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Ten Weeks with Green Beans...And Then Some: Determination of Bisphenol-A (BPA) in Canned Goods from Arkansas Markets Using Fluorescence Spectrophotometry

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SENIOR THESIS APPROVAL

This Honors thesis entitled

"Ten Weeks with Green Beans…And Then Some"

Determination of Bisphenol-A (BPA) in Canned Goods from Arkansas Markets Using Fluorescence Spectrophotometry

written by

Rachel H. Pruett

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

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Ten Weeks with Green Beans...

And Then Some

Determination of Bisphenol-A (BPA) in Canned Goods

from Arkansas Markets Using Fluorescence

Spectrophotometry

Rachel H. Pruett

Abstract:

Bisphenol A (BPA) is a chemical widely used in production of consumer goods. It has come under scrutiny recently after being labeled as an endocrine disruptor (ED), mostly causing adverse effects in infants and young children. It has been associated with diabetes, cardiovascular disease, and abnormal maturation. Because it is so commonly used in product development, humans are exposed to BPA through various means, such as ingestion or dermal absorption. It is a concern that the combined exposure could cause serious effects even in small doses.

In canned foods, the chemical is made into an epoxy resin to provide a protective lining along the inside of the can. BPA migration occurs when the free BPA moves from the lining into the contents of the can. This study used the standard addition method in order to determine the concentration of BPA in canned goods, as well as the effect of heat on the migration of the chemical from the can's epoxy lining to its liquid contents. Fluorescence spectrophotometry was also used to quantify BPA concentrations, as BPA is a fluorescing molecule. The limit of detection (LOD) for the instrument was 0.3844 μg/mL.

After removing the can's original contents and replacing it with HPLC-grade H_2O, BPA levels ranged from 0.7 ± 0.5 to 1.2 ± 0.5 μg/mL. In the original liquid though, concentrations vacillated between 43.5 ± 0.7 and 95 ± 4 μg/mL. After the application of heat, values stretched from 39 ± 2 to 94 ± 12 μg/mL. The results confirmed the presence of BPA in all of the cans, and indicated that there was no effect on migration of BPA after heating the samples.

Dedicated

To

My Parents,

For putting up with me being away from home for an entire summer

My Friends,

For providing an audience for all of my practice presentations

Dr. Sara Hubbard,

For being my mentor for the entirety of my research and writing experience

You are all awesome.

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Introduction:

Bisphenol-A (BPA) is a monomer commonly found in polycarbonate plastics and epoxy linings (Fig. 1). Because of its effectiveness and durability, BPA is a widely used chemical in many consumer goods, including baby bottles, water bottles, and thermal receipt paper.[1-4]

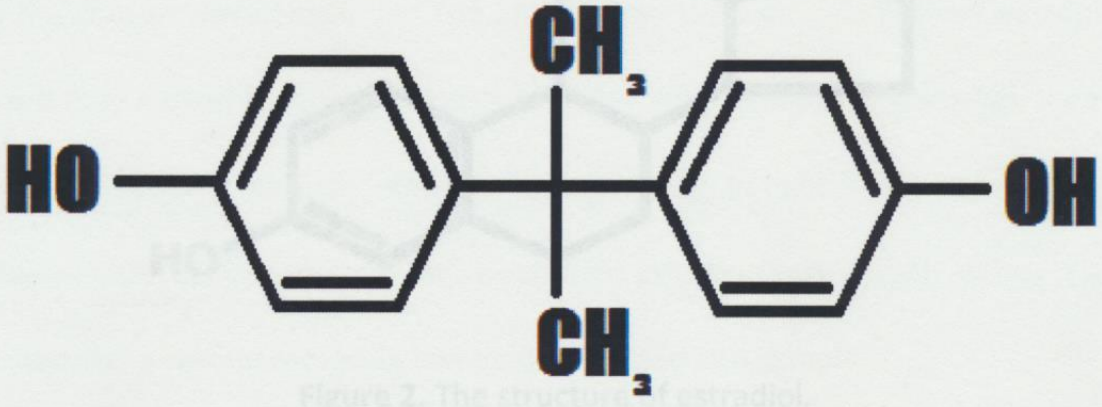


Figure 1. Structure of BPA.

It is also used in the protective linings of canned foods.[5-7] In order to guard the can from accidental erosion and the food from contamination, a shielding film made up of a BPA-containing epoxy resin is placed along the inner surface of the can. The creation of these resins is not always complete, however, and can lead to what is called BPA migration, or BPA leaching, as free BPA moves into the contents of the can.

In recent years this act of leaching has been brought under review, because of the possible medical problems associated with BPA exposure. BPA is classified as an endocrine disruptor, and has been linked to breast cancer, cardiovascular issues, diabetes, and abnormal liver enzymes.[8-10] These problems are largely due to the structural similarities

between BPA and estradiol, an estrogen. While inside the body, BPA will bind to estrogen receptors throughout the body and block actual estrogen molecules from attaching.[1-10] The hormone balance is the human body is a very delicate system, and an estrogen mimic like BPA has the potential to cause havoc in multiple areas.

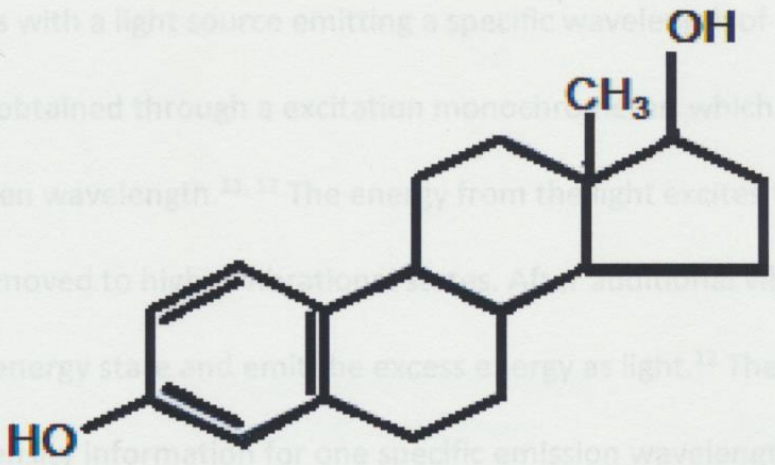


Figure 2. The structure of estradiol.

Because it is so commonly used in product development, humans are exposed to BPA through various means, such as ingestion and dermal absorption. It is a concern that the combined exposure could cause serious effects even in small doses. The greatest danger is for infants and young children because of underdeveloped immune and endocrine systems, though adults can still experience adverse effects.[8-10] In order to protect the general public from such hazards, it is necessary to assess the amount of BPA in various consumer products to ensure that the consumer would not ingest too much on a daily basis.

The aim of the experimental part of this study was to report on the BPA concentration in canned foods from Arkansas markets. Warehouse conditions were replicated in order to determine the effect of varying temperature conditions on BPA

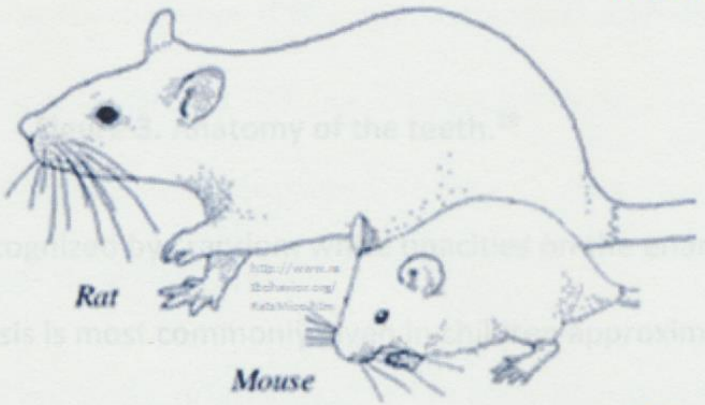
leaching and to monitor how much BPA is being introduced into the liquid surrounding the contents of the can from the protective lining while the food is waiting to be consumed. Migration was assessed using fluorescence, as BPA is a fluorescing molecule.

Fluorescence is often quantified using a fluorescence spectrophotometer. The instrument begins with a light source emitting a specific wavelength of light onto a sample. This specificity is obtained through a excitation monochrometer, which filters the light down to the chosen wavelength.[11, 12] The energy from the light excites the electrons in the sample and they moved to higher vibrational states. After additional vibration, they fall back down to a lower energy state and emit the excess energy as light.[12] The instrument can be set to receive intensity information for one specific emission wavelength, which is how the concentration of a certain molecule can be determined in a sample.

BPA leaching has been studied in various countries, including the United States, Italy, Turkey, and Mexico.[13-16] Researchers have investigated the effect of damage, temperature, and storage time on BPA migration, though there has been much controversy over the results found between the studies. As a precautionary measure, the European Food Safety Authority (EFSA), along with the United States Environmental Protection Agency (EPA) and the U.S. Food and Drug Administration (FDA) has set BPA intake limit of 0.05 mg/kg (bodyweight)/day, although some effects have been noticed with smaller values[17]. Some of the various health effects are examined in greater detail in the next section. They are divided into groups based on what species of animal was used for the experiment.

Health Effects:

Mice and Rats



Disrupted Passive Avoidance Learning and Memory

Inhibition of Ventricular Heart Function

Enhanced Fear Memory

Impaired Hepatic Glucose Sensing

Demasculization

Enamel Defects

Disturbed Spermatogenesis and Apoptosis in Testes

Epigenetic Effects – CpG Hypomethylation

Enamel Defects[18]:

Molar incisor hypomineralization (MIH) is a medical disorder that is becoming a more and more frequent diagnosis. It takes its name from the fact that it most often affects the permanent first molars and incisors (Fig. 2).

