

Diffusion of Paraben Across a Synthetic Membrane After Multiple Cosmetic Applications

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Abstract

The overuse of cosmetics containing parabens can lead to alterations in the consumer's skin and is thought to cause other health disruptions such as breast cancer and the disruption of natural hormonal processes. The focus of this project is to test the amount of methylparaben, propylparaben, and ethylparaben (the three most used and present parabens in cosmetics) in various cosmetic products and whether the chemicals in the cosmetics will diffuse across a synthetic membrane, such as a Strat-M membrane, when a dosage is applied. A Franz Cell apparatus was obtained from PermeGear to resemble a realistic situation of consistent cosmetic application to a human epidermis, and an HPLC machine was used to determine the substances that had diffused across the membrane. Results revealed that two out of the three tested chemicals (methylparaben and ethylparaben) passed through the membrane, which suggests the accumulation of methylparaben and ethylparaben on the skin, while propylparaben poses that it is the safer of the three parabens due to its inability to pass through the Strat-M synthetic membranes after applications.

Purpose

The overuse of cosmetics containing parabens can lead to alterations, such as slight skin damage and allergic reactions, in the consumer's skin. The purpose of this project is to find if paraben can absorb and penetrate through a membrane and if so how much.

Hypothesis

After three days of continuous application of cosmetic products containing the paraben chemicals, the receptor fluid will include a small amount of paraben from the cosmetics and indicate that the chemicals have diffused across the membrane.

Null Hypothesis

After three days of testing using the Franz cells the cosmetic will pass through the membrane, but there will be no paraben indicated in the receptor fluid because of the small amount in the cosmetics being tested.

Question

Will ethylparaben, methylparaben, and propylparaben diffuse across the synthetic Strat-M membranes and be detected in the receptor fluid in the Franz cells?

Introduction

Parabens, short for “para-hydroxybenzoate,” are antimicrobial substances that are often added into cosmetics, foods, beverages, and pharmaceutical drugs. They are used as a preservative that prevents microbial and mold growth in products in order to prolong a product’s shelf life [2]. Out of all cosmetic products, about 90% of these products contain at least one of the six most used parabens - methylparaben (MP), propylparaben (PP), butylparaben (BP), isobutylparaben, benzylparaben, and ethylparaben (EP) [6].

Currently, the U.S. Food and Drug Administration (FDA) only has few guidelines for the use and amount of parabens or preservatives that can be added into a cosmetic product, but the European Union (EU) has put the restriction of 0.4% for individual paraben compounds and 0.8% for the total amount of paraben in a cosmetic product being sold [8]. The FDA declares parabens as “generally recognized as safe” (GRAS) and has set guidelines for MP and PP. MP has been approved at a maximum amount of 1.8 mg in a solid tablet formulation and 0.15% for an oral solution [5]. While PP has been approved for the maximum usage of 0.22 mg in a tablet formulation and 10% in an oral solution [5].

In 1990, parabens were deemed xenoestrogens, which imitates the hormone estrogen and thus causing estrogenic disruption. This disruption has been linked to influence breast cancer development if there is regular cosmetic application to the underarm, breast area [6]. In a long term study done by Dr. Mercola, an osteopathic physician with granted fellowship status by the American College of Nutrition (ACN) since 2012, 99% of paraben esters were found in breast cancer tissue and showed accumulation of paraben in the tissue [10]. The long term study examined 40 women’s tissues who were being examined for breast cancer and revealed that five of the paraben esters were present in 60 percent of the examined tissues.

Parabens also possess oestrogenic activity, a female hormone that is responsible for female reproduction [13]. Chemicals, such as parabens, mimic our natural hormones and as a result corrupt the natural hormonal process, which can then lead to worse health effects, such as breast cancer.

Usage of parabens can also lead to allergic reactions. These types of reactions are referred to as the “paraben paradox”. The “paraben paradox” states that the application of

paraben containing cosmetics is well tolerated when the product is applied to normal and non damaged skin, which is less sensitive and prone to reactions when compared to an irritated area of skin [2]. If a high containing paraben cosmetic product comes into contact with damaged skin the effects could be worse than if that same product and dose were applied to undamaged or altered skin.

Because of these possible health effects more consumers are becoming aware of the possible danger these chemicals possess and many companies are trying to advertise themselves as paraben-free brands in order to attract more customers and promote themselves as a better product. A few of these companies include Aveda, Burt's Bees, Dr. Hauschka, John Masters Organics, Josie Maran Cosmetics, Korres, Origins, and Pangea Organics.

