The Effect of Incubation Time on the Generation of Benzene in Sierra Mist Free

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This experiment performed was to determine the effect of the length of incubation time on the generation of benzene from ascorbic acid and potassium benzoate in Sierra Mist Free. The results would provide useful information on storage methods for soda which might allow people to reduce their benzene intake from soda, thus decreasing their carcinogen accumulation and possibly lowering their risk for developing associated cancers. A headspace gas chromatography and mass spectrometry method was used for the analysis of benzene. Quantitation was preformed using Agilent Enhanced Chem Station D.01.02. A calibration curve was generated by creating dilutions of benzene in deionized water. The range of detection was between 0.52 ng/mL and 20.0 ng/mL. Each sample of Sierra Mist Free was prepared for incubation by adding 10 mL of Sierra Mist Free to a headspace vial. There were 4 samples per group. Groups 1-8 were incubated at 50 °C for a certain amount of time over a 14 day period. Group 9 was the control group, incubated at room temperature. The data showed that no benzene was generated in any sample for any given period of incubation time at 50 °C or room temperature. The hypothesis, which stated that samples incubated at for a longer time at 50 °C would generate more benzene than samples incubated at 50 °C for a shorter period of time or at room temperature, was rejected, and the null hypothesis, which stated that incubation time would have no effect on the amount of benzene generated, was accepted.

Introduction

Anyone who indulges in Coke, Sprite, Mountain Dew, or other sodas generally recognizes the negative effects of drinking carbonated drinks: for example, the caffeine, the high sugar, the empty calories, the possibility of cavities, and so on. But it is generally thought that drinking soda in moderation can be acceptable in terms of health. Recently, however, it has come under speculation that drinking soda—particularly diet soda containing both sodium benzoate or potassium benzoate and ascorbic acid—may lead to an increased risk for associated cancers such as leukemia, (8). Sodium benzoate and potassium benzoate are both salts that occur in nature, but they are more than often produced chemically when added as preservatives in foods and beverages. They are very effective at killing bacteria, yeast, and fungi, but they only work when the pH is less than 3.6. They are most commonly ingredients in foods and drinks with a high acid content, like soda, vinegar, fruit juice, and salad dressing, (6). Ascorbic acid is more commonly known as Vitamin C, and it is found naturally in fruits and vegetables like oranges, tomatoes, strawberries, and broccoli. Humans have to consume foods with Vitamin C, because the human body does not produce it. Vitamin C is needed to carry out many chemical reactions in the body, and it also acts as an antioxidant, bonding to free radicals (atoms, molecules, or ions with unpaired electrons that are very reactive) that "might otherwise damage healthy tissue" or harm "molecules like DNA, lipids, proteins, and carbohydrates," (5). Vitamin C is often added to foods and beverages to prevent spoilage or to add nutrients. Sodium benzoate, potassium benzoate, and ascorbic acid are not harmful by themselves, but in sodas, juice, and even flavored water, sodium benzoate or potassium benzoate can react with ascorbic acid in a metal catalyzed hydroxyl radical reaction to form benzene, (3). Herein lies the problem: "The US Department of Health and Human Services (DHHS) classifies benzene as a human carcinogen—a compound that promotes cancer," (9).

Benzene has the chemical formula $C_6H_{6,}$ and it is the simplest aromatic hydrocarbon. Only slightly soluble in water, benzene is a clear, colorless liquid with a characteristic odor that boils at 80.1°C (176.2°F) and solidifies at 5.5°C (41.9°F), (1). Although benzene does occur as an uncombined molecule, it is more threatening when it is manmade and used in industry. Benzene is often used as an industrial solvent, and it is involved in the production of rubber, plastic, detergents, dyes, and synthetic fibers, (8, 9). Prolonged exposure has been linked to the development of several different types of cancer, particularly AML leukemia, (8). People are exposed to benzene everyday from auto exhaust, industrial emission, and second hand smoke, and in reality, the amount of benzene exposure from soda and other beverages is very small. Both the US Environmental Protection Agency (EPA) and the FDA have established the "maximum allowable level (MCL) for benzene in drinking water at 5 ppb", and, since the identification of this problem in 1990, over 200 drinks have been tested, only 10 of which have been reported to contain benzene levels exceeding 5 ppb, (2). As with many carcinogens, however, accumulation versus exposure may be the key to cancer causing effects. Over a life time, the human body comes in contact with countless carcinogens, so even if the benzene levels in one can of soda isn't "that high" it still makes sense for an individual to attempt to reduce or eliminate his or her beverage-related benzene intake.

