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HPLC Determination of Methylxanthines and Polyphenols Levels In Cocoa and Chocolate Products

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Abstract: . Commercial cocoa and chocolate products such as cocoa powder, cocoa beans, cocoa liquor and chocolate were analyzed for total polyphenols, (-)-epicatechin, (+)-catechin, theobromine and caffeine contents by High Performance Liquid Chromatography (HPLC). The methylxanthines were identified and quantified with the use of -Bondapak column and mobile phase of methanol:water:acetic acid (20:79:1). C.V. values were relatively low (<5%) except for caffeine. This is an indication of good reproducibility of the extraction method used and gave a relatively high percentage of recoveries (>90%). Total polyphenols ranged from 45 52 mg/g in cocoa liquor, 34 60 mg/g cocoa beans, 20 62 mg/g in cocoa powder. For (-)-epicatechin contents, the avarage are 3.81 mg/g in cocoa powder, 2.53 mg/g in cocoa liquor and 4.61 mg/g in cocoa beans. Whereas the avarage for (+)-catechin contents are 4.28 mg/g in cocoa powder, 3.49 mg/g in cocoa liquor and 3.02 mg/g in cocoa beans. Levels of caffein and theobromine in 32 samples of chocolate products averaged 0.62-1.14 mg/g and 0.026-0.153 mg/g respectively. Mean values for theobromine and caffeine contents for chocolate coating were 0.82 and 0.07 mg/g respectively. The chocolate coating made from fat substitute had theobromine and caffeine levels ranged from 0.36 0.70 mg/g and 0.027-0.061 mg/g respectively, with mean values of 0.49 mg theobromine/g and 0.039 mg caffeine/g. In local chocolate, the mean theobromine and caffeine levels respectively were 0.72 mg/g and 0.04mg/g in milk chocolate, and 0.85 mg/g and 0.06 mg/g in dark chocolate. As in imported chocolate, the mean theobromine and caffeine levels respectively were 1.05 mg/g and 0.12 mg/g in dark chocolate; 0.76 mg/g and 0.04 mg/g in milk chocolate; and 0.74 mg/g and 0.03 mg/g in white chocolate. Compared with the local chocolate, imported chocolate has higher level of theobromine and caffeine.

Abstrak: Koko komersil dan produk coklat yang merangkumi serbuk koko, biji koko, likur koko dan coklat telah ditentukan kandungan jumlah polifenol, (-)-epikatekin, (+)-katekin, teobromina and kafeina dengan menggunakan Kromatografi Cecair Bertekanan Tinggi. Sebatian metilxanthin di tentukan dengan menggunakan kolum μ-Bondapak dan fasa bergerak yang terdiri daripada metanol:air:asid asetik (20:79:1). Pekali koefisi adalah rendah (<5%) kecuali untuk kafeina. Ini menunjukkan kebolehulangan kaedah pengekstrakan yang baik kadar kebolehdapatan yang tinggi (90%). Purata kandungan polifenol jumlah ialah 45-52mg/g untuk likur koko, biji koko 34-60mg/g dan serbuk koko 20-62mg/g. Bagi (-)-epikatekin pula ialah 3.81mg/g, 2.53mg/g, 4.61mg/g manakala bagi (+)-katekin pula puratanya adalah 4.28 mg/g, 3.49mg/g dan 3.02mg/g bagi setiap sampel serbuk koko, likur koko dan biji koko. Purata kandungan kafeina dan teobromina ke atas 32 sampel coklat ialah 0.62-1.14mg/g dan 0.027-0.061mg/g setiap satunya. Manakala untuk coklat salutan pula 0.82 dan 0.07mg/g untuk kafeina dan teobromina. Coklat salutan yang diperbuat dari lemak gantian pula mempunyai nilai purata antara 0.36- 0.70mg/g dan 0.027-0.061mg/g. Coklat tempatan mempunyai nilai purata teobromina dan kafeina 0.72mg/g, 0.04mg/g bagi jenis coklat susu dan 0.85mg/g, 0.06mg/g coklat gelap bagi kedua-dua sebatian tersebut. Nilai teobromina dan kafeina untuk coklat import pula mempunyai nilai purata pada 1.05mg/g, 0.12mg/g bagi jenis coklat gelap dan 0.76mg/g, 0.04mg/g coklat susu dan 0.74mg/g, 0.03mg/g untuk coklat putih. Ini menunjukkan coklat import mempunyai nilai teobromina dan kafeina yang lebih tinggi dari coklat tempatan.

Key words: HPLC, Methylxanthines, polyphenol compounds, Cocoa Products, Chocolate

Introduction

A cocoa based food are consumed worldwide and have been shown to be very nutritious, containing substantial amounts of amino acids, except methionine and arginine. Vitamins, minerals and fat are also presence a high proportion. Cocoa products contain many physiologically active compounds. The high level of fat contributes to the high gross energy content of the cocoa bean. Despite its high nutritional value, however, the presence of caffeine and thebromine alkoloids may limit its potential as a nourishing food [1].

