International Journal of Agriculture Innovations and Research
Volume 4, Issue 5, ISSN (Online) 2319-1473
Manuscript Processing Details (dd/mm/yyyy) :
Received : 25/03/2016 | Accepted on : 09/04/2016 | Published : 13/04/2016

Fast, Reliable and Simultaneous Determination of Theobromine and Caffeine in Fermented and Unfermented Cacao Beans and in Cocoa Products using Reverse Phase HPLC

Ian Marc G. Cabugsa, Kim Ryan A. Won

***Abstract* – Fast, reliable and simultaneous HPLC analysis of theobromine and caffeine in cacao and cocoa products was optimized in this study. The samples tested were raw, fermented, and roasted cacao beans as well as commercially available cocoa products. The HPLC analysis was carried out using step gradient solvent system with acetonitrile and water buffered with H_3PO_4 as mobile phase. The HPLC system was optimized using 273 nm wavelength at 35°C for the column temperature with a flow rate of 1.0 mL/min. Using this method, the theobromine percent recovery mean, Limit of Detection (LOD) and Limit of Quantification (LOQ) is 118.68(±3.38)%, 0.727 and 1.05 respectively. The percent recovery mean, LOD and LOQ for caffeine is 105.53(±3.25)%, 2.42 and 3.50 respectively. The inter-day and intra-day precision for theobromine is 4.31% and 4.48% respectively, while 7.02% and 7.03% was for caffeine respectively. Compared to the standard method in AOAC using methanol in isocratic solvent system, the results of the study produced lesser chromatogram noise with emphasis on theobromine and caffeine. The method is readily usable for cacao and cocoa substances analyses using HPLC.**

***Keywords* – Caffeine, Theobromine, Step Gradient Solvent System, HPLC, Cacao.**

I. INTRODUCTION

Theobromine and caffeine are alkaloids naturally found in tea, cacao beans and chocolate products [1]-[2].Both substances are methylxantines and have many beneficial properties. Theobromine (3,7-dimethylxanthine) occurs almost exclusively in cacao and its products [3]. Theobromine can be used to lower blood pressure [4]-[5], inhibit sensory nerve activation [6], uric acid crystallization [7], and remineralize the artificial enamel lesions [8]. Theobromine has also been studied to inhibit phosphodiesterase and suppressing the action of adenosine receptors [9] which has regulatory role in coronary blood flow.

Caffeine (1,3,7-trimethylxantine) is a widely used chemical both industrially and pharmaceutically. The compound can prevent Parkinson's disease and Alzheimer's disease [10]-[11]. It can reduce the occurrence of chronic liver disease and improves immune functions and anti-inflammatory properties [11]. Caffeine is added in small quantities to beverages to add slight bitterness.

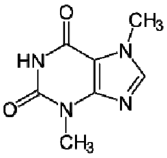


Fig. 1. Structural formula of Theobromine
Source: https://en.wikipedia.org/wiki/Theobromine

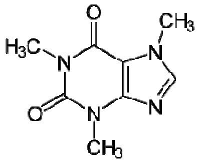


Fig. 2. Structural formula of Caffeine
Source: https://en.wikipedia.org/wiki/Caffeine

The increase in popularity of both theobromine and caffeine created several methodologies for its determination[1]. Several methodologies were made for the determination of theobromine, caffeine and other similar compounds [12]-[13] to which the most popular means of analysis is with the use of High Performance Liquid Chromatography (HPLC) [13]. The relative ease of the use of the machine along with short analysis time and the high accuracy and reproducibility makes HPLC a popular choice. The methods developed in HPLC varies from solvent extraction, mobile phase, wavelength, and even the type of sample to be analysed. Newer versions of HPLC can use step gradient solvent system as opposed to the traditional isocratic solvent systems. The advances in technology made chromatographic studies more accurate and reliable. The objectives of this study are 1) to make use of the solvent gradient system in the simultaneous determination of theobromine and caffeine using acetonitrile as mobile phase. The method will be optimized to have efficient analysis of the target compounds using the Agilent 1260 infinity and 2) to completely isolate the chromatograms of caffeine and theobromine for easy concentration and further analysis.

II. MATERIALS AND METHODS

Apparatus

The Agilent 1260 Infinity was the HPLC system was utilized for this study which was equipped with Diode Array Detector (DAD) and Ultraviolet (UV). However, we only used the DAD detector for this method. The column used was the Zorbax Eclipse XDB –C18 Analytical 4.6 x 250 mm 5-micron. The Analytical balanced used to weigh samples was Mettler AE 260 Delta Range.

