

Extraction and Detection of the Antibiotic Sulfamethoxazole in Milk Using High Pressure Liquid Chromatography and a High School Classroom Simulation

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Background Information

Introduction- 10 min lecture

Bacteria that have become immune to many of the antibiotics that are commonly used today have earned the name super bugs. The overuse of antibiotics in the agricultural, medical, cleaning industries are the ones the blame (February,2007). Bacteria multiply exponentially and are highly adaptable. By constantly exposing bacteria to agents that will destroy them they will respond by becoming immune to their once killer. The purpose of this study is to see if antibiotics can be detected in Milk. If antibiotics are shown to be present in milk it will indicate that the antibiotics are not only being confined to the cow that has ingested them but also the person who drinks the milk of that cow.

Antibiotics are not specific, when ingested by a cow or patient they will kill the pathogenic bacteria but also the healthy bacteria within the organisms. Since all bacteria are highly adaptable all of them will eventually mutate and become resistant to that antibiotic. Instead of having one type of bacteria becoming resistant to the antibiotic all of the bacteria that were affected by the antibiotic will mutate in order to survive. Sometimes these mutations lead to more pathogenetic bacteria that are now resistant to that bacteria (Modern Biology, 2006). This has been the trend since the 1970's with the appearance of bacteria becoming resistant to Penicillin. (Survival of Sickest, 2006).

Our study hopes to detect the presence of antibiotics in the foods we eat. We will be using High Pressure Liquid Chromatography (HPLC) in order detect the presence of the antibiotic. We will also be using various extraction methods to prepare the milk for the HPLC.

Extraction- 15 min lecture

Milk is **homogeneous** mixture that contains many different compounds. In order for us to detect the antibiotic in milk we must first isolate and extract the drug from the milk. An **extraction** is a series of procedures that hopes to isolate a certain compound. Depending on your mixture (milk) and what you are trying to isolate (antibiotics) a scientist may use various methods. For our experiment, the milk was first centrifuged, the heaviest compounds (most likely lipids) that settled at the bottom of the centrifuge tube was discarded. The liquid sample then had acetic acid added to it. This formed a solid with the proteins that was filtered out using a syringe and micro filter paper. The left over extract was then tested for antibiotics.

Detection- 45 min lecture

Chromatography:

HPLC is a method of detecting a compound in a liquid mixture. It was developed and perfected in 1964, by Calvin Giddings. Before HPLC scientist used **Gas Chromatography** as a detection tool, unfortunately **GC** cannot use volatile samples because they would explode in the machine once they were heated to become a gas. HPLC solved this problem because substances did not need to be vaporized in order to work. Instead the substance can be in liquid form (Chromatography, 2007). Like all chromatography methods HPLC consists of a **stationary phase** as well as a **mobile phase**. The mobile phase takes the sample along the stationary phase and the compounds of that mixture will separate along that stationary phase. For example, If I were to separate the colors of a black marker using paper chromatography. I would draw a small dot on a piece of paper about 1cm before the edge. The paper is our stationary phase in this example seeing that it does not move. I will then submerge the edge closer to the marked end of the paper in some alcohol. The alcohol will be the mobile phase because when the paper is placed in the alcohol, the alcohol travels up the paper. Since it is moving it is called the mobile phase. Now the colors in the black marker will separate along the stationary phase depending on their size. The smaller the particle the faster it will travel and therefore be higher on the paper than the other colors in the black marker. Look at figure 1.1 below for clarification.



Figure 1.1

Depending on your mobile phase and stationary phase you can manipulate what compounds will separate on the stationary phase.

HPLC:

A special device is needed to perform HPLC. All will have share certain characteristics. HPLC uses high pressure liquid as its mobile phase and a column as its stationary phase. The liquid that is commonly used in experiments is either water, methanol or acetonitrile. Depending on your sample you can choose one of these or a combination of them. For our experiment we used a 50% solution of water and a 50% solution of acetonitrile. The column or stationary phase while using HPLC is a small metallic cylinder see (fig 1.2) that is filled with packing. The speed at which the sample is forced through the column will be read on a detector which uses wavelengths to measure the absorbance of each compound.



