two layers should be present. I will repeat the addition of 6 mL of hexane, manually invert the tubes and centrifuge for 10 more minutes at 5,500 rpm. I will remove the organic layer on the top and transfer as much of the aqueous layer (ACN/water) as possible into a new 15 mL glass centrifuge tube while avoiding transferring any lipids or organic molecules. The aqueous solution in the clean tubes will be evaporated down to about 1 mL in a 37ºC water bath under N_2 gas.

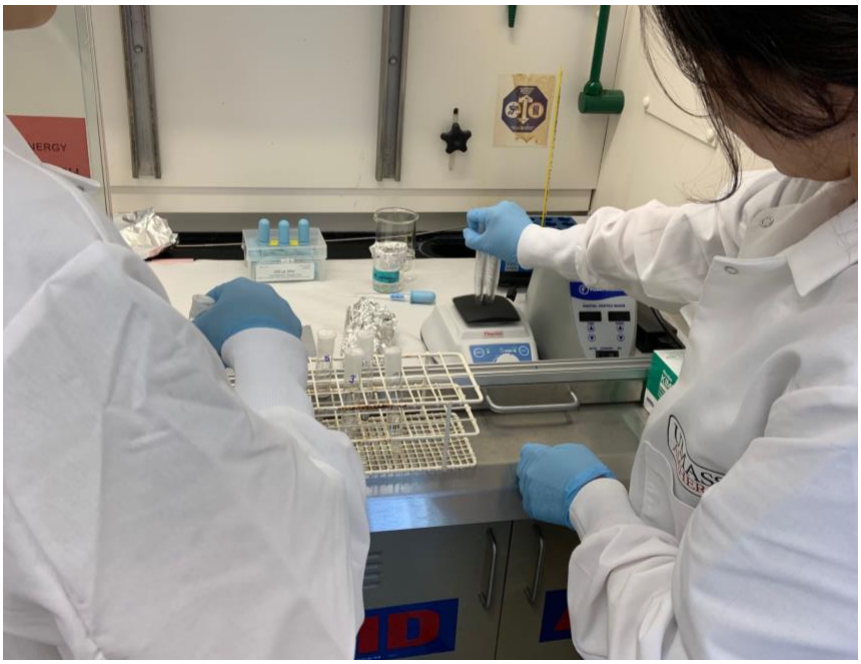


Figure 5. Two researchers vortex samples after adding 6 mL hexane.

Solid Phase Extraction (SPE):

Solid phase extractions purify, extract, and concentrate target analytes in a mixture. While waiting for the 12 samples to evaporate in the water bath, I will initiate the solid phase extraction by first setting up the SPE manifold. Twelve Oasis HLB cartridges will be numbered and placed into their correspondingly numbered locations in the manifold. Each cartridge needs to be conditioned by the addition of the following solvents in this order: 4 mL MTBE, 3 mL MeOH, 5 mL MiliQ water. Each solvent must completely filter through the cartridge without the vacuum until the next solvent can be added. Once the samples have evaporated down to 1 mL, I will dilute them by adding 9 mL of 1:8 LC/MS MeOH: MiliQ water. I will then load the diluted samples into their corresponding columns, about 5 mL at a time. The samples will filter through without a vacuum. To completely rinse the sample tubes of BPA, I will add 5 mL of MiliQ water and load each rinse into its corresponding column; again, without vacuum. The SPE columns will then be rinsed with 3 mL of 50:50 methanol:water followed by 3 mL of 10:2:88

methanol:ammonium hydroxide:water and dried for 5 seconds under N_2 with medium vacuum. This will be followed by a final rinse of 3 mL of dichloromethane followed by 1 min drying time under N_2 with high vacuum. Lastly, BPA will be eluted from the cartridge with 4 mL MTBE without vacuum into a graduated conical tube.

Figure 6. Vac Elut SPS 24 manifold.