Reagents

The solvent used for the extraction of theobromine and caffeine from the cacao and chocolate sample was distilled water that is HPLC grade further purified by Sartorius Arium Pro. The mobile phase was acetonitrile, HPLC grade from Tedia and the solvent for the fat extraction was methanol from Sharlau. The theobromine standard was T400 from Sigma-Aldrich and the caffeine standard was C0750 also from Sigma-Aldrich.

Sample Preparation

The procedure for the sample preparation was taken from AOAC with slight modification. The samples used in the study were cacao beans in various forms and cocoa products. A 0.5g of the sample was placed in a 125 mL Erlenmeyer flask where 100 mL of HPLC grade water that previously passed through a Nessler tube was added. The mixture was heated for 25 minutes at 100°C. It was then cooled and filtered through an Agilent 0.2µm syringe filter into an HPLC vial.

2.1 *Method Development*

Mobile Phase and Solvent System

The researchers first used the method from AOAC which uses the isocratic solvent system. The image in Figure 3 shows the results from the analysis of theobromine and caffeine. The substance at 2.6 minutes is theobromine while caffeine is at 3.725 minutes. The procedure is very efficient in the analysis and quantification of theobromine and caffeine with a very short run time. However, the resolution could have been made better to completely separate the theobromine and caffeine for easy separation.

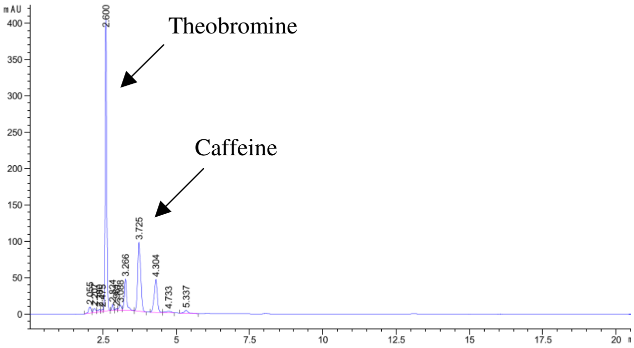


Fig. 3. HPLC Chromatograph of Cacao beans using Isocratic solvent system for analysis of theobromine and caffeine using the method from AOAC. The mAU is more than 400 because of the large amount of sample used.

The first few runs also had very high mAU (Mass Absortion Unit) because we used relatively more cacao samples for the runs. The future runs use of lesser cacao beans to better quantify the amount of the compounds in the sample.

The researchers proceeded in using line-gradient system with acetonitrile and water as the solvents. The concentration of acetonitrile was increasing as the run time was progressing. The image in Figure 4 was the result of the line system gradient. The line gradient system was able to completely separate theobromine at 4.23 minutes however, caffeine was completely immersed with the other peaks and possible separation was impossible.

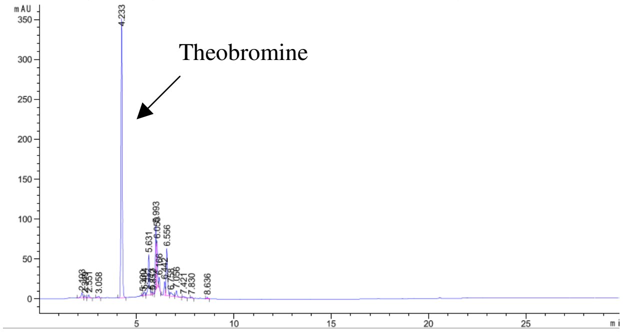


Fig. 4. HPLC Chromatograph of Cacao beans using line-gradient solvent system for analysis of theobromine and caffeine.

The researchers proceeded in using step-gradient solvent system. The method was able to completely separate the theobromine and caffeine from other chromatograms. Considering complete separation with efficient runtime, Step gradient was able to address the two objectives. Figure 5 shows the chromatograph of different step gradient solvent systems. The first from the bottom chromatogram had a gradient system with the best result for the analysis of theobromine and caffeine. The said chromatograph showed very good separation of the target compounds. The step gradient solvent system with optimized efficiency is in table 1.

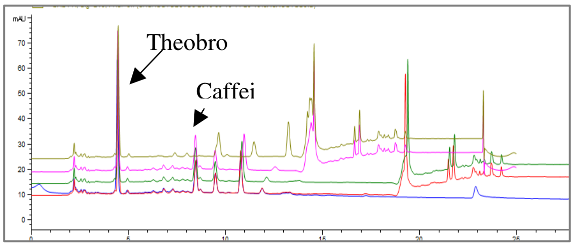


Fig. 5. Chromatographs of cacao beans using different step gradient solvent systems at 210nm for analysis of theobromine and caffeine.