The determination of the levels of methylxanthines and polyphenols in cocoa products is becoming increasingly important in the light of recent concern about the health effects of these compounds and their widespread consumption of the public. Methylxanthines such as theobromine and caffeine are

typical compounds present in coffee, tea, chocolate and products made from them. Cocoa bean polyphenols comprising 12-18% of whole dry bean weight have long been associated with the flavor and color of chocolate [1]. Through complex chemical interactions of polyphenol with protein, astringency is reduced and the burnt feather character of roasted protein depressed. Approximately 35% of the polyphenol content of cocoa beans is (-)-epicatechin ([2S,3S]-2-[3,4-dihydroxyphenyl]-3,4-dihydro-1[2H]-benzopyran-3,5,7-triol) and catechin (Cynidol-3; [2R,3S]-2-[dihydroxyphenyl-3,4-dihydro-1[2H]-benzopyran-3,5,7-triol) [2]. Oxidation of polyphenol compound in chocolate will effect the browning colour and flavour of fermented cocoa beans. A great variety of techniques for the analysis of methylxhantine in food product has been reported ranging from the Kjeldal method [10], UV spectrophotometry [20], gas chromatography [24] and potentiometric titrations [8] to high-performance liquid chromatography (HPLC) using either normal-phase [23] or reversed-phase modes [13].

Many health professionals have recently expressed some concerns regarding the effects of caffeine on health. This subject has been reviewed recently by a number of authors [9, 11, 13,15]. Theobromine (3,7 dehydro- 3,7 dimethyl-IH-purine-2,6-dione) commonly called 3,7-dimethylxnthine and caffeine (3,7 dihydro-1,3,7 trimethyl-1H-purine-2,6-dione) also known as 1,3,7-trimethylxanthine are major compounds in the methylxanthine group in the plant *Theobroma cacao*. Theobromine and caffeine are found in chocolate products and cocoa, and they are responsible for the bitterness of these products. Consumers are increasingly interested in the theobromine and caffeine contents in chocolate and cocoa-based food and beverages due to the increasing awareness of their effects on health. Both theobromine and caffeine have pharmacological effects in that caffeine stimulates the nervous system, and theobromine is a diuretic.

Recent interest in these two alkoloids, however, is centered on their potential reproductive toxicities [5, 22]. Theobromine and caffeine are now known to cross the placental and blood brain barrier thus potentially inducing fetus malformation by affecting the expression of genes vital in development [18]. The average daily consumption of caffeine is estimated at 200-300 mg/day, which is equivalent to 2-3 cups of brewed coffee per day [19]. Although cocoa may supply 230-280 mg of theobromine per cup, there is a lack of data on the average daily consumption levels or intakes of theobromine in the diet. Caffeine content data on chocolate products are fairly limited but also show quite variable levels. Burg [3] noted the differences in caffeine content of American cocoa and South American cocoa (6 vs 42 mg/5-oz cup, respectively). FDA [6] cites a single value of 5mg/cup and Gilbert [7] states that cocoa usually contains less than 40 mg/cup. Zoumas et al. [26] also report average caffeine contents of 6 mg/1-oz serving for milk chocolate, 20 mg/1-oz serving for sweet chocolate (dark, bittersweet or semi-sweet chocolate).

High pressure liquid chromatography has recently been utilised to identify and quantify simultaneously methylxhanthins and polyphenols levels in cocoa and chocolate products [2, 12, 14]. Most of the research carried out involved the analysis of commercial cocoa, chocolate liquors, different types of chocolate and cocoa beverages [25]. Due to the wide range of reported values for methylxanthines and polyphenols content in cocoa products especially in cocoa powder and cocoa liquor, this study was undertaken to determine the theobromine and caffeine contents in popular brands of local and imported chocolates, cocoa products such as cocoa beans, cocoa liquor and cocoa powder.

Material And Method

Samples preparation

Cocoa products such as cocoa beans, cocoa liquor and cocoa powder were obtained from cocoa grinders in Malaysia. Popular brands of chocolate products were purchased in a local supermarkets and retail food in the Kuala Lumpur. The criteria for chocolate product must be not expired at least 6 months after manufactured and keep in dry and cool conditions. Chocolate samples were divided into four groups. Group I comprise 8 samples of local commercial chocolate, group 2 consists of 8 samples of imported chocolate, group 3 has 8 samples of chocolate courverture and group 4 consists of 8 samples of chocolate substitute. All samples were defatted before analysis using soxhlet apparatus.

Determination of total polyphenol

Total polyphenol of cocoa products were determined by a modified method of Cros et al., [4]. A 0.5g sample of defatted cocoa products and 40 ml of acetone:water mixture (80:20) were placed in a 125 ml conical flask, and the mixture was sonicated for 30 min. During sonication, the extraction mixture was kept cold by filling the sonicator vessel with cool

water. Sonication was preferred over shearing as an aid in solubilizing the polyphenol since shearing promoted browning of the polyphenol extract by oxidation. The clear extract was obtained by vacuum filtration through Whatman No. 1 filter paper. The residue and all glassware were washed with the 80% aqueous acetone and the total volume of filtrate was made up to 100 ml in a volumetric flask. One ml of extract was pipette into 10 ml volumetric flask and diluted with 7 ml of water to give known concentrations 0 g/ml, 5 g/ml, 10 g/ml, 25 g/ml, 40 g/ml dan 50 g/ml. The polyphenol were then reacted with 0.5 ml of 2N Folin-Ciocalteaus reagent for 3 min. Then 1 ml of saturated Na2CO3 solution was added to stabilize the colour formed. The blue colour was allowed to develop for at least 2 hrs and its absorbance was measured at 760 nm using digital spectrophotometer.

