

## UHPLC-HRMS Analysis of Theobromine in *Theobroma cacao* and its Products

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### Abstract

*Theobroma cacao* seed is the major ingredient in all types of chocolate products and contains Theobromine. Theobromine is a bitter alkaloid beneficial in the treatment of hypertension, arteriosclerosis and angina pectoris. Of all chocolate brand samples, Sample J containing 70% Cacao had the highest amount of theobromine. Sample A containing 11% Cacao had the least amount of theobromine. The 100% Cacao Chocolate Bar had the highest concentration of theobromine in comparison to roasted cocoa having the lowest concentration of theobromine. Quantitative analysis of Theobromine was completed on the Thermo Scientific UHPLC and LTQ Orbit rap Discovery equipped with an ESI ion source. A three-minute gradient method with a flow rate of 300  $\mu$ L/min was developed on the UHPLC-HRMS using HPLC-grade water and acetonitrile. Ethyl ether was used to remove cacao fats and water was used to isolate theobromine. To obtain the precision of the theobromine extraction process, the recovery analysis was 86%.

**Keywords:** *Theobromine cacao*; Hypertension; Smooth muscle; Psychological effects; Carbohydrates

### Introduction

*Theobroma cacao*, the source of chocolate, has been economically important plant in the cacao producing countries in central and South America for centuries. Cacao has been used in trading, ceremonial purposes and chocolate production [1]. Although chocolate contains fats, carbohydrates, and proteins, the *Theobroma cacao* (cocoa beans) beans are the main component chocolate bar production [1].

Certain chocolate manufacturers have specific methods of drying and roasting cocoa beans. *Theobroma cacao* beans of different processing methods can influence the final chocolate product flavor [2]. Companies are concerned whether their chocolate processing has been effective in preserving fine flavor and aroma [2]. Depending on the manufacturer's cocoa processing, certain methylxanthines (such as theobromine) could be lost through production [3].

Common chocolate manufacturing begins with clean, dried cocoa beans undergoing micronization. Through the process of winnowing and breaking cocoa beans, alkalization takes place. The dried cocoa beans will undergo roasting and grinding, eventually becoming cocoa liquor. Eventually, cocoa liquor will be pressed resulting in cocoa butter and cocoa cake. The cocoa cake is pulverized, leading to the final chocolate bar product [4].

Theobromine is known to possess bitter flavor present in cocoa and chocolate products [5]. It is a metabolite of caffeine and is found in relatively high concentrations in chocolate products [6]. Several studies have shown that theobromine can increase HDL cholesterol, stimulation of heart muscle and relaxation of smooth muscle within lungs [2]. Psychological effects of theobromine had shown positive changes in behavior: increased motivation/alertness and higher energy [7]. In addition, methylxanthines such as theobromine is known to contribute to chocolate cravings and preference for dark chocolate tastes [8].

A novel aspect of the study was a rapid, sensitive UHPLC-HRMS gradient method for theobromine was developed for analysis of *Theobroma cacao* and its products. Within 3.30 minutes, different stages of cacao processing and chocolate samples were successfully analyzed for theobromine.

Frosty Pod Rot (*Moniliasis*) devastated *Theobroma cacao* species in Central/South America by 80% [9]. Within the first 90 days of growth cocoa pods are most susceptible to infection via *Candida* penetration into pod surface. After two weeks, rapid sporulation occurs, giving the creamy-colored spore surface appearance [10]. CATIE organization (The Tropical Agricultural Research and Higher Education Center), aimed to reduce the occurrence of Frosty Pod Rot within Costa Rica. Certain approaches taken to eradicate the infection include elimination of dead sporulating pods (the source of Frosty Pod Rot), shortening tree length, spraying bio control agents and introducing of new species of cacao [10].

Despite eradication of Frosty Pod Rot in *Theobroma cacao*, excess use of pesticides can pose as a toxic health risk. Currently, 58% of nations use pesticides in agriculture, where the most common is glyphosate [11]. After health risk analysis, the theoretical maximum dose intake was 30% higher than the accepted daily intake [11]. Detection of pesticides via UHPLC-HRMS can help farmers control the amount of pesticides/insecticides added to preservation of cacao crops. This can alleviate any possible long term health complications due to pesticide use.