The absorbance of the paraben is determined by its solubility and lipophilicity. Lipophilicity is the ability to combine or dissolve with lipids or fats and substances that are considered lipophilic, insoluble substances. Solubility is the quality or ability to be soluble, dissolved, or liquefied. The higher the lipophilicity or solubility of the chemical, the higher the possibility for the paraben to absorb and pass through the membrane. Lipophilic chemical exposure can also cause cardiovascular diseases such as neurological and neurodegenerative diseases; metabolic disorders, syndrome or obesity; and reproductive and developmental disorders. After exposure to chemicals with high lipophilicity, accumulation in body serum has been shown, and suggests that because the lipophilic chemicals penetrate the membrane this allows for the passage of other toxic chemicals that would otherwise not be absorbed to be absorbed [16]. The highest absorbance to the lowest paraben absorbance of the three most used

parabens is MP, EP, to PP [13]. The other types of parabens (butylparaben, isobutylparaben, and benzylparaben) are not used as often in cosmetics.

The amount of parabens in cosmetics has already been tested, but has only addressed a situation when one product was used continuously for a few days. A gap in this field of study is to determine the results of absorption if at least two products from a commercial drug store were tested for a continuous 3 days, with 24 hour intervals of application. This type of study is more realistic because multiple cosmetic products that contain parabens are applied daily due to their abundance in makeup, lotions, body and hair washes, and some toothpastes. Although humans can be exposed to chemicals, such as parabens, through dermal absorption other factors that lead to increased exposure are through ingestion and inhalation [5].

To determine absorbance and diffusion of parabens across a membrane in cosmetic products, diffusion methods such as Franz Cells and Side-Bi-Side Cells have been used. Franz and Side-Bi-Side Cells are equipment used for studying membrane transport and drug diffusion through biological or synthetic membranes. These diffusion cells are commonly used in experimentation in drug transport and screening, dermatology, toxicology, and determining absorption [4]. Both of these diffusion methods require a type of biological or synthetic membrane, such as the Strat-M membranes. Strat-M membranes are a synthetic and non-animal based model for diffusion testing that is predictive of diffusion of human skin, without safety or storage limitations [14]. In the receptor chamber, bovine serum albumin (BSA) was employed. BSA is a serum albumin protein that is derived from cows and is often used as a protein concentration standard in lab experimentations. The purpose of BSA in an experiment like this is

that it is used to prevent adhesion of an enzyme to reaction tubes, pipet tips, or other vessels. BSA is also considered a universal blocking reagent in many applications because of the protein's ability to not affect other proteins or enzymes that do not require it for the stabilization. Another purpose for the usage of BSA is to determine the amount of other proteins present by comparing the unknown amount to the known amount of BSA in a solution.

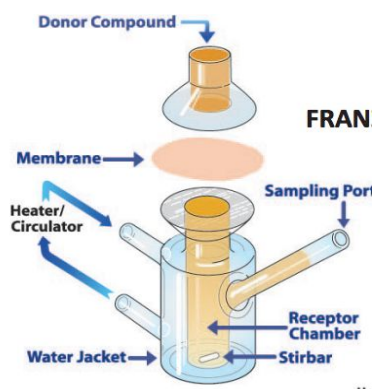


Figure 1. Franz cell diagram.

The determination of paraben absorption can be obtained through Franz diffusion cells and Side-by-side cells, accompanied with the use of an High Performance Liquid Chromatography (HPLC) method to separate mixtures and detect paraben concentration of various cosmetic products ranging from creams and lotions to makeup. HPLC has the ability to separate, identify, and quantify compounds that are present in any sample that has been dissolved in a liquid. The high pressure in this form of liquid chromatography indicates the usage of high pressure to generate the desired flow required for liquid chromatography in packed columns. This technique has been applied to pharmaceutical drugs, food, cosmetics, nutraceutical, forensic samples, environmental matrices, and industrial chemicals. The HPLC consists of multiple components, as seen in figure 2.

