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Determination of BPA in Infant Oral Hygiene Products using Fluorescence Spectrophotometry

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SENIOR THESIS APPROVAL

This Honors thesis entitled

"Determination of BPA in Infant Oral Hygiene Products using Fluorescence Spectrophotometry"

written by

Mallory K. Mayfield

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the requirements for completion of
the Carl Goodson Honors Program
meets the criteria for acceptance
and has been approved by the undersigned readers.

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24 April 2019

Ouachita Baptist University

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Acknowledgements:

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Abstract:

Bisphenol A (BPA) is commonly used to make plastics, and it is also used to make epoxy resin linings for metal food containers. People are exposed to this chemical daily through bottles, metal food containers, and other plastic products. BPA exposure has been linked to negative effects on the body including cardiovascular diseases, diabetes, and reproductive problems. Special caution should be taken for children because their systems are not fully developed, and they are often more susceptible to the negative effects. BPA can bind to estrogen receptors and activate them. It has this ability because of structural similarities to estradiol (Figure 1).

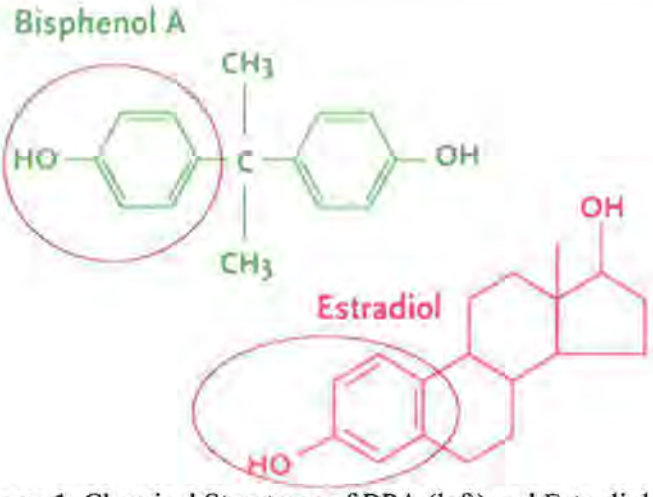


Figure 1. Chemical Structures of BPA (left) and Estradiol (right).
Both compounds contain a phenol group, which can react with estrogen receptors.

While BPA is now regulated in many plastic products, it is not regulated in toothbrushes. This summer's research tested for BPA in several infant oral hygeine products. BPA is a fluorescent compound with an excitation wavelength of 277 nm and an emission wavelength of 304 nm. The presence of BPA was determined through the use of the FS-5 Spectrofluorometer from Edinburgh Intruments. A calibration curve and analytical figures of merit (linear range, limit of detection, and limit of quantitation) were determined for BPA. This information was

then utilized to monitor the concentrations of BPA that leached from several brands of infant toothbrushes over time. Products labeled as "BPA free" were compared to products that did not have a corresponding label.

Introduction:

BPA, or bisphenol-A, is a chemical that is used in the manufacturing of plastics, linings of cans, and many other products. Although it is a component in many items, it has been found to have negative effects on the human body. The first signs of BPA toxicity were founded in the 1930's. When first discovered, BPA was used as a pharmaceutical hormone replacement. The chemical industry first started using it in plastics around the 1940's, which is how it was commonly used, even today. However, the first law to regulate BPA was not made until 1976. The topic of whether BPA is harmful has been a widely debated issue.[i] The evidence of the regulation can be seen in the commonly seen "BPA free" labels on items like water bottles, plastic containers, and other items. It is not commonly found on most popular infant toothbrushes.

The negative effects of BPA are because it is and endocrine disrupter. An endocrine disrupter has a similar structure to the hormone that it is interfering with. Because of the structural similarities, the endocrine disrupter can bind to the hormone binding sites. This leads to the "turning on" of pathways that are not supposed to be started and inhibiting the normal function of the hormone (Figure 2). BPA specifically disrupts the regulation of the hormone estrogen (estradiol).[ii] BPA is capable of causing this disruption because it has a group that resembles a group on estrogen, and that makes it able to bind to the same type of endocrine

receptors. Some of the negative effects of the BPA binding to the estrogen receptors are: male and female infertility, premature puberty, breast and prostate tumors, polycystic ovarian syndrome, type 2 diabetes, and fetal brain development.[iii] Infants and young children are especially susceptible to the negative effects of BPA. The susceptibility makes it especially important to study the products that infants and young children are using.[iv]

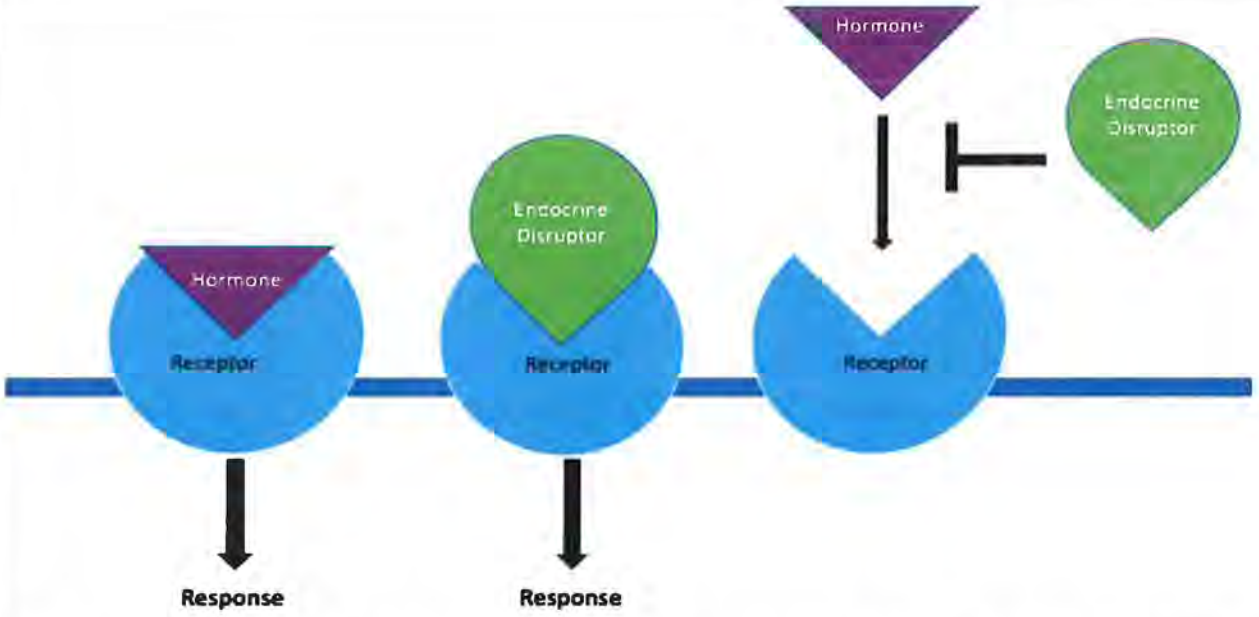


Figure 2. A hormone binds to the receptor and causes a response. However, and endocrine disruptor can bind to the receptor due to structural similarities and cause the same response. This could also lead to the inhibition of a response.