Table 1. Step Gradient Solvent System for analysis of theobromine and Caffeine in cacao beans using Agilent 1260 Infinity.

Time (min)	1% H_3PO_4 in H_2O	Acetonitrile
0	90	10
5	85	15
10	85	15
15	0	100
20	0	100
21	90	10
25	90	10

Wavelength

The wavelength that would provide the greatest peak heights and areas for theobromine and caffeine and minimize the number of undesired peaks detected was also determined. The initial analysis tested the cacao samples and standards at 210, 230, 240, 250, 260, 270, and 278 (as prescribe in AOAC, [14]-[17]) nm. The results showed that the greatest peak heights and areas with minimal undesired peaks were detected at 270 nm. Though greater peak areas were observed for theobromine and caffeine at 210 nm (Figure 6, bottom chromatograph) in the samples, there were several other peaks detected that interfered with the signals for theobromine and caffeine. The interferences were not resolved using the existing solvent system.

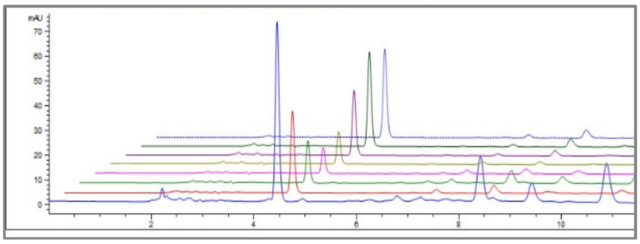


Fig. 6. Overlay of Chromatographs of Cacao Sample for determining theobromine and caffeine at 210-280 nm Legend. Wavelength of Chromotographs 1-8 starting from the bottom; (1) 210 nm, (2) 280 nm, (3) 230 nm, (4) 240 nm, (5) 250 nm, (6) 260 nm, (7) 270 nm and (8) 278 nm.

Additional tests were done at the 265-280 range at 1-nm intervals to further pinpoint the optimal wavelength. Figures 7 and 8 illustrates the peak heights of the chromatograms at different wavelengths. The optimal wavelength was found to be at 273 nm as shown in Figure 8 based on the peak areas for theobromine and caffeine.

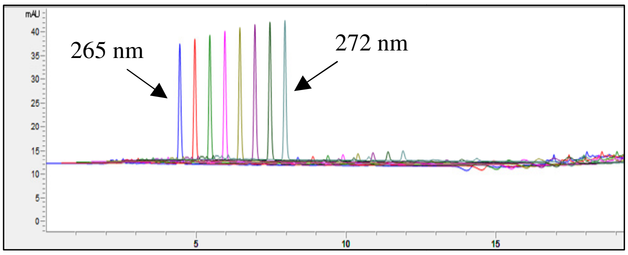


Fig. 7. Overlay of Chromatographs of Cacao Sample for determination of theobromine and caffeine at 265-272 nm (time offset for comparison)

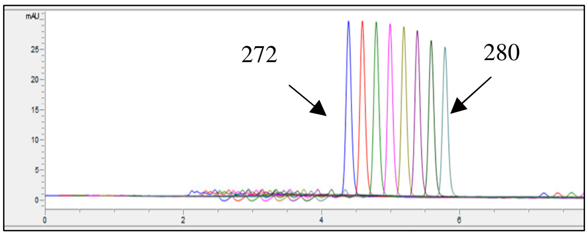


Fig. 8. Overlay of Chromatographs of Cacao Sample for determination of theobromine and caffeine at 272-280 nm (time offset for comparison)

Temperature

The column temperature for the HPLC analysis was also tested to determine its effect on peak areas and retention times of theobromine and caffeine. Column temperatures of 20, 25, 30, 35, 40, and 50°C were tested. Figure 9 shows the chromatographs of a cacao sample at the different temperatures.

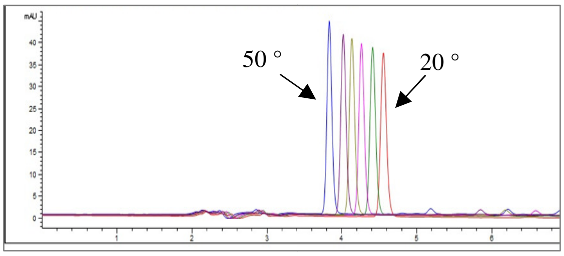


Fig. 9. Overlay of theobromine Chromatograms of Cacao Sample at Different Temperatures at 273nm (time offset for comparison). The chromatograms are ordered from 50, 40, 35, 30, 25 and 20 degrees centigrade starting from the left.