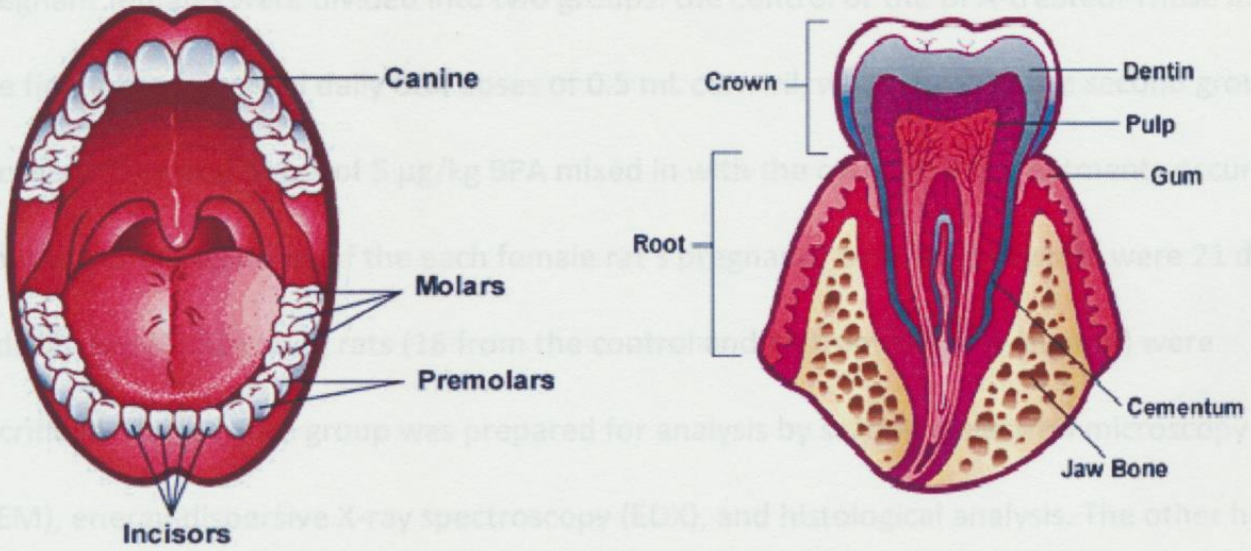


Figure 3. Anatomy of the teeth.[19]

The enamel condition is recognized by "random white opacities on the enamel of affected teeth." Because the diagnosis is most commonly given in children approximately six to eight years of age, scientists theorize that there is a small window of time occurring early on in normal development in which humans are most susceptible to enamel growth defects.

Amelogenesis, or the formation of enamel on teeth, occurs through a series of stages including the secretory and maturation phases. In the first, various proteins (amelogenins, enamelin, ameloblastin, and amelotin) form a matrix that will deposit enamel on the teeth until the full thickness has been reached. At this point, the maturation phase takes over and the serine protease kallikrein 4 (KLK4) begins to break down the enamel

matrix proteins. Teeth can become hypomineralized at this point if KLK4 fails to degrade all of the right proteins.

In the study led by Katia Jedeon[18], researchers used rodents to investigate the effects of BPA treatment on development of MIH. In the primary portion of the experiment, pregnant females were divided into two groups: the control or the BPA-treated. Those in the first group received daily oral doses of 0.5 mL corn oil, while those in the second group received daily oral doses of 5 µg/kg BPA mixed in with the corn oil. The treatments occurred from the very beginning of the each female rat's pregnancy until the baby rats were 21 days old. On day 30, 32 young rats (16 from the control and 16 from the BPA-treated) were sacrificed. Half of each group was prepared for analysis by scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDX), and histological analysis. The other half underwent mandibular dissections. On day 100, the remaining 32 young rats (16 from the control and 16 from the BPA-treated) were sacrificed. These rats were divided up like the previous group and their fates were the same.

During the course of the study, researchers found similar phenotypic results in the molars and incisors of the BPA-treated rats as would be found in a patient diagnosed with MIH. They also found an increase in the albumin and enamelin content in the affected teeth, along with a decrease in KLK4 expression. This supports the idea that BPA could be a potential cause of MIH, although there are many other factors that could also play a role. These results also provide new research into the pathology of this condition and further serve to identify a possible time window of human susceptibility to BPA.

Enhanced Fear Memory[20]:

Fear is the most common instinctual reaction of an animal to a dangerous situation. This response has been passed down through the generations because it enhances defensive behaviors and protects the ones that possess it. Too much of a good thing, however, can become a bad thing. If this emotion is expressed too often, animals can develop a number of problems such as anxiety and depression. In the brain, scientists have linked fear memory and learning to the amygdala, hippocampus, and prefrontal cortex (Fig. 3). These areas are analyzed after fear conditioning and testing occurs in order to determine the molecular effects of certain chemicals on the fear response.

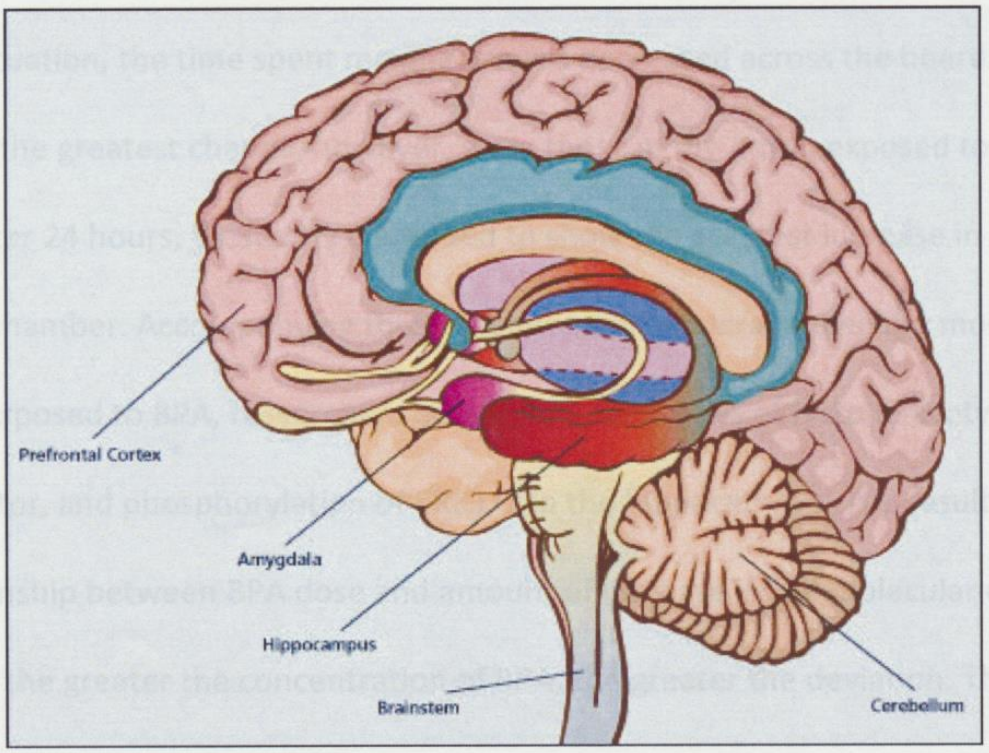


Figure 4. Location of the hippocampus, amygdala, and prefrontal cortex in the brain.[21]

In the study led by Qin Zhang[20], Chinese investigators wanted to determine if long-term exposure to BPA during early development could result in a change in fear memory. They began by assigning 104 nine-week-old male mice to four different treatment groups: control (sesame oil only), 0.4 mg/kg, 4 mg/kg, and 40 mg/kg BPA. All groups received their daily dose through oral administration for 90 days followed by three days of no BPA exposure before being subjected to the fear-conditioning test. After the results from this test were recorded, the mice were sacrificed for hippocampal examination and Western blotting.

During the fear memory test, the emotion was measured by how long the animals spent immobile in the testing chamber. In the beginning, the mice from all the groups spent the majority of their time in motion. After receiving brief foot shocks to simulate a possible dangerous situation, the time spent moving around decreased across the board. The mice that showed the greatest change, however, were those in the group exposed to 40 mg/kg BPA. Even after 24 hours, these rats continued to show the greatest increase in stillness while in the chamber. Accompanying these changes were several molecular modifications. In the mice exposed to BPA, researchers noted increased levels of histone acetylation, NMDA receptor, and phosphorylation of ERK1/2 in the hippocampus. The results reflected a direct relationship between BPA dose and amount of increase in the molecular changes; in other words, the greater the concentration of BPA, the greater the deviation. This supports the authors' hypothesis that long-term exposure to BPA would result in an enhanced fear response that extends for a longer period time after initial stimulation.

Inhibition of Ventricular Heart Function[22]:

Reactive oxygen species (ROS) are molecules that have had incomplete electron reduction (Fig. 4). They are extremely reactive and can steal electrons from other molecules in order to complete their reduction, thereby causing oxidative stress in the cell.

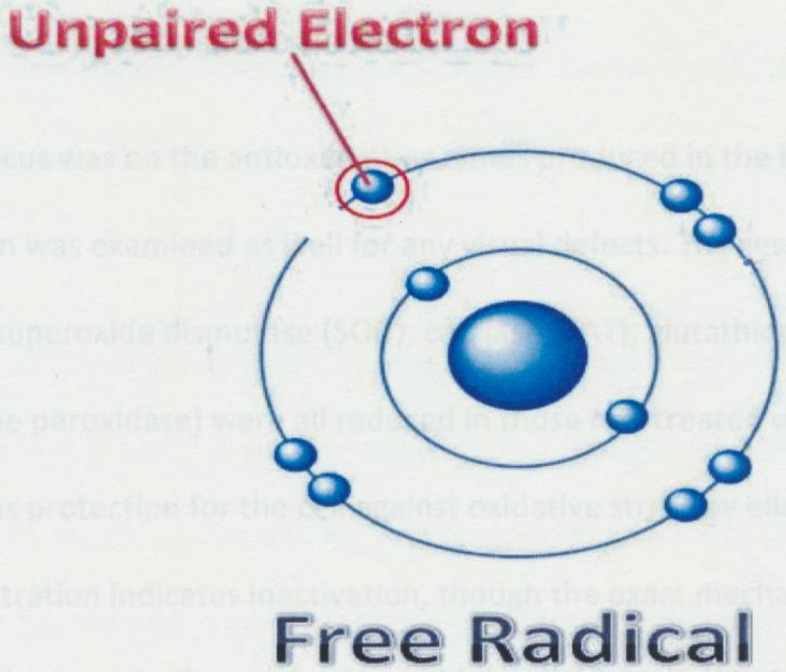


Figure 5. Structure of a ROS.[23]

ROS occur naturally in the body and can be used for cell signaling, but can also pose a threat if there is too much in one place. There are several antioxidants that work in the cell to alleviate this hazard, so that the cell does not have to resort to apoptosis but they can be overwhelmed. Previous research[22] has indicated that BPA can increase oxidative stress in the cell by prompting greater formation of ROS in the cells. Despite the fact that oxidative stress has been linked to several cardiovascular issues, there is little to no research investigating the effect of BPA on the cardiovascular system.