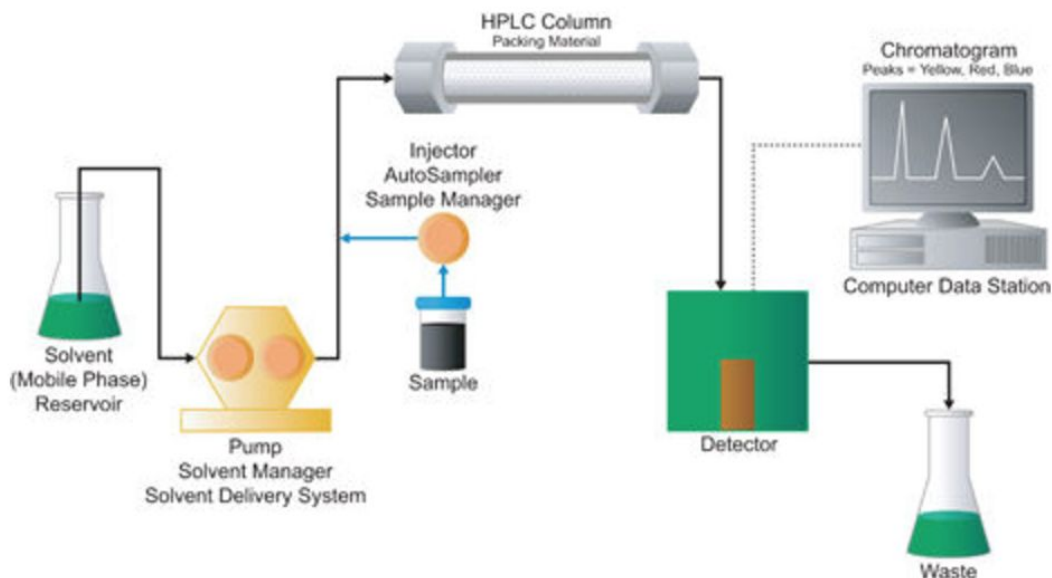


Figure 2. High Performance Liquid Chromatography (HPLC) system.

Various forms of HPLC include the separation through ion exchange, size exclusion, and normal and reverse phase chromatography. In ion exchange chromatography the sample will be separated according to the charge of the components in the sample. The stronger the charge, the more it will be attracted to the ionic surface of the column, and the longer it will take to elute. In size exclusion chromatography the column is composed of material with controlled pore sizes. Larger molecules in the sample will be rapidly washed through the column, while the smaller molecules will penetrate inside the pores and elute later. In normal phase chromatography the column is strongly polar and the sample is separated by the components polarity. The stronger the polarity the longer it will take for the molecule to elute through the column than the nonpolar molecules. Reverse phase chromatography works the opposite as normal phase chromatography,

the column attracting nonpolar molecules while the polar molecules eluting faster. My experimentation uses a reverse phase liquid chromatography method with a C18 column.

The quantitation of the amount of parabens diffused across a synthetic membrane will be determined through the use of a Franz cell apparatus and reverse phase HPLC method. The principal objective of this experiment is to test whether if the three parabens being tested, EP, MP, and PP, will be detected in the receptor fluids of the Franz cells and show accumulation over time after various applications of the cosmetic.

Safety Issues

Methanol is a highly toxic chemical and is not fit for human consumption or direct contact with skin of eyes because of its known trait to be an irritant. This substance is also toxic to the blood, kidneys, liver, brain, upper respiratory tract, skin, central nervous system (CNS), and optic nerve. Repeated or continuous exposure to methanol can result in organs damage or in larger cases deterioration of health. Methanol also is also a flammable substance. The pure paraben powders purchased from Sigma Aldrich should be handled with gloves and avoid contact with skin and eye also due to possible irritation. Throughout the experiment gloves, goggles, and a lab coat will be worn at all times when in the lab area and handling substances.

When using the HPLC, disposal of the solvents should be separate from one another and into correct containers. Also, since the machine using high pressure, goggles, lab coat, and gloves must be worn when handling the machine.

Materials

- 2 commonly used cosmetic products and brands that contain at least one paraben from commercial drug stores
 - Neutrogena Visibly Even Daily Moisturizer with SPF 30 (contains PP, EP, MP)
 - Banana Boat Sport Performance Sunscreen Lotion with SPF 30 (contains PP and MP)
- Pure paraben powders from Sigma-Aldrich: (about 0.5 g each)
 - Methylparaben (MP)
 - Linear formula: $\text{HOC}_6\text{H}_4\text{CO}_2\text{CH}_3$
 - Ethylparaben (EP)
 - Linear formula: $\text{HOC}_6\text{H}_4\text{CO}_2\text{C}_2\text{H}_5$
 - Propylparaben (PP)
 - Linear formula: $\text{HOC}_6\text{H}_4\text{CO}_2\text{CH}_2\text{CH}_2\text{CH}_3$

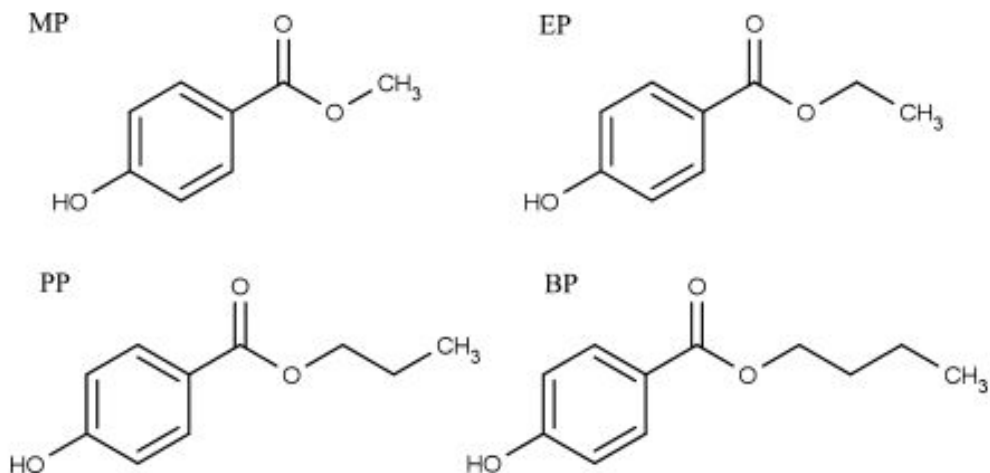


Figure 3. The structures of methylparaben, ethylparaben, propylparaben, and butylparaben.