The second objective was to perform quantitative analysis of theobromine in 10 different popular chocolate brands within U.S. As Americans were becoming increasingly aware of health-related effects based on diet, chocolate manufacturers could alter their products to be appropriate for natural health product ingredients or supplements.

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## Methods

### Samples

*Theobroma cacao* beans from four different stages of cacao processing (raw, dried, roasted, and 100% cacao bar) were sampled from Costa Rica and analyzed in triplicates. Each commercial chocolate sample was purchased in the U.S. based on consumption popularity.

### Chemicals

The analyte of interest was theobromine and the internal standard used in the experiment was 7-(2-Hydroxyethyl) theophylline. Both chemicals were purchased from TCI (Portland, Oregon). All solvents used as mobile phases were purchased from Pharmco-Aaper (Brookfield, CT) and Optima LC/MS grade formic acid was purchased from Fisher Chemical (Fair Lawn, NJ). ACS grade anhydrous ethyl ether was used in the extraction of theobromine and was purchased from EMD Millipore Corporation (Darmstadt, Germany).

### Sample preparation

Cacao samples that were provided and several brands of chocolate samples were extracted via HPLC grade water and ethyl ether. The extraction process was taken from Caudle G, Gu Yifang, Bell N Leonard [12].

### Instrumentation

The Thermo Fisher UHPLC system included the Accela Auto sampler system and Accela 1250 pump (San Jose, California, U.S.). The detector used was the LTQ Orbitrap Discovery, a high-resolution mass spectrometer (Bremen, Germany). Infusion and tuning parameters

were completed for the mass spectrometer for each analyte of interest. The mass to charge ratio for theobromine was 181.0720 m/z. The mass range used was between 100-1000 m/z in a positive ionization mode. The capillary voltage was 5.50 and the tube lens was at 48.00 V. The sheath gas flow was 45.00 arb and the auxiliary gas flow was 13.00 arb. The heated capillary temperature was 350.00°C.

The column used was Waters Acquity UPLC BEH C18 1.7 µm pore size and 2.1 mm by 50 mm dimensions with a temperature set at 50°C. A gradient program was set up with HPLC-grade water and acetonitrile both containing 0.1% formic acid. At 0 minutes and 1.00 minutes, 100% of water and 0% organic ratio were used. However, 100% organic was used at 1.50 and 2.50 minutes. At 2.80 and 3.30 minutes, the ratio of 100% water and 0% organic was used again.

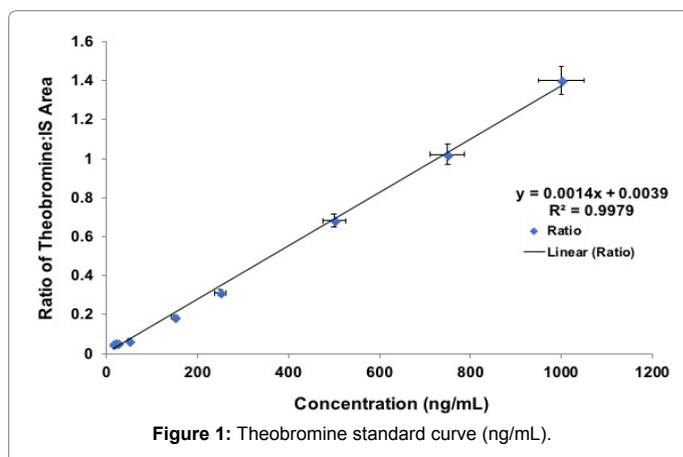


Figure 1: Theobromine standard curve (ng/mL).