The tests showed an increase in peak area and a decrease in retention time with increasing temperature, with the greatest peak areas and lowest retention times at 50°C (first chromatogram). However, this temperature cannot be used for routine analysis since it approaches the operational limit of the column which is at 60°C. Thus, 50°C and 40°C were not considered for the optimized method. Based from the result, the optimized temperature was set at 35°C (3rd chromatograph).

Flow Rate

The flow rate was also tested to determine if analysis time could be decreased without loss of resolution. Flow rates at 0.5, 1.0, and 1.5 mL/min were tested.

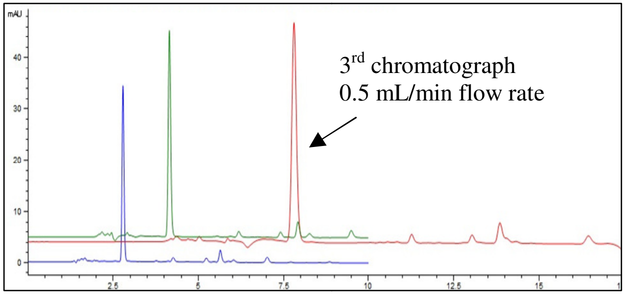


Fig. 10. Overlay of Chromatographs for Cacao Sample with Different Flow Rates at 1.5 mL/min, 1.0 mL/min and 0.5 mL/min using 273 nm(absorbance offset for comparison)

The analysis at a flow rate of 1.5 mL/minute (1st chromatograph) offered the least analysis time, with theobromine eluting at 2.8 minutes and caffeine at 5.6 minutes; however it provided poorer resolution, particularly for caffeine. The analysis at 0.5 mL/min (3rd chromatograph) increases the elution time of theobromine to 8.3 minutes and caffeine to 14.0 minutes, without a significant increase in resolution. Thus, the 1.0 mL/minute flow rate was selected as the optimal flow rate since it allowed a reasonable analysis time as well as satisfactory resolution of the target analytes.

Optimized Parameters

Combining all the data, the optimized condition for the analysis of theobromine and caffeine in the cacao samples are shown in Table 2. The table summarizes the final optimized parameters for the analytical method for the determination of theobromine and caffeine in cacao samples using acetonitrile and 1% phosphoric acid.

Table 2. Optimized Parameters for the Analysis of Theobromine and Caffeine

Mobile Phase	1% H_3PO_4 in H_2O (solvent A), acetonitrile (solvent B)
Solvent Gradient System	0-5 min, 10% B 5-10 min, 15% B 10-15 min 15% B 15-20 min, 100% B 20-21 min, 100% B 21-25 min, 10% B
Detection Wavelength	273 nm
Temperature	35°C
Flow Rate	1.0 mL/min

III. EVALUATION OF THE DEVELOPED METHOD

To test the reliability and efficiency of the developed method, various test for reliability were also conducted. Table 3 and 4 shows the linearity data for theobromine and caffeine respectively. Four different concentrations of the analytes were investigated using the developed method. The average mAU of the different concentration per analyte were graphed and the R2 of both substances were 0.9999 indicating reliability of results.

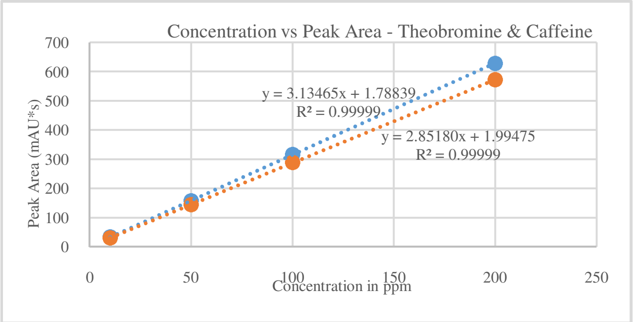
Table 3. Linearity Data for Theobromine. mAU of standard theobromine at different concentrations

Concentration in ppm	Mass Absorbance Unit (mAU)			
	Trial 1	Trial 2	Trial 3	Average
10	33.91476	32.8846	32.78236	33.19391
50	158.1911	158.9367	156.5513	157.893
100	314.2866	316.5281	317.4337	316.0828
200	635.4204	628.1606	621.7911	628.4574