- Hewlett Packard Series 1100 HPLC system

- C18 Column (4.6 x 100 mm)
- UV/Vis detector
- 9 mm clear jacketed Franz Cells with flat ground joint, 5 ml receptor volume, pinch clamp and stir bar. All from PermeGear (3)
- Isotemp 2150 Fisher Scientific immersion circulator for Water Bath from PermeGear
- A 3-station V3-A magnetic stirrer Franz cell apparatus from PermeGear
- EMD Millipore 25 mm discs Strat-M membranes for transdermal diffusion testing from PermeGear (60)
- 50:50 Methanol-HPLC grade (1.5 L)
- Methanol water (0.5 L)
- 30% Bovine Serum Albumin (15 mL)
- Eppendorf Centrifuge 5418
- Vortex
- HPLC vials and caps
- HPLC computer data program
- 125-500 mL beaker
- Analytical balance
- 3 mL syringes
- Syringe needle tips
- 0.078 "I.D. x 0.125 "O.D. Silastic RX-50 Medical Grade Tubing (located in room E8)
- 0.45- μ m syringe-tip filters
- 100-500 mL graduated cylinder

Methods

The following experiment will take place in room E8 at Thousand Oaks High School under the supervision of Dr. Nikki Malhotra.

The method includes two different experimental groups, both utilizing the Franz cell apparatus, to best represent a realistic situation with cosmetic application within 3 days and to determine the absorbance. The second experimental group was compared to the first experimental group. The samples taken from each experimental group was taken for HPLC reverse phase analysis.

Experimental Group 1: Single application of each product

The two cosmetic products containing parabens (Neutrogena Visibly Even Daily Moisturizer with SPF 30 and Banana Boat Sport Performance Sunscreen Lotion with SPF 30) were dissolved into a 10 mL pure methanol solution then placed into the Franz cells over the Strat-M membranes, in the donor compound. The products were applied only once in 200 μ l volume each throughout this test of three days and the receptor fluid from the Franz Cells was obtained and recorded after 72 hours had passed. The purpose of having no reapplications of the products in this experiment is to act as base information to compare with Experiment Group 2 and a simple result showing the diffusion after one application.

Experimental Group 2: Reapplication of products

The same cosmetic products (Neutrogena and Banana Boat) and amount (200 μ l) that were in Experiment Group 1 were used, but were reapplied every 24 hours into the donor

compound and with the usage of the same Strat-M membrane over the span of three days, in order to mimic a realistic situation of cosmetic usage within 3 days. The receptor fluid from the Franz Cell was recorded at the initial state (0 hours) and before any additional applications (24, 48, 72). This continued for 72 hours (3 days).

Prepare parabens powders and cosmetic products for the HPLC: standard solutions

500.0 mg of pure paraben was dissolved in 100 mL of filtered 50:50 methanol/HPLC-grade water. 5 standards were created, ranging from 10 ppm paraben to 500 ppm paraben. 500 ppm solution: 10 mL of the paraben stock solution was poured into a vial labeled 500 ppm. 250 ppm: 5 mL of the previous paraben solution (500 ppm) was combined with 5 mL of the 50:50 methanol water and was poured into a vial labeled 250 ppm. 100 ppm: 4 mL of previous paraben solution (250 ppm) was combined with 6 mL of the 50:50 methanol water and was poured into a vial labeled 100 ppm. 50 ppm: 5 mL of previous paraben solution (100 ppm) was combined with 5 mL of the 50:50 methanol water and was poured into a vial labeled 50 ppm. 10 ppm: 2 mL of previous paraben solution (50 ppm) was combined with 8 mL of the 50:50 methanol water and was poured into a vial labeled 10 ppm. This was continued for each type of paraben; EP, MP and PP. Then 1 mL of each solution was put into a 3 mL syringe with a 0.45- μ m tip filter, then put into a cleaned HPLC vial. The stock solution for each cosmetic was made by dissolving 1 gram of the cosmetic product into 10 mL of 50:50 methanol water. The solution was centrifuged for 10 minutes at 1700 g, then filtered using the same materials and method as the paraben standards. 1 mL of the solution was then set into the HPLC.

