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Effects of Storage Conditions on BPA Leaching from Infant Oral Hygiene Products using Fluorescence Spectroscopy

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SENIOR THESIS APPROVAL

This Honors thesis entitled

"Effects of Storage Conditions on BPA Leaching from Infant Oral Hygiene Products using Fluorescence Spectroscopy"

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meets the criteria for acceptance
and has been approved by the undersigned readers.

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Abstract

Bisphenol-A (BPA) is a structural component in many plastic products, which acts as an endocrine-disruptor mimicking estrogen. This hormonal disruption has been linked to obesity, reproductive issues, cardiovascular problems, and neurodevelopment disorders. Infants are at the highest risk of BPA exposure compared to any other stage of life. Because an infant's endocrine system is developing, exposure to an endocrine-disruptor, such as BPA, can be especially harmful. While the FDA monitors products like baby bottles, canned goods, and plastic containers for BPA, infant oral hygiene products are not closely monitored.

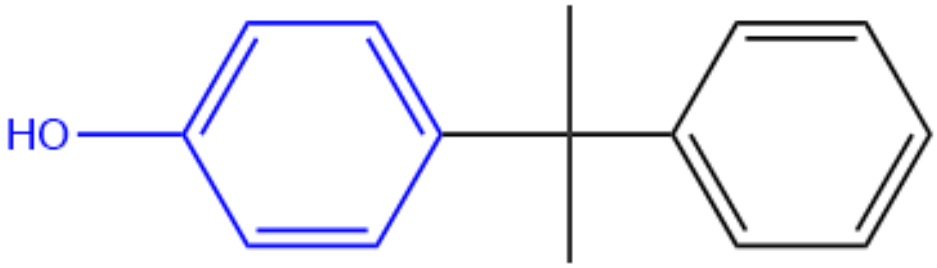
Previous research in our lab used fluorescence spectroscopy to test several brands of infant toothbrushes and found that many contain and leach BPA into their surroundings. Bisphenol-A fluoresces at an excitation wavelength of 278 nm and an emission wavelength of 304 nm. When tested at the average body temperature, 37 °C, research showed an increased amount of BPA leaching from the toothbrushes. For this project, toothbrushes were tested by storing them at higher temperatures, 50 °C, prior to testing for time increments similar to that of shipment in semi-trucks. Large shipments spend an average of 3-7 days in semi-truck containers, and the shipment temperature, on average, reaches 50 °C. Toothbrushes were then placed in 1:1 methanol/water for several hours. Aliquots were removed over time, and the amount of BPA leaching from the samples was monitored using an FS5 Spectrofluorometer from Edinburgh instruments. The results of this experiment revealed an insignificant increase in the amount of BPA leaching out of toothbrushes at increased duration and temperature.

Introduction

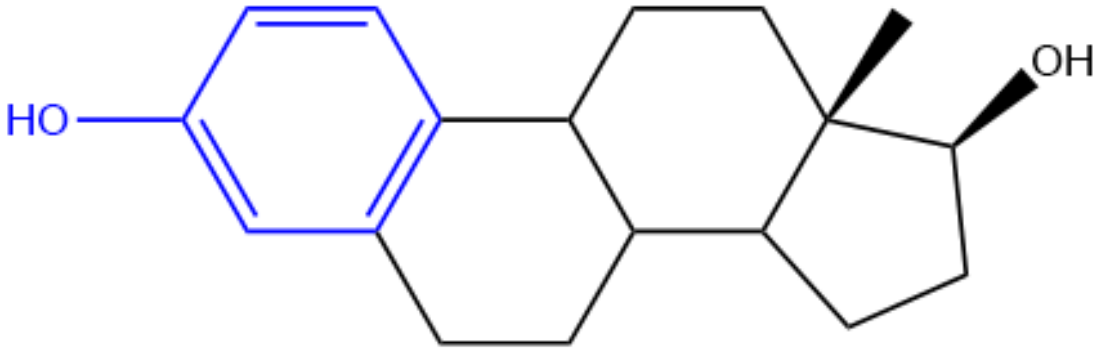
Bisphenol A (BPA) is a man-made monomer used in the synthesis of polycarbonate and epoxy resins. This synthetic chemical has been in use since the 1940s for flame retardants, can linings, and hard plastics, such as baby bottles and sippy cups[xv]. The usage of BPA, and its analogues, in plastics has been a focus for many researchers in sustainable and environmental chemistry. The increase in plastic waste raises concerns over the physiological and environmental effects of the chemicals utilized in plastics. Research into the upcycling of BPA from bisphenol A carbonate (BPA-PC) shows promising results to combat the environmental pollution brought by BPA[xxix]. Further research has been performed to show the concentrations of BPA in canned foods and baby items[xiii]. To continue the research into the concentration of BPA in baby items, this research will investigate the concentrations of BPA in infant oral hygiene products and the effects of exposure to this endocrine-disrupting compound.

While BPA is industrially beneficial, BPA is a known endocrine disruptor, mimicking estradiol, the precursor to estrogen. This hormonal disruption has been linked to obesity, reproductive issues, cardiovascular problems, and neurodevelopment disorders[vii]. Due to this health threat, the Food and Drug Administration (FDA) has placed industrial regulations on the usage of BPA. This regulation, however, does not prevent the usage of BPA in infant oral hygiene products.

Molecularly, BPA consists of two benzene rings, two hydroxide groups, and two methyl groups. Due to the terminal hydroxide groups, BPA acts as a competitive inhibitor for estradiol (Figure 1). The hydroxide functional groups on BPA bind to the estrogen receptors in place of estradiol. This signals to other tissues the incorrect concentration of estrogen within the body. Ultimately, BPA inhibits the production of estrogen, disrupting functions in the endocrine system.



Bisphenol-A



Estradiol

Figure 1. Chemical structures of BPA (top) and estradiol (bottom). Both compounds contain a phenol group (blue), which can react with estrogen receptors.

