SWEET MYSTERIES: ANALYSIS OF PHARMACOLOGICALLY ACTIVE INGREDIENTS IN CHOCOLATE

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ABSTRACT

Chocolate contains pharmacologically active substances that affect people. The two main active components, theobromine and caffeine, were extracted from different types of chocolates, separated by High Performance Liquid Chromatography (HPLC), and amounts of each determined. All chocolate samples examined except one contained these two substances. Milk chocolate appears to contain smaller amounts of theobromine and caffeine, but distinctions among the different dark chocolates were not possible. Two subjective tests were conducted to see if there is a correlation between alertness and concentrations of pharmacologically active ingredients in the different chocolate samples. Neither subjective test showed a clear correlation. Further study, refinement of both the chemical and subjective tests, and statistical analysis are necessary to establish the physiological impact this food has on humans.

INTRODUCTION AND BACKGROUND

Hypotheses

The first hypothesis is that more theobromine will be found than caffeine in all types of chocolate, and that dark chocolate will have more of both active ingredients than milk chocolate, which would have more of both ingredients than white chocolate. The second hypothesis is that subjects' ratings of the chocolate would directly correspond with their chocolate preference, and that the subjects' alertness level would increase (as determined by a self-rating) after tasting the dark chocolate sample. The goal of this study is to show whether chocolate acts as a stimulant, with the higher concentrations of active ingredients in chocolate producing greater alertness.

The History of Chocolate

The story of chocolate begins in South America, in the 15th century where the Aztecs discovered cocoa beans. The Aztecs introduced chocolate to the Europeans whose initial reaction was negative. Chocolate, or *chocolatl* to the Aztecs, was bitter and unappetizing to the tongues of the Europeans. One of the major reasons the chocolate was not appetizing to the Spaniards was that the Aztecs' added pepper to it. Hernando Cortes, the leader of the conquering Spaniards, sweetened the bitter food with cane sugar, making it sweet instead of spicy. Chocolate in this form was brought back to Spain, where it became an instant success. [1].

Chocolate houses soon sprung up all across Europe, and they were a big success among the elite classes. It came to the United States in the mid 18th century, and in 1765 the first chocolate factory was established. The chocolate industry grew throughout the years, and new variations of chocolate, such as milk and dark chocolate, were created. In 1895, Milton Hershey perfected a mass-production process, making chocolate available to the masses. Today, the chocolate industry is very well developed, and chocolate is one of the most popular confections [1].

Chocolate Manufacturing Process

Chocolate-making is a science, requiring a multi-step refining process (Figure 1) to achieve the perfect blend for the desired end product. The first step in manufacturing chocolate is the cleaning process. A machine passes the cocoa beans through and removes dried cacao pulp, pieces of leftover pod, or other worthless material otherwise not removed. After being thoroughly cleaned, the desired beans are carefully weighed, measured, and blended directly to the factory's direct specifications. These formulas are often company-specific, reflecting their respective idea of the ideal flavor, blend, and texture. The selection of beans is vital to the final chocolate's flavor. To bring out the robust chocolate aroma and ambiance, the beans are then roasted in very large, rotating cylinders. As the method proceeds, the beans turn over and over, losing excess moisture, their color turning a deep, dark brown; the characteristic chocolate aroma becomes heavily apparent at this point.

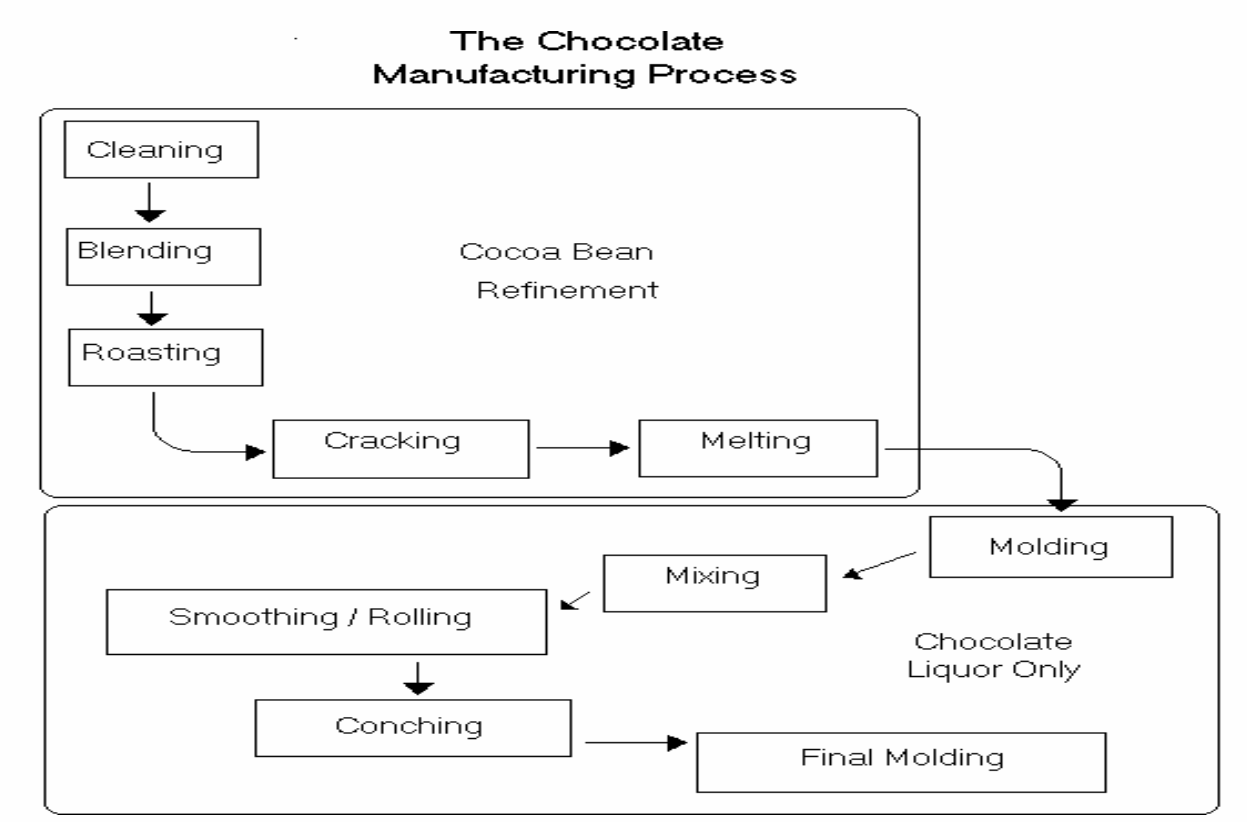


Figure 1: A diagram of the chocolate manufacturing process.