HPLC preparations:

All of the experiments were conducted at 35 °C with a C18 (4.6 x 100 mm) stationary phase, at a wavelength of 254 nm, and with an isocratic method. The mobile phase consisted of methanol:water (50:50) and the flow rate was 0.5 mL/min. Each sample ran for 30 minutes with a 50 µL injection volume and with no post time. The experiment resulted in a total of 30 samples set into the HPLC with normal phase liquid chromatography.

Receptor Fluids:

The receptor chamber was filled with 3 mL of BSA solution (3%). This solution was made by combining 4.5 g of the pure powder and 10.5 mL of Deionized (DI) water. About 15 mL of the 3% BSA solution was created. To determine the calibration curve of the receptor fluids, about 2 mL was extracted using a 3 mL syringe, with a syringe needle tip and medical grade tubing connected to the end through the sampling port. This obtained sample was well shaken and centrifuged for 10 minutes. The supernatant most likely containing diffused parabens was removed and put into clean tubes and evaporated in a fume hood for three days, until the total volume was reduced to half than before. 200 µL of the mobile phase was added to the remaining sample and then vortexed for a few seconds. 1 mL of the sample was filtered and injected into the HPLC.

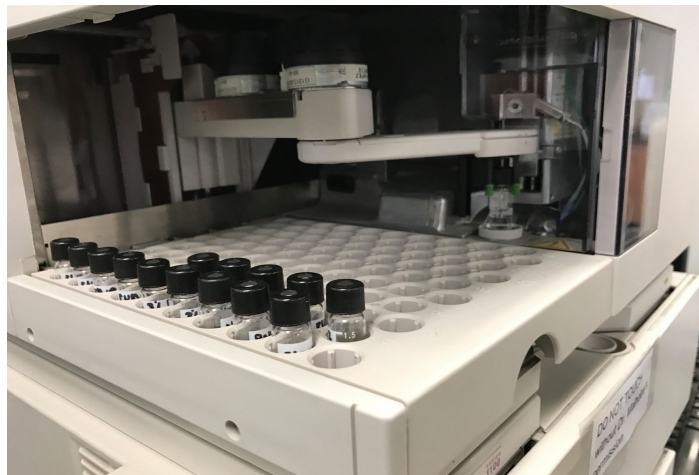


Figure 4. Samples and standards set up for HPLC run.

Results and Discussion

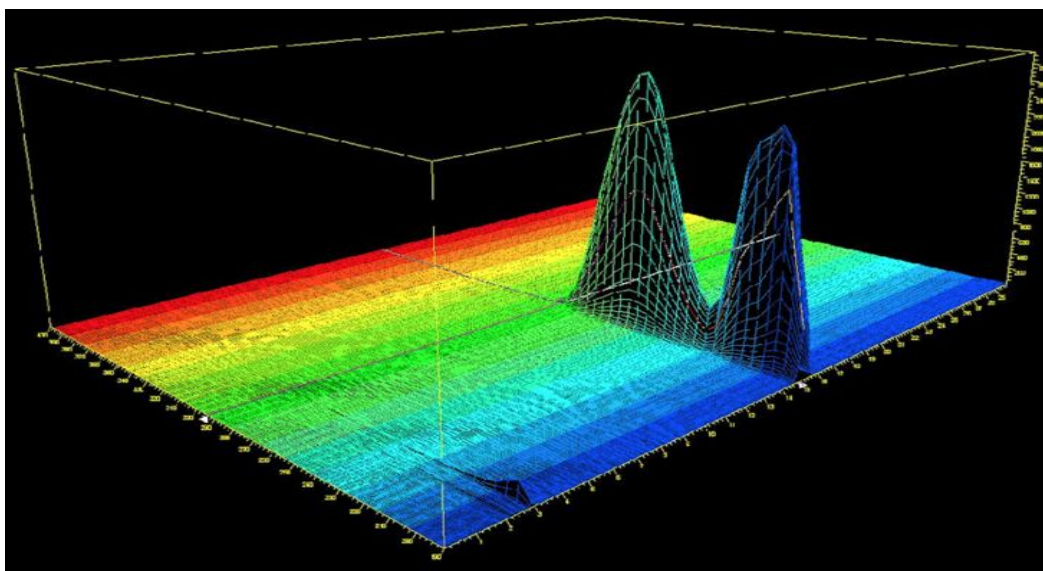


Figure 5. Peak of 500 ppm standard of propylparaben.

Parabens diffusion through a membrane has been investigated by multiple authors. In those studies skin fragments of different origins were used for determining the relative diffusion

of the chemical. The skin fragments consisted of testing on rat skin, pig skin, and human skin. Also in other studies using a Franz cell apparatus, the receptor fluid employed ranges from saline phosphate buffer, ethanol/water solution, and bovine serum albumin, as used in this experiment.

This experiment was done to test whether if any of the three tested parabens in the Neutrogena cosmetic or Banana Boat sunscreen would be detected in the receptor fluid after a certain amount of cosmetic applications. The experiment was tested in triplicate and included two experimental groups that differed in the amount of cosmetic applications. HPLC analysis revealed that the retention time for MP was around 2 minutes, EP around 12 minutes, and PP around the end of 14 and early 15 minutes.