Standard gallic acid samples (Sigma Chem. Co) of five known concentrations (0.00 to 1.00 ml/100ml) were prepared, reacted with Folin-Ciocalteaus reagent in duplicate and their absorbance were measured at 760nm. Linear regression was determined by the Least Squares method and the correlation of determination of absorbance versus concentrations was calculated. Absorbance readings of total polyphenols in cocoa products were compared to a standard curve for quantitation. The amounts of total polyphenols were reported as gallic acid equivalents per gram defatted cocoa.

High Pressure Liquid Chromatography Method

Analysis was carried out by liquid chromatography using a Model M6000A solvent

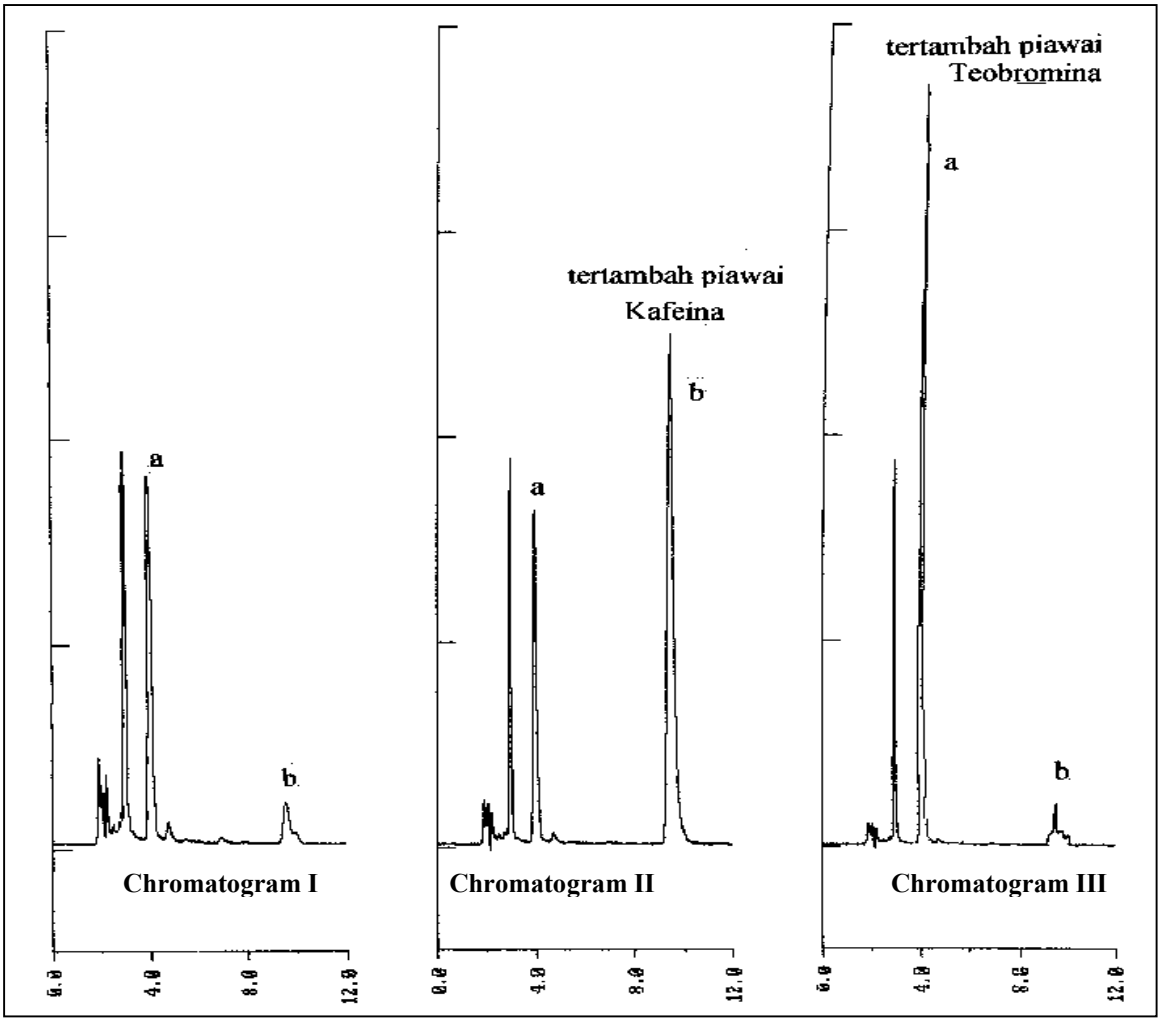

Figure 1. Chromatography of chocolate samples and standard on a µ-Bondapak 10µm column (30 cm x 4.0 mm) HPLC column. Mobile phase: methanol-acetic acid-water (20:1:79, v/v); flow rate = 1.0 ml/min; UV Detector, 280 nm. Standard containing of 20 µg of theobromine and caffeine. Chromatogram I- chocolate extracts, Chromatogram II - standard of caffeine and chocolate samples, Chromatogram III - standard theobromine and chocolate samples

delivery system (Waters Associates, Inc.) and a Model 7120 sample injector system (Rheodyne Inc., CA) with a 20 µl sample loop. A µ-Bondapak 10µm column (30 cm x 4.0 mm) was used to separate the methylxanthines. The solvent flow rate was 1.0 ml/min. The detector used was Waters spectrophotometer Model M440, wavelength of 280 nm. The mobile phase used for analysis of the chocolate was methanol: acetic acid: water (20:1:79, v/v). Quantitation analyses were carried out using the individual standard curves for each type of methylxanthines and polyphenols. Methylxanthine peak heights were obtained from the chart recorder after injection of each sample filtrate into the HPLC. Approximate retention times for theobromine was 8.95 min and for caffeine was 3.82 min.