The cocoa beans, now brittle after roasting, are sent through a large cracking machine, which breaks the beans into pieces. These cracked "nibs," containing about 53% cacao butter, are conveyed to mills, where they are crushed between huge grinding stones or heavy steel discs. This process generates so much heat from friction that it melts the nibs into liquid chocolate "liquor." The liquid is then poured into molds, making bars of unsweetened or "bitter" chocolate. Cocoa is now processed separately; its main by-product, cacao butter, is an essential component of all chocolate, taking up to 25% of any chocolate bar's weight.

All chocolate, be it dark, milk, or bittersweet, utilizes cacao butter. Besides amplifying the flavor, it also makes the chocolate more fluid and prone to melting in the mouth. Common eating chocolate, sweet chocolate, is a combination of unsweetened chocolate, sugar, cacao butter, and a little vanilla. Milk chocolate is very similar, though it uses less unsweetened chocolate and also has milk as a main ingredient. Making any chocolate entails melting and combining the ingredients in a large mixer until it takes on the consistency of dough. The mixture, regardless of the type of chocolate, is passed through heavy steel rollers, becomes a smooth paste, and is ready for "conching," a type of kneading process. This method aerates and agitates the chocolate, creating various degrees of flavor and texture. Often, there is an emulsifying operation that goes along with "conching," giving the chocolate a fine, velvety smoothness. The mixture then goes through a tempering interval, a heating, cooling, and reheating that readies it for the final moulds. It is then that the chocolate finally takes the shape of its final commercial product. The molded chocolate next reaches the cooling chamber, where cooling proceeds at a fixed rate to keep the robust flavor intact. Thus chocolate's birth process ends, and it is packaged, wrapped, and shipped off to distributors and confectioners throughout the country [1].

Previous Research on Chocolate

Chocolate has attracted a wide group of fans since its discovery hundreds of years ago. Many studies have been conducted in hopes of linking its gratifying "kick" with an increase in health. Traveling back a few centuries, Grivetti, an Italian physician, prescribed chocolate to improve anemia, stimulate appetite, strengthen the brain, increase breast milk production, cure kidney stones, increase sexual performance, and even lengthen one's life. Recently, however, researchers have found large amounts of polyphenol antioxidants, a chemical used by the body to "mop up" the blood streams, clearing them of the free radicals, small reactive molecules that cause damage to the body and have a hypothesized connection with cancer. Furthermore, at a symposium at the 17th World Congress of the International Society for Heart Research, scientists have proven that consumption of certain chocolates can affect platelet function positively. Chocolate was found to contain high amounts of flavanol, a naturally-occurring component that aids platelet function [2].

Pharmacologically Active Ingredients in Chocolate

Chocolate is made up of many components, and has a stimulatory effect. One way the bodies of human beings respond to chocolate is by producing natural opiates. Various receptors in the brain respond to the components in chocolate by releasing chemicals that create a sense of euphoria for the chocolate taster. These endorphins make chocolate eaters feel good about

themselves. Along with these endorphins, chocolate induces anandamides, lipids found in the brain, to create a sense of being high. Although our bodies already have these anandamides, chocolate perpetuates the intensity of the feeling that one gets from eating it by directly feeding it into our systems. Another one of chocolate's key components is phenylethylamine, which is able to increase blood pressure that results in higher states of alertness. Chocolate has recently been discovered to release a chemical substance called serotonin into our bodies. Low levels of this compound make the chocolate taster crave for more similar products in sugar and taste. Chocolate is a "drug." This "drug" is highly affective in making its tasters more awake and happier [3].

Xanthines

Xanthines are chemicals or derivatives of composition, $C_5H_4N_4O_2$, that are often found in blood, urine, muscle tissue, and certain plants. Theobromine and caffeine are derivatives of xanthine, only possessing extra methyl groups. In chocolate, xanthines comprise the main components of chocolates [4].

Theobromine – $C_7H_8N_4O_2$ (3, 7-dihydro-3, 7-dimethyl-1H-purine-2, 6-dione)

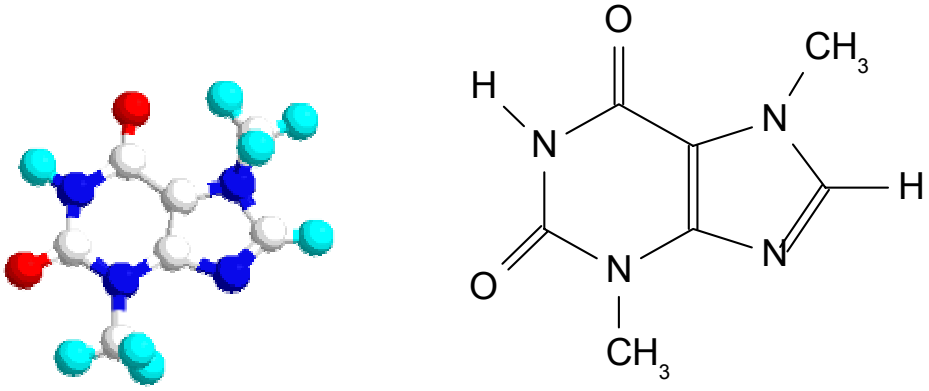


Figure 2: (left) A ball and stick representation of theobromine. (right) An atomic, skeletal representation of the structure of theobromine.

A natural ingredient of chocolate, theobromine occurs naturally in cocoa beans and is present in all chocolates, barely in tea, and never in coffee. Milk chocolate, however, contains less theobromine than dark, or semi-sweet chocolates do. While it both shares similar structure (compare Figure 2 and Figure 3) and stimulates urination and heart muscle like caffeine, theobromine does not sharpen one's alertness. It is often used in treatment for high-blood pressure. When isolated, theobromine must be used carefully since it is a Group 3 carcinogen, able to cause cardiac and central nervous system distress in dogs. Molecularly, water-soluble theobromine sublimes at 290-295°C and has a molecular weight of 180.17 grams [5].