The study led by Panchali Tarafder[22] attempted to remedy this deficiency by using rats to measure ventricular injury due to BPA. They used 28 rats, from 14-16 weeks old, and divided them up into four groups: the 20 day control (with 0.5 mL of 20% DMSO), the 20 day 50 mg/kg BPA-treated, the 30 day control (with 0.5 mL of 20% DMSO), and the 30 day 50 mg/kg BPA-treated. The rats received daily oral administration for the prescribed days before being sacrificed for histological studies.

The main focus was on the antioxidant enzymes produced in the hearts of the rats, but the actual organ was examined as well for any visual defects. The researchers found that expression of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and GP_x (glutathione peroxidase) were all reduced in those rats treated with BPA. These enzymes function as protection for the cell against oxidative stress by eliminating ROS. A decrease in concentration indicates inactivation, though the exact mechanism is unknown. They also found an increase in the production of Malondialdehyde (MDA), which reflects an increase in ROS. These results indicate an overall loss of ability to effectively battle oxidative stress in the cell. On examination of the ventricular tissue samples under a microscope, the authors could distinguish visible deterioration in those rat hearts that ingested the BPA. Together these results suggest that BPA damages the heart by removing its protective combatants.

Impaired Hepatic Glucose Sensing[24]:

One of the most prevalent diseases in the world today, especially in the United States, is diabetes. The prevailing theory has been that "dietary over-consumption and physical inactivity are the single determinants of weight gain." Because of this theory, the disease has been treated mainly with dietary alterations and increased exercise, or the occasional prescribed medicine. As more information is uncovered about the original cause of diabetes in various people, scientists have proposed the idea that environmental factors can also play a role.

In the study led by Leigh Perreault[24], researchers noted the similar relationship between the rise of BPA in plastics and the rise of Type 2 diabetes in America. Type 2 diabetes is marked by insulin resistance; for that reason, people with this disease still produce insulin but cannot take it into the cells. The authors limited their area of research to glucokinase activity in the liver, as "BPA is hepatically metabolized, [and] its toxicity in the liver appears to be particularly prominent." Using six month old male mice, the effects of acute and chronic BPA exposure were analyzed. The acute group received a bolus of BPA before being sacrificed two hours later. The chronic group received approximately 50 µg/kg BPA daily through oral administration for two weeks before being sacrificed. Urine samples were also obtained in order to confirm the presence of BPA in the mice. After the point of euthanization, the livers of all mice were dissected and the glucokinase activity was measured for each.

The authors found that all of the mice exposed to BPA experienced a decrease in glucokinase activity. Those that received the BPA bolus registered an extreme reduction in activity compared to the control, even to the point of inactivity in some cases. Those that were exposed over the two weeks also experienced a significant drop in activity, though no inactivity was seen. This supports the idea that BPA exposure may lead to development of diabetes through the influence of environmental factors. One key piece of information for this study that the authors highlight is that all of the research was conducted with a concentration of BPA that the EPA has deemed as being a "safe dose." Though a normal human is unlikely to come into contact with that large of an amount of BPA in a single dose (as the mice did), this research brings up the idea that "this dose is not safe and has physiological ramification relevant to the development of diabetes, at least in mice."

Disturbed Spermatogenesis and Apoptosis in Testes[25]:

Spermatogenesis is the process by which males produce the sperm cells required for reproduction. This arrangement requires proper maturation of spermatogenic cells in order to pass along the genetic code of a species (Fig. 5).

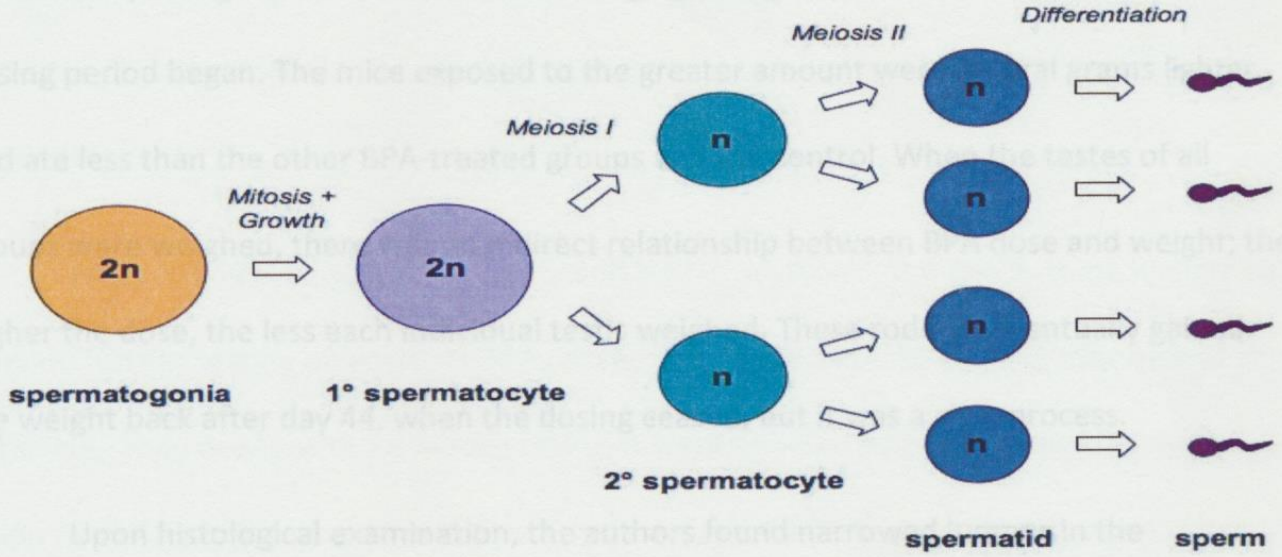


Figure 6. Process of spermatogenesis.[26]

In mammals, this development occurs in the seminiferous tubules of the testes. If, however, the cell encounters a problem during its growth the body will send a signal to destroy it. This cellular signaling can occur through many pathways, some of which have shown evidence of a possibility of being influenced by BPA.

In the study led by Yuan-Jie Li[25], Chinese researchers wanted to discover the effect of BPA on the male reproduction system. They obtained 40 male mice and divided them up into four different groups: the control, 160 mg/kg, 480 mg/kg, and 960 mg/kg BPA. For the first 30 days of their lives, the mice were allowed to develop normally in environmentally controlled rooms. At day 31, the groups received their individual doses of BPA and

continued to receive daily oral administration until day 44. After that time, the mice were allowed to return to normal conditions until being sacrificed on day 90.

Before the BPA treatment began, the mice were all of indistinguishable size, in both body weight and appearance. Physical differences in weight and food intake, however, were immediately recognizable between the 960 mg/kg BPA group and the control once the dosing period began. The mice exposed to the greater amount were several grams lighter and ate less than the other BPA-treated groups and the control. When the testes of all groups were weighed, there was an indirect relationship between BPA dose and weight; the higher the dose, the less each individual testis weighed. These rodents eventually gained the weight back after day 44, when the dosing ceased, but it was a slow process.

Upon histological examination, the authors found narrowed lumens in the seminiferous tubules of the 480 mg/kg and 960 mg/kg BPA-treated groups, as well as signs of underdevelopment in the entirety of the testes. They also found increased marks of apoptosis in spermatogenic cells, as indicated by the increased levels of expression of Fas, FasL, and active caspase-3. The aforementioned are all involved in cellular signaling for apoptosis, and upregulation of them will cause increased cell death, though the exact effect of BPA on this signaling is unknown. These results reveal the danger of high doses of BPA on the reproductive system of male animals. Though the values tested were far above the "safe dose" set by the EPA, BPA can accumulate in the body, especially for those that work in fields where the chemical is handled in large amounts daily.

Demasculinization[27]:

In mice, sex differences have been reliable reported in several forms of testing but most especially for those for anxiety and depression. For this experiment, the most relevant findings are that males usually demonstrate larger amounts of anxiety on the Elevated Plus Maze (EPM). While there has been some research to determine the effects of a number of estrogen-like chemicals on these sex differences, the results have been controversial and conflicting[27].

In the study run by Bryan A. Jones and Neil V. Watson[27], the two authors wanted to provide further testing on whether BPA causes an effect on eliminating the determined sex variances. They began with 15 female rats that were handled daily and trained to drink liquid from a syringe in order to reduce any anxiety that would come from the experiment. These rats were impregnated and divided into five different groups: control (with corn oil), 5 μg/kg, 50 μg/kg, 500 μg/kg, and 5000 μg/kg BPA. They received their daily doses through oral syringe administration from gestational day 7 until the 14th day after they delivered their babies. Each group was allowed to have a total of 12 males and 12 females, which means that several baby rats were eliminated without study. Those that lived were examined for their performances on the Morris Water Maze (MWM), the EPM, and the Forced Swim Test (FST) (Fig. 6).

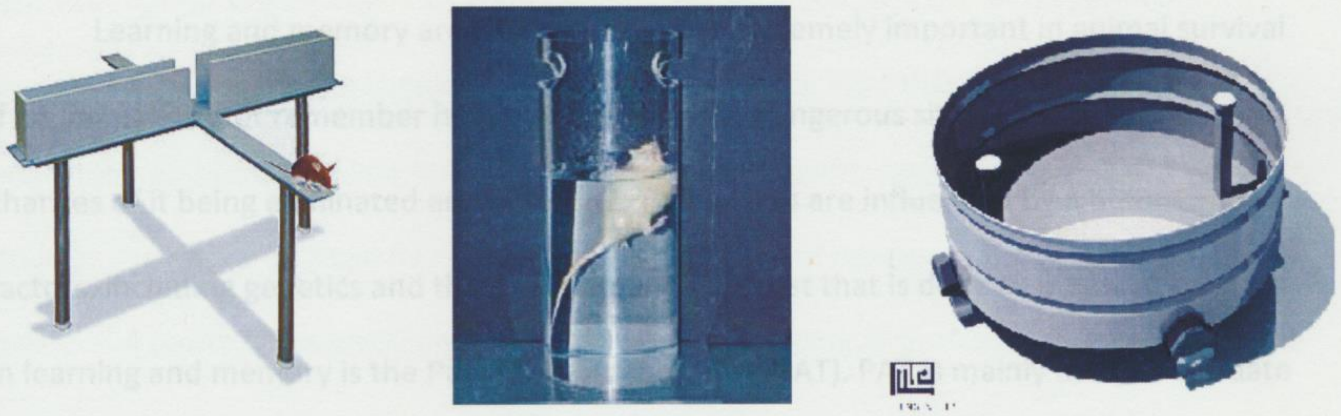


Figure 7. Setups for EPM (left), FST (middle) and MWM (right).[28, 29, 30]

The sex differences detected in the MWM were not eliminated through BPA dosing, nor were there any changes in learning and memory. The same results were observed in the FST, where the traditional dissimilarities were made known. This was not the case for the EPM. As stated before, male rats typically display greater anxiety on the EPM, as reflected by greater amounts of time spent in the closed portion of the apparatus and fewer hub entries. At the 5 μg/kg level, this sex difference was eradicated through the demasculinization of the male rats. This showed in the reduced anxiety levels of those males, putting them on even terms with the females in that group. These results show that rats have a "non-monotonic dose response in the ability to eliminate these sex differences." This experiment also revealed that BPA can cause effects on babies through maternal contact. Further testing will need to be done to determine the exact mechanism, as well as the long-term effects of this type of exposure.