Derivatization:

The derivatization process forms a pyridine sulfonyl derivative to increase the sensitivity of detection by the LC/MS/MS analysis. The final step in the extraction process is the synthesis of a pyridine sulfonyl derivative. First, the eluted samples will be completely evaporated in a 37°C water bath under N_2 gas flow. Once completely evaporated, 100µL PS chloride (1.07 mg/mL in Acetone) will be added to each sample using a glass Drummond pipet. Next, 100 µL 0.1M sodium bicarbonate will be added to the same vial. The glass Drummond pipet will be rinsed with MeOH between each sample to prevent any cross contamination. All samples will be

vortexed for 30 seconds then set on a heating block to react for 5 minutes. Once finished reacting, all samples will be placed on ice for 8-10 minutes before hand warming for 5 minutes to reach room temperature. In the final steps of the derivatization, 1 mL of hexane will be added to all samples and vortexed. Two layers will be visible after vortexing. The top layer – the organic layer – will be transferred using a glass Pasteur pipet into a 2 mL reacti-vial. Adding hexane and transferring the organic layer into a new reacti-vial will be repeated. I will evaporate all samples to dryness under N_2 gas in a 37°C water bath and reconstitute with 1 mL of 50:50 water:acetonitrile for LC/MS analysis.

LC/MS/MS Analysis

BPA concentrations in each sample and control will be analyzed under high performance liquid chromatography and mass spectrometry. Ph.D. student Soon-Mi Kim has extensive knowledge and experience with the instruments used for this analysis and will aid me in this step. Samples will be run through the LC/MS instrument as demonstrated in Figure 7.

For BPA, I will be looking for a parent mass to charge ratio of 511 *m/z* with the instrument ESI set to positive mode. The mobile phases used for liquid chromatography are 0.1% formic acid in water and 0.1% formic acid in ACN.

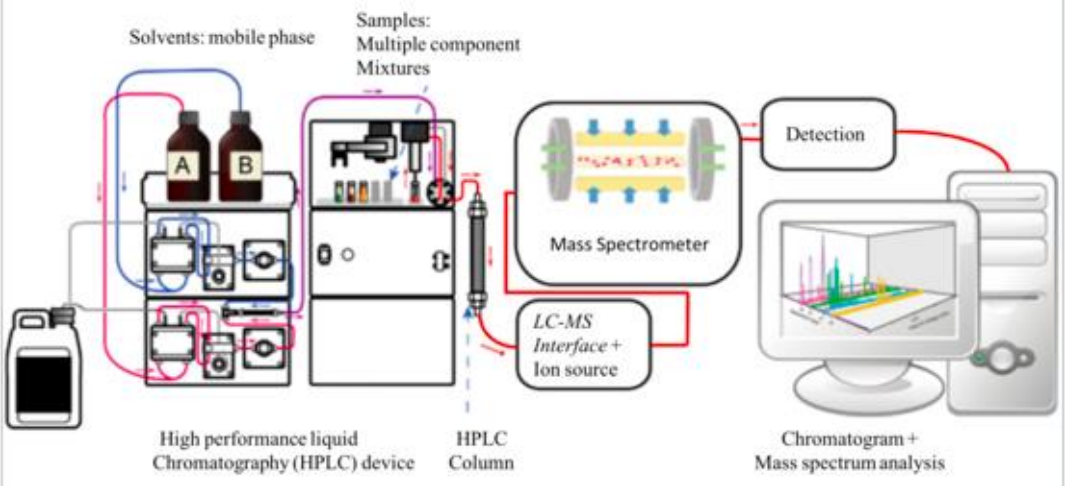


Figure 7. A simplified model of a liquid chromatography and mass spectrometry instrument. Each sample is first separated by polarity using two mobile phases (A and B). Each separated phase flows through the mass spectrometer. During this step, the mass spectrometer generates ions from different chemical components in each sample and separates them based on their mass to charge ratio (*m/z*). We can detect the abundance of these different mass to charge ratios using analytical software corresponding to the device (From https://en.wikipedia.org/wiki/Liquid_chromatography%E2%80%93mass_spectrometry).

Results

Calibrating ESI

In the beginning phases of trial runs, I wanted to test instrumental sensitivity to both underivatized and derivatized BPA. This would also allow us to test whether I could detect BPA in our stock solutions as well as if our derivatization procedure was effective. Underivatized BPA is detected in ESI negative mode and derivatized BPA is detected in ESI positive mode. Using reference chemicals ibuprofen and caffeine that ionize under negative mode and positive mode conditions, respectively, I referred to their known retention times and concentrations to calibrate the LC-MS/MS according to its measurements. The calibration curves of each reference chemical are shown in Figure 8.