On average, an adult intakes 30 to 70 ng/kg of body weight of BPA per day, while an infant intakes 300 to 500 ng/kg of body weight of BPA per day[xviii]. While both these concentrations are lower than the FDA-determined tolerable daily intake (TDI), 5 μg/kg-day, the effects of BPA make an identifiable impact to the health of adults and infants. The FDA found that BPA is primarily ingested orally, in which BPA is quickly metabolized into an inactive form. The TDI was determined based on this mechanism and accounts for the gap in the average intake and tolerable intake[viii]. Infants, however, may ingest 100 times more BPA per body weight compared to the rest of the population. This high concentration is due to the widespread exposure from infant formula, breast milk from the mother carrying concentrations of BPA, pacifiers, oral hygiene products, and more. The endocrine system of an infant is not fully developed; therefore, this increased exposure is especially harmful. Prolonged BPA exposure has been linked to many health issues.

In the United States, the distribution industry never stops. Shipments are exposed to a wide range of uncontrolled temperatures for the duration of the delivery time, an average of three to seven days. Depending on the materials of the shipping container, the inside temperature of a shipping container can rise to 15 °C higher than the ambient temperature. With US ambient temperature reaching as high as 43 °C, this increases the temperature of the internal environment to 58 °C, or 136.4 °F[xvi]. This is a cause for concern, since at 25 °C, BPA releases at 0.62-22.5 ng/L and at 70 °C, BPA releases at 2.89-38.9 ng/L[vii]. Furthermore, BPA release rate increases for a period of four weeks in storage, then stabilizes in long term storage. Exposure to the uncontrolled temperatures during shipment and storage causes BPA to break down and release from plastics products, such as infant oral hygiene products, at an increased rate.

From previous research, BPA leaches from infant toothbrushes during storage at room temperature, under oral conditions, and during usage[xxiii]. As infant oral hygiene products and toothbrushes travel in shipment containers across the US, they are exposed to intense heat during the three-to-seven-day delivery time. This significantly increases the risk of BPA leaching from the toothbrushes. Because of the long-term health effects of BPA on the endocrine system, this risk directly concerns infants.

Health Effects of BPA- Neurodevelopmental

Environment and genes both directly affect an individual's neurodevelopment and their susceptibility to neurodevelopmental disabilities. 17β-estradiol (E2) is the hormone often referred to as estrogen[xxvii]. The synthesis of E2 follows the steroidogenic pathway within the brain[xxvii]. This pathway converts cholesterol to testosterone via enzymes, then aromatizes testosterone via aromatase to 17β-estradiol. However, female fetal ovaries do not produce E2 until after birth. In both male and female fetal brains, E2 can also be produced through the metabolization of dihydrotestosterone (DHT) to 3βAdiol[xxvii]. During gestation, the primary form of circulating estrogen that encourages neurodevelopment is 3βAdiol. For hormones to be effective, they require a receptor. In the case of 17β-estradiol and 3βAdiol, the receptors are estrogen receptor alpha, ERα, and estrogen receptor beta, ERβ; 3βAdiol will primarily bind to ERβ[xxvii]. ERβ is widely distributed in the brain. Specifically, ERβ is distributed in the "hippocampus, cerebral cortex, dorsal raphe, substantia nigra, amygdala, microglia, and oligodendrocytes" [xxvii]. Therefore, ERβ is the main estrogen regulator associated with cognitive and affective behaviors.

The mechanisms behind the relationship between the estrogen regulator and behavior is associated to other hormones, serotonin and dopamine. Serotonergic neurons express ERβ,

where it mediates the rate limiting enzyme for serotonin synthesis, E2-dependent tryptophan hydroxylase (TPH) [xxvii]. In the substantia nigra, dopamine is produced. ERβ is the main estrogen receptor isoform, resulting in the promotion of neuroprotection of dopaminergic neurons via 17β-estradiol (E2).

Furthermore, ERβ has been connected to DNA de-methylation, which is an important pathway in embryonic development and differentiation. In DNA de-methylation, ERβ functions at different levels during neurodevelopment primarily by two mechanisms: promoting gene transcription or directing the mechanism to different gene regulatory regions[xxvii]. Genes CYP19A1 and DYX1C1 potentially link the two components. In normal gene regulation, the aromatase enzyme converts testosterone to E2 and is encoded by CYP19A1. Researchers currently hypothesize that when the CYP19A1 gene is dysregulated, due to mutation or environment, aromatase does not convert androgens to E2, causing overexposure of the brain to androgens[xxvii]. This increase in testosterone promotes apoptosis in the right brain hemisphere and slows the development of the left hemisphere [xxvii]. Ultimately, as the levels of testosterone increase in an individual, the risk increases for a neurodevelopmental disability. In relation to the DYX1C1 gene, ERβ and DYX1C1 genes are associated through feedback regulation. ERβ promotes transcription of the gene when E2 is present, and DYX1C1 promotes proteasomal degradation of ERs[xxvii]. This association shows the impact of the endocrine system on the expression of DYX1C1. Both components, environment and genetics, play important roles in the development of a fetus' neural system.

Neurodevelopmental disorders are "disabilities associated primarily with the functioning of the neurological system"[iv]. These disabilities include attention-deficit/hyperactivity disorder (ADHD), autism, learning disabilities, intellectual disabilities,

conduct disorders, cerebral palsy, and impairments in vision and hearing[iv]. Neuro-developmental disorders affect many adults and children. Globally, 6.7 % of the population, ranging 5-17 years old, is diagnosed with a neurodevelopmental disorder. In the past decade, the percentage of children diagnosed with ADHD, specifically, has increased by 4.4%[xxvi]. On average, in a US class, one in five students has a neurodevelopmental disorder[xxv]. With many stigmas surrounding neurodevelopmental disabilities, these students are often overlooked and underserved.

These neurodevelopmental abilities have many potential genetic components but are also affected by the environmental components. As mentioned earlier, disruption in the gene CYP19A1 increases the levels of testosterone. High levels of testosterone alter the hemispheric lateralization of the brain and put the individual at an increased risk for dyslexia and ADHD[xxvii]. Fetal female ovaries, however, protect the fetus from the loss of local E2 production. Prevalence rates of dyslexia further support this as males are more often affected than females in almost all intellectual and developmental disabilities. DYX1C1 is associated with neural abnormalities, such as cortical ectopias and heterotopias, which are common attributes of a human dyslectic brain[xxvii]. The increased levels of testosterone dysregulate the DYX1C1 expression and could predispose an individual to ADHD. The genomic location of these two genes contains areas susceptible to ADHD[xxvii].