Disrupted Passive Avoidance Learning and Memory[31]:

Learning and memory are two skills that are extremely important in animal survival. If an animal cannot remember how or when to avoid dangerous situations, then the chances of it being eliminated are increased. These tools are influenced by a number of factors, including genetics and the environment. One test that is done to measure changes in learning and memory is the Passive Avoidance Test (PAT). PAT is mainly used to evaluate how a certain chemical will alter the time it takes for an animal to learn how to avoid a certain environment and how well that memory sticks.

In the study led my Mahnaz Teherianfard[31], Iranian scientists used PAT to assess the effects of BPA. They randomly assigned 36 male rats of similar weight to six different groups: the control (no treatment), the sham (with sesame oil), 5 mg/kg, 50 mg/kg, 100 mg/kg, and 150 mg/kg BPA. All groups were subjected to five days of testing. The first few days were used as time for the rats to adjust to the shuttle box, while the last were set up to evaluate learning, memory consolidation, and memory retention. The painful stimulation occurred in the dark side of the box, so the animals "were considered as completely learned, if they did not move to [the] dark compartment after 120 sec."

The researchers found no statistical differences between the control, the sham, and the 5 mg/kg groups, indicating that low exposure to BPA had no effect on learning and memory. At the 50 mg/kg level, they began to see a decrease in the amount of time that group spent in the light. Because this change occurred on the last day of testing, it reflected impaired memory retention and meant that the rats in that set could not remember the

pain that had initially steered them away from the dark. The remaining groups (100 mg/kg and 150 mg/kg) began showing problems even earlier. Even with the same painful stimulation as the others, these rats spent less time in the light than the control. This indicated complications with learning, memory consolidation, and memory retention. These findings show that BPA can have an effect on learning and memory when present at certain levels. The authors theorized that it was due to "remodeling of prefrontal and hippocampal spine synapses," but further testing will need to be done to confirm or reject this hypothesis.

Epigenetic Effects – CpG Hypomethylation[32]:

The field of epigenetics is a relatively new area of study and it looks at certain molecular traits that are not related to physical changes in the DNA. The primary influences come from "DNA methylation, histone modification, and expression of non-coding RNAs" (Fig. 7). It is an important area of study because these influences can produce changes that last throughout the rest of one's life, and even on to future children. Recent evidence has revealed that environmental chemicals like BPA can affect a person's health by altering one or more of the three primary influences of epigenetics.

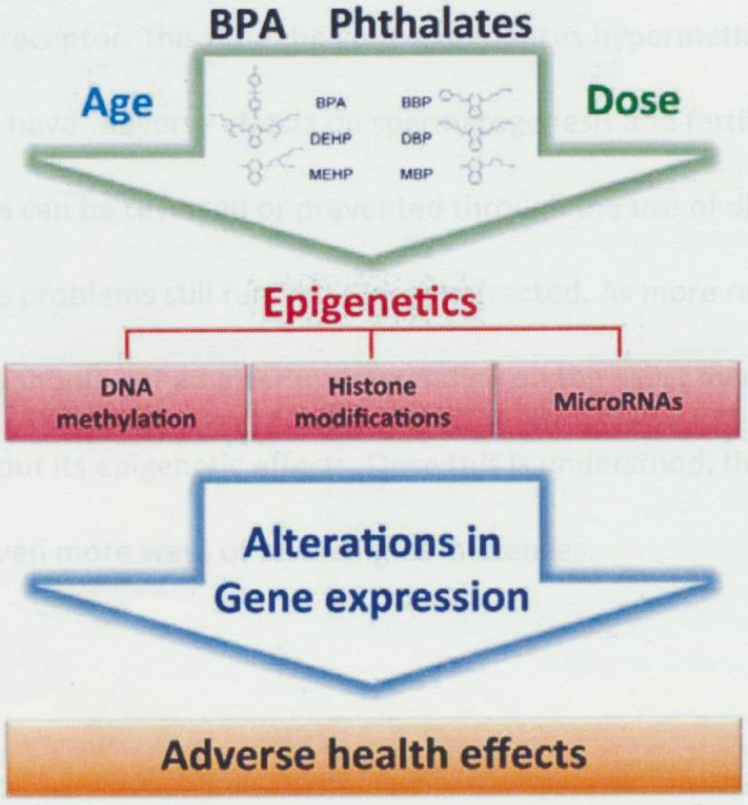


Figure 8. Epigenetic effect of BPA and phthalates.[32]

In the article written by Sher Singh and Steven Shoei-Lung Li[32], the authors compile numerous reports of the epigenetic effects of BPA and some other phthalates. These chemicals work by interacting with common genes and proteins in the body and either lowering or increasing their expression. Scientists have identified approximately 89 of these genes/proteins which they hope "will serve as biomarkers to assay the toxicity of environmental chemicals." BPA is known to affect all three of the primary influences in various ways, depending on the type of gene.

For example, the Agouti gene on mice is often hypomethylated (suppressed) by BPA. This gene controls coat color in mice and, when influenced by BPA, causes the offspring coat color to shift more toward a yellow color. Another gene that has been intensively studied is that of the estrogen receptor. This time the gene experiences hypermethylation (activation) from BPA, which can have "adverse effects on spermatogenesis and fertility." Fortunately, some of these effects can be reversed or prevented through the use of dietary supplements, but the problems still remains unless corrected. As more research is conducted, scientists should get a better understanding on the exact mechanisms through which BPA brings about its epigenetic effects. Once this is understood, they stand a better chance of creating even more ways of reversing its influences.

Health Effects:

Non-Human Primates

Rhesus Macaque

African Green Monkey

Accelerated Secretory Cell Maturation[33]:

The lungs of mammals are multi-lobed organs surrounded by a pleural cavity that protects and insulates it. Epithelial cells line the interior and allow for greater surface area in which to expand and circulate air (Fig. 8). They also control the flow of mucus in and out of the body.

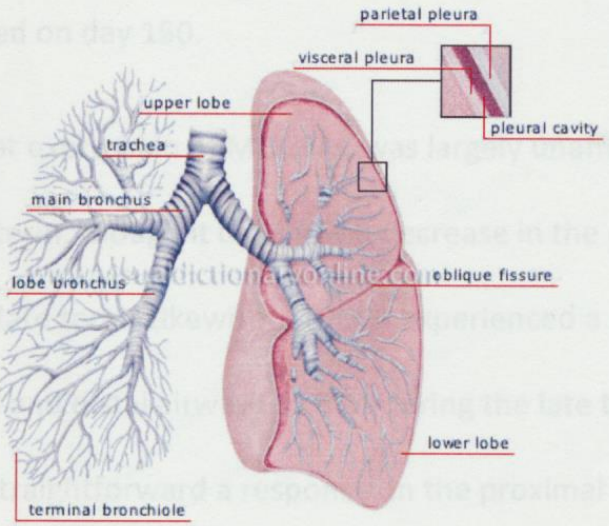


Figure 9. Anatomy of a lung.[34]

Many diseases affecting the pulmonary system are characterized by abnormal lung development. More specifically, people face issues with their lungs when the epithelial cells do not mature as they should.

In the study led by Laura S. Van Winkle[33], researchers used rhesus monkey to emulate the effects of BPA on human lung development. Monkeys were chosen over rats or mice for this particular experiment because the lungs of the former animal are more closely related to human lungs as far as secretory cells are concerned. The scientists focused on three specific proteins (CCSP, MUC5AC, and MUC5B) that play different roles on the body's

immune response and mucus expression. They divided up female monkeys into four different groups: the sham control early term, the sham control late term, the BPA early term, and the BPA late term. Treatments were delivered to the body through subcutaneous placements of implants in the scapular region of the animal, ensuring constant chemical exposure. Monkeys in the early term groups (comparable to a human's second trimester) were sacrificed on day 100, while those in the late term groups (comparable to a human's third trimester) were sacrificed on day 150.

The authors found that expression of MUC5AC was largely unaffected by the presence of BPA in the early term, though it did show a decrease in the distal airway in the group exposed to BPA in the late term. Likewise, MUC5B experienced a significantly increase in both the proximal and distal airways by BPA during the late term. CCSP, on the other hand, did not have as straightforward a response. In the proximal airway during the late term, BPA caused an incredibly significant increase in expression compared to the control. In the distal airway during the late term, BPA caused the exact opposite response.

A number of diseases feature overly abundant amounts of secretion, as could be seen with overexpression of these proteins. The authors link this type of overexpression with BPA exposure in the late term in their article. Also, because all of the changes resulted from BPA exposure during late-term pregnancies, they claim to have "identified a critical window of timing in development for BPA alteration of the human lung." Unfortunately the study did not continue studying the exposed animals into adulthood, which limits how accurate their data can be used to predict later-onset pulmonary diseases.

Decreased Progesterone Receptor Expression[35]:

The balance between estrogen and progesterone in a woman's body is extremely important during pregnancy (Fig. 9). Both chemicals work to maintain the development of the baby as well as the health of the mother. Changes in the equilibrium can lead to several different problems, such as a miscarriage or endometrial defects.