Figure 1.2

The absorbance of the detector is shown on what is called a chromatogram that is projected on a computer. The peaks indicate the presence of a compound and its concentration in the mixture. See figure 1.3 for an example of the chromatogram. In this particular chromatogram since there are 9 different peaks, this indicates there are 9 different compounds in the solution sample. The time at which the mobile phase carries the sample through the stationary phase (column) is what is measured in the chromatogram. The time it took for each of the compounds to go through the machine is indicated on the chromatogram.

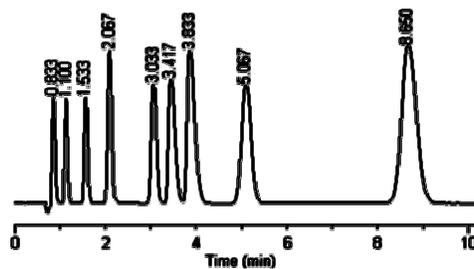


Figure 1.3

In this particular chromatogram all of the peaks are differentiated by the time. However if the proper mobile phase solvent is not used correctly this can make your chromatogram look like one huge peak or no peak at all. Therefore before you are trying to identify any compound you must research the proper solvent ratios for the mobile phase. The detector uses U.V. rays for detection. In order to correctly use the HPLC this too must be researched. For our experiment we used a 50% solution of water and a 50% solution of acetonitrile. We also set the detector at a wavelength of 214. Below in figure 1.4 is a diagram of a complete HPLC device.

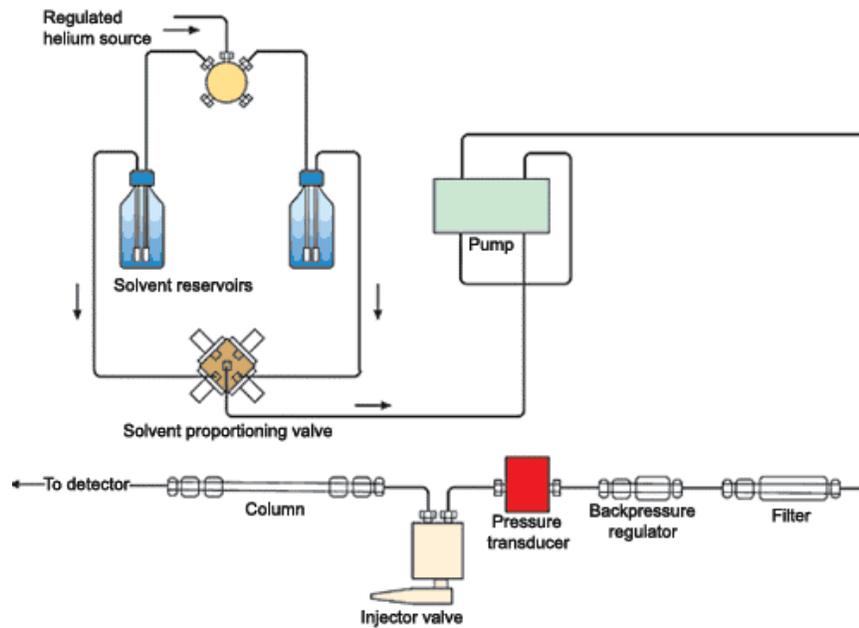


Figure 1.