Statistical analysis

Statistical significance was determined using analysis of variance and Duncan's multiple range test using SAS (ver. 6.04), Cary, North Carolina. Differences were considered when the *P*-value was <0.05. Values are given as means ± SD are presented in the text and tables.

Results And Discussion

Determination of theobromine and caffeine compounds

Figure 1 shows the chromatogram for theobromine and caffeine compound that were separated in the mobile phase. The result showed both types of methylxanthines analysed gave a good separation resolution. Chromatogram 1 is the result of chocolate sample extraction analysis. Co elution peak 'a' with standard theobromine can be seen clearly in chromatogram III. Peak 'b' was identified as caffeine through the spiking test in chromatogram II.

Table 1. Effect of the defatting treatment on the recoveries (Rv) and coefficient of variance (C.V) of cocoa products

Sample	Theobromine		Caffeine		(-)-epicatechin		(+)-catechin	
	Rv(%)	C.V.(%)	Rv(%)	C.V. (%)	Rv(%)	C.V.(%)	Rv(%)	C.V.(%)
Cocoa liquor	91.59	2.90	92.71	9.56	93.01	6.12	90.11	7.09
Cocoa bean	94.65	2.98	93.54	10.2	90.96	5.88	89.11	6.99
Cocoa powder	93.21	3.12	90.02	9.66	92.71	5.98	90.89	7.15
Chocolate	92.58	4.12	91.09	5.6	93.57	4.27	90.83	7.52

Table 2. Regression equations and coefficient of determinations (r^2)

Concentration of standards (ppm)	Standards of methylxhanthin compounds (% peak area)				
	Theobromine	Caffeine	(+)-catechin	(-)-epicatechin	Total polyphenol
5	-	-	-	-	0.09
10	-	-	-	-	0.16
20	-	32.69	20.08	-	0.39
30	-	-	-	-	0.49
40	153.74	60.45	28.14	-	0.78
50	-	-	-	-	-
60	190.97	88.02	42.22	-	-
80	300.13	127.86	63.93	-	-
100	343.35	152.45	78.26	79.02	-
120	422.72	-	-	-	-
200	-	-	-	145.74	-
300	-	-	-	200.73	-
400	-	-	-	304.03	-
500	-	-	-	38.48	-
y	0.2839x-0.1117	0.6488x+0.1426	1.2861x+0.1618	1.2926x+0.8864	0.0192x-0.0188
r^2	0.98	0.9951	0.9785	0.9917	0.9807

Reproducibility and Recovery

Table 1 shows the reproducibility and recovery of the extraction method in determining theobromine caffeine, (-e)–epicatechin and (+)-catechin. All C.V. values were relatively low (<5%) except for caffeine. This is an indication of good reproducibility of the extraction method used. As for the recovery, the method used also gave a relatively high percentage of recoveries (>90%). Overall, the data collected from this study show the extraction method by Kim and Keeney [12] is reliable.

Regression equations and coefficient of determinations (r^2)

The regression equations and r^s for the standards curves of different methyxanthine compounds are shown in Table 2. The r^2 values were very good for caffeine (0.9951) and (-)-epicatechin (0.9917) standards. However, the r^2 for theobromine, (+)-catechin and total polyphenol were only 0.980, 0.979 and 0.981 respectively.

Total polyphenol levels in cocoa products

The status of total polyphenol contents in different type of cocoa liquor, bean and powder is given in Table 3. Among the cocoa powder, Malaysian cocoa powder (PAKB and CPNKLK) have the highest total polyphenols contents. However, Ghana cocoa beans (CBG) contained nearly twice the amount of total polyphenol as compared to the Malaysian beans.

Table 3. Total polyphenol levels in cocoa products[1]

Code	Origin	Sample	Total Polyphenols (mg/g)[2]
CLNM	Malaysia	Cocoa liquor	45.75 (±1.37)) [b]
CLG	Ghana	Cocoa liquor	52.31 (±1.78)) [ab]
CBSQB	Malaysia	Cocoa bean	36.82 (±3.21)) [bc]
CBIWQ	Malaysia	Cocoa bean	34.93 (±4.89)) [bc]
CBG	Ghana	Cocoa bean	60.22 (±2.71)) [a]
CPNKLK	Malaysia	Cocoa powder (Natural)	58.87 (±2.31) [a]
		Cocoa powder (Alkali 1)	32.50 (±0.18) [bc]
		Cocoa powder (Alkali 2)	30.24 (±1.52) [bc]
CPAKB	Malaysia	Cocoa powder (Alkali 1)	61.58 (±1.32)[a]
		Cocoa powder (Alkali 2)	29.11 (±5.30) [c]
CPAMCM	Malaysia	Cocoa powder (Alkali 1)	50.89 (±6.21) [ab]
		Cocoa powder (Alkali 2)	33.31 (±2.13) [bc]
CPECI	Malaysia	Cocoa powder (Natural)	43.01 (±0.65) [b]
		Cocoa powder (Alkali)	41.15 (±2.10) [b]
		Cocoa powder (dark)	27.90 (±3.91) [c]
CPCPM	Malaysia	Cocoa powder (Alkali)	20.59 (±1.22) [cd]
CPLKT	Malaysia	Cocoa powder (Natural)	53.21 (±0.66) [ab]