Caffeine – $C_8H_{10}N_4O_2$ (trimethylxanthine)

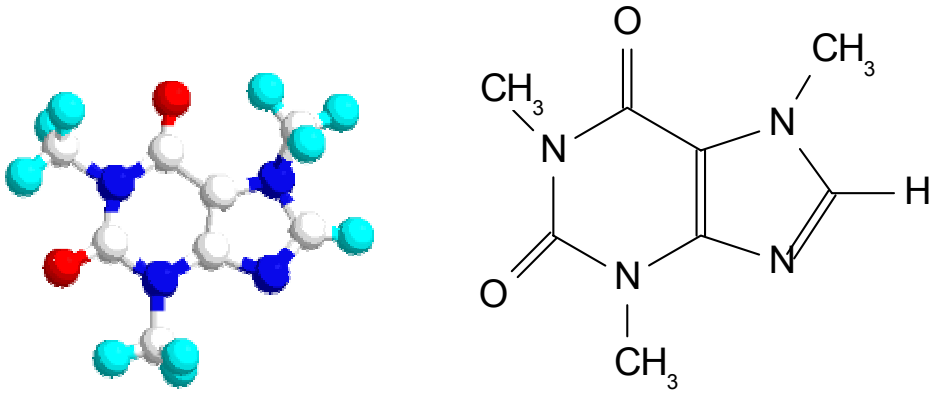


Figure 3: (left) A ball and stick representation of caffeine. (right) An atomic, skeletal representation of the structure of caffeine.

Caffeine has dated back to approximately 850 AD, sprouting legends of its magical capability of alerting the mind. Today, America's most popular drug, caffeine exists as a white, bitter crystalline powder that is purely extracted from the decaffeination of coffee and tea. Medically, caffeine serves as a cardiac stimulant and also as a mild diuretic. Caffeine also stimulates mental awareness, supplying an addictive "boost" to one's day. Such a boost often takes fifteen minutes to reach the consumer, sometimes remaining in the body for 12 hours. The xanthine chemical also affects the reticular formation of the brain, increases heart rate, constricts blood vessels, relaxes air passages to improve breathing and allows some muscles to contract more easily [6].

MATERIALS AND METHODS

High Pressure Liquid Chromatography (HPLC)

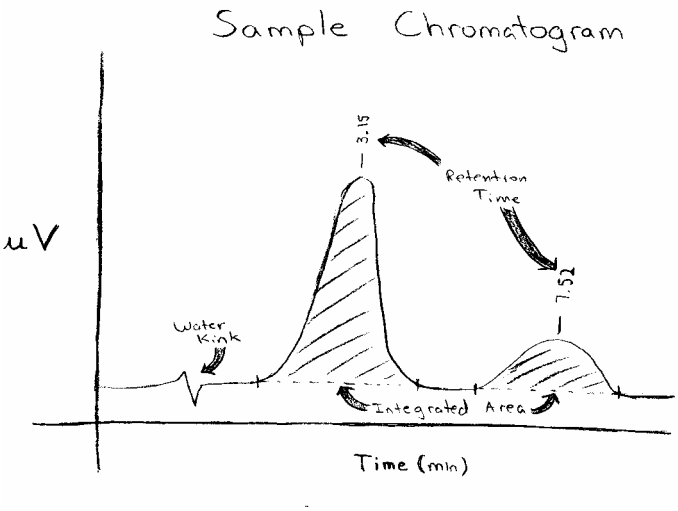
In isolating and analyzing the pharmacologically active components in chocolate, high pressure liquid chromatography (HPLC) is an effective industry-standard method. Using extracted samples of caffeine, vanillin, and theobromine in solution, the HPLC procedure yielded chromatograms that displayed the identities and concentrations of the compounds in different kinds of chocolate.

Chemical Principles Involved in Chromatography

The column used silica microspheres with hydriphobic tails as the stationary phase. Molecules in the fluid phase interact with the stationary phase based on their polarity. Non-polar molecules, attracted to the non-polar C_8 and C_{18} tails, and pass through the tube slower than polar molecules which are repelled by the hydrocarbon tails. These interactions reach equilibrium following the injection; the mobile phase and the pressure applied to the column disrupts the equilibrium and force the compounds to pass separately through the column depending on the strength of the initial equilibrium formed. Because the caffeine has one more methyl group than the theobromine, it will leave the tube more slowly because it interacts more with the non-polar tails. In this case (Figure 4), the sample of chocolate expresses its two components, theobromine

and caffeine, in a ten minute trial [7]. The area under the peak curves is proportional to the concentrations of the two chemicals relative to each other, and can be quantified further by comparing them with a solution of known concentration of pure substance (known as "standards) that is run through HPLC.

Figure 4: An example of a HPLC chromatogram.



Chemical equilibrium occurs when two opposing reactions are taking place at equal rates and producing no net change within the system. It is a dynamic balance, which is re-established if any factor forces the system out of equilibrium at any given moment. For HPLC, the equilibrium is established by the extracted chemicals, and the particles that make up the column. The constantly flowing mobile phase serves as the force that disturbs the equilibrium [8] (Figure 5).

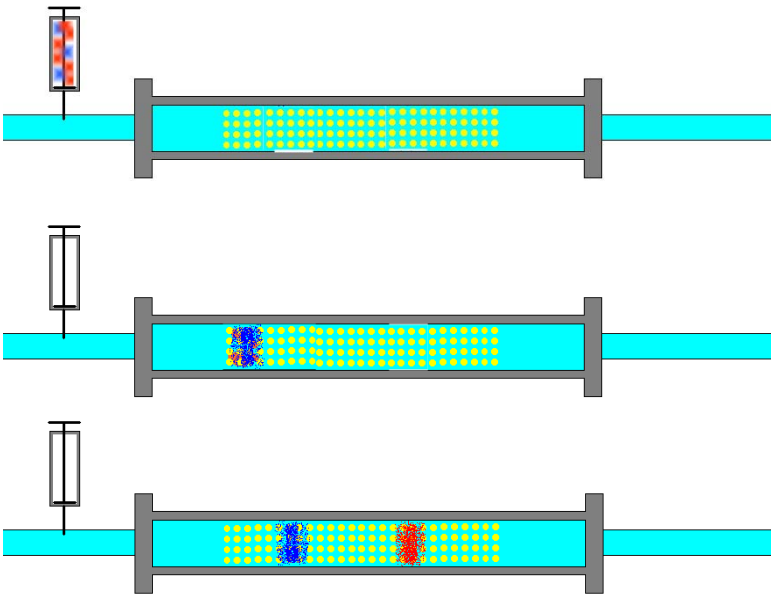


Figure 5: (top) HPLC column flushed with mobile phase before sample solution injected into it. (middle) HPLC column immediately after injection. (bottom) A HPLC column showing the faster movement of the more hydrophilic (red) material as the more hydrophobic (blue) is slowed by its attachment to hydrophobic tails of the beads (yellow) of the stationary phase.

HPLC Technology

There were several important parameters that had to be established before running the samples in the HPLC. The mobile phase, or the liquid component that carries the compound through a column, was selected to allow efficient flow. The column, composed of tightly-packed hydrophobic molecules with carbon tails (stationary phase), interacts with the compounds in the

mobile phase. The varying times needed for the compounds to flow through the column are determined by chemical composition and are known as retention times [9].