Specifics of our HPLC Device and Trials

Type of Machine: Hewlett Packard Series 1050 HPLC

Wavelength used to detect antibiotic- 214nm

Antibiotic used- Sulfamethoxazole

Solvents used- 50% water and 50% Acetonitrile

Column Type- C18 4.6mm 15cm

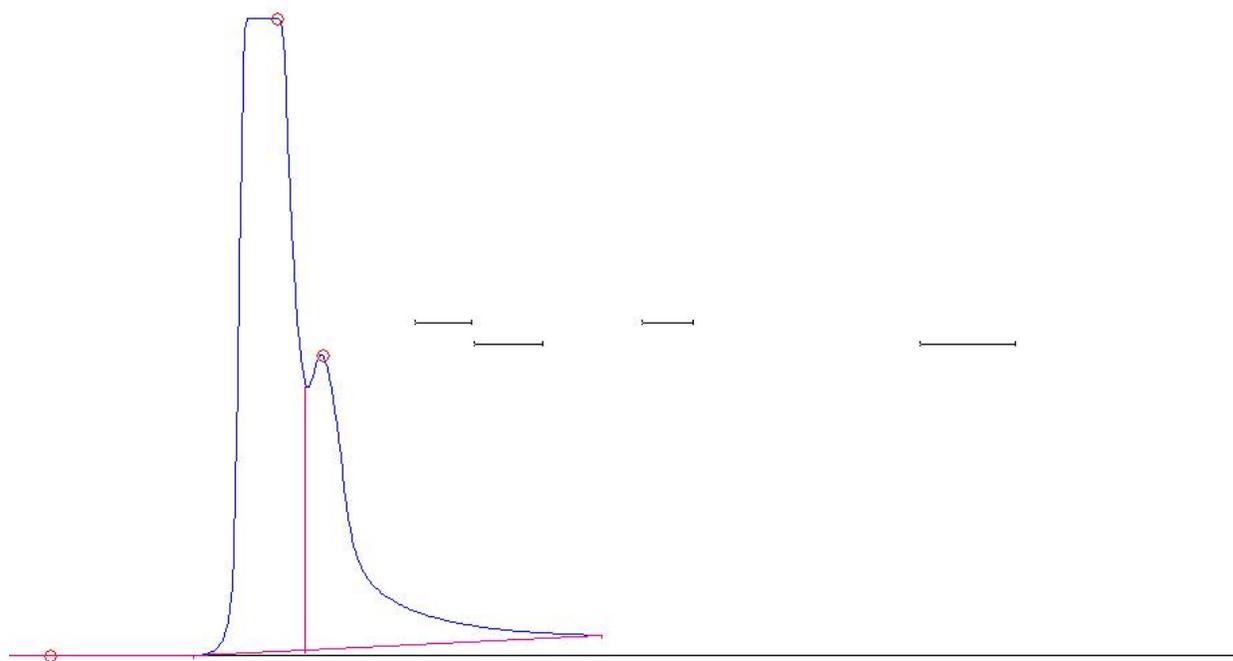
Reverse HPLC

Computer Program- Peak Simple

Results of Our Study- 10 min lecture

With the extraction method used in our experiment we were unable to retrieve any detectable amounts of the antibiotic(Figure 2.1) in the milk. The retention time for sulfamethoxazole is 4.0-4.4. Since there is no peak between those retention times it confirms the absence of said drug.