[1] *Values are mean ± SD; n=3*

[2] *All values with the same letters are not significantly different at p≥0.05*

(-)-Epicatechin and (+)-catechin levels in cocoa products

Table 4 summarizes the contents of (-)-epicatechin and (+)-catechin in Malaysian and Ghana cocoa liquors, beans and powders. As for cocoa powder (-)-epicatechin and (+)-catechin contents, the effect of different alkalization treatments are also shown in the table. Malaysian cocoa powder (CPAKB) presented the highest (-)-epicatechin and (+)-catechin levels. Additionally, Malaysian cocoa powder (CPAKB) and (CPNKLK) showed the highest theobromine and caffeine levels respectively (Table 5).

Table 4(-)-epicatechin and catechin levels in cocoa products[1]

Code	Origin	Sample	(-)-epicatechin (mg/g)[2]	(-)-catechin (mg/g) [2]
CLNM	Malaysia	Cocoa liquor	1.41 (±1.32) [b]	4.16 (±0.83) [a]
CLG	Ghana	Cocoa liquor	3.65 (±0.22) [ab]	2.81 (±1.33) [ab]
CBSQB	Malaysia	Cocoa bean	5.08 (±0.69) [a]	3.91 (±0.05) [a]
CBIWQ	Malaysia	Cocoa bean	5.27 (±0.15) [a]	2.25 (±0.17) [ab]
CBG	Ghana	Cocoa bean	3.49 (±0.38) [ab]	2.90 (±0.30) [ab]
CPNKLK	Malaysia	Cocoa powder (Natural)	4.78 (±0.26) [ab]	5.23 (±0.26) [ab]
		Cocoa powder (Alkali 1)	0.48 (±0.26) [c]	1.74 (±0.26) [c]
		Cocoa powder (Alkali 2)	1.76 (±0.12) [bc]	2.99 (±0.85) [bc]
CPAKB	Malaysia	Cocoa powder (Alkali 1)	7.78 (±1.04) [a]	7.53 (±2.33) [a]
		Cocoa powder (Alkali 2)	5.14 (±0.19) [ab]	4.20 (±0.55) [b]
CPAMCM	Malaysia	Cocoa powder (Alkali 1)	4.47 (±0.99) [ab]	4.20 (±0.13) [b]
		Cocoa powder (Alkali 2)	1.47 (±0.09) [bc]	4.41 (±0.45) [b]
CPECI	Malaysia	Cocoa powder (Natural)	6.32 (±0.55)[a]	5.12 (±2.30)[ab]
		Cocoa powder (Alkali)	3.99 (±0.21) [b]	4.10 (±1.81) [b]
		Cocoa powder (dark)	3.00 (±0.87) [b]	4.18 (±1.23) [b]
CPCPM	Malaysia	Cocoa powder (Alkali)	2.12 (±1.01) [bc]	2.78 (±0.14) [bc]
CPLKT	Malaysia	Cocoa powder (Natural)	4.37 (±0.11) [ab]	4.82 (±0.70) [ab]

[1]*Values are mean ± SD; n=3*
[2]*All values with in a column with same superscript letters are not significantly different at ≥0.05*

Table 5. Theobromine and caffeine levels in cocoa products[1]

Code	Origin	Sample	Theobromine (mg/g)[2]	Caffeine (mg/g)[2]
CLNM	Malaysia	Cocoa liquor	17.72 (±1.66) [bc]	3.13 (±0.54) [bc]
CLG	Ghana	Cocoa liquor	25.28 (±0.56) [ab]	3.77 (±0.48) [b]
CBSQB	Malaysia	Cocoa bean	16.23 (±1.33) [bc]	4.12 (±0.04) [b]
CBIWQ	Malaysia	Cocoa bean	17.54 (±2.36)[bc]	2.52 (±0.13) [c]
CBG	Ghana	Cocoa bean	26.64 (±2.78) [ab]	4.98 (±0.66) [ab]
CPNKLK	Malaysia	Cocoa powder	27.69 (±2.14)[ab]	6.58 (±0.66) [a]
	Alkali 1	(Natural)	19.63 (±1.23) [b]	5.86 (±0.23) [ab]
	Alkali 2	Cocoa powder (Alkali 1)	21.70 (±6.20) [b]	4.32 (±0.99) [ab]
		Cocoa powder (Alkali 2)		
CPAKB	Malaysia	Cocoa powder	29.38 (±2.96) [a]	5.46 (±1.03) [ab]
	Alkali 2	(Alkali 1)	26.87 (±0.28) [ab]	4.98 (±1.22) [ab]
		Cocoa powder (Alkali 2)		
CPAMCM	Malaysia	Cocoa powder (Alkali 1)	22.81 (±3.02) [b]	4.91 (±1.06) [ab]
		Cocoa powder (Alkali 2)	26.18 (±1.79) [ab]	5.69 (±0.20) [ab]
CPECI	Malaysia	Cocoa powder (Natural)	20.73 (±3.50) [b]	3.27 (±0.65) [bc]
		Cocoa powder (Alkali)	23.36 (±1.25) [b]	3.47 (±2.01) [b]
		Cocoa powder (dark)	19.80 (±0.16) [b]	4.12 (±0.78) [ab]
CPCPM	Malaysia	Cocoa powder (Alkali)	24.79 (±1.36) [ab]	4.27 (±0.38) [ab]
CPLKT	Malaysia	Cocoa powder (Natural)	26.62 (±0.65) [ab]	4.04 (±1.40) [ab]