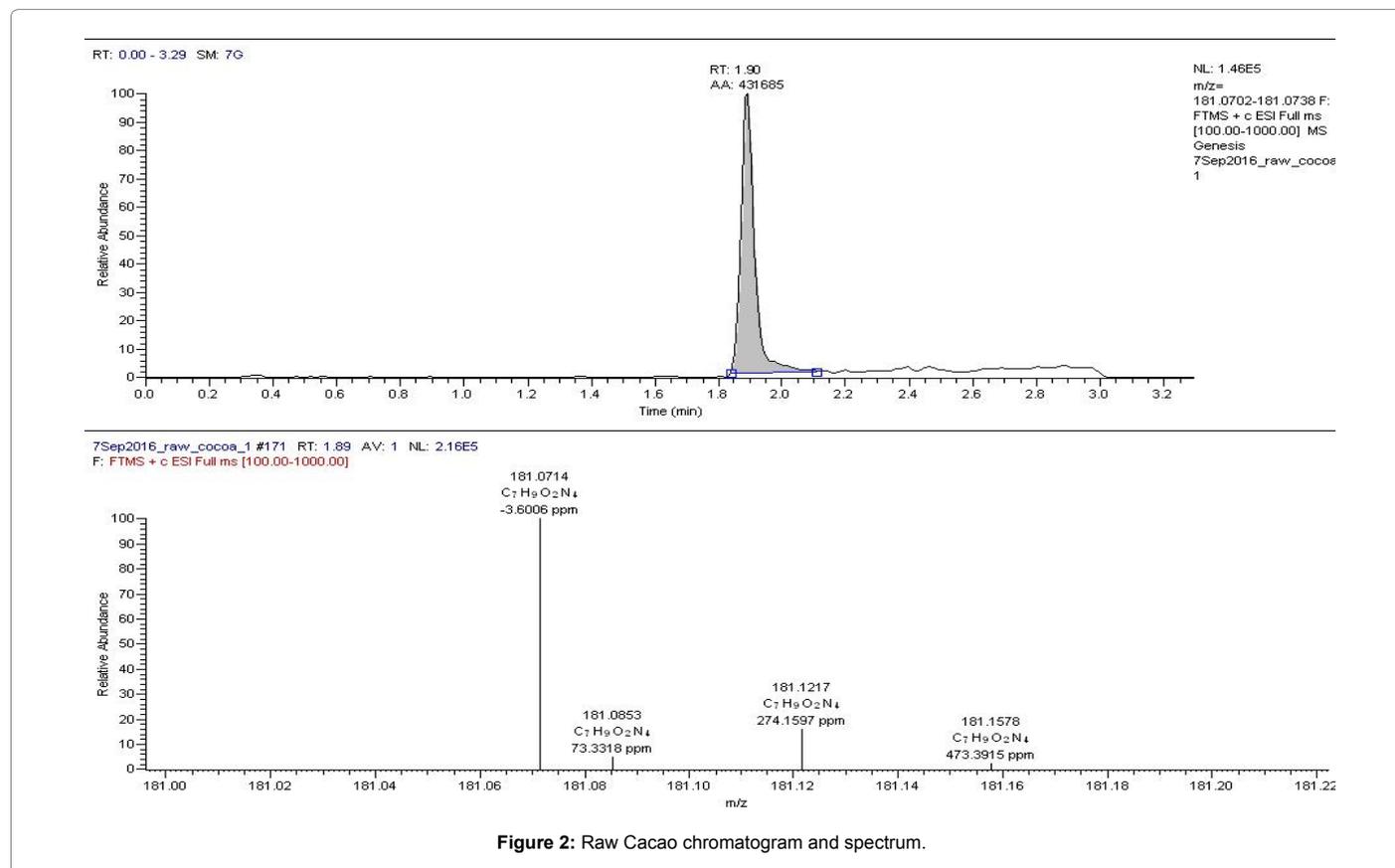


Figure 2: Raw Cacao chromatogram and spectrum.