BPA in infant oral hygiene products is not a widely researched topic and the presence of BPA in the toothbrushes is not highly regulated. When looking at the "big name" brands of infant toothbrushes, it is not clearly labeled whether the product is BPA free or not. For my thesis research project, the presence of BPA in different oral hygiene products was investigated. BPA was present in different brands of infant toothbrushes. This information will help arm parents and guardians with information so they can avoid exposing their children to BPA.

Negative Health Effects of Bisphenol-A:

To better understand the negative health effects of BPA, it is important to look at the pathways that BPA interrupts or starts. As previously mentioned, BPA is an endocrine disrupter for the estrogen hormone (estradiol). BPA is found in many polycarbonate plastics and is used in food and drink packaging, water and infant bottles, compact disc, impact resistant safety equipment, and medical devices. Most exposure to BPA occurs through the diet.[v] Most of the exposure in the diet comes from the containers that the food is stored in, such as plastic containers or the lining of cans. The BPA leeches out of those containers and ultimately, we consume the leeched BPA in the food or liquid that is stored in the containers. There has been a lot of evidence showing that BPA has an impact even at low doses.[vi] BPA is metabolized in the body and that metabolite binds to the estrogen receptor more strongly than BPA itself. The metabolite is called methyl-bis-pentene (MPB) and is shown in Figure 3.[vii]

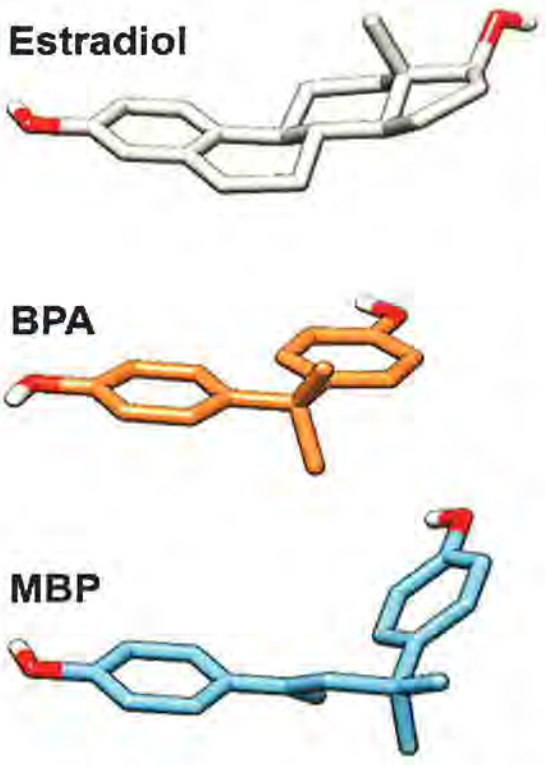


Figure 3. Estradiol, BPA, and MBP, the metabolite of BPA.

As mentioned above, Bisphenol A mainly enters into the body through our diet; however, it can enter though dust, water, and air as well.[v] When BPA enters the body it can cause negative effects, such as diabetes, cardiovascular diseases, and female infertility, which will be

discussed later in detail. The negative health effects that are caused by BPA are especially harmful for children. They are growing and developing and hormones play an important role in developing. BPA would potentially disrupt the normal developing because it endocrine disrupter.

According to Normiri and Ambrosino, there have been several studies that show that BPA is linked to many chronic health conditions. BPA deregulates cell signaling pathways associated with cell growth, proliferation, migration, invasion, and apoptosis. This is what can lead to the cancerous effects of BPA. BPA has specifically been linked to diabetes. The potential link between BPA exposure and type 2 diabetes and insulin resistance have been something that several researchers are studying. A study from 2013 suggests that BPA exposure is related to B-cell dysfunction. In this study, they tested the effect of BPA on INS-1 cells. INS-1 cells are insulin secreting cells, and therefore, linked to diabetes.[viii, ix] They found that when exposed to BPA INS-1 cell viability is decreased.

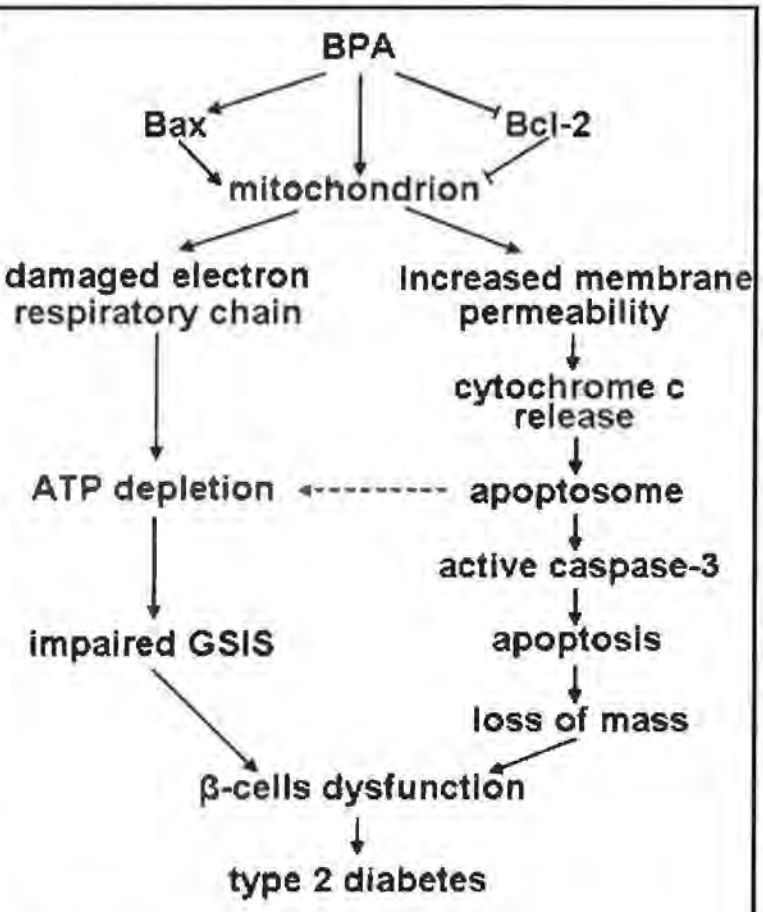


Figure 4. BPA can bind to estrogen receptors and cause apoptosis or ATP depletion. Both eventually lead to the dysfunction of the B-cells and ultimately type 2 diabetes. This is a proposed pathway.