The basic idea for this project came from a research experiment conducted by Robert Fray and a team from Scientific Instrument Services, Inc, (7). The team tested three brands of orange sodas purchased from a local grocery store: Brand X (a diet carbonated drink), Brand Y (a non-diet carbonated drink), and Brand Z (a non-diet non-carbonated drink). Their purpose was to study the effect of heat on the formation of benzene. (Studies have shown that benzene forms more readily at higher temperatures and in bright light. Sugar has also been shown to inhibit the production of benzene.) The team determined the benzene quantity with purge and trap thermal desorption gas chromatography for separation and mass spectrometry for

analysis. Samples of each brand of orange soda were incubated at 20°C, 50°C and 90°C. See "Table 1: Benzene Levels in Soda at 20, 50, and 90°C" for their results, (7):

Sample	Purge & Trap Temperature(°C)	Benzene (pg/µl)
X	20	0.36
X	50	0.60
X	90	1.45
Y	20	0.70
Y	50	0.90
Y	90	0.14
Z	20	5.5
Z	50	7.4
Z	90	10.1

Table 1:Benzene levels in soda at 20, 50 and 90°C

After observing the data, the team concluded that there is a "definite trend towards higher benzene levels found in these beverages as the incubation temperature is increased," (7). The exception was Brand Y incubated at 90°C. The team said this error was most likely a result of some difficulties they had in keeping the lid on the flask when Brand Y, which was found to be more carbonated than Brand X and Z, was heated to 90°C. The pressure produced from Brand Y at that heat level kept making the lid pop off.

The experiment to be conducted for this project is different from the experiment conducted by Robert Fray's team. Only one brand of soda will be used. The soda used in this experiment will be Sierra Mist Free because it contains both ascorbic acid and potassium benzoate, has no sugar, and because the FDA has previously detected benzene in Sierra Mist Free. A headspace gas chromatography and mass spectrometry method will be used for analysis of benzene in this experiment. According to Henry's Law, "the solubility (C) of a gas or volatile substance in a liquid is proportional to the partial pressure (P) of the substance over the liquid," (10). In mathematical terms, Henry's law can be stated as:

$$P=kC$$

where k is a constant called "Henry's law constant and it is characteristic of the solvent and the solute, (10). The amount of benzene dissolved in each sample of Sierra Mist Free can be determined by analyzing a sample of headspace gas from each of the headspace-vials containing Sierra Mist Free. The headspace sampler will remove a sample of gas from each of the headspace vials, and then a gas chromatography will be used to separate the benzene and internal standard. Finally, a mass spectrometer will be used to detect and quantify the amount of benzene present, (4). The purpose will be to determine the effect of incubation time on the amount of benzene formed at 50°C over a period of 14 days. The data found in this experiment will be useful information regarding how people can reduce their benzene intake from soda, thus decreasing their carcinogen accumulation and possibly lowering their risk for developing leukemia. It is hypothesized that the amount of benzene detected in Sierra Mist Free samples incubated at 50°C for a longer period of time will be higher than the amount of benzene detected in samples incubated at 50°C for a shorter period of time or in samples incubated at room temperature. The null hypothesis is that there will be no difference in the amount of benzene detected in samples of Sierra Mist Free that were incubated at 50 °C or at room temperature for different periods of time.

Materials and Methods

This experiment was conducted at the Toxicology lab of the South Carolina Enforcement Division, using cans of Sierra Mist Free obtained from a local grocery store, 99% pure benzene (Fisher Scientific), tert-butanol HPLC-grade (Aldrich Chemical Company), methanol (Fisher Scientific), and deionized water. Safety goggles and gloves were worn at all times, and a fume hood was used during the preparation of stock solutions and calibrators to limit exposure to benzene fumes. An Agilent Technologies G1888 Headspace Sampler, 6890N Gas Chromatographer, and 5973N Mass Selective Detector were used to process the samples

First, a 1 mg/mL stock solution of benzene in methanol was prepared. Approximately 80 mL of methanol was added to a 100 mL volumetric flask. Considering that the benzene was only 99% pure and knowing that the density of benzene is 0.88 g/mL, it was calculated that 115 µL of 99% pure benzene would be needed to create the stock solution. A micropipeter was used to add 115 µL of 99% pure benzene to the volumetric flask, and then the solution was QS-ed to 100 mL with methanol. Second, a 1 ng/mL working standard was prepared by creating a 1/1000 dilution of the stock solution in deionized water: 100 µL of the stock solution was diluted in 100 mL of deionized water. Third, the calibrators were created by diluting specific amounts of the working standard in 25 mL of deionized water: 13 µL of working standard were added to 25 mL of water to create a 0.52 ng/mL calibrator, 25 µL of working standard were added to 25mL of water to create a 1.0 ng/mL calibrator, 50 µL of working standard were added to 25 mL of water to create a 2.0 ng/mL calibrator, 125 µL of working standard were added to 25 mL of water to create a 5.0 ng/mL calibrator, 250 µL of working standard were added to 25 mL of water to create a 10.0 ng/mL calibrator, and 500 µL of working standard were added to 25 mL of water to create a 20.0 ng/mL calibrator. The positive control was a 10 ng/mL solution of working standard in deionized water, and the negative control was 10 mL of deionized water. As an internal standard, 50 µL of 0.01% tert-butanol was added to each calibrator.