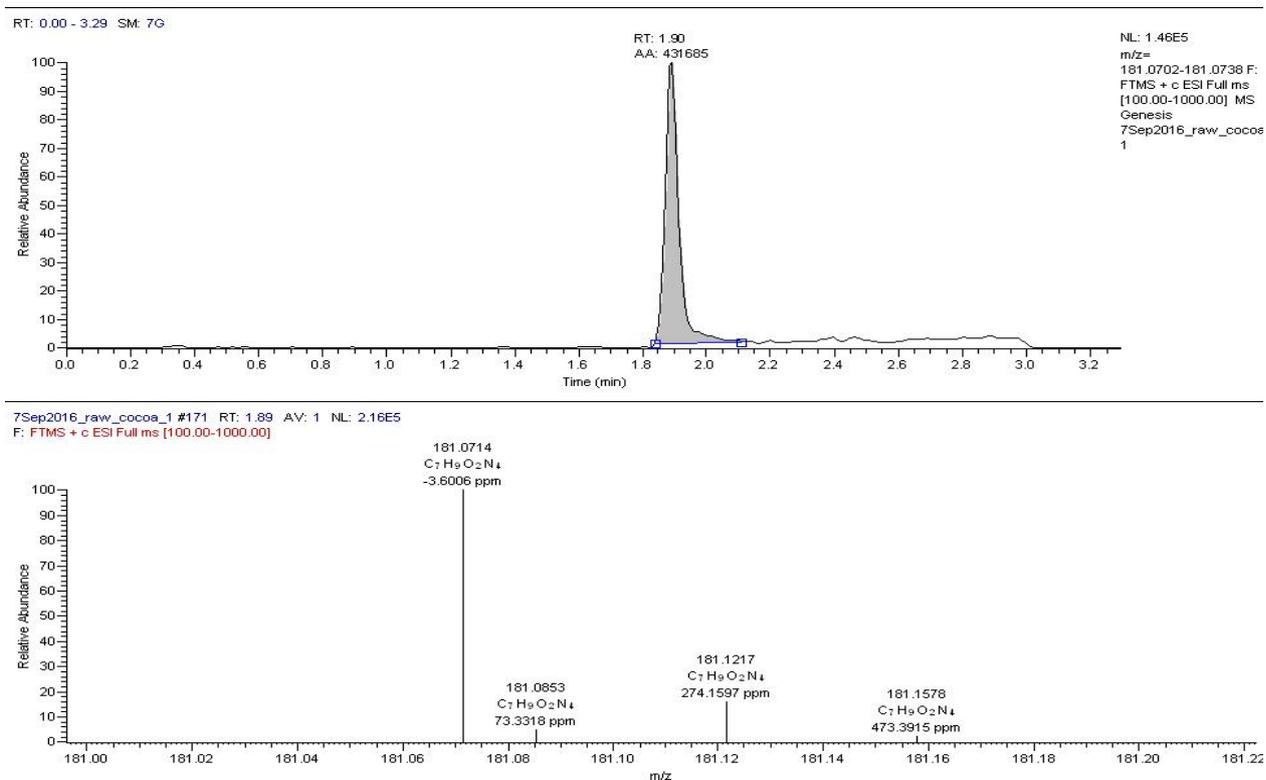


Figure 3: Dried Cacao chromatogram and spectrum.