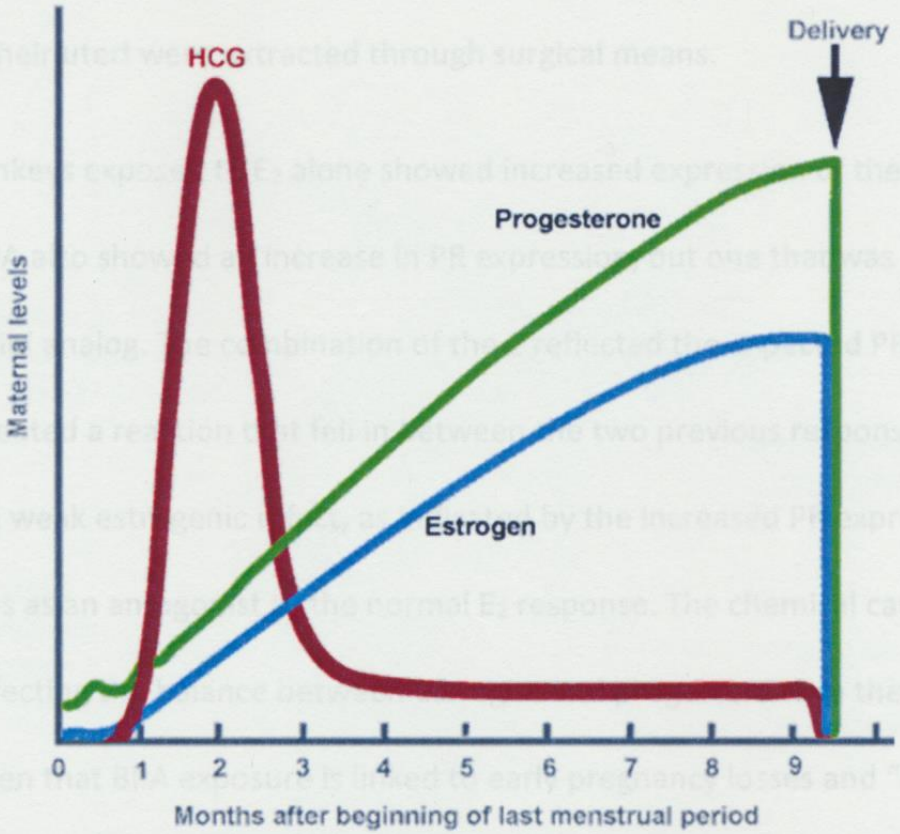


Figure 10. Levels of hCG, estrogen, and progesterone during pregnancy.[36]

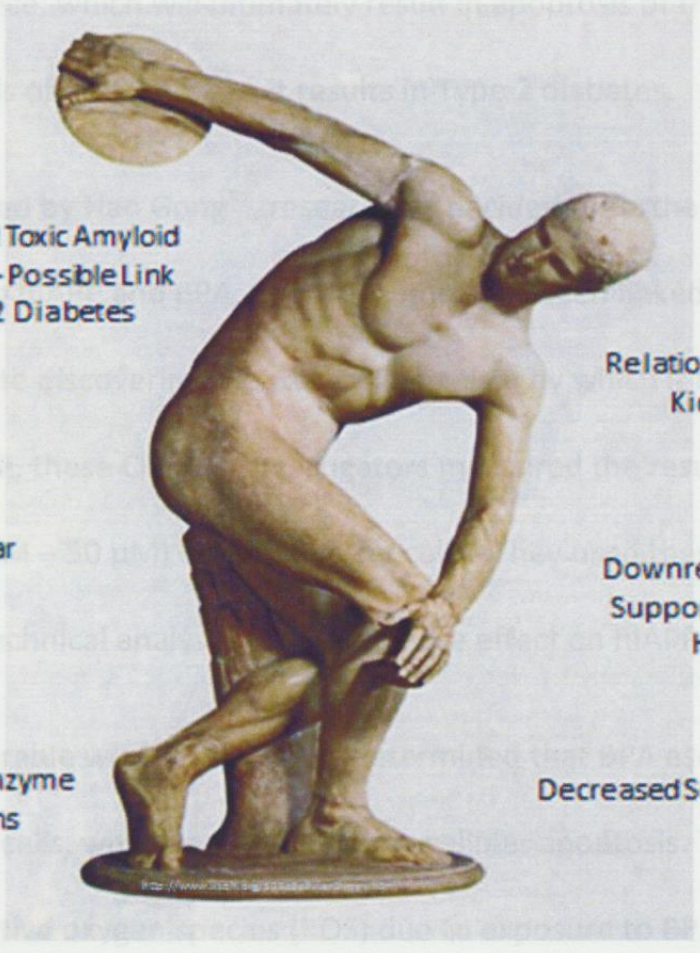
BPA has been reported to have an estrogen-like effect in humans, though its effect in the body is constantly being examined. Exposure to BPA during one's pregnancy could then possibly cause an imbalance between estrogen and progesterone and lead to some serious consequences for both mother and child.

In the study led by Tamir S. Aldad[35], American scientists used African green monkeys in order to analyze the effect of BPA on progesterone receptor (PR) expression. They selected 10 animals and divided them up into four different groups: control (1 monkey), an estrogen (E_2) benzoate (3 monkeys), 50 µg/kg BPA per day (3 monkeys), and a combination of E_2 benzoate and BPA (3 monkeys). Treatments were given through a Silastic capsule implanted subcutaneously in the back. After 29 days of exposure, the animals were sacrificed and their uteri were extracted through surgical means.

The monkeys exposed to E_2 alone showed increased expression of the PR. Those treated with BPA also showed an increase in PR expression, but one that was much lower than the estradiol analog. The combination of the 2 reflected the expected PR expression increase and created a reaction that fell in between the two previous responses. Although BPA produces a weak estrogenic effect, as indicated by the increased PR expression, it mainly functions as an antagonist to the normal E_2 response. The chemical causes damage indirectly by affecting the balance between estrogen and progesterone in the body. It makes sense then that BPA exposure is linked to early pregnancy losses and "diseases associated with increased endometrial proliferation," More research will need to be done in order to confirm the results found in this study, as well as to analyze in more depth the exact mechanism through which BPA alters PR expression.

Health Effects:

Humans



Accelerated Toxic Amyloid Formation – Possible Link to Type 2 Diabetes[37]:

As stated before, diabetes is a disease that severely affects humans worldwide. There are two types of this disease, with Type 2 diabetes accounting "for more than 90% of diagnosed diabetes." One reason for the development of diabetes is the "misfolding of human islet amyloid polypeptide (hIAPP)," which is a pancreatic protein that has a tendency to clump together and form toxic bundles. The formation of these aggregations can lead to membrane disturbance, which will ultimately result in apoptosis of the cell. Because this happens in the β-cells of the pancreas, it results in Type 2 diabetes.

In the study led by Hao Gong[37], researchers decided to further examine the relationship between hIAPP and BPA. BPA has commonly been linked to diabetes and much research has gone into discovering the exact mechanism by which it brings about its effects. Using synthetic hIAPP, these Chinese investigators measured the results of increasing BPA concentrations (20 μM – 50 μM) on human islet cells. They used these mixtures to perform various assays and technical analyses to examine the effect on hIAPP development.

After considerable work, the authors determined that BPA escalates the formation of toxic hIAPP in the cells, which in turn increases cellular apoptosis. They also confirmed the elevation of reactive oxygen species (ROS) due to exposure to BPA that other scientists have discovered. Taken together, this delivers an explanation as to why so many pancreatic β-cells are destroyed by BPA exposure. The authors also suggest that this provides concise evidence for a link between the rise in BPA use and the rise in prevalence of Type 2 diabetes.

Associated Cardiovascular Diagnoses/Abnormal Liver Enzyme Concentrations[10]:

Due to the ethical concerns of experimenting on humans, there is not a large amount of research available on the exact mechanisms of BPA in actual humans. Most scientists use animal models or human cell lines when they want to test a certain response, but the accuracy of these investigations is always in question. The other avenue that they take is to analyze the concentration of BPA in urine or blood and compare it to the health concerns expressed in the individual.

In a study led by Iain A. Lang[10], researchers gathered volunteers and questioned them about their medical histories. They gathered a total of 1455 adults (694 men and 761 women) that were between the ages of 18 and 74 and took urine samples. The scientists tested the urine for BPA as well as certain liver enzymes (such as γ-glutamyltransferase and alkaline phosphatase).

What they found was that a higher concentration of BPA in the urine was correlated with a higher risk of a cardiovascular diagnosis. This means that that those patients that possessed a history of a heart problem such as coronary heart disease, heart attack, and/or angina also had the highest BPA levels in their urine. The same was true for the patients with a history of diabetes. In some cases, these two diagnoses overlapped. The other common association was between high BPA levels in the urine and "clinically abnormal concentrations of the two previously mentioned liver enzymes." After completing their examination, the authors concluded that "higher BPA exposure...may be associated with avoidable morbidity in the community-dwelling adult population."

Relationship with Chronic Kidney Disease[38]:

The kidneys are bean-shaped organs situated on either side of the spine of a human, on the dorsal side of the abdominal cavity (Fig. 10). They normally function as part of the urinary system in order to help filter the blood and maintain proper blood pressure.