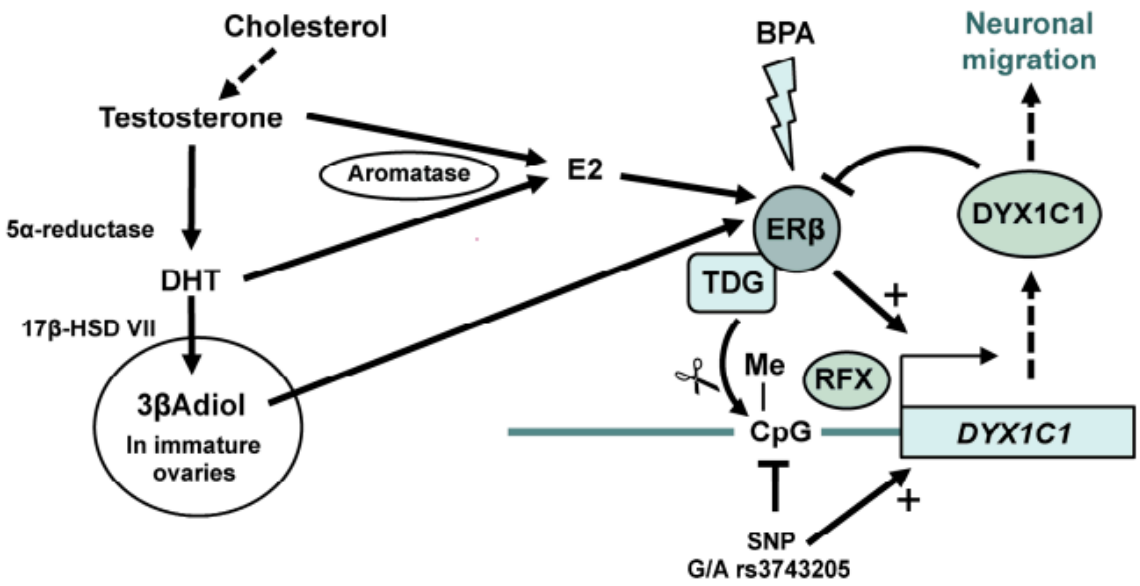


Figure 2. Genetic regulation model showing how BPA effects the interaction with DYX1C1 gene expression[xxvii].

Environmental exposures, such as endocrine-disrupting compound (EDC), affect developing neuroendocrine systems. To further compound these effects, environmental stressors such as socioeconomic hardship and poor parental behavior can greatly increase a child's exposure to EDCs[xvix]. Because of the steroidogenic mechanism and its systemic effects, estrogen, E2, helps protect the brain in all stages of life. Disruption of this mechanism from EDCs can lead to neurodevelopmental consequences. Endocrine imbalances created by aromatase dysregulation and EDCs heavily impact DYX1C1 expression (Figure 2). Bisphenol A is a major EDC used in hard plastics and epoxy resins. As mentioned earlier, Bisphenol A has been associated with a variety of health problems following exposure, due to its endocrine disrupting properties. Neurodevelopmental disorders have increased in prevalence over the past decades, which coincides with the significant increase in BPA exposure. The research performed on the link between estrogen and neurodevelopment

shows a positive correlation between endocrine-disrupting compounds and neurodevelopmental disorders[xxvii].

Based on the research over the neurodevelopmental effects of endocrine disrupting compounds (EDCs), specifically BPA, many companies have attempted to create replacement analogues of BPA. These include Bisphenol S (BPS), Bisphenol AF (BPAF), Bisphenol C (BPC), Bisphenol E (BPE), Bisphenol FL (BPFL), and Bisphenol Z (BPZ) [xii]. Because of the structural similarity to estrogen, these analogues present similar or higher toxic biological effects than BPA. Bisphenol S, specifically, can be detected in fetal cord blood, meaning it crosses the placental barrier[ix]. Bisphenol S does not show estrogenic activity but does show progestogenic activity. While this BPA analogue is greatly under-researched, the same basic problem remains; exposure to Bisphenol S negatively impacts placental developmental and endocrine function.

Fluorescent Spectroscopy

Fluorescent spectroscopy is a useful method for determining the distribution of small molecules within a solution[iii]. Both excitation and emission spectrums can provide important information about a chemical solution. Absorption wavelengths and emission wavelengths are independent of each other[xxviii]. Therefore, these two spectrums are uniquely individual in their technics for analyzing chemical substances. Absorption occurs when an electron transfers to a higher energy level state by absorbing the light energy at a specific wavelength (Figure 3)[iii]. This spectrum shows the excitation intensities. Absorption is a strong experimental technique when impurities are removed. In this experiment, however, impurities were not removed due the delicacy of BPA in solution. Due to this, an emission spectrum was more effective in the solution analysis. The emission spectrum was measured on the FS5 Spectrofluorometer from Edinburgh instrument as electrons were transferred to a lower energy level, releasing energy in the form of photons at a specific wavelength (Figure 4). This experiment utilized emission intensities because BPA is fluorescent and will release photons while falling from a higher energy level to a lower energy level. This is measured by sending the excitation light at a 90 ° angle to the sample[i]. Furthermore, the emission spectrums will be polychromatic as the emission light spreads across many wavelengths[xxviii]. The emission intensities are averaged

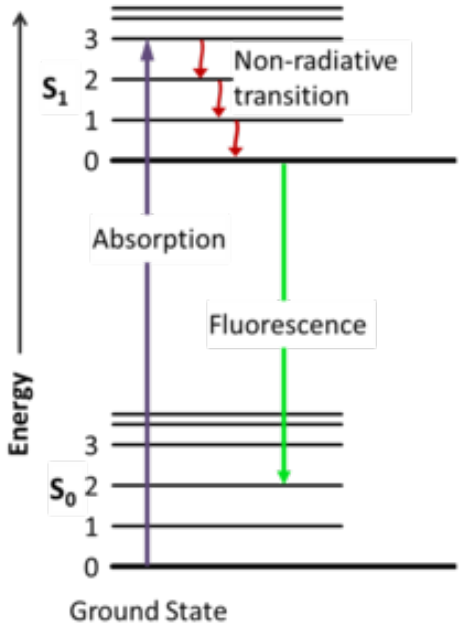


Figure 3. The Jablonski Diagram. Used as a model in Florescence Spectroscopy to illustrate the absorption versus emission in relation to energy levels[iii].

per sample. These average emission intensities were correlated to varying concentrations of BPA via a calibration curve.