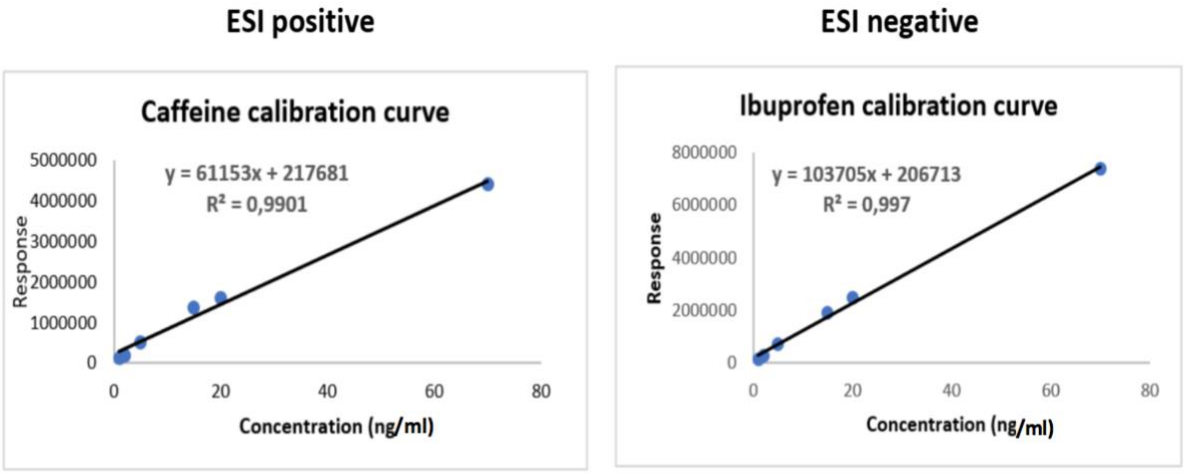


Figure 8. Calibration curves for LC-MS/MS set in ESI positive mode with reference chemical caffeine and in ESI negative mode with reference chemical ibuprofen.

Calibration curves are used to predict concentrations in unknown samples. A linear function (y=mx+b) is calculated based on collected data points to determine the instrumental detection limit of the analyte and is applied to unknown sample data to predict concentration values. The R_2 value of a linear curve represents the sensitivity of the instrument to a specific analyte, that is, it tells us how sensitivity changes as concentration changes. An R_2 value of 1is a perfectly linear line, and R_2 values of 0.95 are generally acceptable calibration curve values. Figure 8 calibration curves are both linear and show an R_2 value of at least 0.99, representing consistent instrumental sensitiviy in each setting. Each ESI setting was tested with 6 samples of known concentrations in duplicate.

Appearance of Sodium Adduct

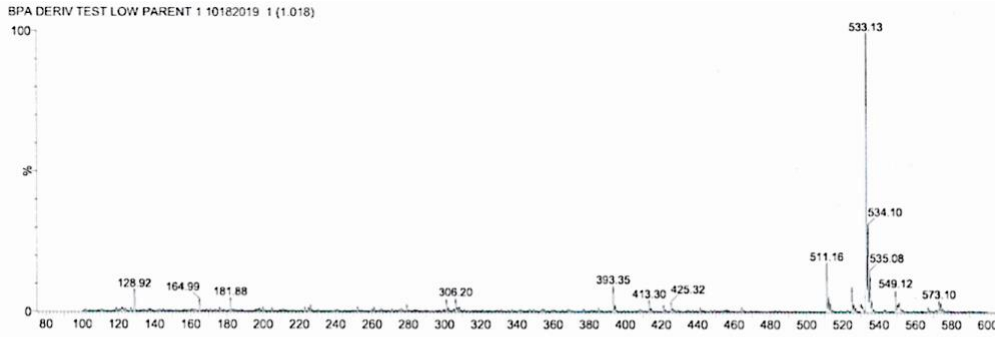
Throughout the process of detecting the presence of BPA-diPS in our derivatized practice samples, I was looking for peaks of *m/z* values of 511 with a daughter fragment 354 (refer to Figure 4). While creating our derivatized calibration standards (Table 1), I consistently saw higher abundance peaks at *m/z* value of 533, both higher than our target parent ratio by a

difference of 22 (Figure 9). For simplicity, I focused on the ‘low level’ and ‘high level’ standard mass spectra, their initial concentrations indicated in Table 1.