Another parameter to set up was the HPLC detector, which detects the compounds as they elute from the column. The information from the detector is sent to the chromatogram software and displayed as a peak on the graph. The peak's intensity and duration will vary depending on the given organic compound's retention time and the detector being used. In chocolate extractions, an ultraviolet (UV) detector was set to 258 nanometers [10].

In order to run the samples through the HPLC, caffeine and theobromine had to be extracted from the chocolate. The extraction solutions were injected into the mobile phase and pushed through the stationary phase under high pressure conditions (Figure 5). Using computer software, integration, and theoretical standards, the peaks were analyzed to provide data for the concentrations of the compounds in chocolate.

Equipment Setup and Optimization of HPLC runs

The experimental setup consisted of a PC loaded with TotalChrom software, a device controller, an auto sampler with a tray allowing testing of up to 100 vials, an HPLC column, and a UV detector. Various parameters must be optimized before actual measurements are possible [11]. This include determining the best solvents to use in extracting the compounds from the original material, the optimal amount of initial sample to be injected, and the length of the run that will give the best separation of the compounds being analyzed. The method development proceeds through series of trial runs that discovers the optimum conditions for the runs. In this study, 10 uL of a solution at a concentration of 10 mg of solute per 100 mL of solvent running for 10 minutes yielded good results for the known standards, and these conditions were used through out this study.

Preparation and Running of Standards

To prepare standard solutions, 10% acetonitrile was used as the solvent and mobile phase. Acetonitrile is a commonly used organic solvent for reserve-phase HPLC [12]. Compounds from the unknown extracted samples were identified by matching their retention times to the retention times of the known standards, and the amount of compound determined by measuring the area under the curve produced on the chromatogram. It was important to determine an amount that produces a peak that is neither insignificant nor so large that it was cut off on the chromatogram. Once an initial amount was determined for each active component we were studying (caffeine and theobromine), each ingredient was mixed in varying proportions so as to produce a chromatogram with distinct peaks. Two and 5 mg of caffeine and 2 and 5 mg of theobromine in 100 ml of 10% ACN were used as standard amounts. 500 µL of standard solutions was the standard amount used in the HPLC runs.

Programming the HPLC Instrument

In order to run an HPLC test of a compound, a series of instructions must be given to the instrument that dictates all aspects of a run via numbers and percentages, including its total length, the injection volume, the needle level, the flush volume, frequency, interval, and the maximum pressure. All of these variables are essential to a successful run, which would ideally produce a relatively clean chromatogram, with a high signal to noise ratio. As each run consisted of many samples, the methods are then imported into the sequence portion of the software. Thus, a sequence can be seen as a list of methods that determines the order and the manner in which the compounds are to be tested.

The TotalChrom software was used to manage the PerkinElmer HPLC instrument. A new method was created for each run (including standards). The sampling rate for each run was set at 2.5 points/second, the injection volume was set at 10.0 µL, needle level was set at 10%, and flush volume was set at 700 µL. The HPLC instrument had an automatic shut down mechanism, should the pressure on the column exceed 6100 PSI. 0.5 minutes was set as the flush time before the initial run, and a total run-time of 10 minutes was set. The method was then imported into the sequence portion of the program, and once a method had been imported for every single vial to be tested, the pump was started. The data points taken by the detector signify the amount of UV light absorbed by the sample leaving the column at a given point in time; as the absorption is directly proportional to the concentration of the sample, the absorption measures the relative amount of sample that leaves the HPLC-column at a given time. Thus, the peaks produced on the chromatogram correspond to the amount of absorption measured by the detector.

<u>Extraction of Pharmacologically Active Ingredients in Chocolate</u>

The following procedure was used for routine chocolate extraction throughout this experiment. In some cases the nature of chocolate sample required modification of this standard procedure. Pieces of chocolate were counted out, and placed into a mortar and pestle. Liquid nitrogen was poured over the chocolate to freeze it, allowing it to be ground into a fine powder. One gram of this chocolate powder was measured out using an analytical balance, and five milliliters of methyl chloride was added in an Erlenmeyer flask. The solution was stirred using a magnetic stir bar until the solution became a suspension of very small particles.

A syringe, needle, and a 0.22 µm filter was used to remove any large or precipitated particles. 500 uL of the filtered extracted solution was transferred using a micropipette and deposited into another vial. The methylene chloride was evaporated by placing the vial into a warm water bath and blowing an air stream over it. Five hundred micro liters of ethanol was added to the vial following the evaporation, and gentle swirling was applied to dissolve the crystals of the compounds in the ethanol. The 500 uL of the dissolved ethanol solution was placed into an HPLC- vial using a micropipette, with care being taken to avoid transferring any of the cocoa butter (insoluble in ethanol) into the vial. The vial was then labeled and placed into the auto sampler for analysis.

RESULTS

Chromatographic Analysis

Figure 6a shows a chromatograph of a standard sample where theobromine is the greater concentration and caffeine the lesser. The taller, theobromine peak came out first with a retention time of 2.86 minutes, while the smaller caffeine peak came out later with a retention time of 6.76 minutes.

Figure 6b is a chromatogram of an actual chocolate sample. Here the first peak (theobromine) had a retention time of 3.14 minutes with the second peak (caffeine) coming out at a retention time of 6.83 minutes. All of the samples analyzed showed these two peaks with retention times very close to the standards (Table 1 and 2). The standard deviations for the retention times were very small, indicating that our identification of what substance was in which peaks was correct. The fact that both peaks were not perfectly symmetrical showed the presence of other substances in the chocolate extracts may have influenced how theobromine and caffeine interacted with the stationary phase.

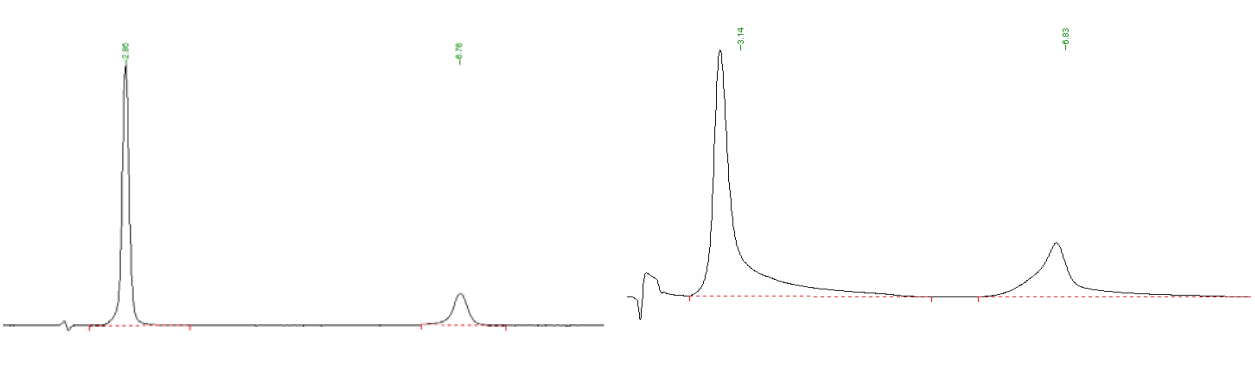


Figure 6: (left: a) The standard containing 5mg theobromine and 2mg Caffeine (right: b) Sample chromatogram from chocolate extract showing theobromine and caffeine peaks.