Based on the calibration curve, the excitation wavelength was 278 nm, and the emission wavelength was 304 nm (Figure 5). Fluorescent spectroscopy was utilized to measure the emission intensities from concentrations of BPA in solution.

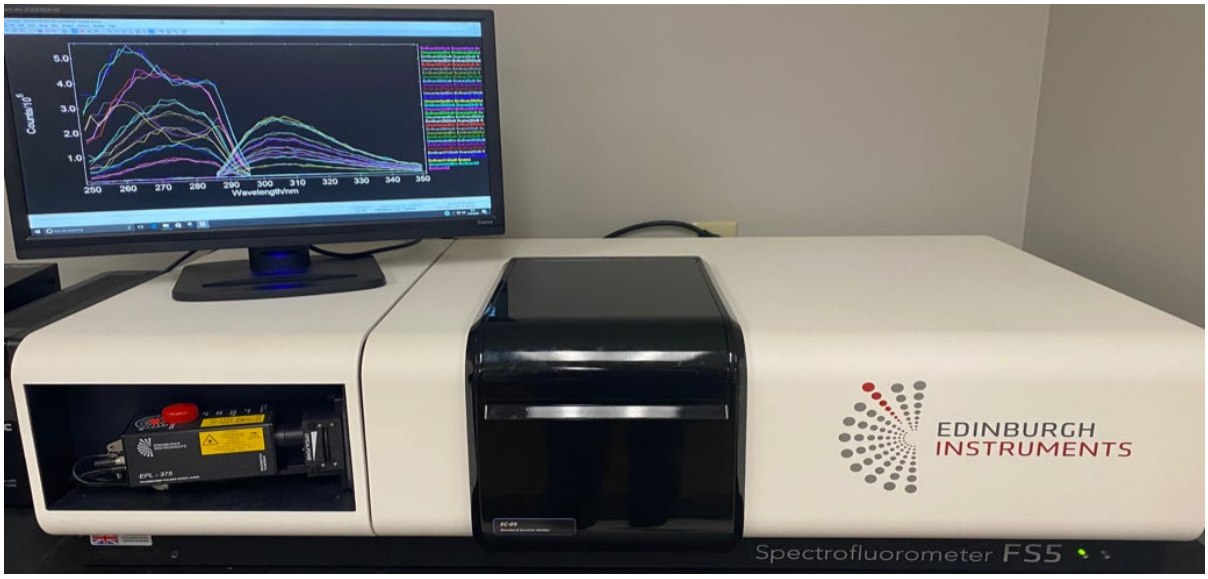


Figure 4. Edinburgh Instruments Spectrofluorometer. Instrument used to measure emission intensities of solutions containing BPA[xxiii].

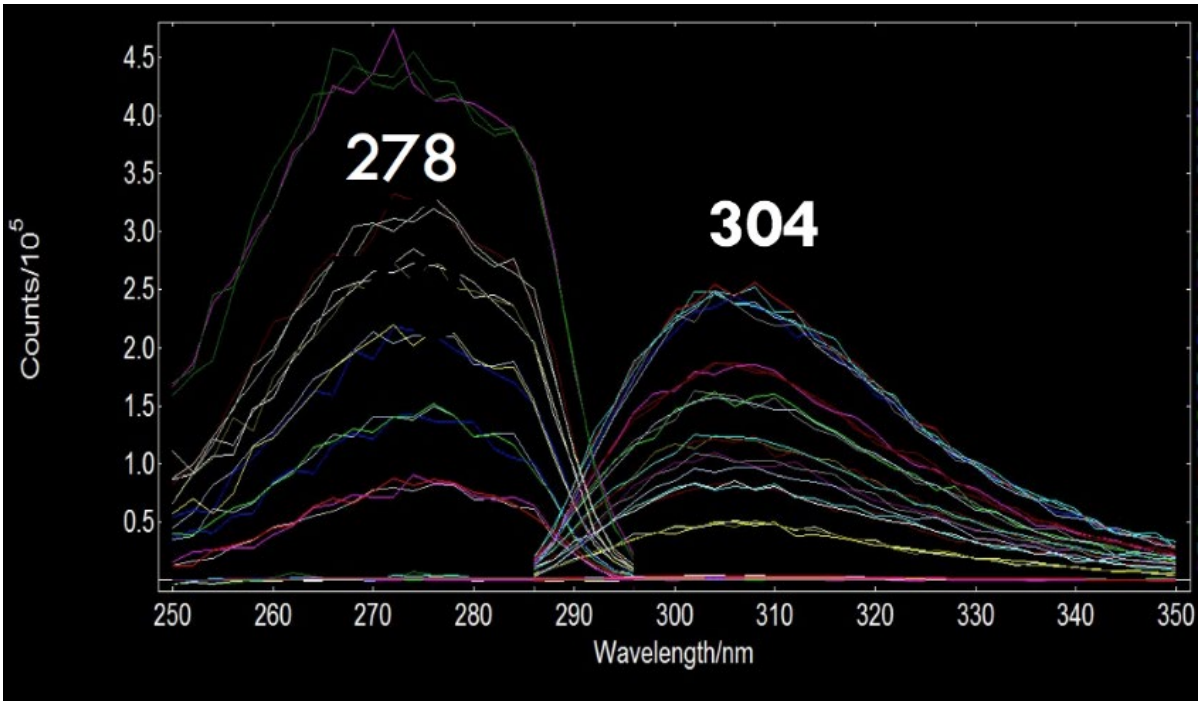


Figure 5. Fluorescence Excitation and Emission spectra obtained using the FS5 Spectrofluorometer from Edinburgh Instruments. The excitation wavelength is on the left (around 278 nm) and the emission wavelength is on the right (around 304 nm)[xxiii].