Table 1. Calibration standards sample composition

Sample Name		Conc. of native-BPA (ng/mL)	Conc. of 13C12-BPA (ng/mL)
Calibration standards	STD 0	0	6
	STD 0.1	0.1	6
	STD 0.2	0.2	6
	STD 0.5	0.5	6
	STD 1	1	6
	STD 2	2	6
	STD 5	5	6
	STD 10	10	6
	STD 20	20	6
	Control	0	0
	Low level	1000	0
	High level	5000	0

A



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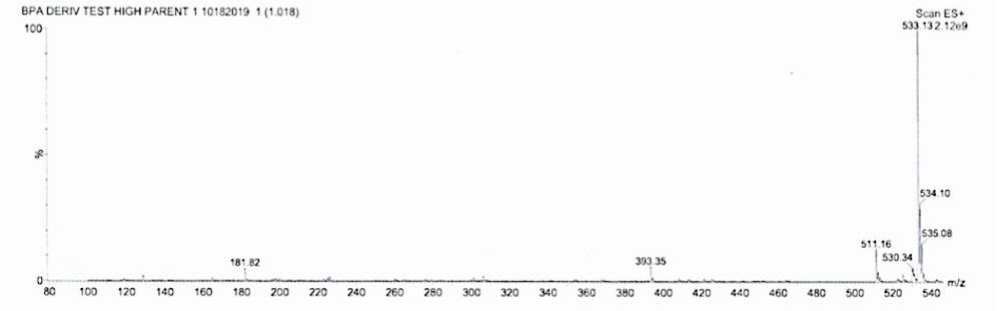

Figure 9. (A) High 533 parent peak abundance for the low concentration derivative standard. (B) High 533 parent peak abundance for the high concentration derivative

In addition to our calibration standard samples, the labeled standard samples ($^{13}C_{12}$-BPA) also displayed an unexpected peak at 545 where normally a peak should appear at 523 (Figure 10). This ratio was also higher than the normal value by a difference of 22.

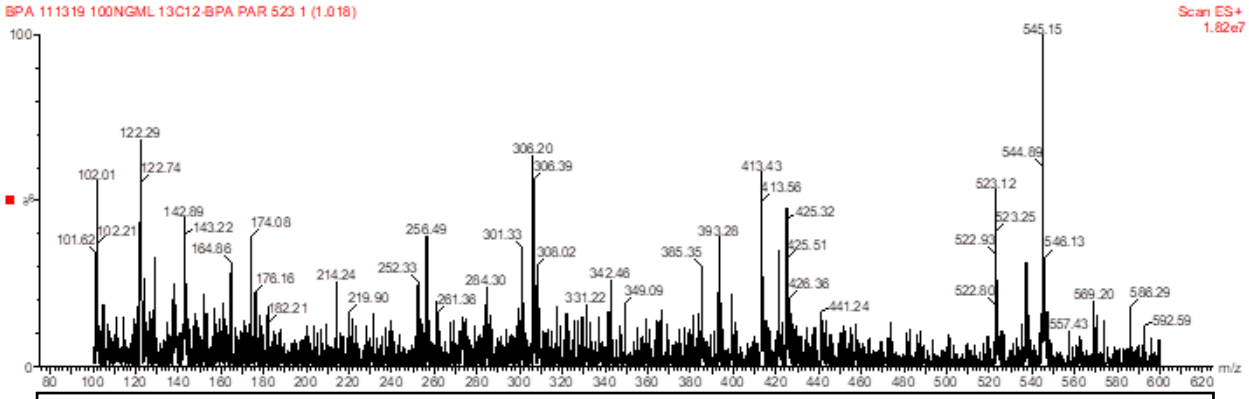

Figure 10. High 545 parent peak abundance in 100 ng/ml of $^{13}C_{12}$-BPA labeled standard.

