Improved High-Performance Liquid Chromatography Method to Determine Theobromine and Caffeine in Cocoa and Cocoa Products

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At present, the commonly used HPLC method for the analysis of caffeine and theobromine contents in aqueous cocoa extracts employs direct application of the extracts on the column. This practice gradually reduces the efficiency of the column and shortens its life. Also, this method gives inflated values due to interfering substances and difficulty in achieving baseline resolution. In the improved method, the interfering cocoa pigments are effectively removed by passing the aqueous extract through a Sep-pak C_{18} cartridge. Subsequent injection on a C_{18} reverse-phase column employing acetonitrile and water (20:80) as the mobile phase reduces the analysis time without affecting either resolution of the peak or the accuracy of caffeine and theobromine determination or achieving baseline resolution. Therefore, this method is ideally suited for rapid routine analysis of cocoa and its products.

Keywords: *Theobromine; caffeine; Cocoa; Sep-pak C_{18}; fermented cocoa beans; HPLC*

INTRODUCTION

Cocoa (*Theobroma cacao* L.) is a popular and important flavoring ingredient in the preparation of beverages, confectionery, ice cream, baked products, and general foods. The stimulating effect of cocoa is due to the presence of purine bases such as theobromine, caffeine, and theophylline. Theobromine is the major alkaloid of cocoa, present to the extent of 3.7% on a fat-free basis, and the caffeine content is about 0.2% (*1*). Trace amounts of theophylline (*2*) and salsolinol (*3*) have also been reported. The alkaloids of cocoa and its products are quantified by a number of methods such as spectrophotometric, titrametric, and HPLC techniques. The purine alkaloid content of defatted unsweetened chocolate is relatively constant at 3.2% (*4*). Determination of cocoa content is important in cocoa-based products, for this the theobromine and caffeine ratio in cocoa and cocoa products can be used as the criteria in calculating the cocoa content.

In 1921, Wadsworth (*5*) developed a titramatric method for the determination of theobromine in cocoa. Gerritsma and Koers (*6*) improved this method by using chloroform in an ammonical medium, instead of trichloroethane, for the alkaloid extraction. Holmes (*7*) used water-boiled extracts clarified with lead acetate in determining purine bases by the titrametric method. Jalal and Collin (*8*) extracted alkaloids using chloroform and ammonia, but separated the cocoa alkaloids by TLC prior to spectrophotometric measurement. Senanayake and Wijesekera (*9*) extracted the alkaloids with chloroform using a Soxhlet extractor and estimated the alkoloid concentration by spot area method on TLC plates. Knapp and Wadsworth (*10*) and Senayake and Wijesekera (*11*) showed that variety, ripeness of fruit, and the fermentation process affect alkaloid content. The advantages of HPLC in the analysis of cocoa and cocoa products are its efficiency, sensitivity, and specificity as compared to the methods previously described for theobromine. Also, by using a HPLC technique the number of alkaloids can be quantified with a single run.

Kazi et al. (*12*) estimated the caffeine from tea by refluxing the material with MgO, passing the filtrate through a heavy MgO column, and injecting the elute onto a HPLC. Dultzky et al. (*13*) quantified the total caffeine and other purine alkaloids in coffee, tea, and cocoa by HPLC. This method involves refluxing the sample with water and MgO for 1 h in a tector digestion tube (normally used in determining nitrogen), followed by extraction with solvent, and subjection to HPLC. Kreiser and Martin (*14*) developed a HPLC method to determine theobromine and caffeine from cocoa and chocolate products. This method involves injection of a hot-water extract on HPLC reverse-phase C_{18} column using a mobile phase of methanol/water/acetic acid (20:79:1). Timbie et al., Liang et al., and Watanabe et al. (*1*, *15*, *16*) have also developed HPLC methods for caffeine estimation in cocoa, tea, and their products. These methods involve refluxing of samples with water and aqueous alcohol, filtration, and HPLC.

Blauch and Stanley (*17*) have described a simple and accurate method for the determination of caffeine in coffee, tea, and cocoa beverages. Blauch's method involves sample preparation by adding 3.0 g instant cocoa mix to 125 mL of boiling water and mixing with a magnetic stirrer for 30 min. The following HPLC conditions were used: (1) HPLC model M-45 solvent delivery system (Waters, Milford, MA) and model 7125 sample injection system (Rheodyne Inc. Berkeley, CA) with a 25-μL sample loop; (2) A Waters μ-Bandapak C_{18} column (3.9 mm × 30 cm); (3) mobile phase of acetonitrile and water (8:92); (4) Waters model 450 variable wavelength detector at a sensitivity of 0.04 AUFS and a wavelength of 245 nm. However, in this method the extract was directly injected without removing the pigments, resulting in shortening of column life and inaccuracy in

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quantitation due to interfering substances (*17*). Assessment of the accuracy of published data on the alkaloids of cocoa is complicated because of the disparity in analytical methods employed. The most widely used method of determination is spectrophotometry which gives inflated values due to interfering substances (*19*).