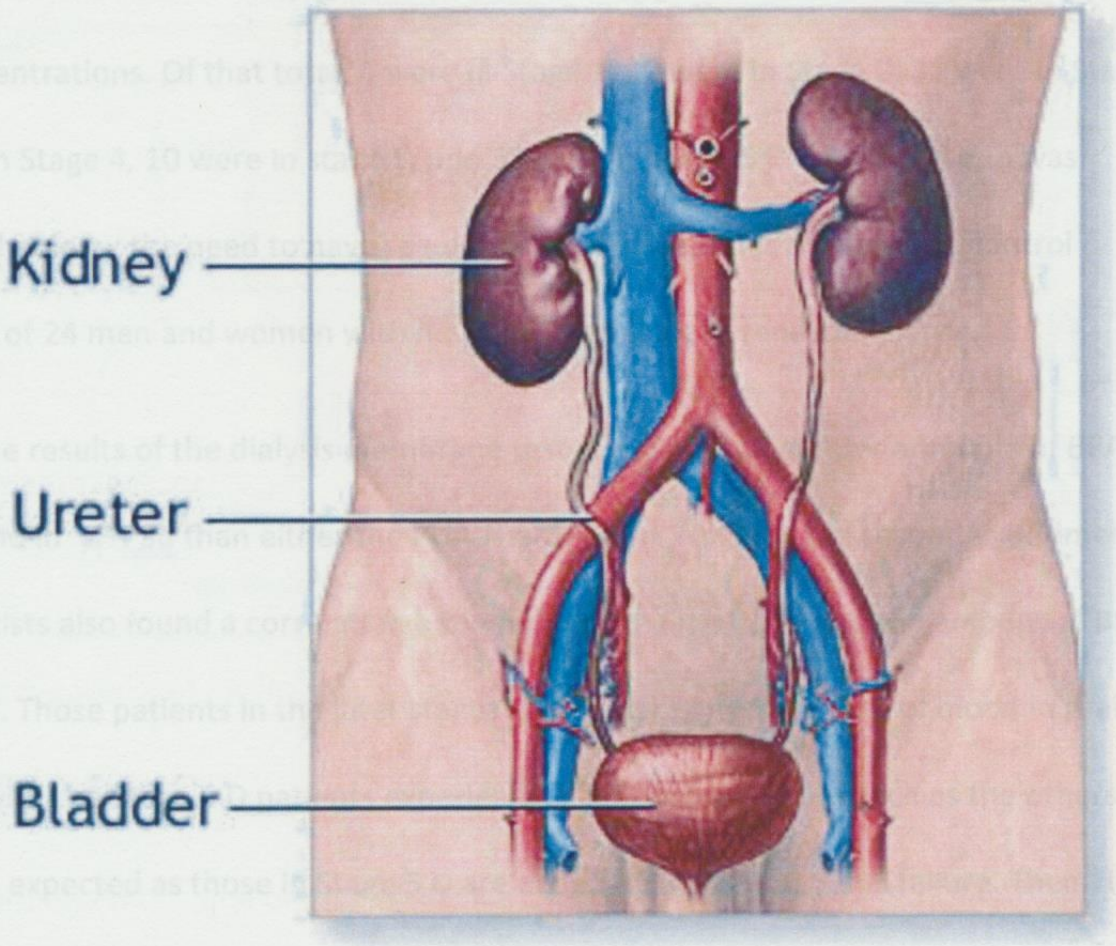


Figure 11. Placement of kidneys in the body and urinary system.[39]

One of the items filtered from the blood is BPA. In the average human, this chemical normally has "a half-life in blood of less than 2 h after oral ingestion." In patients that have impaired renal function, however, the ability to remove foreign chemicals is severely diminished.

In the study led by Detlef H. Krieter[38], the researchers ran two experiments. In the first, they randomly assigned patients to three different groups for four weeks of hemodialysis treatment. Each group had a different dialysis membrane that would elute various amounts of BPA: a high-flux polyethersulfone PUREMA H (PUR-H), a high-flux polysulfone (HF-PSu), and a low-flux polysulfone (LF-PSu). In the second trial, 152 patients diagnosed with chronic kidney disease (CKD) volunteered to have their blood monitored for BPA concentrations. Of that total, 6 were in Stage 1, 12 were in Stage 2, 31 were in Stage 3, 40 were in Stage 4, 10 were in stage 5, and 53 were in Stage 5 D. The last group was distinguishable by the need to have regular dialysis three times a week. The control consisted of 24 men and women with no previous history of renal deficiency.

The results of the dialysis membrane test revealed that greater amounts of BPA were found in LF-PSu than either the PUR-H or HF-PSu. Through the second experiment, the scientists also found a correlation between the stages of CKD and the amount of BPA in the blood. Those patients in the later stages had higher concentrations of blood in their plasma, with the Stage 5 D patients experiencing almost 5 times as much as the others. This was to be expected as those in Stage 5 D are experiencing severe renal failure. Their kidneys do not function well at all in these patients, so the BPA present in their bodies remains. Further research would need to be done to see if this BPA accumulation occurs in other peoples, and if there is a way to expedite its progress out of the body.

Downregulation of MMP11 Supports Development of Hypospadias[40]:

Hypospadias (HS) is a genetic disease that is characterized by malformation of the male genitalia. When normal development occurs, the opening of the urethra is established at the distal end of the penis. In infants born with HS, the opening of the urethra is on the underside of the penis and is more proximal to the base of the penis (Fig. 11).

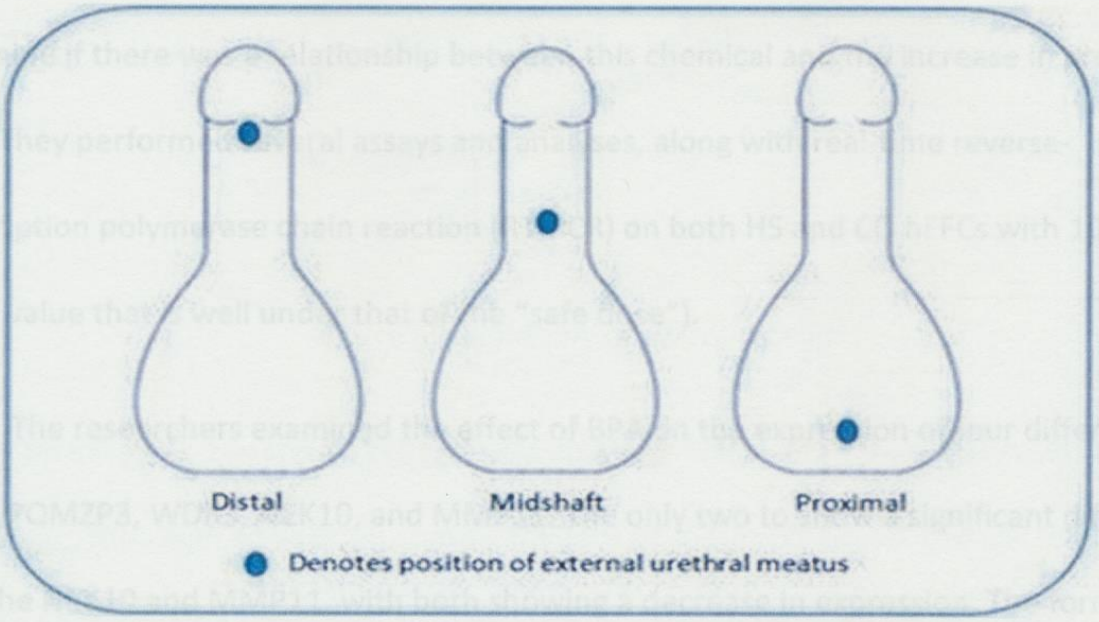


Figure 12. Locations of urethra openings in normal patients (left), HS patients (middle), and severe HS patients (right).[41]

Although this is one of the more common conditions affecting newborns, "with a global prevalence of approximately 0.2-1% at birth in male infants," not much is actually known about the exact causative agent(s). In the past, it has been lumped together with other abnormalities such as cryptorchidism (CO), a disease where one or both of the testes is absent, into one big group called testicular dysgenesis syndrome (TDS). As time has passed and more research has been done, scientists are beginning to realize the uniqueness of this diagnosis.

In the study led by Xian-Yang Qin[40], investigators took samples of human foreskin fibroblast cells (hFFCs) from 23 HS patients in Japan that were going to receive a surgical procedure to correct this problem. The means age of the sampling groups was 2.3 years, as surgical correction is done quite early on so as to give the best chance for continued normal development. They also obtained samples of hFFCs from CO patients in order to determine a clinical relationship. The authors wanted to test the effect of BPA on the hFFCs, in order to determine if there was a relationship between this chemical and the increase in prevalence of HS. They performed several assays and analyses, along with real-time reverse-transcription polymerase chain reaction (RT-PCR) on both HS and CO hFFCs with 10 nM of BPA (a value that is well under that of the "safe dose").

The researchers examined the effect of BPA on the expression of four different genes: POMZP3, WDR3, NEK10, and MMP11. The only two to show a significant difference were the NEK10 and MMP11, with both showing a decrease in expression. The former seemed to gain a greater level of expression as the amount of BPA was increased to 100 nM, but the latter kept declining. This supported the authors' hypothesis that BPA acts in a dose-dependent way on this type of gene. In the body, matrix metallopreteinases like MMP11 are important in early human development, especially in tissue remodeling. The conclusion drawn by the investigators is that BPA acts to downregulate MMP11 expression in the hFFCs. Supporting this idea is the fact that MMP11 expression is significantly lower in HS patients than both the control and CO group. Although further research needs to be done in order to analyze MMP11 in greater detail, this provides a possible explanation for a relationship between BPA and HS.

Declining Semen Quality[42]:

The male reproductive cell is sperm. It is made up of a head that contains the genetic information, a midpiece, and a tail for motility (Fig. 12).

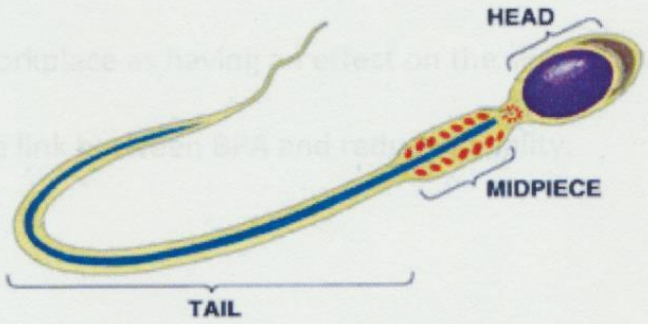


Figure 13. Anatomy of a sperm cell.[43]

During fertilization, the sperm cell releases enzymes which allow it to pass through to the female egg. Although semen can contain millions of sperm cells, only one will make it through to the egg's center. Semen quality is very important when considered

In the study led by De-Kun Li[42], researchers investigated the relationship between the concentration of BPA in human urine and semen quality. They analyzed 218 Chinese laborers that encounter various amounts of BPA in their individual work locations. Semen specimens were obtained before and after the workers went to their jobs in order to evaluate the change. Quality was determined according to six different parameters: volume, total sperm count, concentration, vitality, motility, and morphology.

The scientists tabulated the results and discovered that "participants with more advanced education and longer employment history had relatively lower BPA levels." It is

likely that this is because they hold higher positions in companies and do not do much menial labor. For all those involved in the study, however, the authors did notice that overall semen quality declined as BPA concentrations increased. All in all, the volume and morphology of the individual sperm cells remained the same while the total count, concentration, vitality and motility were reduced. Although other factors could be considered from the workplace as having an effect on the semen, this research provides evidence for a plausible link between BPA and reduced quality.

Experiment on Canned Goods

The following experiment was conducted at Ouachita Baptist University during the summer of 2015. All cans were purchased at local grocery stores during the course of that summer.

Materials and Methods:

Instruments and Technology:

Previous research[4] in our lab determined the excitation and emission wavelengths for Bisphenol-A to be 278nm and 304nm, respectively (Fig. 13). These values were confirmed for this study using a Hewlett-Packard UV-Visible instrument Model 8453, which used UV-Visible ChemStation Software from Agilent Technologies.