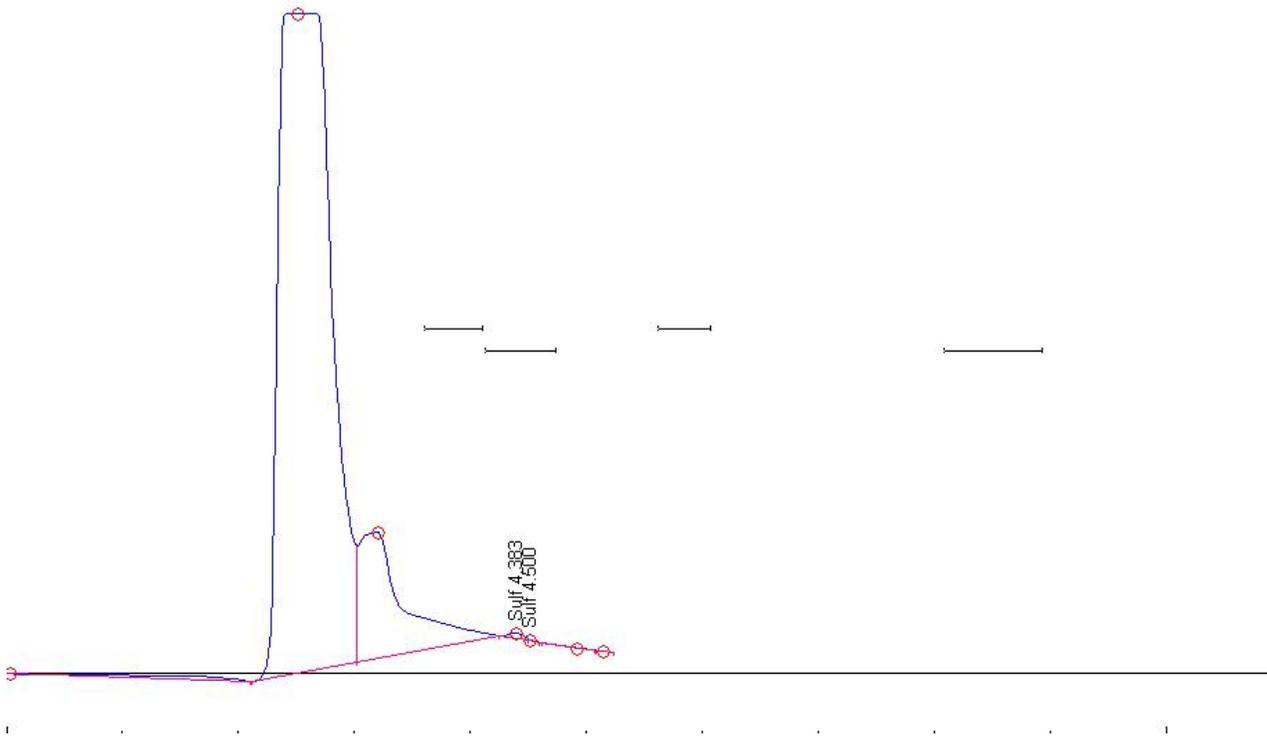
HPLC Data Milk Sample



Retention Time
Figure 2.1

In order to see if our methods were not destroying the antibiotic we spiked a milk sample with sulfamethoxazole with at a solution that was 2ppb of the antibiotic. The spiked sample was detected by HPLC confirming that our methods did not harm the sulfamethoxazole (see figure 2.2 and 2.3).