One possible explanation of the 533 peak could be contamination with BPS-diPS. BPS-diPS has an *m/z* ratio of 533 and a labeled standard *m/z* ratio of approximately 545, accounting for both peaks in Figures 9 and 10. Xevo MS analysis also revealed the presence of an abundant 533 peak, reducing the likelihood that UPLC instrumental contamination played any role. If the peak really was BPS-diPS, this would indicate that somewhere in our methods our samples were contaminated with BPS in order to form the BPS derivative. The chromatogram in Figure 9 shows very low 511 peaks in comparison to the 533 peaks, which would mean that this contamination had to be substantially higher than the spiked concentrations of BPA. Considering the 533 peak did not consistently show up in every practice run and I could not locate the exact source of BPS contamination, the idea that contamination with BPS-diPS was the source of the 533 peak was eliminated.

Another possibility for the appearance of these higher ratio peaks is the formation of an adduct. Not unusual in negative or positive ESI, although more frequent in positive settings, an

adduct may form as a result of trace cationic species from a laboratory procedure or solvent impurities forming with target ions in electrospray form [25]. Sodium ions have an atomic mass of about 22 u, which is the exact difference between the target parent peaks and the unexpected parent peaks. This sodium species may have resulted from contamination during our derivatization step, which uses sodium bicarbonate as a buffering solution and offers a more likely reason as to why a higher mass peak appeared on the chromatogram. The LC-MS/MS instrument was run with water and methanol to eliminate any residue and caution was used regarding sample transferring during the derivatization phase. After placement of these precautions, I did not observe the 533 peak in practice samples, which may have indicated that experimental residue created an observable adduct.

Collision energy

Collision energy is another important factor for increasing both the sensity and specificity of instrumental detection of our target analyte. Collision energy refers to the ion kinetic energy used during the gas phase to fragment the parent ion species into analyte-specific daughter ions. Fragmentation pattern and abundance depend on the energy strength. Low collision energies yield ion rearrangement while higher collision energies are more likely to yield bond breakage. As shown in Figure 11, increasing collision energy increases the abundance of daughter anions (m/z = 354, 290, 276, 212, 197, 134) and decreases the abundance of the parent ion (m/z = 511).