The relationship between the peak area of the parabens and the amount/concentration show a positive correlation for MP, EP, and PP. This means that as the concentration and amount of each paraben in a solution or mixture increases the peak area of the chromatogram also increases. The equations of the standard best fit line displayed with the graphs in Figure 6 were used to determine the amount of each paraben in the cosmetics and the samples from the experiments. Through this method of calculation it was determined that the Neutrogena cosmetic contained a total of 0.2% of paraben with 0.08% of MP, 0.01% of EP, and 0.1% of PP. The Banana Boat sunscreen contained a total of 0.04% of paraben, with 0.0003% of MP, 0.0413% EP, and no PP. In the Neutrogena Visibly Even Daily Moisturizer the most abundant paraben was PP, and in the Banana Boat Sport Performance Sunscreen Lotion the most abundant paraben was EP. In both cosmetics the paraben that diffused across the synthetic membrane the least was used the most in the cosmetic, and the most diffusing paraben was used less.

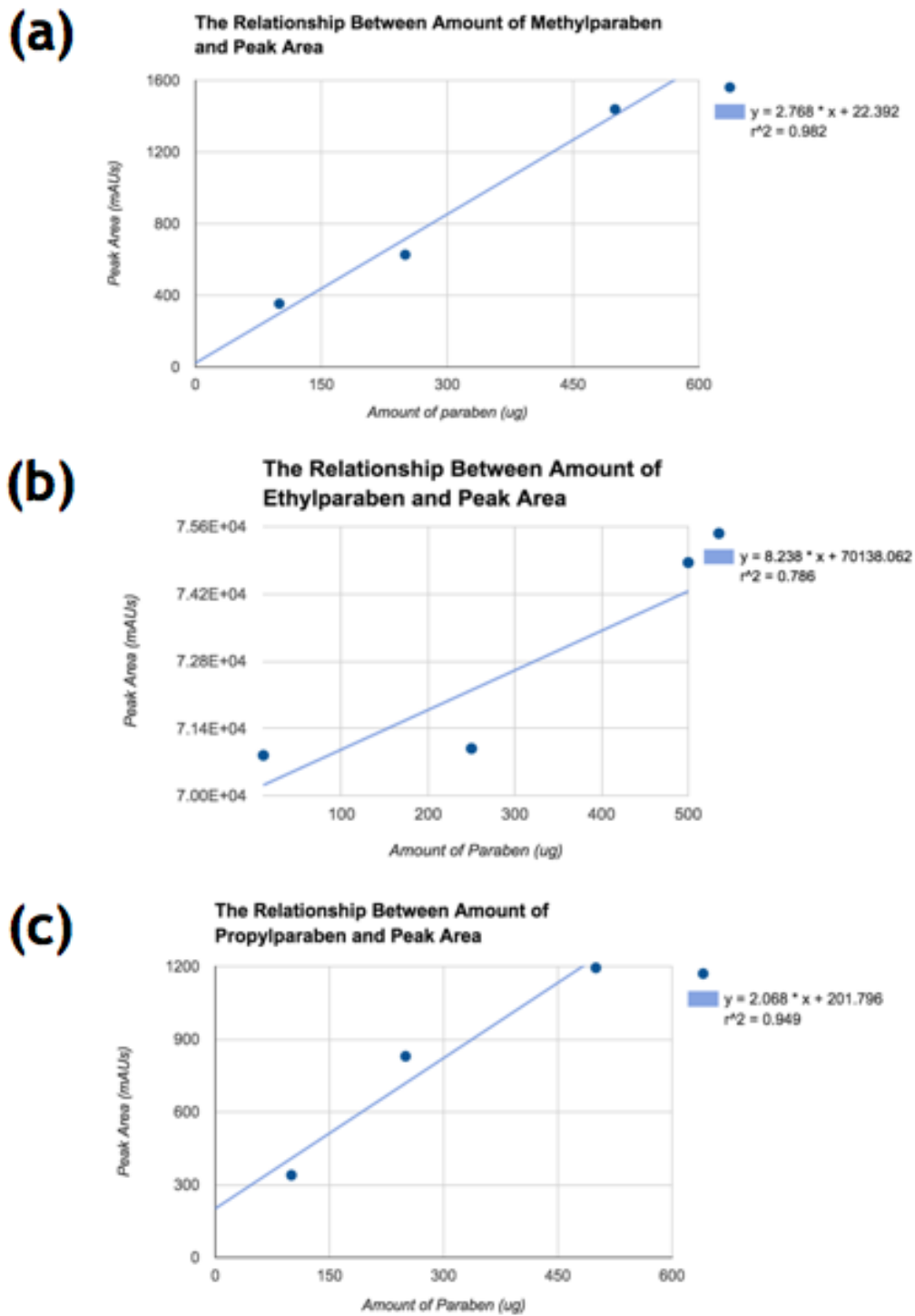


Figure 6. (a) Correlation of methylparaben amount to the peak area. (b) Correlation of ethylparaben amount to the peak area. (c) Correlation of propylparaben amount to the peak area.