Materials/Methods

This experiment utilized the following chemicals: Bisphenol-A (BPA), HPLC water, HPLC methanol, and HPLC nitric acid. Glassware included 10-mL volumetric flasks, 5-ml volumetric flasks, 100-ml beakers, six watch glasses, and three 5-mL pipettes. Between experimental trials, all glassware was washed three times with a 25% nitric acid-water solution, rinsed three times with distilled water, then three times with Milli-Q water. Two instruments were used for this experiment. First, emissions intensities were determined via fluorescence using the FS5 Spectrofluorometer from Edinburgh Instruments. Second, the controlled shipment conditions were generated by the Shake'N'Bake Hybridization Oven from Boekel Scientific (Figure 6). Three toothbrush brands-Colgate, Baby's First Toothbrush, and Dollar General-of varying BPA concentrations, confirmed through past research, were tested in this experiment. Additional materials, such as gloves, goggles, labels, and parafilm, were used for safety measures, minimizing errors, and preventing evaporation or contamination of the samples.

Figure 6: Shake'N'Bake Hybridization Oven from Boekel Scientific. Used to generate shipment environment.

To correlate emission intensities and BPA concentration, a calibration curve was created. Bisphenol A was measured to 0.203 g, then dissolved in 100-mL volumetric flask

with a 1:1 methanol water solution. This stock solution was then serially diluted across 12 25-mL volumetric flasks. The volumes of BPA solution were 0 mL, 1 mL, 2 mL, 3 mL, 4 mL, 5 mL, 7 mL, 10 mL, 13 mL, 15 mL, 20 mL, and 25 mL. Each volume was diluted to a total volume of 25 mL. The final concentrations were 0 mM, 2.06 mM, 4.13 mM, 6.19 mM, 8.26 mM, 10.32 mM, 14.44 mM, 20.64 mM, 26.83 mM, 30.96 mM, 41.28 mM, and 51.6 mM. For the fluorescence measurements, each sample was measured in quintuplicate. The excitation wavelength was set to 278 nm and the emission wavelength at 304 nm. From the calibration curve, the linear range of measurable fluorescence was determined to be between 0-20 μg/mL. The limit of detection was 0.109 μg/mL, and the limit of quantification was 0.363 μg/mL (Figure 7). The FS5 Spectrofluorometer measured the excitation and emission intensities of the varying concentration of BPA in solution.

This experimental control was repeated at 50 °C to test if BPA concentration would be affected by temperature. To do this, samples of varying concentration were placed in a water bath at 50 °C for 1 hour. After the solutions were warmed to 50 °C, the fluorescence was measured again in the same manner, then compared to the standard curve at room temperature.

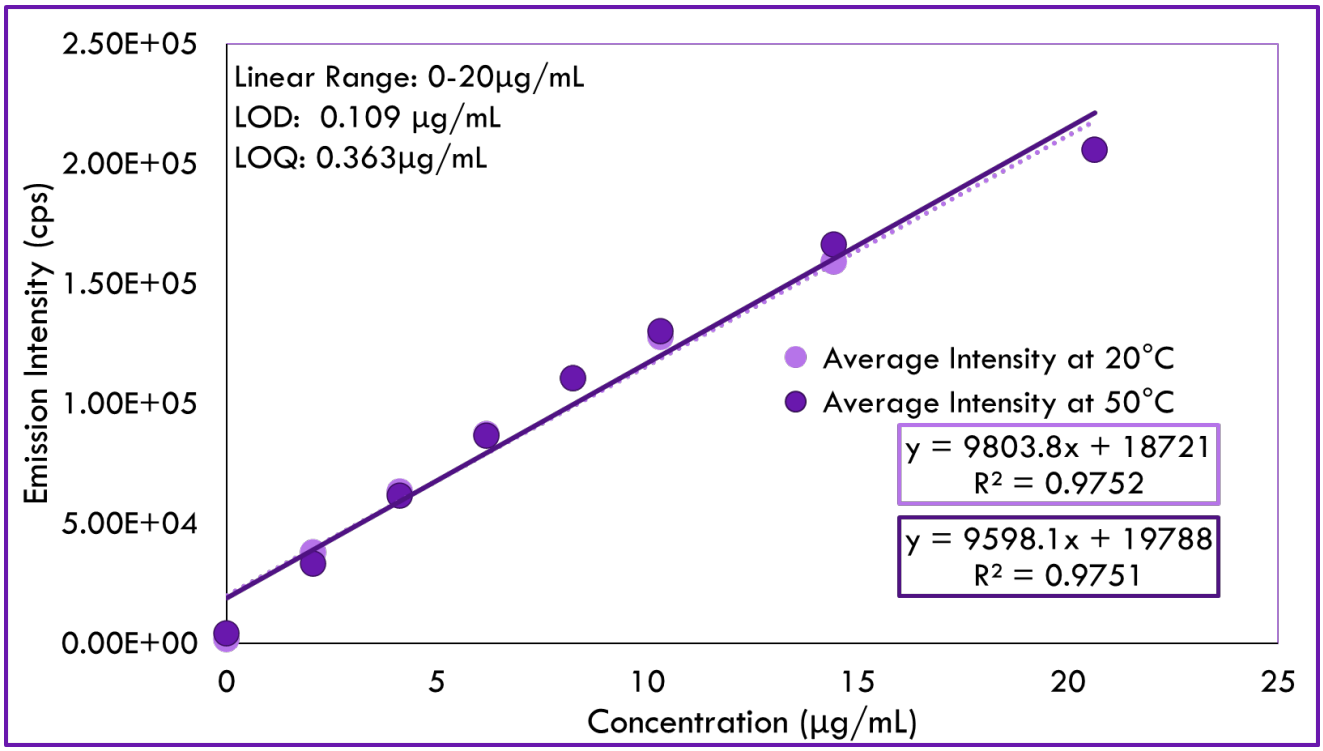


Figure 7: Fluorescence calibration curves of BPA in 1:1 M/W solution obtained at 20 °C and 50 °C.

Three infant toothbrush brands were tested in this experiment. The Control Group was a BPA-free toothbrush, confirmed through previous research. Toothbrush "A" and "B" contain BPA. Previous research showed Toothbrush A containing the highest concentration of BPA of several brands. Toothbrush B was chosen from a mid-level concentration of BPA from previous research. The toothbrushes were labeled accordingly to minimize errors.