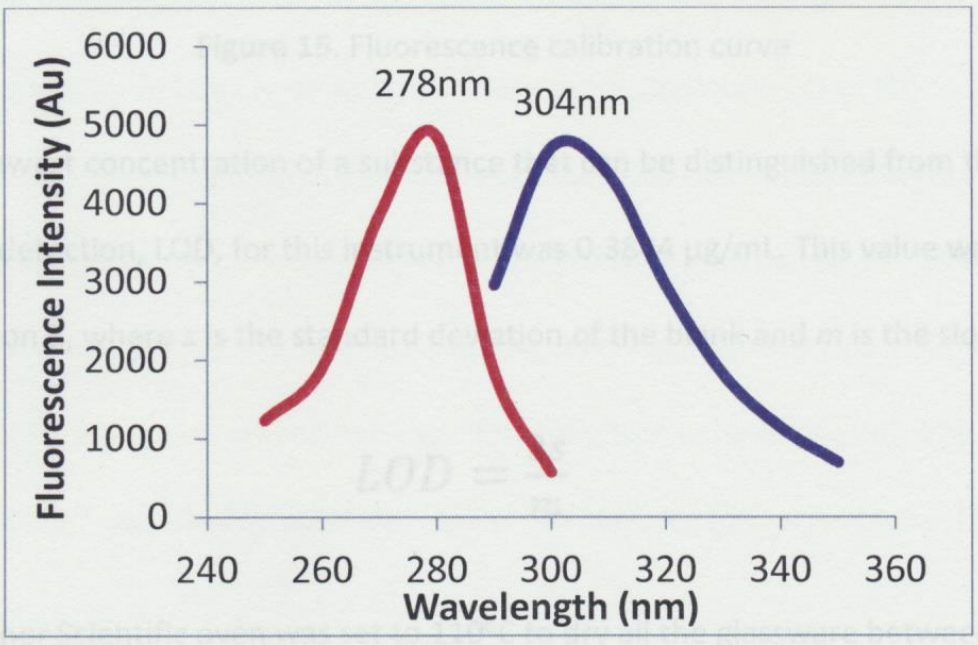


Figure 14. Excitation and emission spectra for BPA

A Hitachi 2000-F Spectrofluorometer was used to measure all concentrations of BPA in liquid, with the previously defined wavelengths of excitation and emission. A calibration curve was produced in order to become familiar with the instrument and calculate the limit of detection (Fig. 14). The R^2 value is the coefficient of determination that reveals how well certain data fit onto the curve; the best data will have a value close to 1.

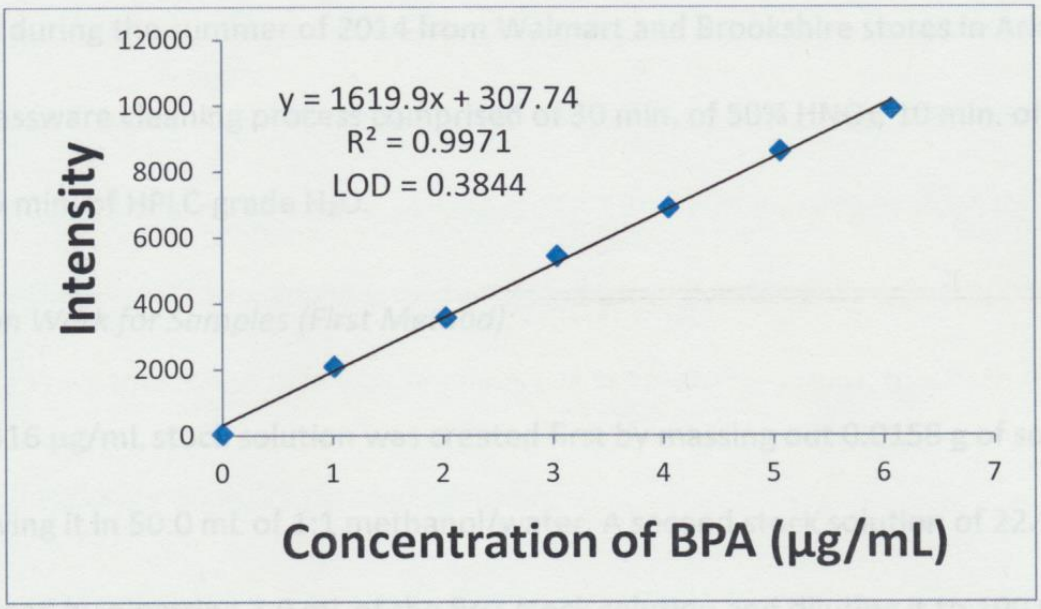


Figure 15. Fluorescence calibration curve

LOD is the lowest concentration of a substance that can be distinguished from the blank. The limit of detection, LOD, for this instrument was 0.3844 µg/mL. This value was calculated using Equation 1, where *s* is the standard deviation of the blank and *m* is the slope.

$$LOD = \frac{3s}{m}$$ Equation 1.

A Fisher Scientific oven was set to 110°C to dry all the glassware between experiments, and Corning hot plates were used to simulate heated conditions in the third method. Microsoft Excel and Microsoft Word were used to calculate and analyze all the data.

Reagents:

BPA stock solutions were created using 99+% Bisphenol-A from Sigma-Aldrich. HPLC-grade water and methanol were also obtained from Sigma-Aldrich. All canned goods were

purchased during the summer of 2014 from Walmart and Brookshire stores in Arkadelphia, AR. The glassware cleaning process comprised of 30 min. of 50% HNO_3, 10 min. of distilled H_2O, and 5 min. of HPLC-grade H_2O.

Preparation Work for Samples (First Method):

A 316 µg/mL stock solution was created first by massing out 0.0158 g of solid BPA and dissolving it in 50.0 mL of 1:1 methanol/water. A second stock solution of 22.12 µg/mL was produced by pipetting 7.0 mL of the first stock solution and diluting it to 100.0 mL with 1:1 methanol/water. For this experiment, six cans were used: one Great Value Tomato Paste, one Hunt's Tomato Paste, one Great Value Diced Tomatoes, one Del Monte Diced Tomatoes, one Great Value Green Beans, and one Del Monte Green Beans. The contents of the cans were removed and the cans were rinsed once with distilled H_2O, then filled with 40.0 mL of HPLC-grade H_2O and allowed to sit overnight. The next morning, the standard addition method[44] was used to create a total of five samples for each can, as shown below (Fig. 15).

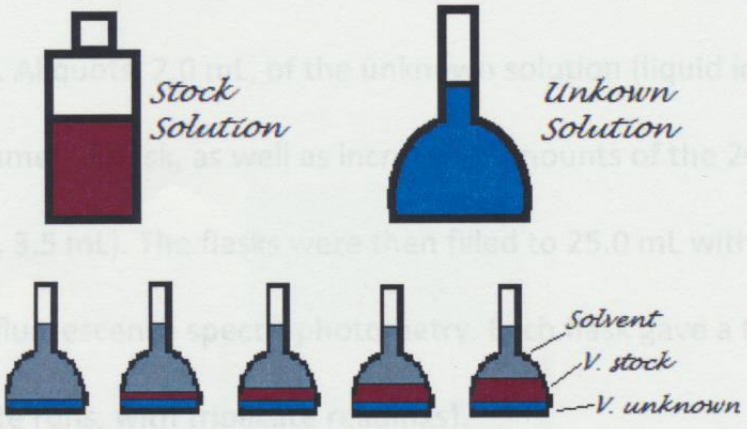


Figure 16. Diagram of the standard addition method

Aliquots, 5.0 mL, of the unknown solution (H_2O in the cans) were placed in each 25.0 mL volumetric flask, along with increasing amounts of the 22.12 µg/mL stock solution (0, 2, 2.5, 3, 3.5 mL) and shaken to ensure homogeneity. The flasks were then filled to 25.0 mL with 1:1 methanol/H_2O (the solvent) and analyzed using fluorescence spectrophotometry. Each flask gave a total of 18 intensity readings (six separate runs, with triplicate readings).

Preparation Work for Samples (Second Method):

A 1.012 mg/mL stock solution was made by massing out 0.506 g of solid BPA and dissolving it in 50.0 mL of 1:1 methanol/H_2O. A second stock solution of 20.24 µg/mL was created by transferring 2 mL of the first stock solution to a new flask and diluting it to 100.0 mL with 1:1 methanol/H_2O. This experiment was performed 3 times, using a total of 18 cans: three Del Monte Cut Green Beans (no added salt), three Del Monte Cut Green Beans (with sea salt), three Great Value Cut Green Beans (no added salt), three Great Value Cut Green Beans (with added salt), three Green Giant Cut Green Beans (with 50% less sodium), and three Green Giant Cut Green Beans (with regular salt). The standard addition method was used again, with samples being drawn directly from the liquid already present in the cans upon purchase. Aliquots, 2.0 mL, of the unknown solution (liquid in cans) were placed in each 25.0 mL volumetric flask, as well as increasing amounts of the 20.24 µg/mL stock solution (0, 2, 2.5, 3, 3.5 mL). The flasks were then filled to 25.0 mL with 1:1 methanol/H_2O and analyzed using fluorescence spectrophotometry. Each flask gave a total of 18 intensity readings (six separate runs, with triplicate readings).

Preparation Work for Samples (Third Method):

A 1.012 mg/mL stock solution was made by massing out 0.506 g of solid BPA and dissolving it in 50.0 mL of 1:1 methanol/H_2O. A second stock solution of 20.24 µg/mL was also created by transferring 2.0 mL of the 1.012 mg/mL solution into a new flask and diluting it to 100.0 mL with 1:1 methanol/H_2O. This experiment was performed three different times, with increasing temperature values, and used a total of 18 cans: three Del Monte Cut Green Beans (no added salt), three Del Monte Cut Green Beans (with sea salt), three Great Value Cut Green Beans (no added salt), three Great Value Cut Green Beans (with added salt), three Green Giant Cut Green Beans (with 50% less sodium), and three Green Giant Cut Green Beans (with regular salt).

After being produced, cans are typically sent across the country to various warehouses by transportation trucks. During this time, the cans experience several stages of heating and cooling. The research on whether or not BPA leaching increases during this time is often contradictory, so analysis of the effect of heat application was examined. Warehouse conditions were mimicked by inserting the cans into water baths heated by hot plates (Fig. 16).