The chromatograms from the Franz cell experimentation showed that some paraben diffused across the membranes and that the amount was more than hypothesized even after a single application (Figure 7c). The increase of peak area from each cosmetic application suggests that there was relative accumulation in the receptor fluid from the prior application. The difference in the results between the two experimental groups were observed with the variety of substances detected and an increase in peak areas (Figure 7d).

During the first experimental group, after 24 hours of the cosmetic application, all of the substance in the donor compound had either diffused across the membrane or possibly evaporated over time. For both groups, there was a visible change in the amount of liquid in the receptor chamber, a significantly larger change in the second experimental group.

When analyzing the chromatograms from both experimental groups, the chemicals that were most present in the receptor fluid was EP and MP (Figure 7c and d). The amount of paraben detected in the first experimental group using the Franz cells was about 1.5% and the second experimental group contained about 2% paraben. The chromatograms also revealed that PP only diffused across the membrane early on in the application of the cosmetics (Figure 7c), then was no longer detected after further application and as testing continued. This suggests that PP has a low lipophilicity because it was barely capable of passing through the membrane and that EP has the highest lipophilicity out of the three parabens tested because it diffused the most. Because PP has a low absorption because of its low lipophilicity, more cosmetic products contain this form of paraben than the others. Also, when considering the chromatograms of the cosmetic products, the paraben with the highest amount is PP, the “safest” of the three parabens

due to its lack passage through the membrane, thus not contributing to the health defects that parabens can cause in consumers.

Sources of error in experimentation include the usage of the HPLC and timeliness with the Franz cells and with the application of cosmetics. The HPLC prompted a troubleshooting process because of the various interruptions throughout multiple runs and possible alterations with the column over time. Due to the numerous runs the column was used many times and over time there would be substances left over on the column, as shown when looking at a chromatogram of the mobile phase (data not shown). The chromatogram should have showed zero peaks, but most still showed various peaks, suggesting that there were substances left on the column throughout various runs. Although this could be an altering factor when determining results, the peaks that were found in the chromatograms of the mobile phase were identified in the samples and subtracted out when determining the peaks for the parabens in the experimental samples.

Consistent timing of application of the cosmetics could be another factor that could lead to altered results. Despite time of application being recorded, some runs would last longer than others because of scheduling issues. Another issue with the time of application of cosmetics in the Franz cells is the time elapsed between each. After about 20 hours after application the donor compound containing the cosmetics would be empty. This suggests that the cosmetics could have completely diffused at an earlier time and that reapplication could have been sooner than 24 hours. Time of application may not significantly alter results because despite the amount of time that had elapsed where there was no cosmetic in the donor compound and no diffusion, the receptor fluid could not have been changed and there could not have been any substances added

or taken away from the receptor fluid in the Franz cells throughout that time, and the only occurrence within the Franz cells would be the continuous flow of 37 °C water from the water bath and the continuous movement of the magnetic stir bars in the Franz cells.