The experimental plan for the toothbrushes consisted of time in the Shake'N'Bake and time in 1:1 methanol water solution. Each experimental toothbrush was baked at 50 °C for 3, 5, and 7 days; this provided conditions similar to a shipment container. In addition to the labels "Control," "A," and "B," the toothbrushes were also labeled according to bake time in days. The full label would then include days:group, i.e., 3A. After the allotted bake time, the toothbrushes were placed in 100 mL of 1:1 methanol water solution at room temperature (20 °C). Using the 5 mL pipette, 5 mL samples were removed at 0 min, 5 min, 10 min, 15 min, 20 min, 40 min, 60 min, 80 min, 100 min, 120 min, 4 hrs, 5 hrs, 6 hrs, 8 hrs, 24 hrs, 48 hrs, and 72 hrs. These samples were run in duplicate in FS5 Spectrofluorometer, measuring the peak emission intensities. Emission intensities were measured in counts per second (cps). This was repeated twice for each toothbrush to confirm results.

All collected data was entered into Excel, where the data was graphed and analyzed. For statistical analysis, the emission intensities were compared through ANOVA between groups (Control, A, B) and days (3, 5, 7). The analysis compared data at a .05 level of significance.

Results and Discussion

Using ANOVA, the emission intensities were compared by bake time and by brand. The graphs compare the emission intensities to the time in solution after the allotted bake time. Average emission intensities were graphed versus time for the control environment from all toothbrushes prior to baking. For the Control Group, the average intensity over time was 3.80E03 cps (SD=1606.85). Toothbrush A's emission intensities averaged at 3.56E03 cps (SD=1106.12), and Toothbrush B's emission intensities averaged at 4.12E03 cps (SD=1694.39) (Figure 8). The ANOVA found that there was no significant difference between the toothbrush brands in the control (20 °C) environment, 6.07E-01(17, 17) = 0.605, $p > .05$ (Table 1).

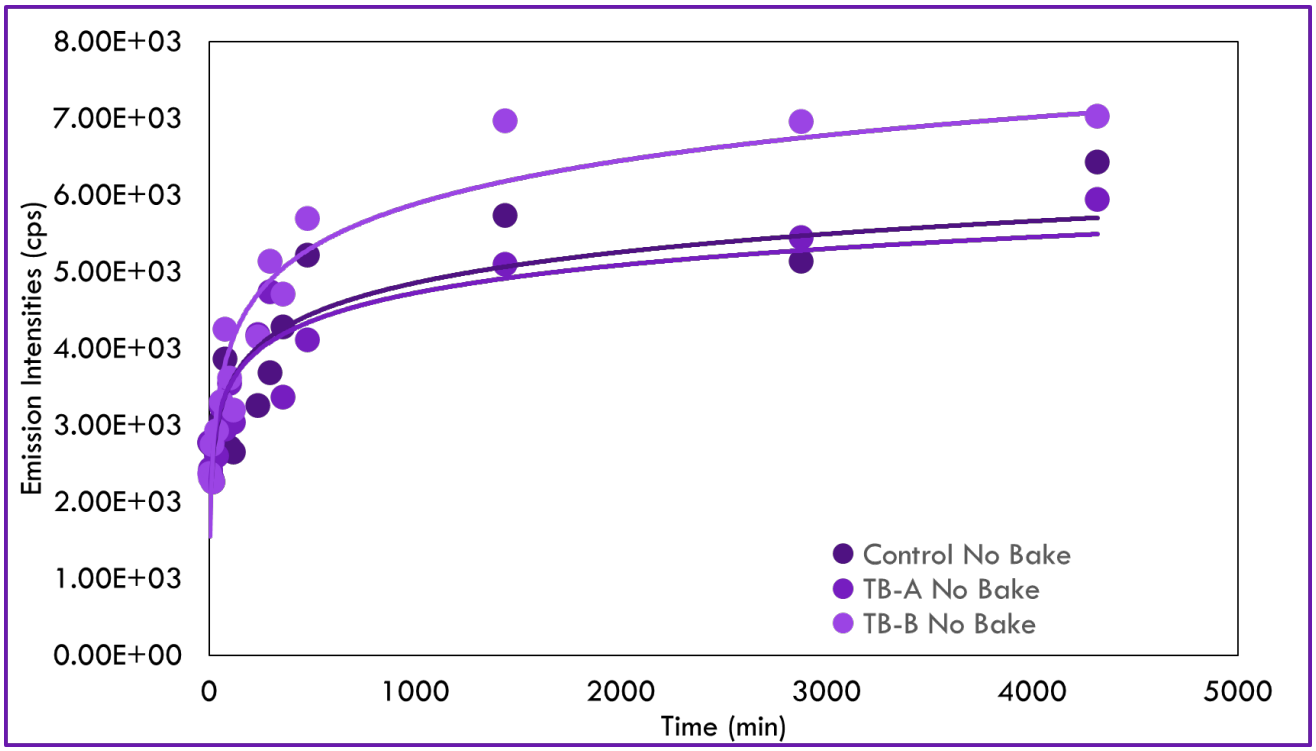


Figure 8: Average fluorescence emission intensities of No Bake Control vs Toothbrush A vs Toothbrush B