The study described above also found that BPA induces mitochondrial dysfunction in INS-1 cells. The mitochondria are a crucial part of the cell. The mitochondrion is involved in

many functions of the cell, such as signaling, cellular differentiation, and cell death. It also plays a large role in the cell cycle and cell growth. The mitochondrion has been referred to as the "powerhouse of the cell". It got this nickname because it supplies cellular energy.[x] They also found that BPA triggers apoptosis and the release of apoptogenic factors in INS-1 cells. This happens when BPA is in high concentrations. Finally, this study found that BPA triggers apoptosis through the intrinsic mitochondrial pathway. The proposed pathway of how BPA induces B-cells dysfunction is shown below in Figure 1. In this figure, it is shown that BPA is affecting the Mitochondria and leading to apoptosis or ATP depletion which in turn leads to the dysfunction of the B-cells and ultimately type 2 diabetes. The article attempts to demonstrate the link between BPA and type 2 diabetes, but as of right now this is a theoretical pathway. [ix]

The next serious condition that has been linked to BPA exposure is cardiovascular disease. Cardiovascular disease is a widely-studied health problem that affects individuals around the world. Researchers have analyzed the genetic, physiological, and pathophysiological causes of cardiovascular disease. However, the effects of different chemicals on the cardiovascular system need to be investigated further. One article suggests that the linkage between cardiovascular disease and BPA exposure is the ability of BPA to alter the cardiac Ca^{2+} handling, ion channel inhibition/activation, oxidative stress, and genome modifications. It has specifically been linked to angina, hypertension, heart attack, and coronary and peripheral arterial disease. High BPA urinary output has also been linked to arrhythmias and atherosclerosis in rodents.[xi]

The final negative health effects linked to BPA are reproductive issues, the literature review for this project focused on female infertility. Female infertility has been defined as a failure to achieve a successful pregnancy after 12 months or more of appropriate, timed

unprotected intercourse.[xii] It has been found to affect 10-15% of couples.[xiii] Since BPA is an endocrine disruptor for the hormone estrogen, the link between female infertility and BPA exposure is not shocking. BPA can interfere with the endocrine system in the female body. It would in turn throw off the hormone balance in the female system. It has actually been found that BPA can accumulate in reproductive organs.[xiv, xv]

BPA can cause functional defects in the hypothalamic system. The hypothalamic system is the link between the nervous system and the endocrine system through the pituitary gland. This could potentially result in the inability to reach reproductive capacity at the time of puberty. This would also lead to issues in maintaining that capacity during adulthood. There has been no definite pathway found for this effect. However, an article mentioned that is could be from the production of kisspeptin. Kisspeptin is a driving signal of gonadotropin release hormone secretion. [xii] This hormone makes the pituitary gland produce and secrete hormones that cause the ovaries to make estrogen and progesterone. [xvi]

BPA can also affect the ovaries. It has been linked to follicle loss. Decreased oocyte survival has also been linked to the exposure of BPA to the body. BPA could also be linked to polycystic ovary syndrome. The symptoms of PCOS include hair loss, oily skin, infertility, weight gain, menstrual problems, and depression. [xvii] It affects 1 in 10 women of childbearing age. Women who have this disease have a hormonal imbalance. [xviii] This hormonal imbalance could potentially be linked to BPA exposure, since BPA has been found to cause hormonal imbalances. Research has shown that women with PCOS had a higher BPA level than those without PCOS. BPA has also been linked to an increase in testosterone concentration. [xii]

Fluorescence Spectrophotometry:

BPA is a fluorescent compound, which means that it will experience excitation. Following the excitation, BPA will emit radiation. Energy is absorbed, and this is what ultimately leads to fluorescence (Figure 5). The excitation wavelength for BPA is around 278nm and the emission wavelength is around 305 nm (Figure 6).

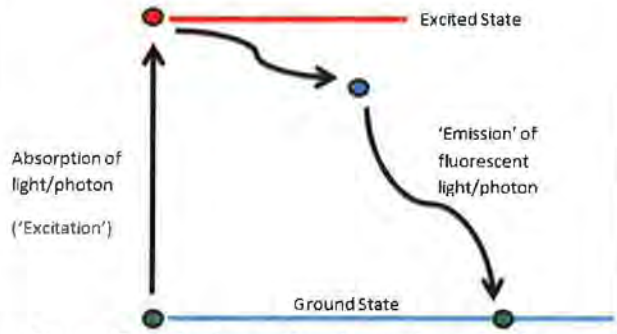


Figure 5. This shows the absorption of light (excitation) and the emission of the fluorescent properties.

Once the emission wavelength is recorded, it can be converted to the corresponding concentration of BPA that is in the M/W sample. Fluorescence can be used to detect small amounts of a substance and small molecules. In this experiment, the FS-5 Spectrofluorometer from Edinburg Instruments was used to detect the emission and excitation of the M/W solution that was being tested.

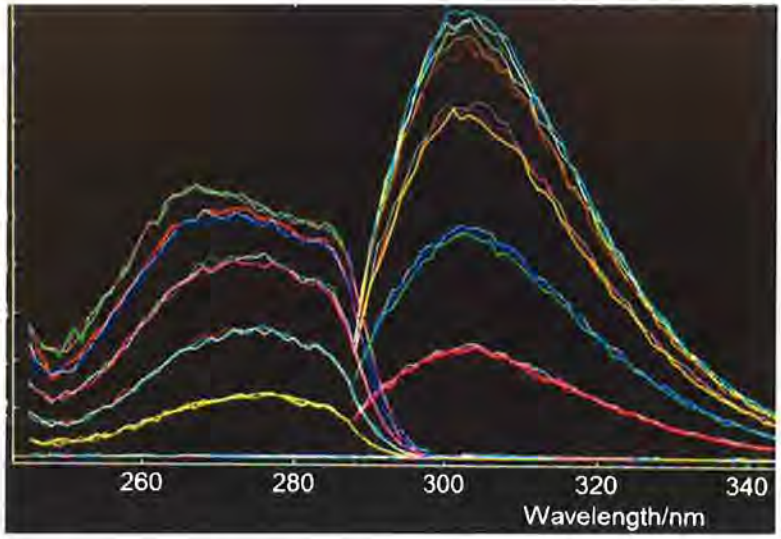


Figure 6. The results from the FS-5 Spectrofluorometer. The excitation wavelength is on the left (around 278nm) and the emission wavelength is see on the right (around 305nm)

Materials and Methods:

Materials:

- 0052g of BPA
- 50 mL of HPLC grade water
- 50mL of HPLC grade methanol
- 100mL volumetric flask
- 25mL volumetric flasks
- FS-5 Spectrofluorometer
- A variety of toothbrushes
- 14 5mL volumetric flasks
- Glass micropipettes
- Plastic micropipettes
- 100mL of HPLC grade methanol
- 100mL of HPLC grade water
- 100mL beaker
- Goggles
- Gloves
- Labeling materials
- 10% Nitric acid
- 12 toothbrushes (BPA free and unlabeled)

Toothbrushes Tested	**Label**
Red Firefly	Not labeled
Pink and Yellow Rexall	Not labeled
Colgate	Not labeled
Colgate	Not labeled
Baby's First Toothbrush	BPA free label
Blue Firefly	Not labeled
Green Rexall	Not labeled
Jordan Super Soft	BPA free label
Orange Reach	Not labeled
Blue Totz Radius	BPA free label
Oral-B	Not labeled
Jordan Super Soft	BPA free label