HPLC Analysis of Active Ingredients

The HPLC data was analyzed by an external standard calibration method to find concentrations of active ingredients in chocolates. The calculations were based on the assumption that the ratio of peak area to concentration for a specific chemical remained constant for a given wavelength in a HPLC detector [13]. For example if the standard concentration of caffeine (N) has an area of A and the unknown concentration (X) is represented by a sample peak with area of B, then:

$$\frac{N}{A} = \frac{X}{B}$$

B is the measured quantity, so the constant N/A needs to be determined. This was done by doing a linear regression through the origin with the areas of the standard peaks. Figure 7 shows the regression analysis for caffeine with constant being 48701 area units/mg caffeine in 100 mls. For theobromine the constant is 24560 area units/mg theobromine in 100 mls. (Figure

8) Note that the 2 mg caffeine points were lower than expected while the theobromine resulted in a good regression.

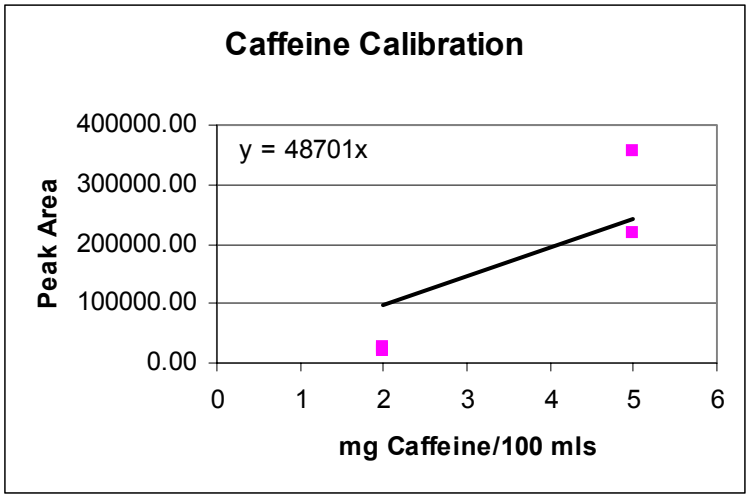


Figure 7 – Regression analysis establishing peak area to caffeine concentration. Analysis indicated that Caffeine concentration (mg/100 ml) = Measured peak area/ 48701 (n= 5)

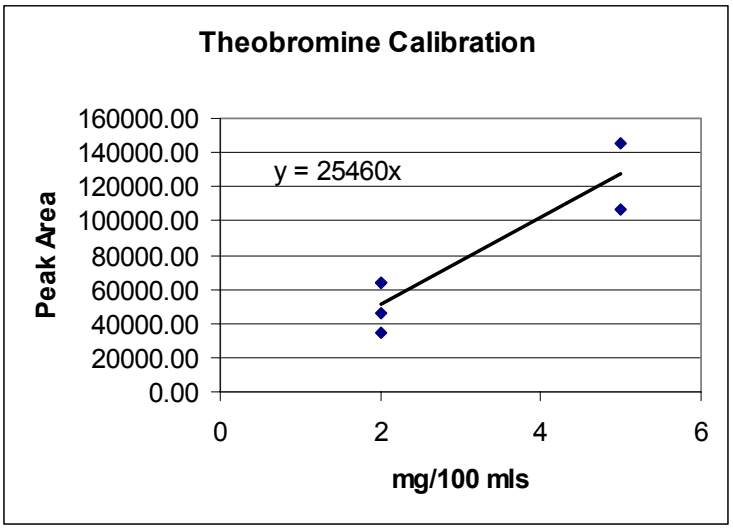


Figure 8. Regression analysis establishing peak area to theobromine concentration. Theobromine concentration = measured peak area/ 25460. (n= 5)

Table 1: Theobromine in Chocolate

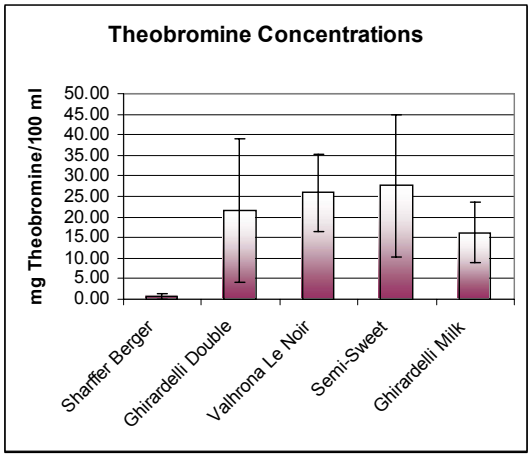
	Retention time		Concentration		
	Average	std	Average	std	n
2 mg Theobromine std	2.85	0.02	2.06	0.57	4
5 mg Theobromine std	2.86	0.04	4.95	1.09	2
Sharffer Berger 70%	2.86	0.04	0.75	0.61	4
Ghirardelli Double Chocolate	2.92	0.07	21.60	17.44	4
Valhrona Le Noir	2.94	0.05	25.89	9.41	4
Semi-sweet	2.92	0.04	27.64	17.24	4
Ghirardelli Milk Chocolate	2.88	0.04	16.19	7.35	3

Std= standards used to calibrate instrument

Table 2: Caffeine in Chocolate

	Retention time		Concentration		
	Average	std	Average	std	n
2 mg Caffeine std	6.73	0.11	0.42	0.07	3.00
5 mg Caffeine std	6.74	0.12	5.95	1.41	2.00
Sharffer Berger 70%	6.64	0.09	2.62	2.64	4.00
Ghirardelli Double Chocolate	6.63	0.09	4.93	3.69	4.00
Valhrona Le Noir	6.64	0.10	4.59	3.49	4.00
Semi-Sweet	6.59	0.08	3.72	2.16	4.00
Ghirardelli Milk Chocolate	6.64	0.09	1.08	1.07	3.00

Std= standards used to calibrate instrument



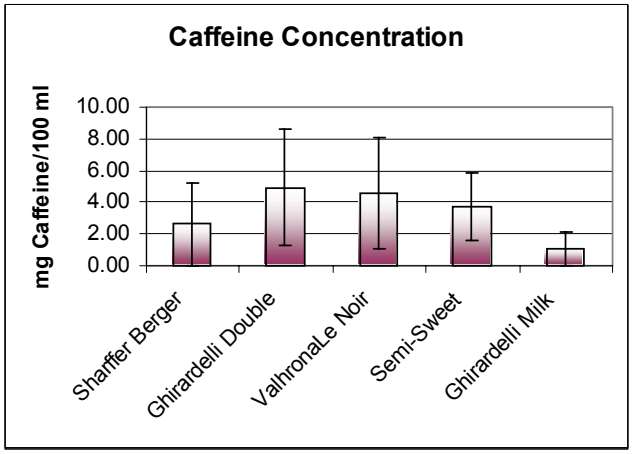


Figure 10: Comparison of measured caffeine concentrations in different chocolates. Chocolates arranged in order of decreasing darkness. Standard deviation shown as error bars.