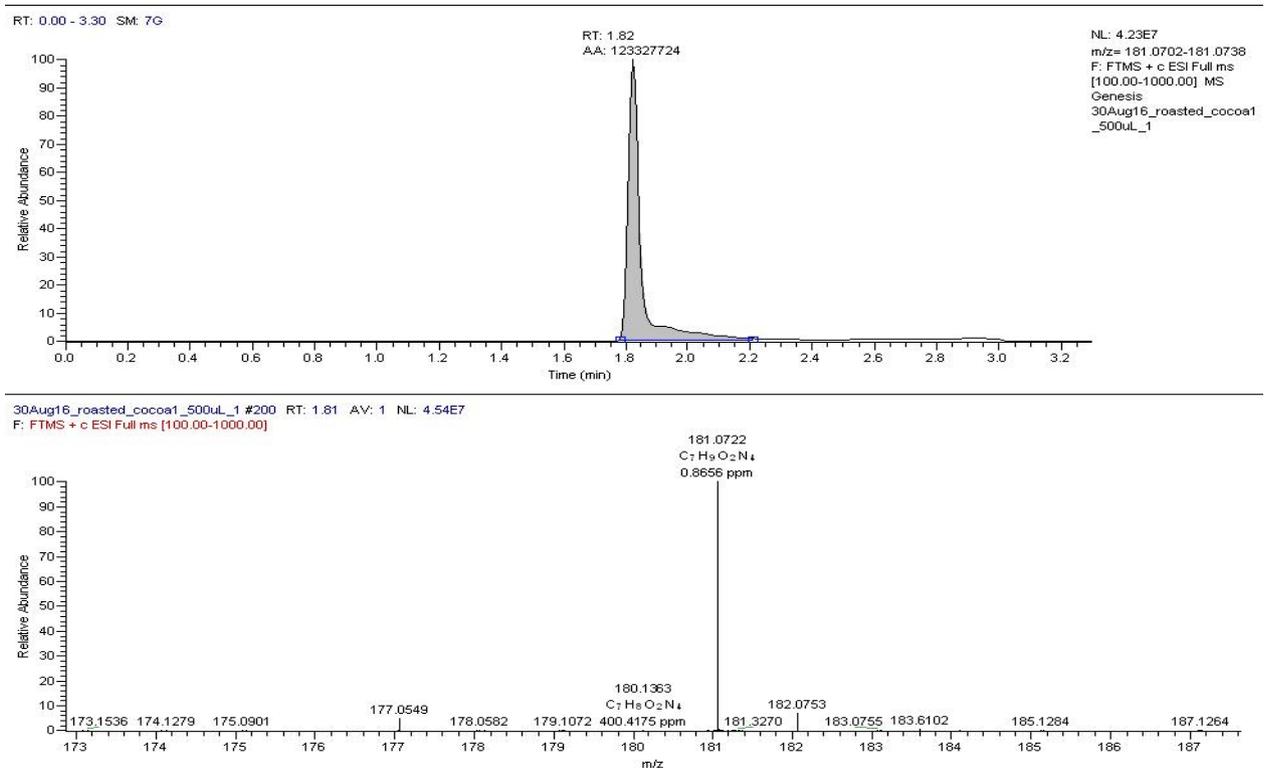


Figure 4: Roasted Cacao chromatogram and spectrum.