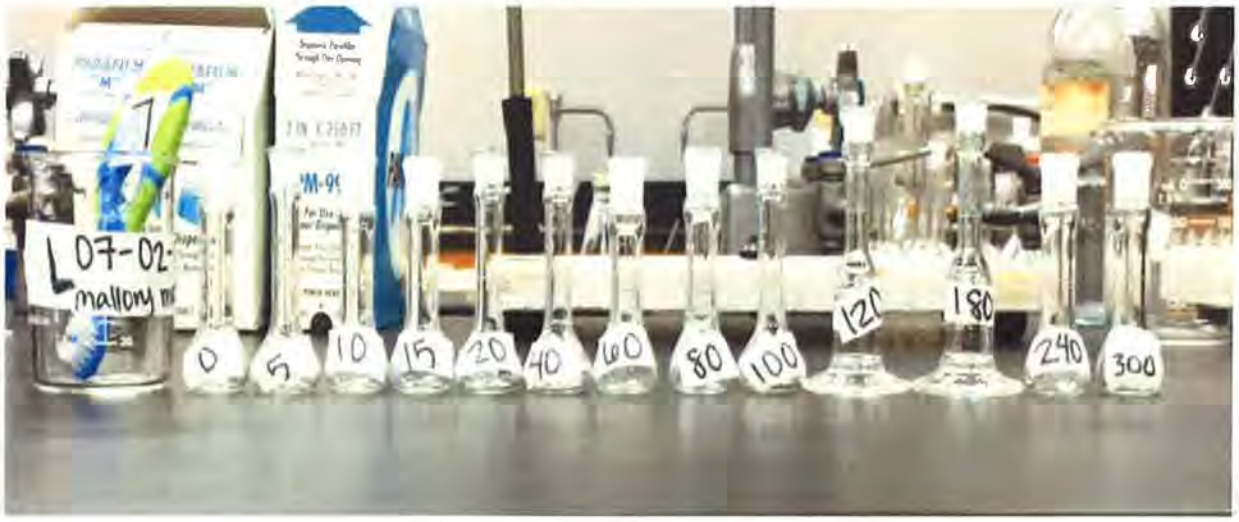
In preparing for the calibration curve, 0.0052 g of BPA was weighed and put in a 100mL volumetric flask and diluted to the mark with 50% methanol/water solution (M/W). The solution was then mixed together by shaking and swirling. The resulting solution concentration was 0.052 mg/mL. This stock solution was used to obtain a fluorescence calibration curve. Different mL volumes of the stock solution were removed and put in 25mL

volumetric flasks. The flasks were then filled to the 25mL line with 50% methanol water and the new solutions were run in the FS-5 Spectrofluorometer. A calibration curve was then prepared using the intensity values obtained from running the samples. The calibration curve was used to formulate the equation that was needed to find the concentration of BPA in each of the samples that were run later in the experiment. The calibration curve and data will be discussed in the next section.

For this experiment, different brands of toothbrushes were tested for the presence of BPA. The toothbrushes were then labeled A-L to keep the brands of the various toothbrushes anonymous and remove potential bias. After obtaining and labeling the toothbrushes, the presence of a BPA free label or not was recorded. The toothbrushes were then placed in a beaker with 100 mL of the methanol water solution. In this experiment, one toothbrush was tested per day. A timer was set and a 5mL sample was taken at 0 minutes, 5

minutes, 10 minutes, 15 minutes, 20 minutes, 40 minutes, 60 minutes, 80 minutes, 100 minutes, 120 minutes, 4 hours, 5 hours, 6 hours, 24 hours, 48 hours, and 1 week after the toothbrush was placed in the solution. These solutions were then run in the FS-5 Spectrofluorometer during the large gaps of time throughout the day. The emission intensity, emission wavelength, excitation intensity, and the excitation wavelength were recorded for each sample collected.

Using the equation from the calibration curve, the intensities were then converted to concentration of BPA. The analytical figures of merit were calculated and used to determine if BPA had in fact leeched from the toothbrushes into the solution. All of this information was graphed using Microsoft Excel, and the toothbrushes with BPA free labels were compared to toothbrushes without BPA free labels.



Results and Discussion:

At the beginning of the experiment, samples were run and a calibration curve was made with the solutions that had a fixed concentration of BPA. The calibration curve is shown below in Figure 7. The equation from the calibration curve was used to calculate the concentrations are later seen in Table I.

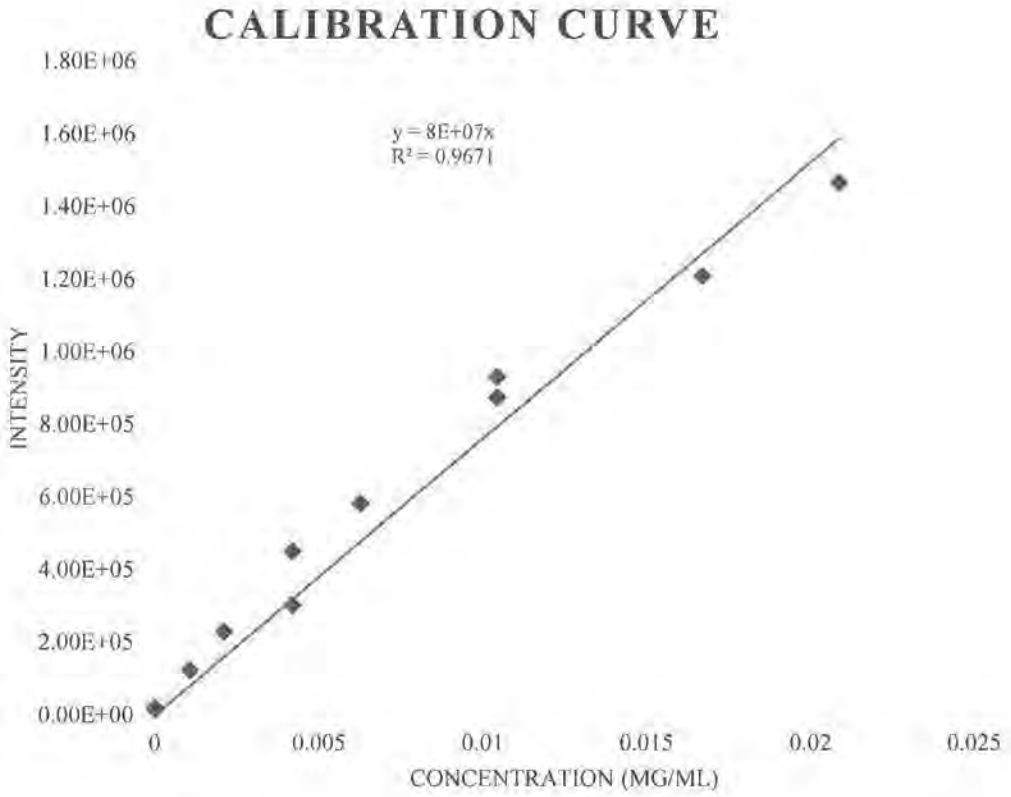


Figure 7. Fluorescence Emission Calibration Curve
For BPA in 1:1 M/W at 308nm

As discussed in the materials and methods section…experiment reminder. Monitoring the release of BPA from toothbrushes into your M/W over time.