HPLC Data Spiked Milk Sample



Retention Time
Figure 2.2

HPLC Data Spiked Sample Magnified

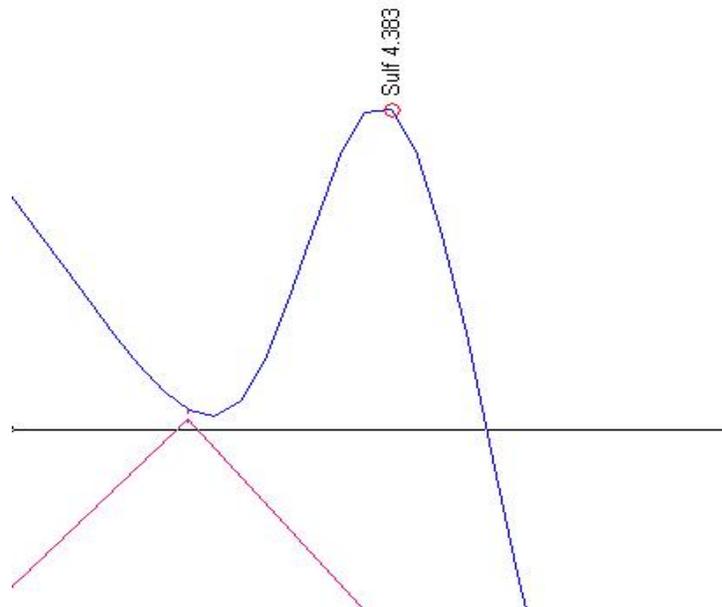


Figure 2.3

Conclusion- 10 min lecture

Our result indicates that there is no detectable amount of sulfamethoxazole in milk using our methods. The concentrations of the antibiotic fell within the calibration curve (Figure 3.1) confirming we extracted the antibiotic at the specific concentration that was added to the sample with _____% error.