Table 4. Linearity Data for Caffeine. mAU of standard caffeine at different concentrations

Concentration in ppm	Mass Absorbance Unit (mAU)			
	Trial 1	Trial 2	Trial 3	Average
10	30.11398	30.44338	29.86698	30.14145
50	144.949	145.2024	142.5794	144.2436
100	286.9882	288.9271	289.2631	288.3928
200	577.9309	572.0636	565.5576	571.8507

Graph 1. Standard Curve Concentrations of Theobromine and Caffeine using peak area



Percent Recovery, Limit of Detection and Limit of Quantification

The table below shows the sensitivity of the instrument using the developed method. Generally, the sensitivity of the instrument is greater for theobromine than in caffeine.

Table 5. Percent recovery, Limit of Detection (LOD) and Limit of Quantification (LOQ) of theobromine and Caffeine using the developed method

Compound	Mean % Recovery	LOD in ng/mL	LOQ in ng/mL
Theobromine	118.68	0.0727	1.0498
Caffeine	105.53	2.4230	3.4994

Application of the Developed Method

The developed method was applied to quantify the amount of theobromine and caffeine in different cacao samples and cocoa products. The method was able to readily quantify the amount of theobromine and caffeine with respect to the total mass of the sample. Table 5. Shows the results of the analysis.

Table 5. Results of the Analysis of Theobromine and Caffeine in various samples

Cacao / Chocolate Product	Theobromine (%)	Caffeine (%)
Alfonso's Hot Chocolate Tablea	0.3239 ± 0.0098	0.0309 ± 0.0006
Antonio Tablea	0.3860 ± 0.0066	0.0907 ± 0.0016
Boy Pure Tablea	1.3458 ± 0.0195	0.2676 ± 0.0037
Cacao de Davao Unsweetened	1.3292 ± 0.0227	0.2804 ± 0.0052
Zamboanga cacao nibs (7 days fermented) unroasted	0.8996 ± 0.0091	0.1247 ± 0.0016
Zamboanga cacao liquor (7 days fermented)	0.9874 ± 0.0759	0.1703 ± 0.0134
Cheding's Pure Tablea	1.3872 ± 0.0300	0.3706 ± 0.0072
Espeso Tablea	0.9034 ± 0.0061	0.1732 ± 0.0025
Kablon Farms Tablea	1.1155 ± 0.0165	0.2495 ± 0.0006
Malagos 65% Dark Chocolate	0.5207 ± 0.0101	0.1393 ± 0.0010
Malagos Premium Unsweetened	0.9271 ± 0.0388	0.2494 ± 0.0104
Pinky's Pure Tablea	1.3399 ± 0.0097	0.2487 ± 0.0033
Rich Tablea Cocoa Powder	0.3718 ± 0.0110	Below detection limit
Surebuy Cocoa Powder	2.0396 ± 0.0200	0.2607 ± 0.0022
Surebuy Cocoa Tablets	1.5063 ± 0.0470	0.1898 ± 0.0048
Cacao nibs (Subasta)	1.1552 ± 0.0007	0.3457 ± 0.0187
Sunny Farm Cocoa Powder	2.0773 ± 0.0928	0.2703 ± 0.0140
Xocolate 100% Pure Unsweetened	0.8022 ± 0.0139	0.1233 ± 0.0021

IV. CONCLUSION

The developed method was able to simultaneously analyze the concentration of theobromine and caffeine using HPLC with high degree of accuracy and reputability. The chromatograms of theobromine and caffeine are readily isolated for further experimentation. The method was also tested to work well with cacao and cocoa products.

V. ACKNOWLEDGEMENT

The researchers would like to thank USAID-STRIDE for the generous support provided to the researchers in developing the method. Furthermore, we would like to thank the Chemistry department and the University Research Council (URC) of Ateneo de Davao University for allowing us to do this research.

REFERENCES

[1] Bispo, M.S.,Veloso, M.C.C., Pinheiro, H.L.C., de Oliveira, R.F.S., Reis, J.O.N., and de Andrade, J.B. Simulataneous Determination of Caffiene, Theobromine, and Theophylline by High-Performance Liquid Chromatography. Journal of Chromatographic Science, Vol.40, pp 45-49 January 2002.