[1]*Values are mean ± SD; n=4*
[2]*All values with in a column with same superscript letters are not significantly different at ≥0.05*

Theobromine and caffeine levels in local and imported chocolate

The result for theobromine levels in 8 of the local samples analysed are presented in Tables 6. Among the local chocolates (Group 1) and imported chocolates (Group 2), imported dark chocolate has the highest level of theobromine and caffeine that was 0.83 mg/g and 0.058 mg/g respectively compared with local chocolate was 0.75 and 0.041 mg/g respectively. This is because dark chocolate contains a higher level of chocolate liquors than in milk chocolate. Theobromine content in a local (Brand LC2) milk chocolate contains almond is less than that plain milk chocolate (Brand LC1). Ingredients in milk chocolate such as almond, peanuts and raisin decrease the theobromine and caffeine compound levels [13]. Furthermore, the higher level of theobromine in Brand LC1 could be due to higher content of cocoa solid in the milk chocolate as compared to Brand LC2. According to Beckett [1], the average content of cocoa solid in milk chocolate is lower (15.7%) than the average content of cocoa solid in bittersweet chocolate and dark chocolate (61.7% and 39.6% respectively). Brand LC2 milk chocolate has the least amount of theobromine content among other local chocolates and all milk chocolates. Kreiser & Martin [13] reported that the difference in theobromine and caffeine levels in milk chocolate influenced more by the type of cocoa beans than the cocoa content itself. Thus the lack of significant differences in the theobromine levels in Brand LC3, LC4, LC5, LC6, LC7 and LC8 could be because the local manufacturers use the same type of cocoa beans.

Table 6. Theobromine and caffeine levels in chocolate products

Code	Origin	Sample	Theobromine (mg/g	Caffeine (mg/g)
Brand LC1	Malaysia	commercial chocolates	0.88 ± 0.05^{a}	0.034 ± 0.01^{a}
Brand LC2	Malaysia	commercial chocolates	0.85 ± 0.03^{a}	0.058 ± 0.02^{a}
Brand LC3	Malaysia	commercial chocolates	0.74 ± 0.03^{a}	0.028 ± 0.02^{a}
Brand LC4	Malaysia	commercial chocolates	0.72 ± 0.05^{a}	0.034 ± 0.01^{a}
Brand LC5	Malaysia	commercial chocolates	0.71 ± 0.05^{a}	0.039 ± 0.01^{a}
Brand LC6	Malaysia	commercial chocolates	0.69 ± 0.06^{a}	0.029 ± 0.01^{a}
Brand LC7	Malaysia	commercial chocolates	0.69 ± 0.07^{a}	0.041 ± 0.01^{a}
Brand LC8	Malaysia	commercial chocolates	0.67 ± 0.06^{a}	0.066 ± 0.08^{a}
Brand IC1	Switzerland	commercial chocolates [1]	1.14 ± 0.01^{b}	0.153 ± 0.02^{b}
Brand IC2	German	commercial chocolates [1]	0.96 ± 0.02^{a}	0.082 ± 0.02^{a}
Brand IC3	Holland	commercial chocolates [1]	0.84 ± 0.02^{a}	0.047 ± 0.02^{a}
Brand IC4	USA	commercial chocolates [1]	0.81 ± 0.04^{a}	0.039 ± 0.01^{a}
Brand IC5	France	*commercial chocolates* [1]	0.76 ± 0.04^{a}	0.051 ± 0.01^{a}
Brand IC6	Switzerland	*commercial chocolates* [1]	0.74 ± 0.01^{a}	0.028 ± 0.01^{a}
Brand IC7	Switzerland	commercial chocolates [1]	0.71 ± 0.02^{a}	0.026 ± 0.01^{a}
Brand IC8	Belgium	commercial chocolates [1]	0.69 ± 0.02^{a}	0.031 ± 0.01^{a}

[1]*Values are mean ± SD; n=4*

[2]*All values with in a column are not significantly different at p≥0.05*

[2]*Within a column, values with same superscripts letters are not significantly different (p≥0.05)*

Bittersweet chocolate Brand IC1 contained the highest caffeine level followed by Brand IC2 dark chocolate at 0.153 mg/g and 0.082 mg/g respectively. Brand IC7 milk chocolate has the lowest caffeine level, even lesser than of white chocolate. Brand IC8 milk chocolate contained at least 25% of white chocolate mixture. White chocolate does not contain any cocoa solid.