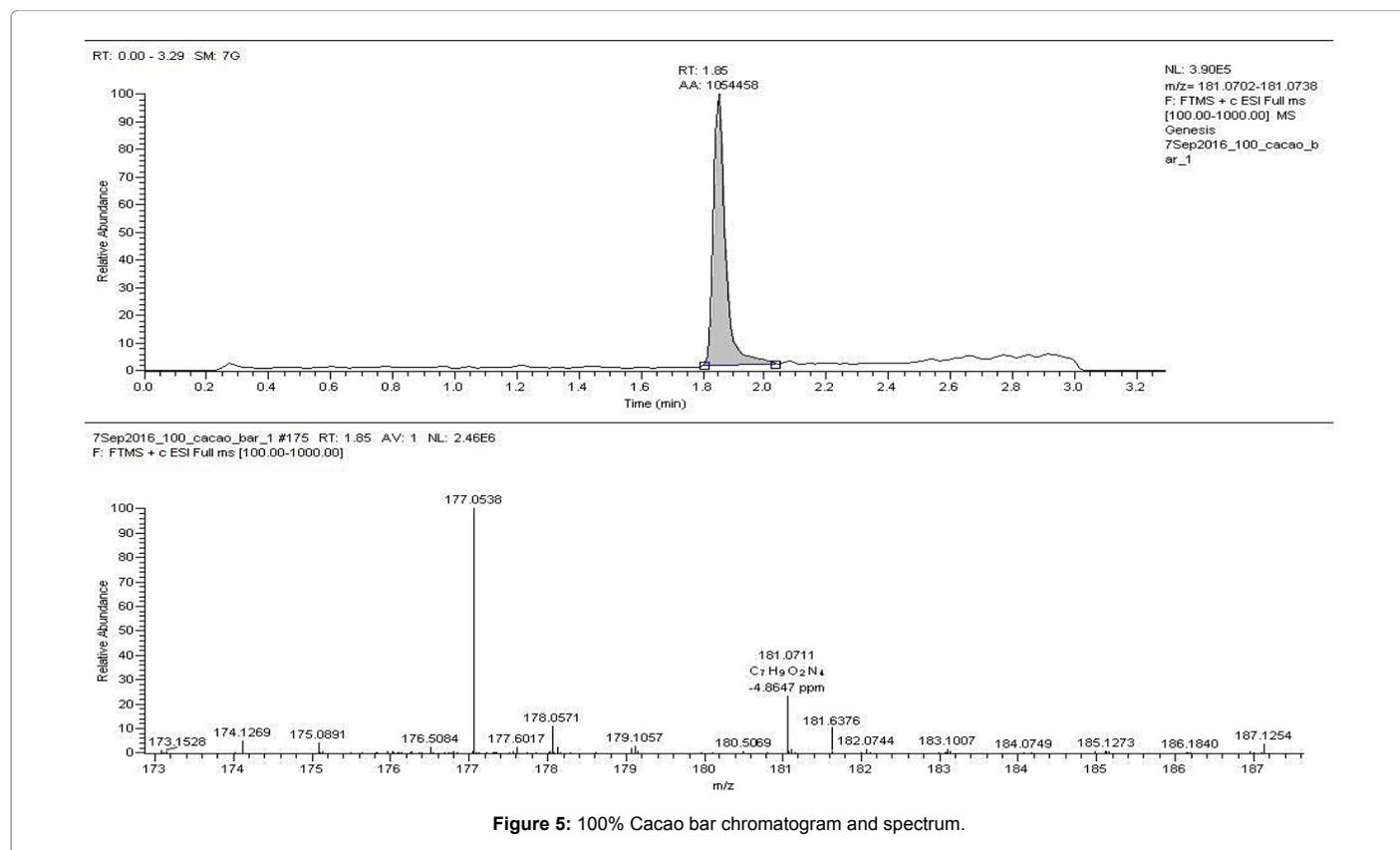


Figure 5: 100% Cacao bar chromatogram and spectrum.

Processing Stage	Theobromine Concentration (ng) +/- SD	SE
Roasted Cacao	371.37 +/- 25.66	12.83
Dried Cacao	401.96 +/- 27.46	15.86
Raw Cacao	420.74 +/- 22.15	11.08
100% Cacao Chocolate Bar	739.93 +/- 12.80	6.40

Data expressed as mean +/- SD = Standard Deviation, SE = Standard Error

Table 1: *Theobroma cacao* theobromine concentration.

Chocolate Brand and % Cacao	Theobromine Concentration (ng) +/- SD	SE
Sample A, 11%	33.70 +/- 7.02	4.05
Sample B, 30%	37.21 +/- 3.51	2.03
Sample C, 31%	42.56 +/- 3.71	2.14
Sample D, 34%	61.29 +/- 3.59	2.07
Sample E, 45%	89.47 +/- 11.12	6.42
Sample F, 72%	173.19 +/- 12.87	7.43
Sample G, 85%	182.52 +/- 8.21	14.22
Sample H, 90%	200.99 +/- 8.43	4.87
Sample I, 70%	230.17 +/- 7.19	12.45
Sample J, 70%	243.21 +/- 13.55	7.82

Data expressed as mean +/- SD = Standard Deviation, SE = Standard Error

Table 2: Commercial chocolate theobromine concentrations.

## Result

A standard curve (ng/mL) was generated to obtain the concentration of theobromine in each sample. With the standard curve, the average ratios of the analyte of interest compared with the internal standard are plotted with its corresponding concentration. Within the standard curve LLOQ (lower limit of quantitation) and ULOQ (upper limit of

quantitation) was found to be 15 ng/mL and 1000 ng/mL respectively Figure 1.

A recovery analysis was completed for theobromine. 86% of theobromine was recovered via extraction technique used in this experiment.