Semi-sweet had the highest theobromine concentration (Figure 9) while Double Chocolate had the highest caffeine concentration (Figure 10). Milk chocolate had lower concentrations of both theobromine and caffeine than the others (Figure 9 & 10). The surprise was that the Scharffer Berger, the darkest chocolate, had very little theobromine and lower caffeine amounts than the other "dark" chocolates. During the extraction process, the Scharffer Berger chocolate contained more cocoa butter than in other samples resulting in problems with filtering and possibly interfering with the chromatography. Standard deviations for all determinations were large (Tables 1 & 2, Figures 9 & 10) making any distinctions impossible and statistical testing of these results was not possible due to the lack of time.

Five other products containing chocolate (Dove Dark, Hershey's Milk Chocolate Kisses, Nestle White Chocolate, Ghirardelli Sweet Ground Chocolate and Cocoa, and W&S Fudge Sauce) were extracted and analyzed, but the standards for this run did not produced satisfactory chromatographs and the concentrations of theobromine and caffeine in these samples could not be determined. Nestle White Chocolate produced no chromatograms and interfered with the chromatography of the next chocolate sample that was run. While the theobromine peak appeared where expected (Figure 11), the caffeine peaks were much later than seen in the first run for both the standards and new chocolate samples (Figure 11). The caffeine peak disappeared when the first set of chocolate extractions (Table 1 & 2) were run again (Figure 11). Examination of the vials showed the presence of crystals which was presumed to be crystallized caffeine.

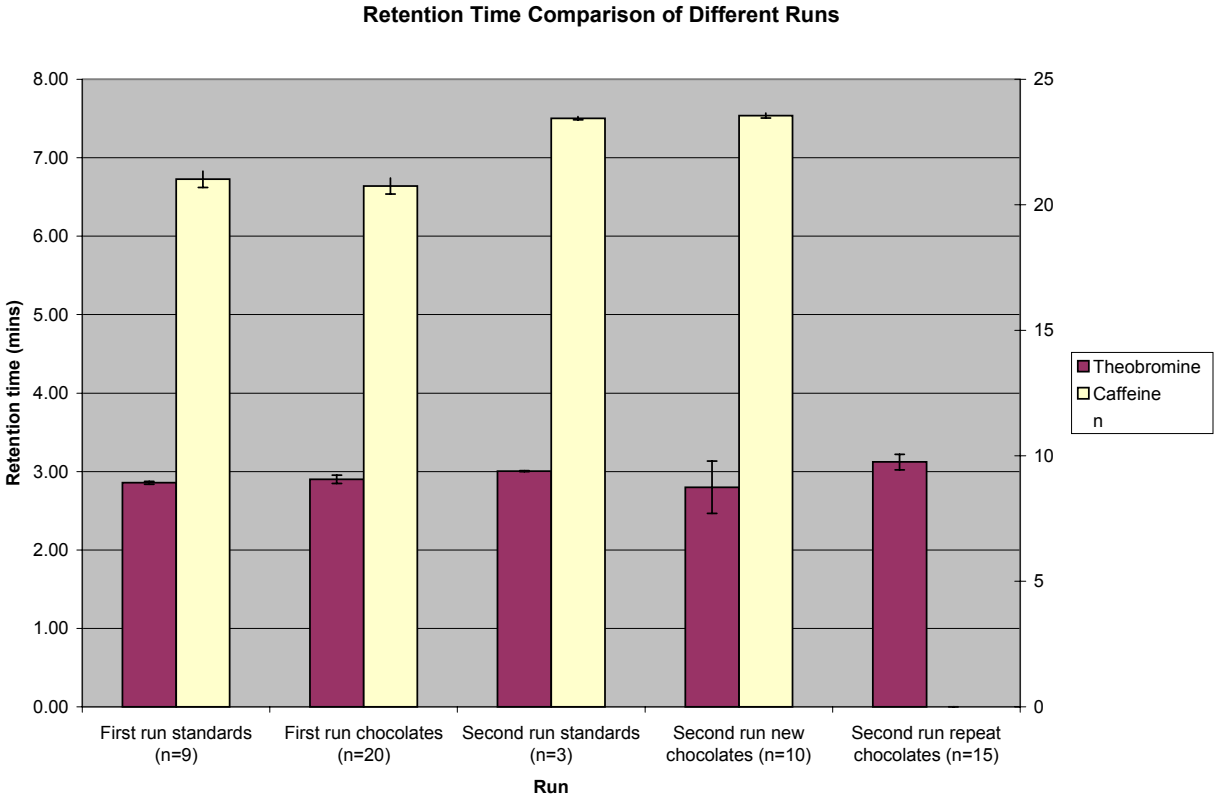


Figure 11. Comparison of retention times between different chromatographic runs. The retention times of theobromine and caffeine peaks are compared between the first and second runs. The retention time for theobromine was constant in both runs, but the caffeine retention time was significantly different between runs. In addition, the caffeine peak was absent when the previous samples were re-chromatographed (Second run, repeat chocolates). n= sample size, error bars show standard deviation.

SUBJECTIVE TESTING

Subjective Test I

A subjective study (see appendix A) was conducted on Thursday, August 8, 2002 to find out if a correlation between alertness and the concentration of pharmacologically active ingredients found in chocolate exists. In establishing the subjective survey procedure, several factors were considered. Mood during test, time between samples, number of questions, type of chocolate, sample size, crushed vs. not crushed, 1 day versus 3 day, and conditions for the subjective study were all considered before deciding the final plan for study. Only a one day study was done due to time constraints. The prime characteristics to be surveyed were taste preference and alertness. Fifty people would be tested to provide a large enough sample size for the results to be significant.