Overall, the means for theobromine content in local and imported chocolates are 0.75 mg/g and 0.83 mg/g consecutively. The mean levels of caffeine in local and imported chocolate are 0.041 mg/g and 0.057mg/g respectively. These results showed that imported chocolates contain higher levels of theobromine and caffeine compared to local chocolates. This could be due to higher amount of cocoa paste used in imported chocolates than in local chocolates. Furthermore, species of cocoa beans used by overseas manufacturers in imported products could be different from those used by local manufacturers. Kreiser & Martin [13,14] has reported that cocoa paste in Malaysian cocoa beans had 1.1% theobromine whereas Trinidad cocoa beans contained 1.2%

theobromine. Different species of cocoa beans contribute to different levels of theobromine and caffeine. This study showed that dark chocolate contains higher theobromine and caffeine content compared to milk chocolate. A similar finding was reported by Kreiser & Martin [14].

Comparison among the types of Chocolate

The theobromine and caffeine contents of the chocolate coating using chocolate couverture and substitute are summarized in Table 7. Chocolate couverture produced from cocoa liquor to which sugar and cocoa butter have been added. However, vegetable fats have been added to produced chocolate substitute as a fat replacer of cocoa butter. Chocolate substitute contained cocoa powder, natural fats subsitute, sugar, milk and emulsifier. Mean values for theobromine and caffeine content for all chocolate coating were 0.82 and 0.07 mg/g respectively. The chocolate coating made from fat substitute had theobromine and caffeine levels that ranged from 0.36 – 0.70 mg/g and 0.027-0.061 mg/g respectively, with mean values of 0.49 mg theobromine/g and 0.039 mg caffeine/g. It is clear that with increasing amounts of cocoa powder added to the chocolate coating, the levels of theobromine and caffeine increased in the final chocolate product. Tables 8, summarized the mean values of theobromine and caffeine contents in different types of chocolates namely, dark chocolate (including bittersweet chocolate), milk chocolate (including milk chocolate mixed with other ingredients) and white chocolate. It was found that the average theobromine and caffeine contents in local dark chocolate were 0.85 mg/g and 0.06 mg/g respectively compared to 1.05 mg/g and 0.12 mg/g in imported dark chocolate. Theobromine and caffeine contents in local milk chocolates were 0.73 and 0.04 mg/g as compared to 0.76 and 0.04 mg/g in imported milk chocolates. As for the white chocolate type, as there are no locally produced white chocolates. Theobromine and caffeine contents in the imported white chocolate were 0.737mg/g and 0.028mg/g respectively. This study showed that theobromine and caffeine levels in local chocolates were lower than those in the imported chocolate.

Table 7. Theobromine and caffeine levels in chocolates coating (couverture) [1]

Code	Origin	Sample	Theobromine (mg/g	Caffeine (mg/g)
Brand CC1	Malaysia	*chocolate couverture*	0.82±0.86[b]	0.082±0.09[a]
Brand CC2	Malaysia	*using cocoa butter*	0.87±0.74[b]	0.077±0.10[a]
Brand CC3	Malaysia		0.95±0.80[a]	0.089±0.09[a]
Brand CC4	Malaysia		1.03±0.95[a]	0.085±0.08[a]
Brand CC5	Malaysia		1.04±1.01[a]	0.093±0.11[a]
Brand CC6	Malaysia		0.84±0.65[b]	0.064±0.07[a]
Brand CC7	Malaysia		0.88±0.94[b]	0.062±0.09[a]
Brand CC8	Malaysia		0.97±0.94[a]	0.074±0.12[a]
Brand SC1	Malaysia	chocolate couverture	0.60±0.43[b]	0.050±0.06[a]
Brand SC2	Malaysia	using cocoa butter	0.40±0.39[b]	0.029±0.05[a]
Brand SC3	Malaysia	substitute	0.55±0.75[b]	0.043±0.08[a]
Brand SC4	Malaysia		0.69±0.38[a]	0.066±0.08[a]
Brand SC5	Malaysia		0.36±0.35[b]	0.027±0.03[a]
Brand SC6	Malaysia		0.70±0.38[a]	0.061±0.06[a]
Brand SC7	Malaysia		0.54±0.32[b]	0.037±0.06[a]
Brand SC8	Malaysia		0.65±0.72[ab]	0.039±0.06[a]

[1] *Values are means ± SD; n=3*

[2] *Within a column, values with same superscripts letters are not significantly different (p≥0.05)*

Table 8. HPLC analysis of theobromine and caffeine levels in chocolates [1]

Sample	Chocolates [1]		Chocolate using CB* [1]		Chocolate using CBS* [1]	
	Theobromine	Caffeine	Theobromine	Caffeine	Theobromine	Caffeine
Average (mg/g)	0.74	0.06	0.93	0.08	0.56	0.04
Range (mg/g)	0.36-1.04	0.03-0.09	0.82-1.04	0.06-0.09	0.36-0.70	0.03-0.07
CV (%)	3.64	5.49	3.73	4.84	3.56	5.52

[1] *Values are average ± SD; n=24*
* CB = cocoa butter; CBS = cocoa butter subsitute

Conclusion

The application of μ-Bondapak with Waters spectrophotometer Detector Model M440, wavelength of 280 nm and mobile phase methanol: acetic acid: water (20:1:79, v/v) to analyze of methylxanthine and polyphenol compounds from cocoa and chocolate products allows for rapid determinations with high reproducibility and linearity over a wide range of both compounds. From this study, it shows that the highest levels of theobromine and caffeine were in imported dark chocolate. Overall, local chocolates have lower amounts of theobromine and caffeine than imported chocolate. Imported chocolate have a higher level of caffeine compared to local chocolates.