After the experiment, the emission intensities were recorded and converted to concentration of BPA in those samples using the relationship between concentration and

intensity found in the calibration curve (Figure 7). The results of this conversion are shown on the next page in Table I. The table also includes if the toothbrushes were labeled as BPA free.

The concentrations were then graphed in Microsoft Excel. Appendix A shows the graphs for all of the toothbrushes tested. The graphs were then used to compare the concentrations of toothbrushes labeled BPA free and those not labeled BPA free. In each example graph below, the concentrations of BPA leached from two toothbrushes over time were compared. In Figure 8, Toothbrush E was labeled BPA free, and toothbrush C was not labeled BPA free. The same pattern was followed in Figure 9 where H and K were compared. The results are shown below.

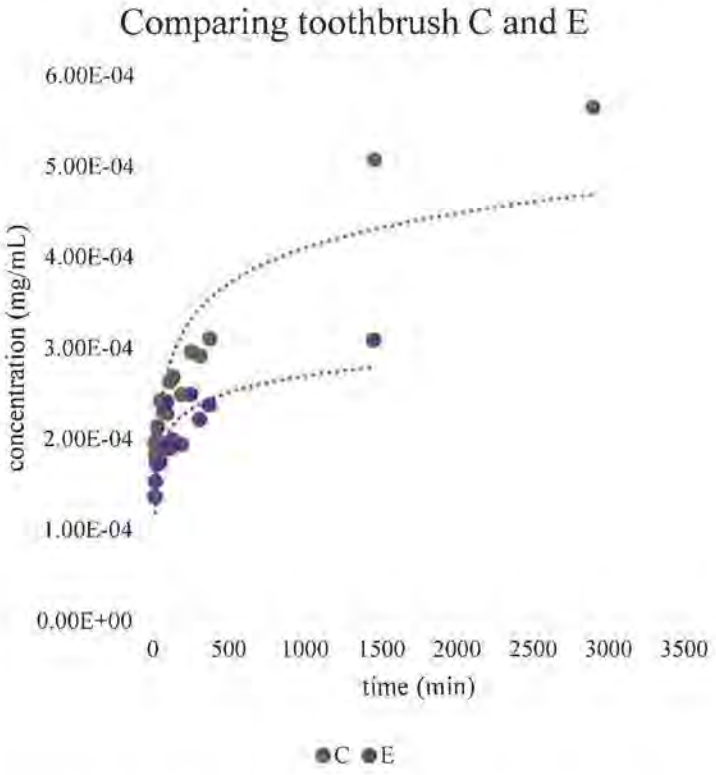


Figure 8. Toothbrush E has the BPA free label, and it is being compared to toothbrush C which has no BPA free label.

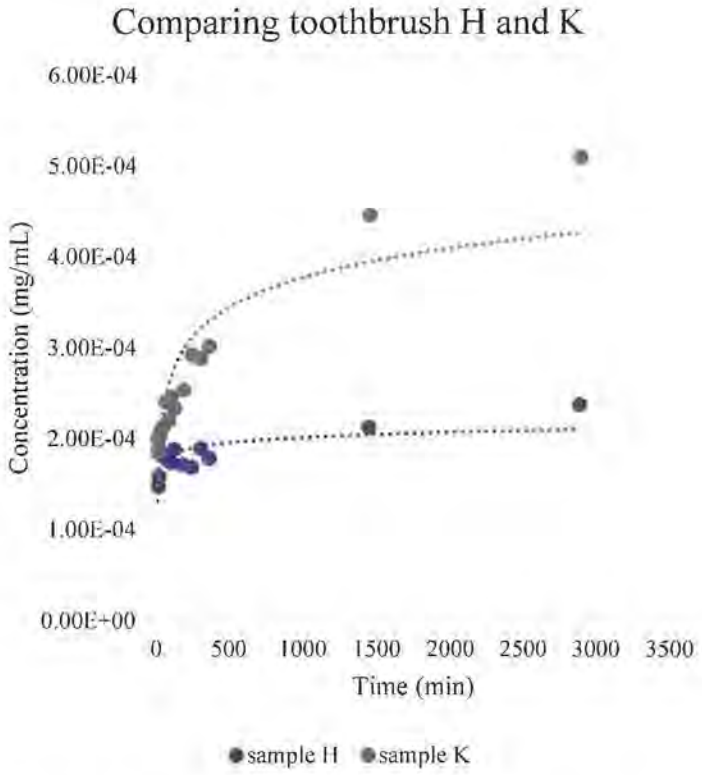


Figure 9. Toothbrush H has the BPA free label, and it is being compared to toothbrush K which has no BPA free label.

As shown in the figures above (figures 8 & 9), the toothbrushes that were labeled as BPA free (E and H) released little to no BPA into the methanol water over a period of time. However, the toothbrushes shown in the graphs that were not labeled BPA free (C and K) did show an increase in the amount of BPA released into the methanol water over a period of time.

T-tests were performed comparing the toothbrushes in the above graphs (C, E, H, and K) and it was found that the toothbrushes without a BPA free label (E and H) had a statistically significant difference in concentrations when compared to the toothbrushes that were labeled BPA free. The changes in concentration of BPA leached into methanol water over time from all of the toothbrushes are summarized in Table I below. The rows that are white (E, H, and J) were labeled BPA free and contained little to no BPA. The rows that are light purple (F, G, and I) also remained low, which indicates that they may also be BPA free. The rows that are dark purple (B, C, D, and K) had a significant increase in BPA concentration, that was statistically significantly different that the lower concentrations, which suggest that these toothbrushes do contain BPA.

Table I. Concentration of BPA leaching from toothbrushes in 1:1 M/W over time
Darker purple: Not labeled and higher concentration.
Lighter purple: Not labeled and lower concentration
White: Labeled as BPA free

Toothbrush	Label on toothbrush	Concentration at 0 hours (mg/ml)	Concentration at 6 hours (mg/ml)	Concentration at 24 hours (mg/ml)
B	No label	.000272	.000292	N/A
C	No label	.000184	.000310	.000508
D	No label	.000174	.000274	.000433
E	BPA free	.000170	.000238	.000309
F	No label	.000203	.000254	.000390
G	No label	.000184	.000233	.000215
H	BPA free	.000208	.000179	.000213
I	No label	.000183	.000273	.000358
J	BPA free	.000195	.000222	.000267
K	No label	.000172	.000302	.000446