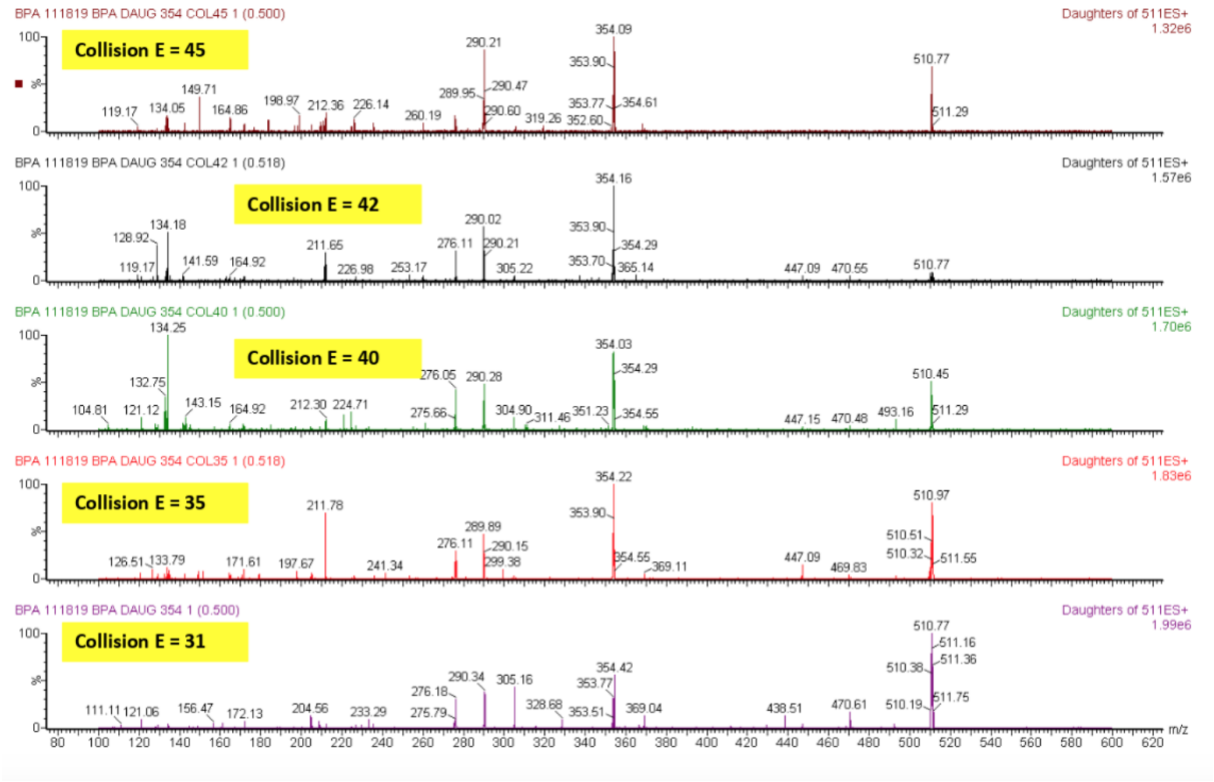

Figure 11. Mass spectrum for each collision energy adjustment, displaying differences in daughter ion abundance. Energy measured in electronvolts.

As an instrumental parameter, this setting is usually based on previous methods that test similar target analytes. The setting used throughout our practice runs and study samples was 36 eV, however Zimmers' methods paper used an energy of 31 eV [21]. Although Figure 6 displays the collision energy of 31 eV with the highest abundance of the parent ion, this parameter value worked best with our temperature and cone energy settings: source temperature of 145 ºC, desolvation temperature of 350 ºC, and cone energy of 45 eV, which differed from the methods paper. With mass spectrometry, spending a significant amount of time to adjust parameters is common because of its trial-and-error-like process as one method for an analyte in one experiment will not always work for the same analyte of another experiment.

Mobile Phase Composition

Mobile phases are important to the separation process of liquid chromatography and generally consist of one aqueous component and one organic component. The mobile phases should differ in some kind of physicochemical component from the target analytes in order for analytes to be detectable, such as pH. In the past, basic mobile phases have altered the sensitivity and stability of the column used in LC. In Zimmers' paper, acetonitrile comprised mobile phase A while 0.1% acetic acid comprised mobile phase B, with parent peaks appearing just under 6 minutes [21]. While assessing instrumental paremeters using these mobile phases along with the new temperature and cone energy parameters, parent peaks did not show up on the chromatogram. As part of the trial-and-error process of optimizing instrumental inputs, different mobile phases were used to separate the sample solvents. I determined the new optimal mobile phase A to consist of 0.1% formic acid in water and the new optimal mobile phase B to consist of 0.1% formic acid in acetonitrile, previously used by Li Xu and David Spink in their analysis of steroidal estrogen derivatives [23]. After implementing mobile phase changes, the parent peaks appeared on the chromatogram at approximately 3.7 minutes.

Discussion

In summary, optimizing a sensitive method for detection of BPA in breast milk relies on the efforts of previous literature and practice run data. While LC/MS/MS has become the preferred method for quantification and analysis of target analytes in a range of matrices, a significant portion of time may be devoted to developing methods for proper extraction and adjusting instrumental input to increase sensitivity. As evident in this study, a trail-and-error approach was chosen for determining methods and instrumental parameters based on literature-sourced solutions.

Diagnosing the source of the 533 and 545 peaks, for example, required numerous practice runs to rule out instrumental contamination and further research to solve this issue. Multiple instruments, the UPLC and Xevo, were used to compare results. Both chromatograms displayed 533 peaks, indicating the source of contamination to be methods-sourced. As mentioned previously, adducts are observed in both negative and postivie ESI settings, and may serve a useful role in increasing the sensitivity to some compounds that may not be directly ionizable, such as sugars. However, this process is not well understood and is complicated to control [26]. It is thought that cations, sodium in this case, carry surface excess charges of ESI nanodroplets, rendering the formation of these species possible. Kruve and Kaupmees (2017) found that mobile phase additives help prevent the formation of these adducts due to their chelating properties competing with the target analyte for any sodium present in the mobile phase. Specifically, the water phase additives of 0.1% formic acid or 0.1% acetic acid were recommended to monitor adduct formation [26]. Although the intended purpose for changing our mobile phases from 0.1% acetic acid to 0.1% formic acid was not specifically for reducing adduct formation, it is possible that in addition to methods precautions and cleaning the instrument, adduct formation was greatly reduced. Reading the literature in this instance filled gaps in my knowledge to further support the mobile phase change as well as prevent this problem from occurring in the future.