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References

1. Beckett S.T., 1988. Industrial chocolate manufacture and use. Van Nostrand Reinhold Co. Inc., New York, NY.
2. Blauch J.L. and Tarka S.M., 1983. HPLC Determination of caffeine and theobromine in coffee, tea and instant hot cocoa mixes. *J Food Sci*. 48:745-748.
3. Burg A.W., 1975. How much caffeine in the cup? *Tea Coffee Trade J.* 1: 40-41
4. Cros, E., Villeneueve, F., Vincent, J.C., 1982. Evaluation of bietterness and astringency of polyphrnolic compounds in cocoa powder. *Food Chemistry*. 60(3) : 365-370
5. Eteng M.U., Eyong E.U., Akpanyung, E.O., Agiang M.A. and Aremu C.Y., 1997. Recent advances in caffeine and theobromine toxicities: A review. *Plant Foods for Human Nutrition.* 51: 231-243.
6. FDA. 1980. Caffeine: Deletion of GRAS status, proposed declaration that no prior sanction exists, and use on an interim basis pending additional study. *Food and Drug Admin*. *Fed*. Reg. 45: 69817
7. Gilbert R.M., 1981. Caffeine-Its identity, dietary sources and biological effects. *Nutr. Rev*. 36♋4): 97-106
8. Greenhow E.J. and Spencer L.E., 1973. Ionic polymerisation as a means of end-point, indication in non-aqueous thermometric titrimetry. IV. The determination of catecholamines. *Analyst* 98(168) : 485-92
9. Hadorn H., 1980. Theobromine, Caffeine and total alkaloid contents of cocoa mass. *CCB Review for Chocolate, Confectionery and Bakery*. 5(3): 26-28
10. Horowitz, W., Ed. 1980. Method of analysis of the asoociation of Official Analytical Chemists, AOAC: Washington, DC. Method 15.022
11. Hurst W.J., Kreiser W.R. and Martin R.A., 1980. The use of HPLC in the chocolate and confectionery industry. *Manufacturing Confectioner*. 60(8): 39-41.
12. Kim H. and Keeney P.G., 1983. Method of analysis for (-) epicatechin content in cocoa beans by high performance liquid chromatography. *J. Food Sci*. 48:548-551.
13. Kreiser W.R. and Martin R.A., 1978. High pressure liquid chromatographic determination of theobromine and caffeine in cocoa and chocolate products. *J. Assoc. Off. Anal.Chem. 61(6):* 1424-1427.
14. Kreiser W.R. and Martin R.A., 1980. High pressure liquid chromatographic determination of theobromine and caffeine in cocoa and chocolate products : A collaborative study. *J. Assoc. Off. Anal.* Chem. 63(30): 591-594.

15. Legator M.S. and Zimmeving S., 1979. Review of genetic effects of caffeine. *J Env Sc and Health*. 13: 135-138
16. Matissek R., 1997. Evaluation of xanthine derivatives in chocolate- Nutritional and chemical aspects. *J. Food Techno*. 205(3): 175-184.
17. McGee M.B., 1980. Caffeine poisoning in 8 year old female. *J. Forensic Sci*. 25: 29-36
18. Morris M.B. and Weinstein L., 1981. Caffeine and fetus: It trouble brewing? *Am J. Obstet. Gynecol*. 140: 607-619
19. Narod S.A., Desanjose S., Victoria C., 1991. Coffee during pregnancy: A reproductive haxard? *Am J Obstet Gynecol*. 164: 1109-1114
20. Newton J.M., 1979. Spectrophotometric determination of caffeine in coffee products: collaborative study. *J Assoc Off Anal Chem*. 62(4) : 705-8
21. Nightingle S.L. and Flamm W.G., 1983. Caffeine and health : Current Status. *Nutr. Update*. 1:4
22. Roberts H.R. and Barone J.J., 1983. Biological effects of caffeine: History and use. *J. Food Tech. 37(9): 40-43.*
23. Sommadossi J.P. and Cano J.P., 1981. Determination of a novel fluoropyrimidine, 5'-deoxy-5-fluorouridine, in plasma by high-performance liquid chromatography. *J Chromatog.*, 225(2) : 516-20
24. Strahl N.R., Lewis H., Fargen R., 1977. Comparison of gas chromatographic and spectrophotometric methods of determination for caffeine in coffees and teas. *J Agric Food Chem*. 25(2) : 233-5
25. Timbie D.J., Sechrist L. and Keeney P.G., 1978. Application of High Pressure Liquid Chromatographic To The study of Variables Affecting Theobromine and Caffeine Concentrations In Cocoa Beans. *J. Food Sci*. 43:560.
26. Zoumas B.L, Kreiser W.R. and Martin R.A., 1980. Theobromine and caffeine content of chocolate products. *J. Food Sci*. 45:314-316.