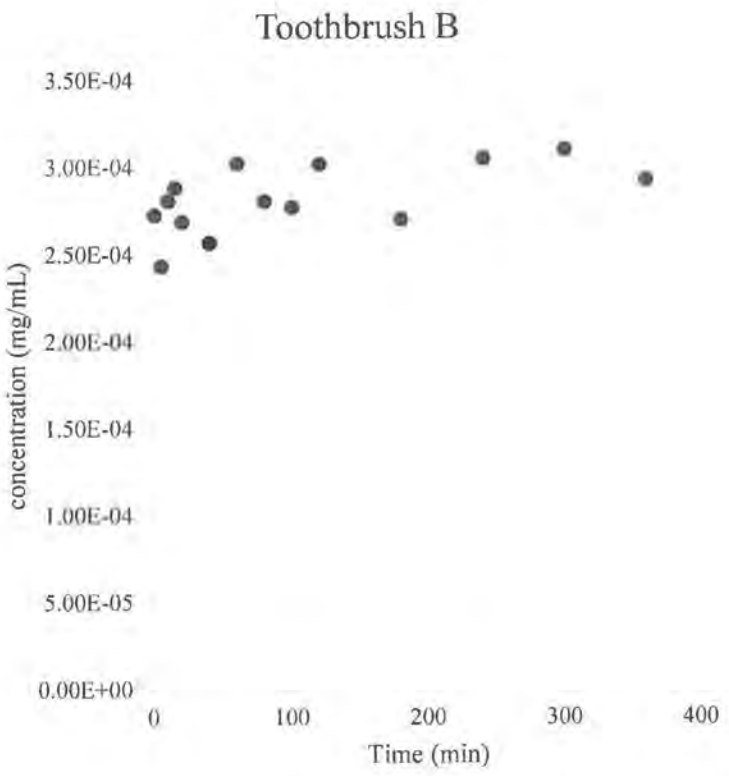
Conclusions:

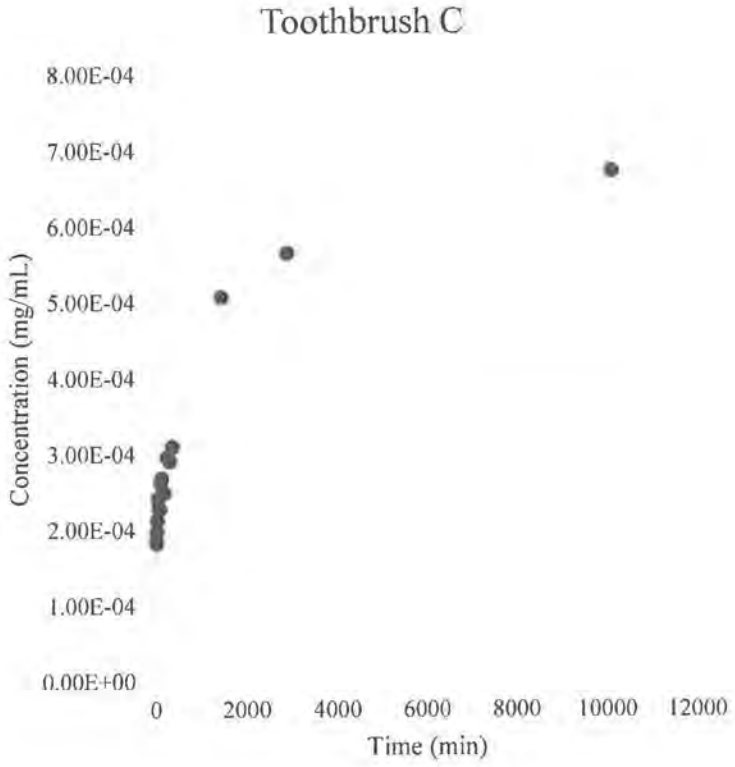
In examining the results of this study, it can be concluded that M/W exposed to toothbrushes with BPA free label have a statistically significantly lower concentration at a 95% confidence level than M/W exposed to the toothbrushes that are not labeled BPA free. A significant increase is seen specifically in toothbrushes B, C, D, and K. The higher concentrations indicate that BPA may be present. Toothbrushes not labeled BPA free that had a lower concentration could be toothbrushes that are not labeled, but that do not contain BPA. The lower concentration toothbrushes are seen in toothbrushes F, H, and J, which are the ones with the "BPA free" label. There is still work to do to quantify the amount of BPA present in the toothbrushes. This is a valid method for the determination of the presence of BPA in toothbrushes because we were able to differentiate between the sample sets.

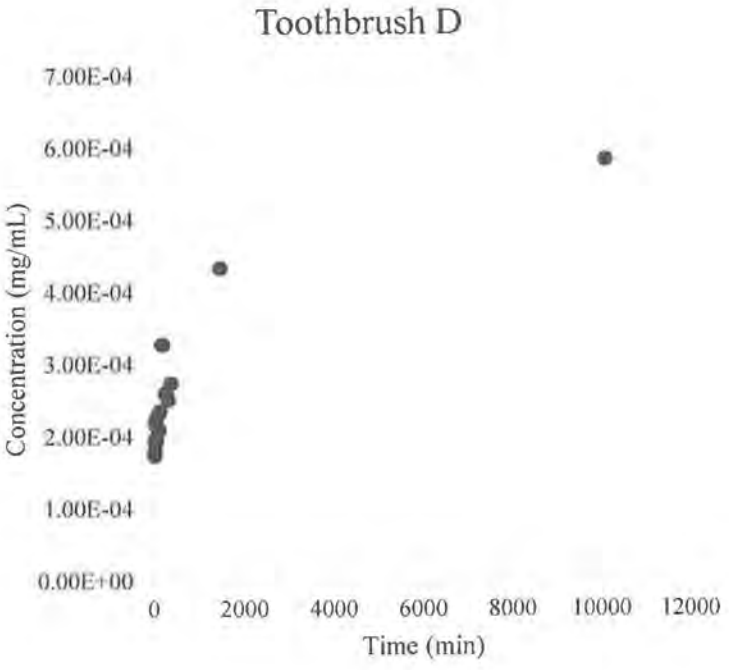
Future Experiments:

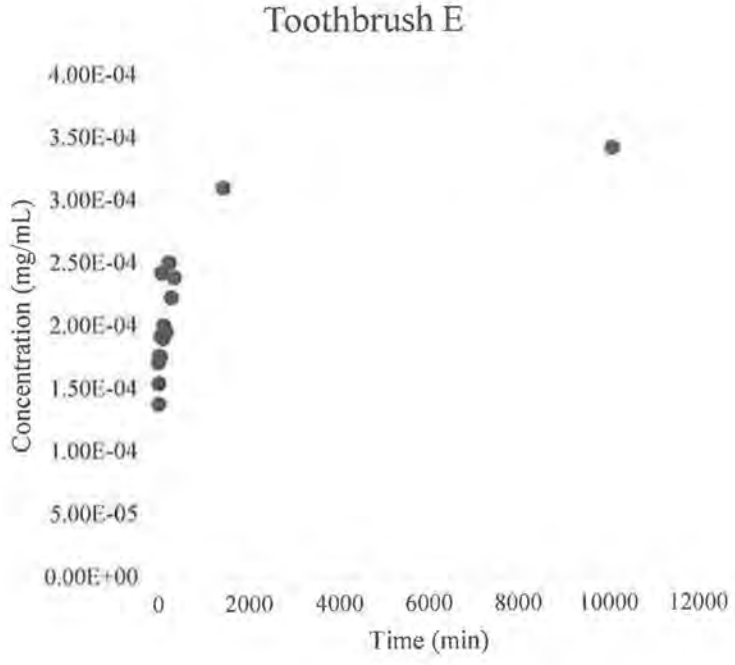
In the future, it would be useful to examine toothbrushes at different pH values. This could mimic the different pHs that the toothbrushes could potentially be exposed to. It would also be helpful to test the toothbrushes at different temperatures. This would mimic the different temperatures that the toothbrushes would be exposed to throughout their existence. They could be exposed to extreme temperatures in the warehouse, the delivery trucks, or even sitting in the sun in the bathroom. These different scenarios are very important to consider, so an accurate measure of the presence of BPA can be obtained. The brushing motion that the toothbrush is put under while individuals brush their teeth needs to be researched as well. Theoretically, this could make a difference in the amount of potential BPA that is leeched off from the toothbrush into children's vulnerable bodies. Finally, like any experiment, this experiment needs to be repeated to ensure the results that were obtained in this experiment were valid and reproducible.