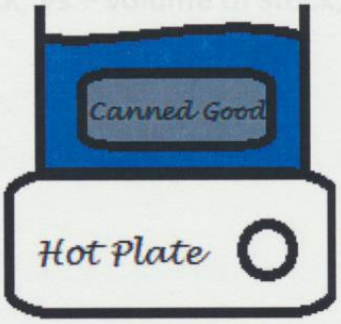


Figure 17. Demonstration of a water bath

The first trial involved six cans (one of each brand listed previously), which were in water that began at 35-37°C, but cooled to 24°C overnight. The second test involved six cans in the water overnight at a constant temperature of 35-37°, and the third involved six cans the water overnight at a constant temperature of 45-47°C. The water bath temperature was increased from trial to trial in order to simulate the various temperatures in which the cans may be stored during cross-country transportation. The morning after the cans had been heated, 2 mL aliquots of the liquid in the cans were put into each 25 mL volumetric flask. The flasks were filled to 25 mL with 1:1 methanol/H_2O after the addition of increasing amounts of the 20.24 µg/mL stock solution (0, 2, 2.5, 3, 3.5 mL). The samples were analyzed using fluorescence spectrophotometry, with each flask giving a total of 18 intensity readings (six separate runs, with triplicate readings).

Calculations:

Data were collected from the different methods and evaluated through the creation of a number of standard addition curves. The intensity averages were calculated per sample, as well as the standard deviation (stdev) and relative standard deviation in percent (RSD, %). A scatter chart was produced with Si*Vs/Vo on the x-axis, and I*Vx/Vo on the y-axis (where Si – initial conc. of stock, Vs – volume of stock, Vo – total volume, I – intensity) (Tab. 1, Fig. 17).

Table I. Experimental values used to create standard addition curve below.

Std. Added (mL)	Avg. Intensity	Stdev	RSD, %	X Values Si*Vs/Vo	Y Values I*Vx/Vo	Linest	
0	1108.8333	65.65081	5.920711	0	88.70667	15.1722	89.30094
2	1405.8333	22.41389	1.594349	1.6192	112.4667	1.308625	2.65923
2.5	1552.3889	45.91954	2.957992	2.024	124.1911	0.978169	2.86251
3	1576.8889	64.11193	4.065723	2.4288	126.1511	134.4208	3
3.5	1626.3333	22.40011	1.377338	2.8336	130.1067	1101.44	24.58189

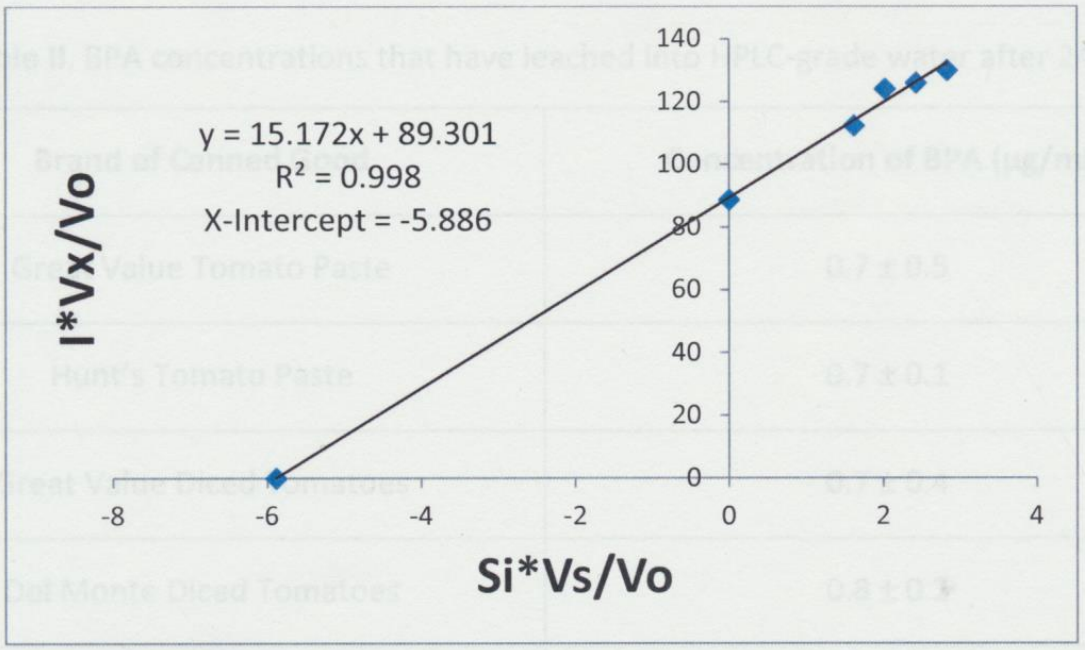


Figure 18. Example of a standard addition curve, with x-intercept calculation

From the graph, as well as LINEST information, an x-intercept could be determined.

Overall concentration of BPA and error for the sample were calculated using the x-intercept.

Results and Discussion:

BPA concentrations found in the first part of this experiment are displayed in Table II. These values ranged from 0.7 ± 0.5 to 1.2 ± 0.5 µg/mL. Within each type of canned good (tomato paste, diced tomatoes, green beans), there was no statistical difference between the two differing brands. The Del Monte Green Beans did show the greatest amount of BPA leaching, at 1.2 ± 0.5 µg/mL, but the other cans were not that much lower, most around 0.7 µg/mL. The relatively low values reflect the belief that the majority of free BPA coming from an incomplete resin has already leached into the can's liquid contents by the time the can is opened.

Table II. BPA concentrations that have leached into HPLC-grade water after 24 h.

Brand of Canned Good	Concentration of BPA (µg/mL)
Great Value Tomato Paste	0.7 ± 0.5
Hunt's Tomato Paste	0.7 ± 0.1
Great Value Diced Tomatoes	0.7 ± 0.4
Del Monte Diced Tomatoes	0.8 ± 0.2
Great Value Green Beans	0.7 ± 0.2
Del Monte Green Beans	1.2 ± 0.5

The concentrations found using the second method are given in Table III. These values are much higher than those found through the first method, which is to be expected. They range from 43.5 ± 0.7 to 95 ± 4 µg/mL, with the highest occurrence being seen in the Del Monte Cut Green Beans (with sea salt). Though this portion of the experiment was done in triplicate, there was a great deal of variance among the sample set. This result is most likely due to the samples being drawn from diverse places in the can. Further research should be done on extracting samples from the same place in order to decrease the error.

Table III. BPA concentrations of investigated foods upon purchase.

Type of Canned Good	Concentration of BPA, 1st Trial (µg/mL)	Concentration of BPA, 2nd Trial (µg/mL)	Concentration of BPA, 3rd Trial (µg/mL)		Average Concentration of BPA (µg/mL)
Del Monte Cut Green Beans (No Salt Added)	55 ± 4	74 ± 7	56 ± 37		62 ± 13
Del Monte Cut Green Beans (With Sea Salt)	127 ± 9	72 ± 7	86 ± 5		95 ± 4
Great Value Cut Green Beans (No Salt Added)	47 ± 2	35 ± 1	46.1 ± .5		43.5 ± 0.7
Great Value Cut Green Beans (With Added Salt)	75 ± 6	59 ± 9	52 ± 3		62 ± 4
Green Giant Cut Green Beans (50% Less Sodium)	62 ± 4	59 ± 4	57 ± 3		59 ± 2
Green Giant Cut Green Beans (With Regular Salt)	52 ± 6	51 ± 8	66 ± 6		56 ± 4

Concentrations of BPA from the third method are reflected in Table IV. For this set of data, the range was from 39 ± 2 to 94 ± 12 µg/mL. Again, the highest values were seen with the Del Monte Cut Green Beans (with sea salt). This portion of the study focused on the effect of heat on the samples. While the average concentrations of BPA changed with the application of heat, there is no statistical significance to that difference. Therefore, these results confirm that the application of heat has no effect on BPA migration from the can's lining to the liquid contents of the can.

Table IV. BPA concentrations of investigated foods after application of heat.

Type of Canned Good	Concentration of BPA, 1st Heating (µg/mL)	Concentration of BPA, 2nd Heating (µg/mL)	Concentration of BPA, 3rd Heating (µg/mL)		Average Concentration of BPA (µg/mL)
Del Monte Cut Green Beans (No Salt Added)	46 ± 3	52 ± 5	39 ± 3		46 ± 2
Del Monte Cut Green Beans (With Sea Salt)	94 ± 35	71 ± 3	116 ± 6		94 ± 12
Great Value Cut Green Beans (No Salt Added)	61 ± 12	59 ± 12	49 ± 3		56 ± 6
Great Value Cut Green Beans (With Added Salt)	44 ± 4	46 ± 2	26 ± 4		39 ± 2
Green Giant Cut Green Beans (50% Less Sodium)	69 ± 2	55 ± 4	51 ± 7		58 ± 3
Green Giant Cut Green Beans (With Regular Salt)	64 ± 4	60 ± 7	54 ± 2		59 ± 3

Conclusions:

The EPA maximum recommended value of BPA intake is 0.05mg/kg (body weight)/day.[17] For a 70 kg person (approximately 154 lbs.), the maximum intake can be as high as 3.5 mg (3500 μg) per day. None of the values found in this study exceed that amount set by the government, though toxic effects have been seen due to chronic exposure from lower doses.[17] Another concern is the synergistic effect of this chemical with other xenoestrogenic chemicals. As an endocrine disruptor, BPA imitates estrogen and interferes with the creation and management of the body's hormones.

While the BPA concentrations found in this study do not immediately cause a sense of alarm, it must be made clear that this is only one source of BPA intake examined. After removing the can's original contents and replacing it with HPLC-grade H_2O, BPA levels ranged from 0.7 ± 0.5 to 1.2 ± 0.5 μg/mL. In the original liquid though, concentrations vacillated between 43.5 ± 0.7 and 95 ± 4 μg/mL. After the application of heat, values stretched from 39 ± 2 to 94 ± 12 μg/mL. Bisphenol-A is a common industrial chemical and is found in a number of different products. These considerations inspire a need for continuous research on this chemical as well as other endocrine disruptors.

Future Research:

To truly assess the amount of BPA that a human would ingest from eating a canned item, it becomes necessary to analyze the concentration of the chemical in the actual food itself. For the large portion of this study, the main focus was on the liquid surrounding the green beans in various cans. The next step would be to analyze the green beans themselves, as well as other canned substances. Another goal would be to assess the concentration of BPA in the food before it was placed into a can, in order to accurately determine how much BPA was coming from the lining of the can and how much was already present.

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