36 samples of Sierra Mist Free were prepared by adding 10 mL of Sierra Mist to headspace vials, and then the vials were sealed. The 36 samples were divided into 9 groups of 4. 32 of the samples (Groups 1-8) went into a headspace oven at 1:00 pm on December 31, 2008 to incubate at 50°C. Group 9 was a control group. Group 1 was removed from the oven at

1:00pm on the following day (January 1, 2009), and those samples were allowed to cool back down to room temperature. The same procedure was repeated for all the samples in Groups 2-7. Group 8 was allowed to incubate for 14 days.

Group #	# of Days in Oven
1	1
2	2
3	3
4	4
5	5
6	6
7	7
8	14
9	0

Table 2: Length of Incubation for soda samples in groups 1-9.

Prior to analysis 50 μL of internal standard was added to each sample with a syringe. Then the samples and calibrators were loaded into the autosampler for analysis. Finally, quantitation was performed using Agilent Enhanced Chem Station D.01.02.

Results

No statistical analysis was run.

Calibrator Concentration (ng/mL)	Response
20.0	49841
10.0	30781
5.00	14188
2.00	4770
1.00	2951
0.52	1170

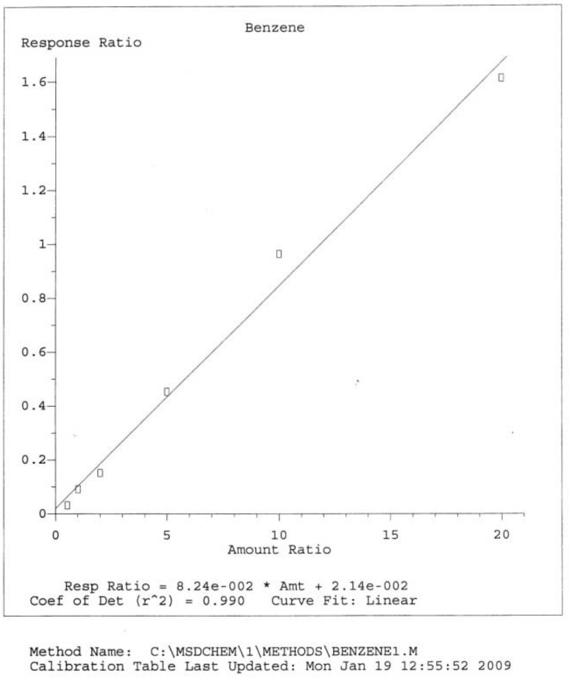
Table 3: The benzene response from each calibrator

Control	Response
Positive Control (10ng/mL)	30931
Negative Control	0

Table 4: The Benzene Response from the Positive and Negative Controls

	0	1	2	3	4	5	6	7	14
1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0

Table 5: The Benzene Response for Samples of Sierra Mist Free Over Time. Down: Headspace Vial Sample Number. Across: Number of Days of Incubation.



Graph 1: Calibration curve

Discussion

The data in Table 3: The Benzene Response from Each Calibrator shows that HS/GC/MS can be used to detect and quantify benzene. This data was used to create the calibration curve seen in Graph 1: Calibration Curve. The range of detection was 0.52 ng/mL to 20.0 ng/mL. The data in Table 4: The Benzene Response from the Positive and Negative Controls shows that the HS/GC/MS was working properly.

The purpose of this project was to determine the effect of incubation time over a period of 14 days on the generation of benzene from the ascorbic acid and potassium benzoate in Sierra Mist Free. It was hypothesized that the amount of benzene detected in Sierra Mist Free samples incubated at 50°C for a longer period of time would be higher than the amount of benzene detected in samples incubated at 50°C for a shorter period of time or in samples incubated at room temperature. The null hypothesis was that there would be no difference in the amount of benzene detected in samples of Sierra Mist Free that were incubated at 50 °C or at room temperature for different periods of time. The data in Table 5: The Benzene Response for

Samples of Sierra Mist Free Samples Over Time shows that no benzene was detected in any sample for any period of incubation at any incubation temperature. Therefore, the hypothesis must be rejected and the null hypothesis must be accepted. With such data it was neither possible nor necessary to run statistical analysis.

There are several possible sources of error. Because the limit of detection achieved by the lowest calibrator was 0.52 ng/mL, trace levels of benzene below 0.52 ng/mL may have gone undetected. Another possibility is that the aluminum can is in some way involved in the generation of benzene from ascorbic acid and potassium benzoate in soda. Samples in this experiment were not incubated in aluminum cans; the samples were incubated in glass vials because the aluminum cans burst when incubated at 50 °C in the headspace oven. A third possibility is that the carbonation in soda plays a roll in the generation of benzene. The can from which all of the samples of Sierra Mist Free were obtained was allowed to sit open for 1 hour to go flat because pipeting an accurate amount of sample was not possible when the drink was first open due to air bubbles created in the pipet from the carbonation. Further research might involve creating a lower limit of detection or finding successful methods for incubating aluminum cans or pipeting carbonated soda samples. Also, other brands of soda might be tested for comparison.

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