[2] International Agency for Research on Cancer. http://monographs.iarc.fr/ENG/Monographs/vol51/mono51-12.pdf. October 22, 2015

[3] Smit, H. J. and Blackburn, R. J. Reinforcing Effects of Caffeine and Theobromine as found in Chocolate. Psychopharmacoloy (2005) 181 pp 101-106

[4] Bogaard, B. V. D., Draijer, R., Westerhof, B.E., Meiracker, A. H. V. D., Montfrans, G. A.V., and Born, B. H. V. D.. Effects of Peripheral and Central Blood Pressure of Cocoa With Natural or High-Dose Theobromine: A Randomized, Double-Blind Crossover Trial. Hypertension 2010. Vol 56 pp 839-846

[5] Kelly, C. J.. Effects of Theobromine Should be Considered in Future Studies. American Journal of Clinical Nutrition. Ajcn.nutrition.org downloaded last October 22,2015 pp 486-487

[6] Usmani, O. S., Belvisi, M. G., Patel, H.J., Crispino, N., Birrell, M. A., Korbonits, M., Korbonits, D., and Barnes, P.. Theobromine Inhibits Sensory Nerve Activation and Cough. The FASEB Journal. 2004

[7] Grases, F., Rodriguez, A., and Costa-Bauza, A.. Theobromine Inhibits Uric Acid Crystallization. Potential Application in the Treatment of Uric Acid Nephrolithiasis. Open access. Plos One. Vol 9. Issue 10. 2014

[8] Amaechi, B.T., Porteous, N., Ramalingam, K., Mensinkai, P.K., Ccahuana Vasqez, R.A., Sadeghpour A., and Nakamoto, T.. Remineralization of Artificial Enamel Lesions by Theobromine. Caries Research 2013; 47 pp 399-405

[9] Martinez-Pinilla, E., Onaatibia-Astibia, A., and Franco, R.. The Relevance of Theobromine for the Beneficial Effects of Cocoa Consumption. Frontiers in Pharmacology. Vol 6, article 30, 2015

[10] Center for Science in the Public Interest. http://www.cspinet.org/reports/caffeine.pdf. November 19, 2015

[11] International Food Information Council Foundation. http://www.foodinsight.org/Content/6/Final%20Revised%20CAF%20CPE%2011-16-09.pdf. November 19, 2015

[12] de Sena, A. R., de Asis, S. A., Branco, A.. Analysis of Theobromine and Related Compounds by Reversed Phase High-Performance Liquid Chromatography with Ultraviolet Detection: An Update (1992-2011). Open Access. Downloaded last October 22, 2015

[13] Brunetto, M.R., Gutierrez, L., Delgado, Y., Gallignani, M., Zambrano, A., Gomez, A., Ramos, G., and Romero, C.. Determination of Theobromine, Theophylline and Caffeine in Cocoa Samples by High-Performance Liquid Chromatographic Method with On-line Sample Clean-up in a switching column System. http://www.rinconartesanal.com/cacaoaroma/pdf/8.pdf. Downloaded last Oct 22, 2015.

[14] Lo Co, F., Lanuzza, F., Micali, G., and Capellano, G.. Determination of Theobromine, Theophylline, and Caffeine in by-Products of Cupuacu and Cacao Seeds by High-Performance Liquid Chromatography. Journal of Chromatographic Science vol 45 2007

[15] Srdjenovic, B., Djordjevic-Milic, V., Grujic, N., Injac, R., and Lepojevic, Z., Simulataneous HPLC Determination of Caffeine, Theobromine, and Theophylline in Food, Drinks and Herbal Products. Journal of Chromatographic Science vol 46 2008

[16] Risner, C.H. Simultaneous Determination of Theobromine, (+)-Catechin, Caffeine, and (-)-Epicatechin in Standard Reference Material Baking Chocolate 2384, Cocoa, Cocoa Beans and Cocoa Butter. Journal of Chromatographic Science vol 46 2008

[17] Dyke, T.M. and Sams, R.A. Detection and Determination of Theobromine and Caffeine in Urine after Administration of Chocolate-Coated Peanuts to Horses. Journal of Analytical Toxicology. Vol 22 1998

AUTHOR'S PROFILE

Ian Marc G. Cabugsa is from Davao City Philippines and is an Asst. Prof. in Chemistry of Ateneo de Davao University. Research interests include agricultural, environmental and analytical chemistry.
Email ID : imgcabugsa@addu.edu.ph

Kim Ryan A. Won is from Davao City Philippines. A graduate of Chemistry in Ateneo de Davao University and is also working for Ateneo de Davao University.
Email ID : kimryan_won@yahoo.com