Troubleshooting the formation of adducts was one of the many examples where normal experimental conditions or day-to-day inconsistencies, such as water quality, may influence a single component of analysis and alter results in their entirety. This was also present in the buffers made months prior to beginning study sample runs. Zimmers' methods paper used a sodium bicarbonate buffer with a pH of 10. A calibrated probe detected the pH of the buffer

made with same concentration of sodium bicarbonate to be 8.5. Although literature supported that as long as the buffer was alkaline, the difference in pH would not make a difference to the reaction of the analyte with PS-chloride. Unfortunately, I did not dive into the literature for this problem, and it resulted in weeks of testing multiple buffers of different pHs, further solidifying the value of spending time reading the literature.

Other inconsistencies were present in communication and organization skills. There were times when deciding the best direction to get to the root of a problem was not always agreed upon among the team. Similar to the buffer problem mentioned before, some obstacles could have been solved by going through the literature instead of conducting time-consuming practice runs for the same problem. Regardless of the frustration and time spent on this portion of the study goal, I have learned more about liquid chromatography and mass spectrometry than I thought I would as an undergraduate. I am appreciative of the myriad of LC/MS/MS applications despite the amount of time needed to tailor its parameters for specific analytes. With the eventual success in setting up the instrument, it has served a incomparable role in the beginnings of our study sample analysis.

Future Directions

The results from practice samples are important for accurately analyzing BPA levels in study breast milk samples. With the setup noted in Appendix A, as determined by this project, a few batches of study samples have already undergone LC/MS/MS analysis without instrumental or method interferences. I am hoping study sample analysis is completed in the future as smoothly as the batches already analyzed. We can then compare these BPA levels to measured cytokine levels collected from the same breast milk samples prior to analysis in order to

determine an association. As the immediate result of this study, this association will then be used to assess for any relationship between BPA and breast density, measured with MRI.

Our study design incorporates samples collected from 16 women at 6 weeks and 8 weeks postpartum. Providing the same two timepoints for all women standardizes sample collection and offers two different points in time to track changes in BPA levels. While this study design had the purpose of removing limitations from previous studies, the samples collected do not accurately represent the BPA exposure of these women and make up a rather small sample size. Originally, the funded study would have included around 400 women. However, instead of relying on one milk collection for the entire day, women should provide multiple samples from different times in a day to improve the accuracy of representation. Better still, a 24-hour urine collection could detail the body's exposure for an entire day. This would vastly improve the interpretation of an association between BPA and cytokine levels by increasing the accuracy of how much BPA women are truly exposed to.

Regardless of study design, LC/MS/MS is a valuable technique to identify and quantify analytes in a given matrix with applications in health, food, and environmental sciences. Going forward, instrumental parameters set in this project may set precedence for avoiding similar issues in other projects analyzing BPA in milk or similar matrices. Although this project has been a slow learning curve, I hope to take the skills I have learned in E Lab II and the Life Sciences Laboratory and apply them to future endeavors.

Acknowledgements

I would like to thank the Arcaro Lab and the Commonwealth Honors College at the University of Massachusetts Amherst for funding this project. I would also like to thank Dr. David Reckhow, Dr. Patrick O'Keefe, Ph.D. candidate Soon-Mi Kim, and Sherrie Webb-

Yagodzinski for their time and effort to help with laboratory procedures and training in carrying out the completion of this project. Lastly, a big thanks to my honors committee board, Dr. Kathleen Arcaro, Dr. Laura Francis, and Dr. Sarah Pallas, for dedicating their time to the process of creating and writing this thesis.

Appendix A. Supplementary Material

Supporting Information
Table S1

S1. ESI+ MS/MS Parameters	
Voltages	
Capillary (kV)	2.5
Cone (V)	45
Extractor (V)	5
RF Lens (V)	0.1
Temperatures	
Source (°C)	150
Desolvation (°C)	450
Gas Flow	
Nebulizer:	
Desolvation (L/hr)	950
Cone (L/hr)	150
Collision (mL/min)	0.2
Collision Cell Voltages (V)	
Parent Ion:	
Entrance	50
Collision	2
Exit	50
Daughter Ions:	
Entrance	3
Collision	36
Exit	1

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