As the subjects came into the waiting area, they were assigned a number that determined which of the 5 survey groups they were placed in. Each survey group consisted of 2 administrators and 10 subjects. Before participating, each of the 50 subjects was asked a series of questions concerning gender, caffeine consumption, chocolate preferences and level of alertness to gain background information. One subject of each group was then blindfolded and brought into the testing area, where he/she was given the first of three spoonful-sized samples of crushed chocolate. Each group administered the three types of chocolate (dark, milk, or white) in a different order. The subject was brought back out to the waiting area, and the process was repeated for each of the remaining nine participants in each group. Five minutes after the first sample was consumed, each subject was brought back into the testing area, where he/she was asked questions concerning the sample's taste and his/her level of alertness. He/she was then given the second sample, and the process was repeated with each successive sample.

Twenty four females and twenty six males were tested. Of these, 34% preferred dark chocolate, 52% preferred milk chocolate, and 14% preferred white chocolate. Of the 24 females, 50% had a dark chocolate preference, 46% had a milk chocolate preference, and 4% had a white chocolate preference. Of the 26 males, 19% had a dark chocolate preference, 58% had a milk chocolate preference, and 23% had a white chocolate preference. The testers drank an average of 2.28 cups of coffee/tea a week, 6.58 cups of other caffeinated beverages a week, and ate chocolate 3.14 times a week. Of the 50 participants, 18% felt less alert after the samples, 32% felt more alert after the samples, 42% felt the same, and 8% had alertness levels that fluctuated between samples (Table 3).

A correlation for alertness could not be found. One possible reason for this is that the elapsed time between eating a sample and answering the corresponding questions was not long enough to produce a noticeable difference. Another possible reason for this is that the subjects were given the three chocolate samples consecutively with too little time separating each sample. Any effect the subjects noted on their alertness could have been the result of the combination of samples and not the result of any one individual chocolate sample. In addition, on rating the samples by taste, the participants were biased as to which chocolate they normally preferred and therefore rated the samples accordingly (Figure 12). It may also be possible that the pharmacologically active ingredients of chocolate have no effect on how much someone likes a

specific kind of chocolate. Other flavoring ingredients such as sugar and cocoa butter may be more significant in attracting consumers on a regular basis. Furthermore, the sample sizes of chocolate may not have been large enough to affect the participants, and perhaps a larger sample size would have produced more accurate results. Perhaps using a different scale with a wider range of ratings/levels would have allowed the subjects to more easily quantify their opinions; also, taking freeform notes in addition to the standard questionnaire may have extracted more usable information from the study. Inclusion of other relevant questions about people's states of alertness may have been useful as well in analyzing the effects of the pharmacologically active components on the subjects. Communication lapses during the study may have affected the results in that the procedure was not carried out uniformly throughout the testing.

Table 3. Results of the first subjective survey

Background Information

	Males	Females	Dark Chocolate Preference	Milk Chocolate Preference	White Chocolate Preference
Number of Participants	26	24	17	26	7

Alertness

	Increased Alertness	Decreased Alertness	Constant Alertness	Fluctuating Alertness
Number of Participants	16	9	21	4

Taste Ratings

	Highest rating of preferred sample	Highest rating of other sample
Number of Participants	27	23

Since the subjective study proved to be somewhat inconclusive, after considering possible errors, further analysis was conducted. It was hypothesized that the subjects' stated chocolate preferences would correspond directly with the chocolate sample that he/she gave the highest rating. After looking over the data (summarized in Figure 8), it was found that the subjects who stated their chocolate preference as dark chocolate did indeed give dark chocolate their best rating. The same was found for those who stated their chocolate preference as milk or white chocolate. Since these relationships were found to be true, it could be concluded that the study was administered correctly, and any errors were most likely caused by the sources discussed above.

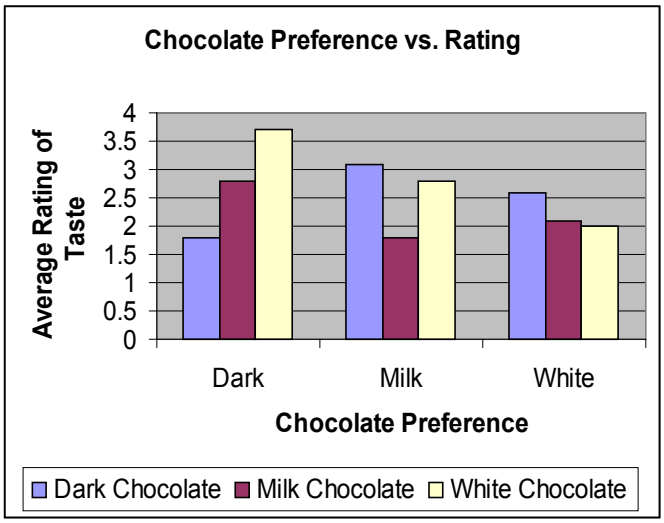


Figure 8: Taste rating is shown to correlate and support original chocolate preference. Note that the lower the rating, the more it was preferred.

Subjective Test II

On Monday, August 12, 2002, the second portion of the subjective test (see appendix B) was conducted. This session consisted of ten subjects each testing five chocolate samples and commenting on taste and any changes and alertness. While the first subjective test tested three common chocolates, the second test tried to compare four "fancy" chocolates with each other and with one brand of common chocolate (Dove Dark). For this test, the procedure was revised to reflect various lessons learned from the first subjective test. For example, the procedure was followed more rigorously, the times between samplings were adhered to more closely, and the taste and alertness scales were increased to 1 to 7 in order to capture more variation.

Each subject was given a sheet with a number on it. The subjects then answered questions regarding chocolate preference, caffeine intake, nightly sleep, and recent caffeine consumption. After the background was complete, the testing began. Each person ate a one teaspoon sample of chocolate and immediately answered questions about taste and alertness change. The subjects then waited 15 minutes between samplings, and then returned. They answered another question about alertness and then sampled another chocolate. This process continued until all five samples were complete.

Table 4: A summary of the second subjective test data.

	Ghiradelli Milk	Ghiradelli Semi-Sweet	Ghiradelli Double	Scharfen Berger 70% Dark	Dove Dark
Average Taste Rating	5.3	4.5	4.7	3.5	5.6
Average Alertness Change	0.5	0.2	0.2	0.4	0.1

Analysis and Results

Ghiradelli milk chocolate appeared to cause the biggest change in alertness followed by Scharffen Berger Dark Chocolate and then Ghiradellia Semi-Sweet and Double Chocolate (Table 4). Dove Dark Chocolate was the best liked chocolate, but appears to cause the smallest change in alertness. The darkest chocolate, Scharfen Berger 70% Dark Chocolate was the least liked of those examined.