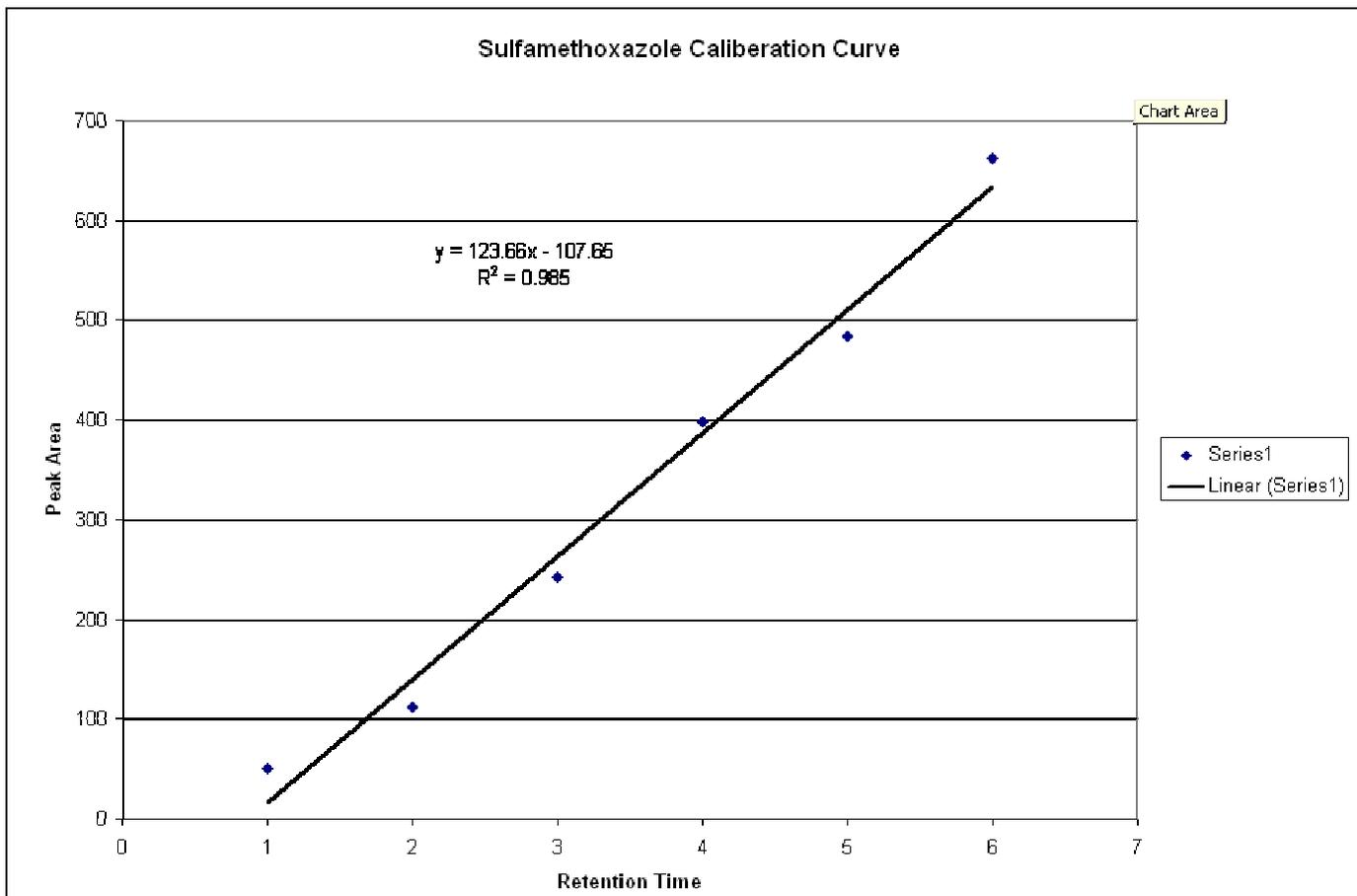


Figure 3.1

Since we were able to detect the antibiotic in the spiked sample it confirms that our method did not destroy the antibiotic. However, our experiment does not confirm that there are no antibiotics in the milk. There could be other types of the antibiotic in the milk since we did not test for them. Also, our experiment does not confirm that there are no other forms of sulfamethoxazole in the milk. Due to metabolic processes from the cow, some of these processes could have changed the chemical structure of the antibiotic enough to not be detected by HPLC but still deadly enough to kill bacteria. Our experiment allowed us to practice using HPLC and understand the intricacies of the machine. The extraction method is extremely useful and can be used in numerous experiments such as DNA extraction from organisms.

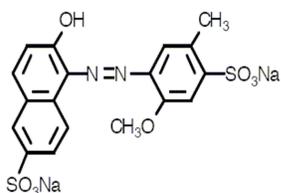
Now knowing how difficult it is to test for trace elements in foods, I will use great care in the things I consume. There are many compounds that are being added to foods that may have detrimental effects. I will encourage my students to consume products that do not have antibiotics, pesticides, or any other potentially harmful compounds.

Extraction and Identification of Antibiotic Simulation Using Column Chromatography

Introduction:

Column chromatography is a useful procedure when trying to separate compounds in a mixture with high volumes. In this demonstration you are to isolate the red compound from the purple mixture using column chromatography. The column will be filled with silica gel (SiO_2). The mobile phase in this procedure will be water. Seeing that silica gel and water are both polar they will have a greater affinity to the compounds in the purple mixture that are charged. The compounds that are not charged will penetrate the membrane and will be collected at the bottom of the column. For our sake let's assume that the purple mixture is soda, and we want to separate the soda from any antibiotics that might have gotten into them through manufacturing. The antibiotics in this situation will be Red 40 color (Figure 4.1). You will have success if you are able to separate the red compound from the purple mixture. Good Luck!

Red 40



Blue 1

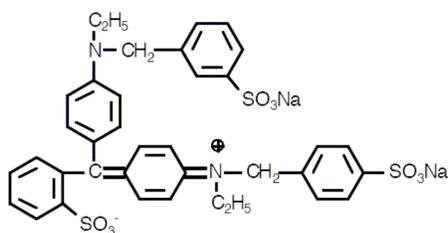


Figure 4.1

Chemical Structure for Red 40 (antibiotic) and blue 1.

Materials:

Goggles
Latex Gloves
Lab Apron
100 ml graduated cylinder
5-100 ml beakers
30 cc syringe
25 ml chromatography column
½ cotton ball
1 Stirring Rod
Column Clamp
Stand
2 inch of 3mm tube to connect column and syringe
5 grams Silica Gel (Caution- Silica gel is toxic when inhaled it must be used under hood)
75 ml Hexane (Caution-Hexane is volatile and must be used under the hood)
75 ml Distilled water
10 drops of red food coloring (red 40)
10 drops of blue food coloring (blue 1)
1 Gallon Organic Waste container

**Note- Do not discard silica gel or hexane down the sink or garbage can.
Discard used portions in designated waste container.**

Procedure:

1. Label 3 beakers, purple, blue and red.
2. Place 25 ml of water in blue and red beaker each.
3. Place 10 drops of blue food coloring in the beaker labeled blue and swirl.
4. Place 10 drops of red food coloring in the beaker labeled red and swirl.
5. Place 10 ml of blue colored liquid in beaker labeled purple.
6. Place 10 ml of red colored liquid in beaker labeled purple.
7. Assemble clamp on stand and lock column into place.
8. Place cotton ball inside column and push it down to the end using the stirring rod.
9. Place beaker under column in order to catch any waste that may go through the column in the upcoming steps.
10. Obtain 75 ml of hexane in graduated cylinder.
11. Weigh out 5 grams of silica gel.
12. Mix silica gel and 30ml of hexane in 100ml beaker with stirring rod. (This should make a slurry white mixture seeing that the silica gel will not completely dissolve in hexane)
13. Pour slurry mixture in column. (The silica will begin to gather on bottom of the column)
 - a. Tap column with finger in order to eliminate any air bubbles that may get trapped with the silica sediment.
14. Repeat steps 5+6 until all of the silica gel is placed into the column.
 - a. Do not over fill column.
 - b. Do not allow silica gel to harden by not constantly exposing it to a solvent (liquid)
15. Attach syringe with rubber tube to column.
 - a. The syringe can be used to add air pressure to aid in the extraction.
16. Pour purple mixture in column and collect any pure colored substances that come from the column in the beaker.

17. Compare your obtained pure colored substance with the beaker labeled red.
a. Take a picture of your results and put them in your data section of your lab report.

Conclusion Questions:

1. Why would the extraction method be useful?
2. Give two examples of compounds that apply to your life that you would like to extract from a mixture?
3. Water is polar and so is silica gel, given this information and by looking at the chemical structures of red 40 and blue 1 on figure 4.1 and SiO₂ and Water on Figure 4.2, why is it that the red compound was able to be separated from the purple mixture.

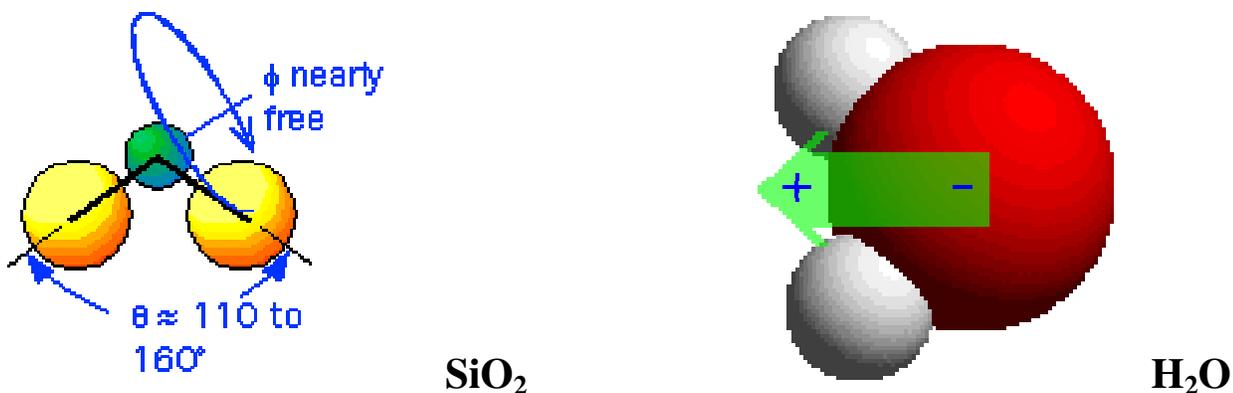


Figure 4.2

4. What purpose did the silica gel serve in this demonstration? Use the internet and research any other substances we could have used instead of the silica gel.

Extra Credit: Explain why we could use another substance instead of silica gel. What properties do the two substances share. Come after school and try the demonstration using the other substance.