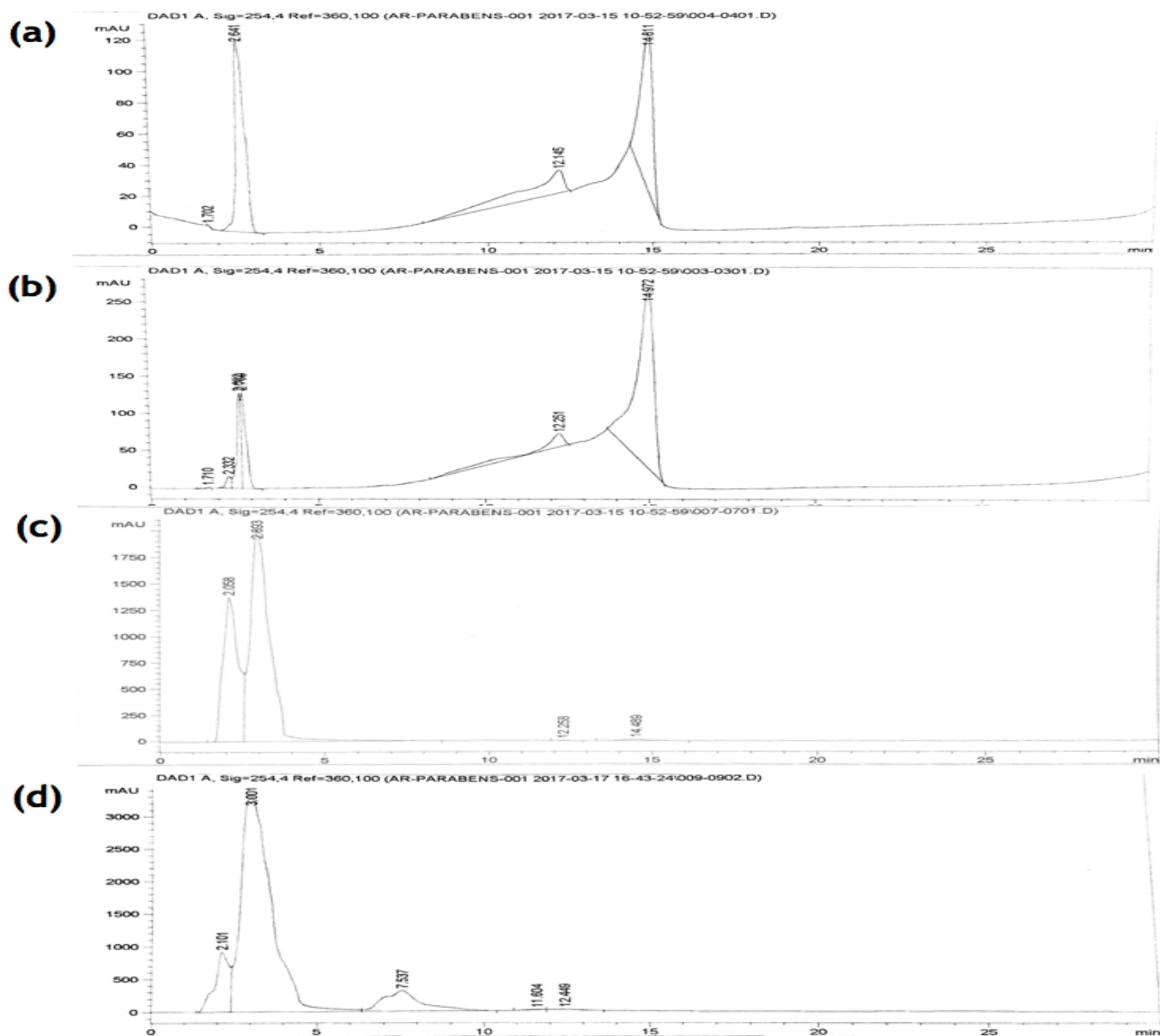


Figure 7. The retention time for MP is around 2 minutes, EP around 12 minutes, and PP around the end of 14 and early 15 minutes. (a) Typical chromatogram showing the parabens detection in

the Neutrogena cosmetic. (b) Chromatogram showing the parabens detection in the Banana Boat cosmetic. (c) Chromatogram showing parabens detection in the receptor fluid after one application of both cosmetics. (d) Chromatogram showing paraben detection after three applications of both cosmetics over the span of 72 hours.

Further Work

Testing on *Caenorhabditis elegans* is another alternative to this method. *C. elegans* have an epidermis similar to that of a human, and because of this various studies have been done on this organism. While this organism is cheaper than the method of using Franz Cells, it still presents reliable results. Also, *C. elegans* are easy to test on because they are easy to maintain and monitor during experimentation.

Using this new approach the same cosmetics would have been directly applied to the surface of the organisms and would include the same concept of the two experimental groups, then homogenized and set into HPLC. The first experimental group would just have the single application and result in one sample for HPLC analysis. The second experimental group would consist of three sections. One section of *C. elegans* for each 24 hours. The first would consist of *C. elegans* with only the single application lasting 24 hours, the second with two applications lasting 48 hours, and the final with three applications lasting 72 hours.

Throughout the tests, the way the *C. elegans* would react to the products, such as their appearance and mobility, would be recorded and considered when analyzing results. After both of the experimental groups were finished, the *C. elegans* would be grinded, combined with the mobile phase, and filtered for injection into the HPLC. Before the *C. elegans* remains would be

added to the mobile phase, the solution would be evaporated and the residue would be combined with the mobile phase. In this case, the mobile phase would be the same that was used in actual experimentation, 50:50 Methanol Water, but could also include the use of acetone, ethanol, or deionized water.

For HPLC analysis, the same type of C18 column would be used, along with the same method of filtration. Tests on the *C. elegans* should reveal similar results to the experimentation done with the Franz cells, that two out of the three parabens diffused across the membrane, and that after each application the accumulation of MP and EP would either double or triple to the amount that was present in the first application of the cosmetics.

Conclusion

The results revealed that the hypothesis was correct in determining if the parabens had diffused across a membrane and the amount of MP in Experiment 2 had almost doubled compared to Experiment 1. The amount of PP was not detected in the receptor fluid and most likely did not diffuse across the membrane, while the amount of EP significantly increased from Experiment 1 to 2.

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