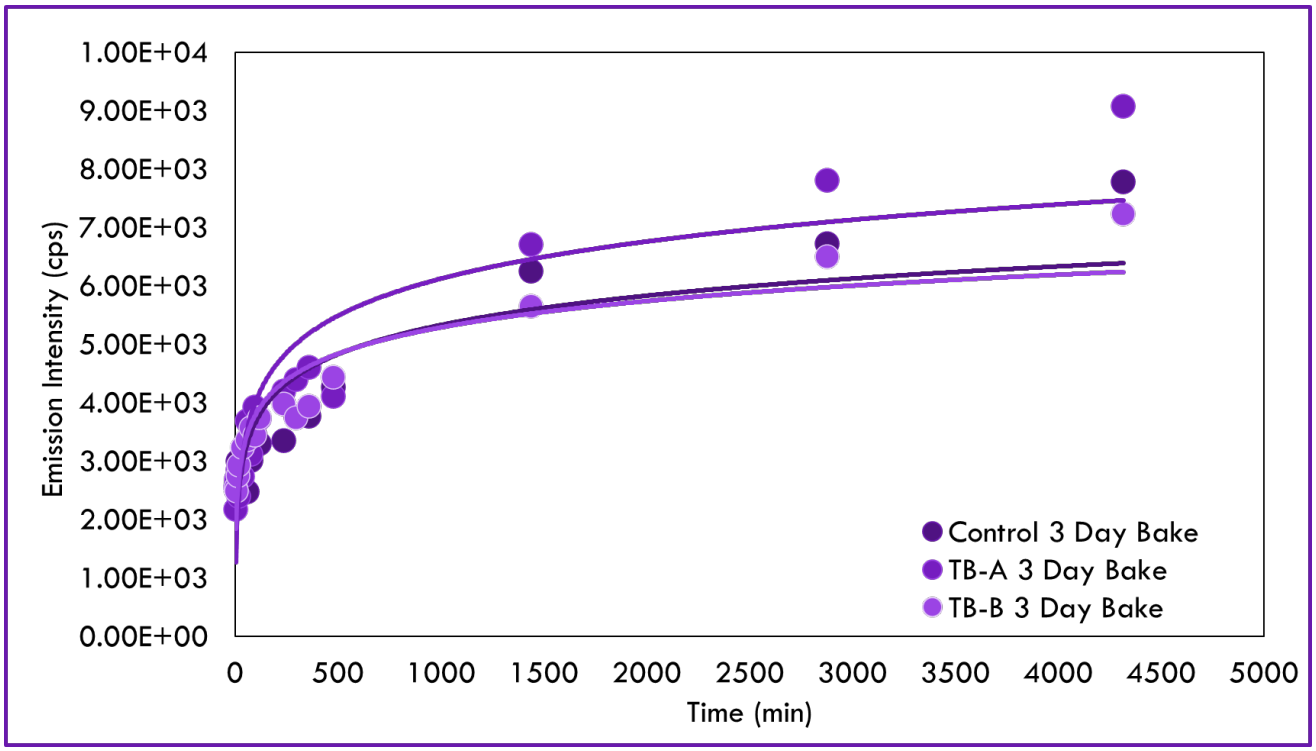


Figure 9: Average fluorescence emission intensities of 3 Day Bake Control vs Toothbrush A vs Toothbrush B

After three days under shipment conditions, the Control Group had emission intensities averaging 3.73E03 cps (SD=1657.26). The emission intensities of Groups A and B averaged 4.17E03 cps (SD=1955.7) and 3.88E03 cps (SD=1383.08), respectively (Figure 9). There 6.07E-01(17, 17) = 0.605, $p > .05$ was no significant difference in the amount of BPA leaching from the infant toothbrushes at 50 °C over three days, 2.88E-01(17,17) =0.400, $p > .05$ (Table 1).

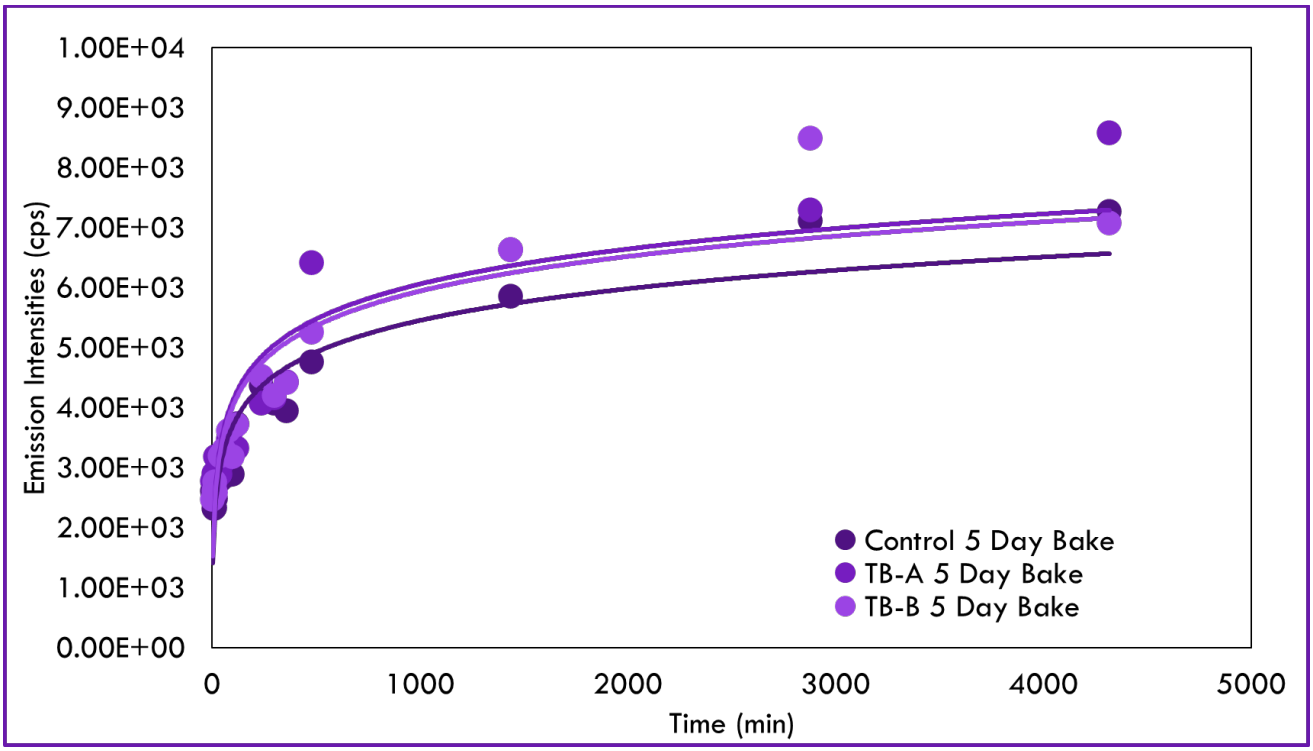


Figure 10: Average fluorescence emission intensities of 5 Day Bake Control vs Toothbrush A vs Toothbrush B

For the five-day shipment trial, the emission intensity average of the Control Group was 3.81E03 cps (SD=1612.28). The average emission intensity of Toothbrush A was 4.28E03 cps (SD=1808.77) and Toothbrush B was 4.12E03 cps (SD=1799.27) (Figure 10). There was no significant difference in the amount of BPA leaching from the infant toothbrushes at 50 °C over 5 days, 3.13E-01(17,17) = 0.392, $p > .05$ (Table 1).