DISCUSSION AND CONCLUSIONS

The basic assumption of this study has been the darker the chocolate, the more unsweetened chocolate is presence and dark chocolate would therefore contain more of the pharmacologically active substances found in unsweetened chocolate. The lighter the chocolate, the more these active agents are diluted by inert materials such as sugar or milk. With the exception of Nestle White Chocolate, all of the samples that were examined could be shown to contain two known pharmacologically active substances, caffeine and theobromine, as evidenced by peaks with identical retention times to their respective standards (Table 1, 2, Figure 11). What we could not conclusively demonstrate was the fact that darker chocolate contains more of these substances than light or milk chocolate. Ghirardelli milk chocolate does appear to contain less theobromine and caffeine than the darker chocolates (Figures 9 & 10), but the standard deviations of our measurements do not permit a more rigorous conclusion nor enable distinctions among the different dark chocolates. The fact that the darkest chocolate examined, Scharffer Berger 70% Dark Chocolate, had the smallest amount of theobromine and less caffeine than other dark chocolates certainly argues against this conclusion. Scharffer Berger, however, was the most difficult to do the standard extraction, and the presence of traces of cocoa butter in this sample may have interfered with its chromatography.

The subjective test data also cannot be used to support this hypothesis. There is no clear pattern that shows a correlation between the darkness of a chocolate is and a clear affect on the subject. In fact Ghirardelli milk chocolate had the greatest affect on alertness, but appear to contain less theobromine and caffeine than the other chocolates tested. Again the large standard deviation makes it impossible to determine whether the patterns shown by the data are correct or whether measurement error is masking the pharmacological effects being exhibited. Certainly the sample size must be larger and statistical testing required to rigorously test this result. As evident by our first trial, the results of the subjective testing are influenced by a large number of factors that must be controlled. Finding clear parameters that clearly measure the effect under study proved to be a difficult task. It is also not clear how long it takes for these agents to have their effect, nor what is the cumulative effect of sequential tastings within a short time period. The subjective tests given over multiple days would be a way of eliminating the cumulative effects of eating various chocolates one right after another.

Time did not allow further exploration of the question of whether dark chocolate has more of the stuff that gives a “kick” to a person. Only one extraction technique was tried and from results obtained it appears that how the chocolate is extracted may have a significant factor on the final results in that the presence of cocoa butter appears to affect the performance of the HPLC. Additional optimalization of the extraction procedure is therefore needed. For example other solvents may do a better job of extracting the active substances than methylene chloride that was used. It is not clear whether the UV wavelength that used was the optimal one, and whether both caffeine and theobromine is best detected by that wavelength. The length of time between samples used for rinsing the column may need to be increased so as to wash out any interfering factors. The fact that caffeine appeared to have crystallized out of solution showed that there is a big difference between immediately running the samples and storing them for any length of time before HPLC analysis. Finally obtaining enough data so as to do statistical analysis of the results should be a greater priority than examining a wide spectrum of different

chocolates. In conclusion, any team that studies chocolate in the future will find the following statement indisputable: chocolate contains a lot of deliciously sweet mysteries.

ACKNOWLEDGEMENTS

The team wishes to extend special thanks to Dr. James Fukunaga and Carrie Williams for their voluntary help with this project and some special acknowledgments to PerkinElmer Corporation, Waters Corporation for donated equipment and Burdick Honeywell for providing solvents and other chemicals. Special thanks goes to William Sonoma for the chocolate that they donated to us to do this study.

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Appendix A – Subjective Test I Questionnaire

Gender_____ Number_____

Before sampling, choose one answer:

1. Chocolate preference: ___Milk Chocolate ___Dark Chocolate ___White Chocolate
2. Frequency of coffee/tea consumption:
   ___ cups per week
3. Frequency of chocolate consumption:
   ___ times per week. (approximate size _____)
4. Consumption of other caffeinated beverages:
   ___ cups per week.
5. Alertness level before consumption: ___
   (1 being very alert, 5 being very tired)

For Samples A through C: After sampling, choose one answer:

1. Chocolate rating based on taste for sample a: ___
   (1 being best, 5 being worst)
2. Alertness level after consumption a: ___
   (1 being very alert, 5 being very tired)

Appendix B – Subjective Test II Questionnaire

Gender_____ Number_____

Before sampling, choose one answer:

1. Chocolate preference: ___Milk Chocolate ___Dark Chocolate ___White Chocolate
2. Frequency of coffee/tea consumption: ___ cups per week
3. Frequency of chocolate consumption: ___ times per week
4. Consumption of other caffeinated beverages: ___ cups per week
5. Alertness level before consumption:

   Tired Awake

   1……2…….3…….4…….5…….6…….7
6. Amount of sleep last night: ___ hours

7. Most recent consumption of caffeine: _____ago

Chocolate Sample A through F

Taste (fill out immediately after, 1- worst, 7 best):

1……2…….3…….4…….5…….6…….7

Initial Alertness Reaction (fill out immediately after, 1- tired, 7 awake):

1……2…….3…….4…….5…….6…….7

Delayed Alertness Reaction (fill out 10 minutes after; before next sample):

Tired Awake

1……2…….3…….4…….5…….6…….7

Appendix C – Subjective Test II Raw Data

Subj. #	Taste A	Taste B	Taste C	Taste D	Taste E	IA*	IR* A	DR* A	IR B	DR B	IR C	DR C	IR D	DR D	IR E	DR E
1	4	5	6	3	6	5	5	5	6	6	6	6	7	6	7	7
2	5	2	3	1	4	6	6	6	6	7	7	7	7	7	7	7
3	5	6	5	4	7	2	2	3	3	3	3	4	4	4	4	5
4	6	3	2	1	2	4	4	4	4	3	4	4	5	5	5	5
5	5	6	7	7	7	3	3	3	3	3	3	3	4	4	4	4
6	7	4	3	4	6	5	6	6	6	6	6	6	6	7	7	7
7	4	5	6	5	7	3	3	3	4	3	4	5	6	6	6	6
8	5	4	4	1	4	2	4	4	4	5	5	6	6	6	6	6
9	7	4	4	2	6	3	4	5	5	6	7	6	6	7	7	7
10	5	6	7	7	7	3	5	6	6	6	6	6	6	7	7	7

*IA=Initial Alertness; Before Any Testing
*IR=Initial Reaction; Alertness Immediately Following Sample
*DR=Delayed Reaction; Alertness 15 Minutes After Sample, Before Next Sample

Chocolate A = Ghiradelli Milk; Chocolate B = Ghiradelli Semi-Sweet; Chocolate C = Ghiradelli Double; Chocolate D = Scharfen Berger 70% Dark; Chocolate E = Dove Dark