Appendix A:


Toothbrush B
3.50E-04
3.00E-04
2.50E-04
2.00E-04
1.50E-04
1.00E-04
5.00E-05
0.00E+00
concentration (mg/mL)
0
100
200
300
400
Time (min)


Toothbrush C
8.00E-04
7.00E-04
6.00E-04
5.00E-04
4.00E-04
3.00E-04
2.00E-04
1.00E-04
0.00E+00
Concentration (mg/mL)
0
2000
4000
6000
8000
10000
12000
Time (min)


Toothbrush D
7.00E-04
6.00E-04
5.00E-04
4.00E-04
3.00E-04
2.00E-04
1.00E-04
0.00E+00
Concentration (mg/mL)
0
2000
4000
6000
8000
10000
12000
Time (min)


Toothbrush E
4.00E-04
3.50E-04
3.00E-04
2.50E-04
2.00E-04
1.50E-04
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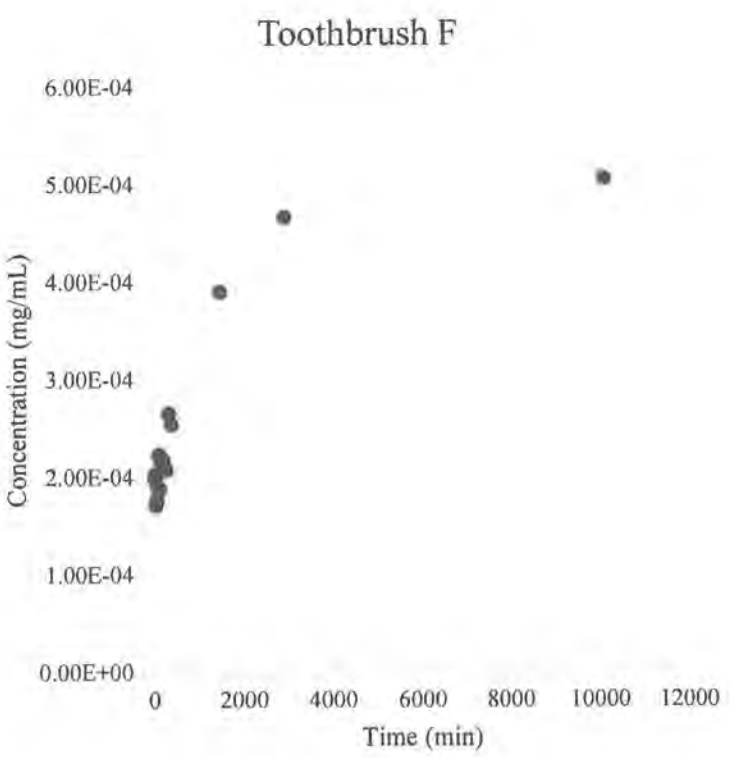
0.00E+00
Concentration (mg/mL)
0
2000
4000
6000
8000
10000
12000
Time (min)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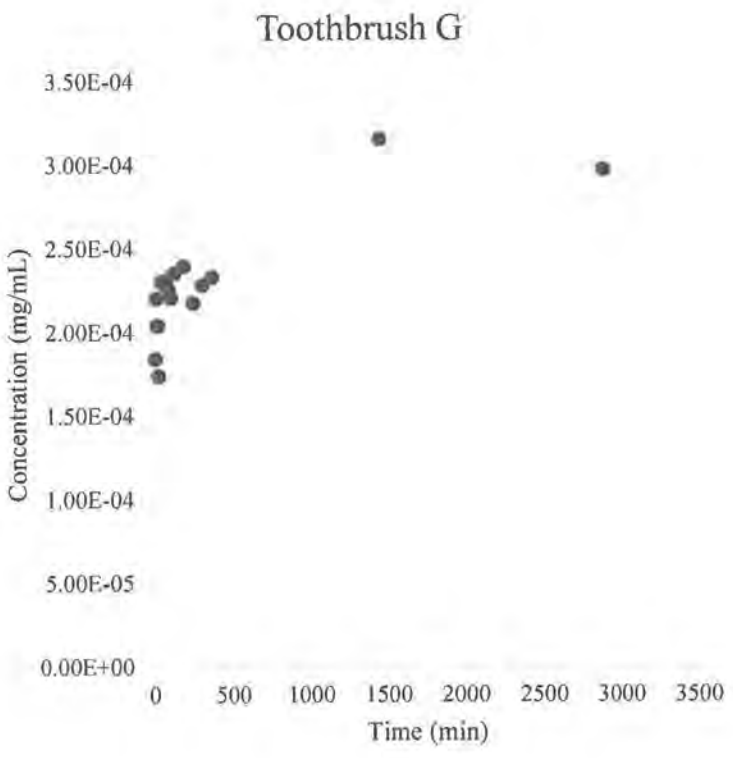
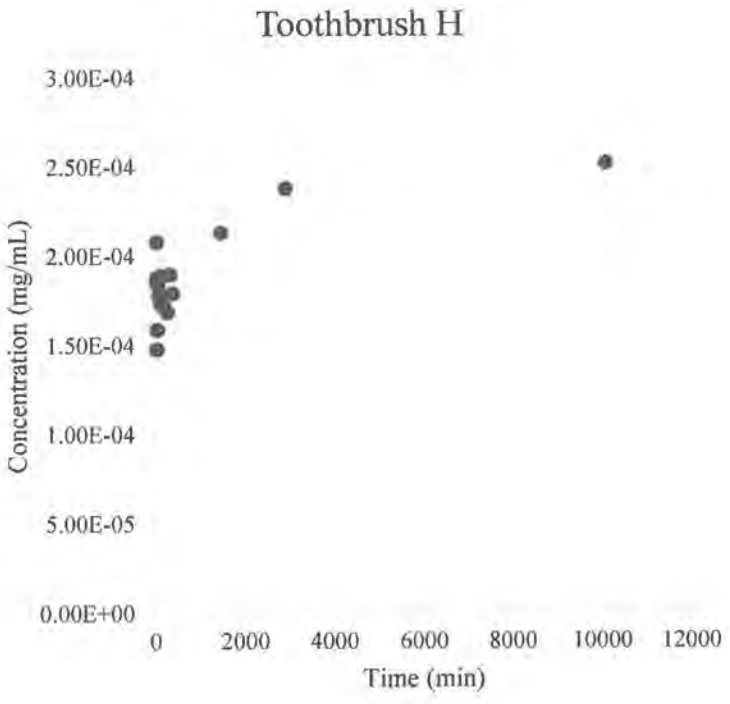
5.00E-04
4.00E-04
3.00E-04
2.00E-04
1.00E-04
0.00E+00
Concentration (mg/mL)
0
2000
4000
6000
8000
10000
12000
Time (min)