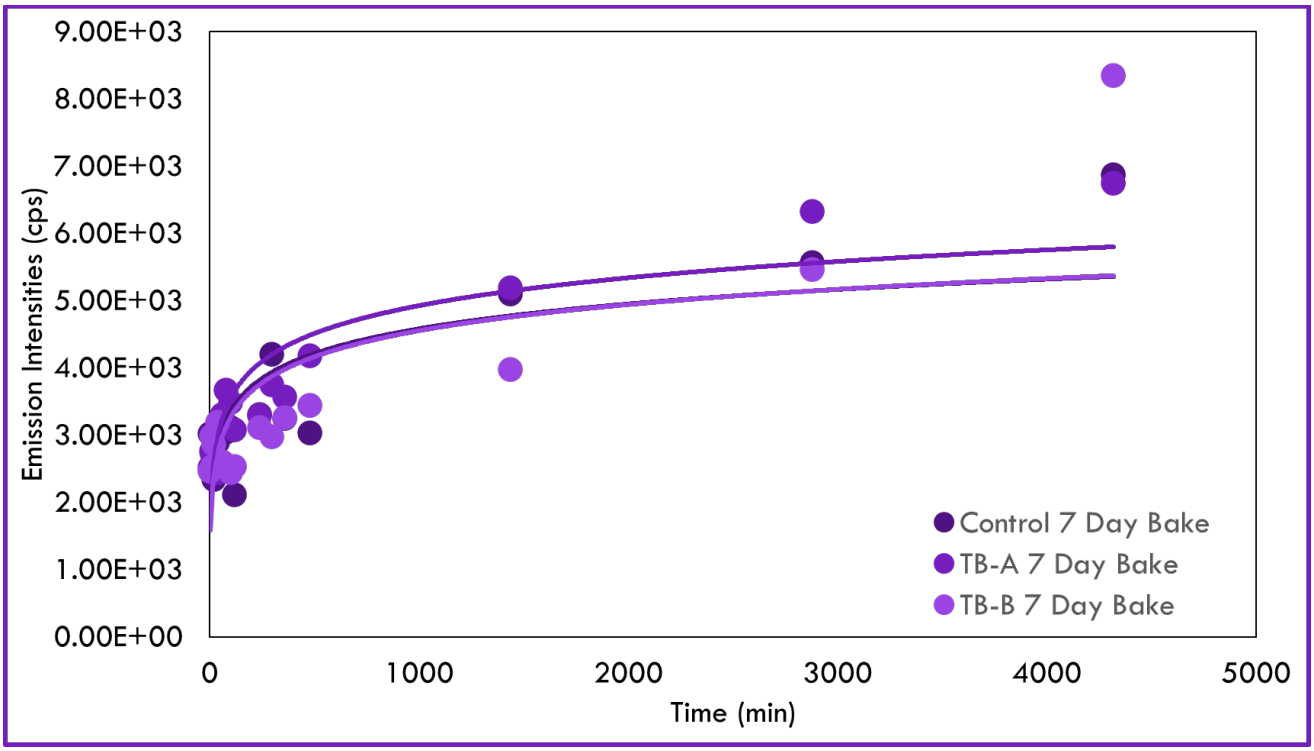


Figure 11: Average fluorescence emission intensities of 7 Day Bake Control vs Toothbrush A vs Toothbrush B

Finally, when placed in shipment condition for seven days, the emission intensity of the Control Group averaged at 3.44E03 cps (SD=1280.63). The emission intensities of toothbrushes A and B averaged at 3.63E03 cps (SD=1312.88) and 3.409E03 cps (SD=1470.03), respectively (Figure 11). The ANOVA revealed there was no significant difference in the amount of BPA leaching from the infant toothbrushes at 50 °C over 7 days, 2.06E-01(17,17) = 0.419, $p > .05$ (Table 1).

	F-Value	P-Value
0 Days: Control vs A vs B	0.607	.605
3 Days: Control vs A vs B	0.288	.400
5 Days: Control vs A vs B	0.312	.392
7 Days: Control vs A vs B	0.206	.419

Table 1: Statistical comparison (ANOVA) of fluorescence emission intensities over time.

A potential error in this experiment should be noted; these toothbrushes are approximately five years old. The effect of time on the concentration of BPA within the toothbrushes was unknown prior to the start of this research. According to previous research, the release rate of Bisphenol A from plastics decreases to insignificant amounts after four to five years. Because of the insignificance of the data from the control experiment, in which the toothbrushes were not under shipment stress, the data presented in this experiment could be skewed. Further testing would be required to determine the validity of this error.

Conclusion

Based on the results of this experiment, shipment temperature and duration does not significantly affect the amount of BPA leaching from infant toothbrushes. If accurate, this information can be comforting to parents of infants, as this shows there is no increase of BPA released from infant toothbrushes due to shipment. This information does not discount previous research showing the significant release of BPA from infant toothbrushes.

Future Work

Future research entails retesting with recently manufactured toothbrushes, additional comparisons with other BPA-containing toothbrushes, and performing this experiment in a 50 °C water bath. The breakdown of BPA in relation to the age of the toothbrushes tested did affect the results of this experiment. By using recently manufactured toothbrushes, the results could reveal a different conclusion. Future work could also entail testing the other brands of

infant toothbrushes containing BPA, as this experiment is limited to only three brands. Finally, future researcher could benefit from testing the leaching of BPA when the toothbrushes are under shipment conditions, 50 °C for 3-7 days. By taking samples prior, during, and shortly after the environmental exposure, the results may reveal BPA to be significantly leaching within the shipment container. Biologically, further research into the effects of BPA on neurodevelopment would be potentially pivotal to infantile health.

Abbreviations

ADHD: Attention-Deficit/Hyperactivity Disorder

ANOVA: Analysis of Variance

BPA: Bisphenol – A

BPAF: Bisphenol AF

BPA-PC: Bisphenol-A Carbonate

BPC: Bisphenol C

BPE: Bisphenol E

BPFL: Bisphenol FL

BPS: Bisphenol S

BPZ: Bisphenol Z

CPS: counts per second

DHT: dihydrotestosterone

E2: 17β-estradiol

EDC: endocrine-disrupting compound

FDA: Food and Drug Administration

SD: Standard Deviation

TDI: Tolerable Daily Intake

TPH: Tryptophan Hydroxylase

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