Raw cacao, dried cacao, roasted cacao, and 100% cacao bar were quantified for theobromine in Table 1. Raw cacao had a theobromine concentration of 420.75 ng and dried cacao had a theobromine concentration of 401.96 ng. Roasted cacao had a theobromine concentration of 371.37 ng and the 100% Cacao Bar had a theobromine concentration of 739.39 ng. The chromatograms (Figures 2-5) demonstrate the detection of theobromine at 86 min.

Ten chocolate samples popular within the U.S. purchased within the U.S. were tested for theobromine quantity (Table 2). Sample A had shown 33.70 ng of theobromine and Sample B had 37.21 ng of theobromine. Sample C had shown 42.56 ng of theobromine and Sample D had 61.29 ng of theobromine. Sample E contained 89.47 ng of theobromine, Sample F had 173.19 ng of theobromine, Sample G had 182.52 ng of theobromine, and Sample H had 200.99 ng of theobromine. Sample I had contained 230.17 ng of theobromine and Sample J had 243.21 ng of theobromine.

## Discussion and Conclusion

A rapid, sensitive UHPLC-HRMS gradient method for theobromine was successfully developed and detected within all samples. Figures 2-5 had shown theobromine detected within a 10 second window. Theobromine mass to charge ratio was identified within each sample.

Of all the cacao processing stages, roasted cacao had demonstrated the least amount of theobromine, whereas the 100% cacao bar had shown

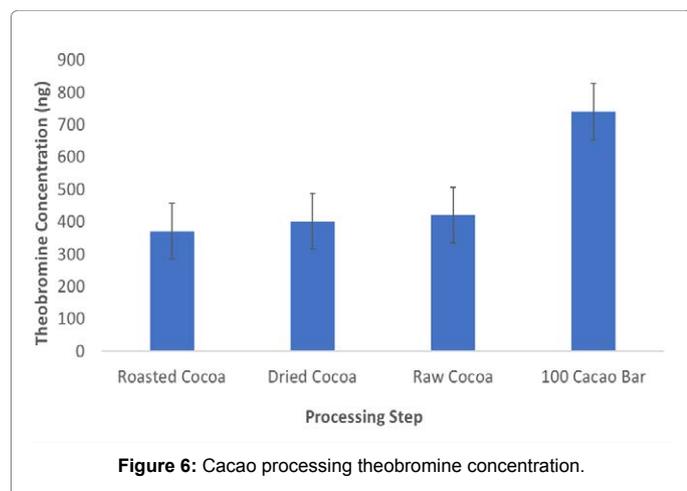


Figure 6: Cacao processing theobromine concentration.

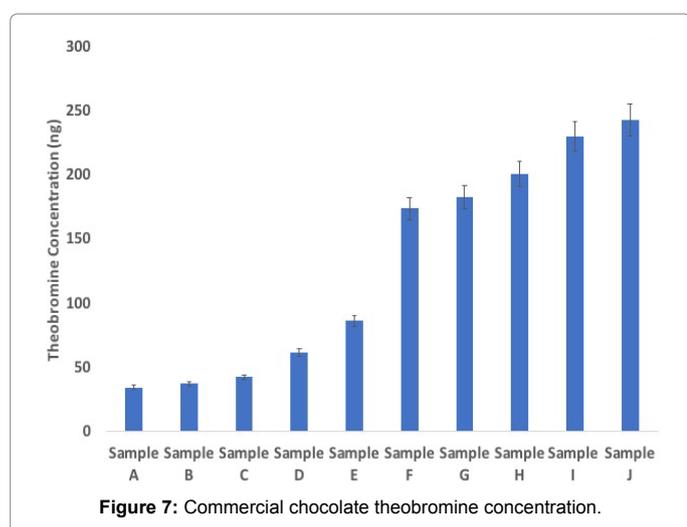


Figure 7: Commercial chocolate theobromine concentration.

the most amount of theobromine, as shown in Figure 6. According to the manufacturing process of cocoa beans, theobromine loss can occur at the roasting stage, due to temperature setting and roasting time [3].