Toothbrush G
3.50E-04
3.00E-04
2.50E-04
2.00E-04
1.50E-04
1.00E-04
5.00E-05
0.00E+00
Concentration (mg/mL)
0
500
1000
1500
2000
2500
3000
3500
Time (min)


Toothbrush H
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2.50E-04
2.00E-04
1.50E-04
1.00E-04
5.00E-05
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Concentration (mg/mL)
0
2000
4000
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8000
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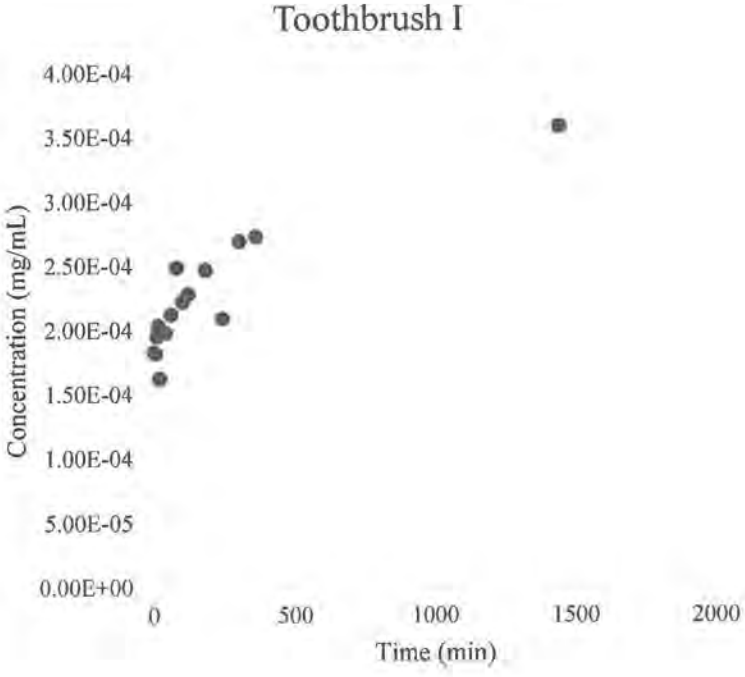
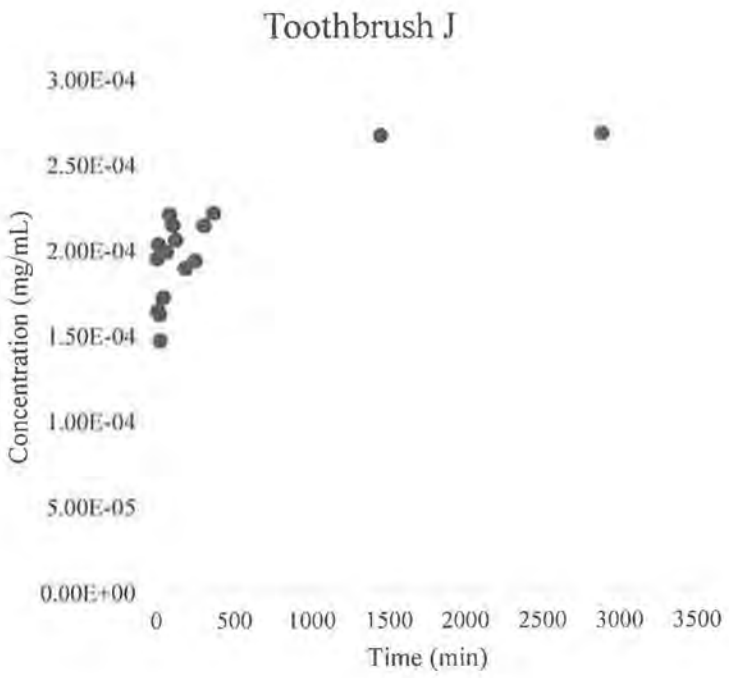
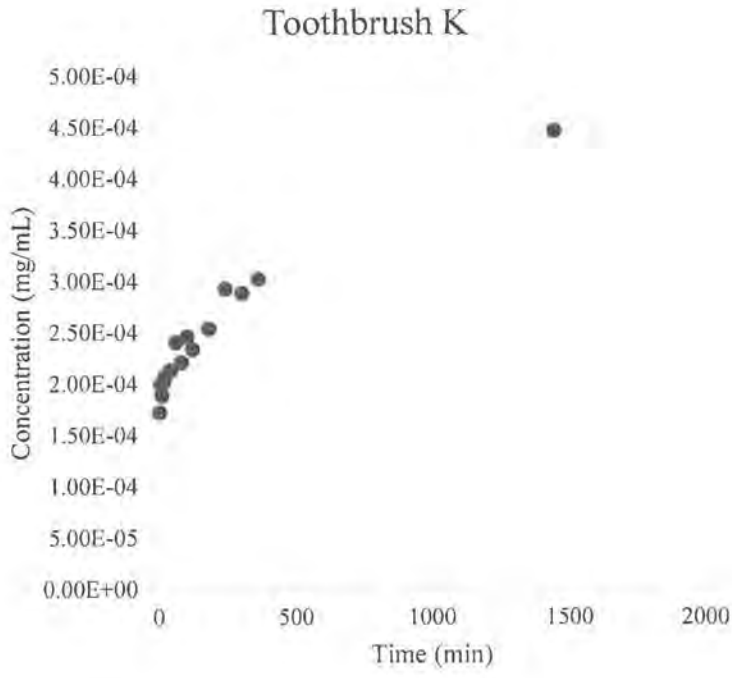
12000
Time (min)


Toothbrush I
4.00E-04
3.50E-04
3.00E-04
2.50E-04
2.00E-04
1.50E-04
1.00E-04
5.00E-05
0.00E+00
Concentration (mg/mL)
0
500
1000
1500
2000
Time (min)


Toothbrush J
3.00E-04
2.50E-04
2.00E-04
1.50E-04
1.00E-04
5.00E-05
0.00E+00
Concentration (mg/mL)
0
500
1000
1500
2000
2500
3000
3500
Time (min)


Toothbrush K
5.00E-04
4.50E-04
4.00E-04
3.50E-04
3.00E-04
2.50E-04
2.00E-04
1.50E-04
1.00E-04
5.00E-05
0.00E+00
Concentration (mg/mL)
0
500
1000
1500
2000
Time (min)

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