Dried cacao had shown the second least amount of theobromine. The process of alkalization requires the use of raw cacao beans to be dried for roasting. However, alkalization influences loss of methyl xanthine compounds [13]. Raw cacao had shown the second most amount of theobromine, as it is the initial stage of cacao processing and taken directly from cacao pod [14].

However, the 100% cacao bar had shown the highest amount of theobromine, as no milk, sugar or additives was used in the process of the final cacao product. The 100% cacao bar claimed to use low-

fat natural cocoa powder, contributing to higher concentrations of theobromine [1].

In Figure 7, Sample A had the least amount of theobromine, whereas Sample J had the most amount of theobromine. Sample A was a popular U.S. milk chocolate brand, containing 11% of cacao of unknown cacao bean species. Sample J was an organic U.S. brand 70% cacao dark chocolate and claimed to use Trinitario cacao beans. Both Sample I and J contained 70% cacao content, however different amounts of theobromine. Further investigation is required to conclude whether per cent cacao in chocolate products influences the final amount of theobromine.

## References

1. Todorovic V, Redovnikovic IR, Todorovic Z, Jankovic G, Dodevska M, et al. (2015) Polyphenols, methylxanthines, and antioxidant capacity of chocolates produced in Serbia. J Food Compost Anal 41: 137-143.
2. Moser A (2015) The manufacturing confectioner 95: 31-38.
3. Jinap S, Rosli WIW, Russly AR, Nordin LM (1998) Effect of roasting time and temperature on volatile component profiles during nib roasting of cocoa beans (*Theobroma cacao*). J Sci Food Agri 77: 441-448.
4. Scapagnini G, Davinelli S, Renzo LD, Lorenzo AD, Olarte H, et al. (2014) Cocoa Bioactive Compounds: Significance and Potential for the Maintenance of Skin Health. Nutrients 6: 3202-3213.
5. Stark T, Bareuther S, Hofmann T (2006) Molecular Definition of the Taste of Roasted Cocoa Nibs (*Theobroma cacao*) by Means of Quantitative Studies and Sensory Experiments. J Agric Food Chem 54: 5530-5539.
6. Mitchell E, Slettenaar M, Meer NV, Transler C, Jans L, et al. (2011) Differential contributions of theobromine and caffeine on mood, psychomotor performance and blood pressure. Physiol Behav 104: 816-822.
7. Yoneda M, Sugimoto N, Katakura M, Matsuzaki K, Tanigami H, et al. (2017) Theobromine up-regulates cerebral brain-derived neurotrophic factor and facilitates motor learning in mice. J Nutr Biochem 39: 110-116.
8. Smit HJ, Blackburn RJ (2005) Reinforcing effects of caffeine and theobromine as found in chocolate. Psychopharmacology 181: 101-106.
9. Krauss U, Hidalgo E, Bateman R, Adonijah V, Arroyo C, et al. (2010) Improving the formulation and timing of application of endophytic biocontrol and chemical agents against frosty pod rot (*Moniliophthora roreri*) in cocoa (*Theobroma cacao*). Biological Control 54: 230-240.
10. Bowers JH, Bailey BA, Hebbar PK, Sanogo S, Lumsden RD, et al. (2001) The Impact of Plant Diseases on World Chocolate Production. Plant Health Prog 2: 1-12.
11. Li Z (2018) Evaluation of regulatory variation and theoretical health risk for pesticide maximum residue limits in food. J Environ Manage 219: 153-167.
12. Caudle AG, Gu Y, Bell LN (2001) Improved analysis of theobromine and caffeine in chocolate food products formulated with cocoa powder. Food Res Int 34: 599-603.
13. Li Y, Feng Y, Zhu S, Luo C, Ma J, et al. (2012) The effect of alkalization on the bioactive and flavor related components in commercial cocoa powder. J Food Compost Anal 25: 17-23.
14. Miller KB, Hurst WJ, Payne MJ, Stuart DA, Apgar J, et al (2008) Impact of Alkalization on the Antioxidant and Flavanol Content of Commercial Cocoa Powders. J Agric Food Chem 56: 8527-8533.