All the HPLC methods reviewed suffer from drawbacks of sample preparation, long analysis time, or tedious or limited applications (only caffeine or only theobromine). Pura Naik and Nagalakshmi (*18*) have effectively removed the interfering pigments by passing the sample through a Sep-pak C_{18} cartridge, but they restricted their study to estimating the soluble caffeine in tea brew which is actually consumed.

Accurate and rapid methods are required for the determination of total theobromine and caffeine in cocoa and cocoa products. The main object of this study is to consolidate these methods to develop a quick and reproducible method for the routine analysis of theobromine and caffeine in cocoa and cocoa products.

MATERIALS AND METHODS

Samples. Samples of fermented and dried cocoa beans were procured from the cocoa drying yard of M/S Cadbury (India), Mysore, and M/S Campco, Puttur, both in Karnataka State, India. Samples of low- and high-fat content cocoa powders (LFCP and HFCP) and cocoa-based products (Chocos flakes) were obtained from the Mumbai market, India.

Equipment. A Sep-pak C_{18} cartridge was obtained from M/S Waters Associates, London, and a Millipore filter Type FH (pore size of 0.5 μm) was obtained from M/S Millipore (India) pvt. ltd.

A Shimadzu HPLC solvent-delivery system controller (model LC-6A) with system controller (model SCL-6A) was used. The injection system used a 20-μL sample loop. Detection was by a 9 UV−Visible spectrophotometer (SPO-6 AV) set at a sensitivity of 0.08 AU FS and a wavelength of 276 nm. A 5-μm C_{18} reverse-phase column pore size A:80, 250 × 4.6 mm size, cartridge type, was used to separate the theobromine and caffeine. The data processor (model CR-4A chromatograph) was set at a chart speed of 2.5 mm/min. The mobile phase consisted of acetonitrile and water (20:80; v/v) at a flow rate of 1 mL/min.

Chemicals. Methanol, chloroform, and acetonitrile, all of GR grade and obtained from M/S Merck (India) Ltd. were used. Solvents were distilled and filtered through a 0.5-μm filter and degassed under vacuum prior to use. Water used was double-distilled in an all-glass apparatus.

Preparation of Standard Caffeine Stock Solution. A stock solution was prepared by dissolving 80 mg of caffeine (BDH) in 100 mL of water to give a concentration of 0.8 mg/mL.

Preparation of Working Standard Caffeine Solution. A working standard was prepared by diluting 10 mL of the caffeine stock solution to 100 mL with water to give a concentration of 0.08 μg/μL.

Preparation of Standard Theobromine Stock Solution. A stock solution was prepared by dissolving 10 mg of theobromine (BDH) in 100 mL of water to give a concentration of 0.1 mg/mL.

Preparation of Working Standard Theobromine Solution. A working standard was prepared by diluting 80 mL of theobromine stock solution to 100 mL with water to give a concentration of 0.08 μg/μL.

Calibration Graph. Working standard solutions (5−20 μL) of caffeine and theobromine were injected onto the HPLC, and peak area responses were obtained. Linear standard curves for caffeine and theobromine were obtained separately by plotting concentration versus area.

Sample Preparation. *Fermented and Dried Beans.* Cocoa nibs were separated from shells and dried in an oven at 105 ± 2 °C for 5 h. They were coarsely ground in a hand-operated mill (Husqvarna, made in Sweden) and extracted in a Soxhlet using petroleum ether (40−60 °C) for 16 h. Then they were ground in a Braun dry grinder to a 200-mesh powder. A subsample of 0.20 g was accurately weighed into a 250-mL flat-bottomed flask, 40 mL of water was added, and the mixture was set to gentle reflux for 30 min. The extract was filtered through a cotton plug, cooled, made up to 50 mL with water, and filtered through Whatman no. 44 paper. The filtrate (2 mL) was subjected to cleanup as described below.

Cocoa Powder. Ten g of cocoa powder was extracted to fat-free in a Soxhlet extractor. This defatted cocoa powder (0.2 g) was extracted as described in the sample preparation section, and 2 mL of this extract was subjected to the cleanup procedure.

Chocos Flakes. Chocos flakes samples were coarsely ground in a hand-operated mill and made moisture- and fat-free as described in the sample preparation section. Then these defatted samples were ground in a Braun dry grinder to a 200-mesh powder. A subsample of 1 g was extracted as described in the sample preparation section, and 2 mL of this extract was subjected to the cleanup procedure.

Cleanup Procedure. A Sep-pak C_{18} cartridge was preconditioned by first passing 2 mL of methanol by means of a 5-mL glass syringe. Then an empty syringe was used to pass air through the cartridge to expel any remaining methanol. The cocoa extract (1−2 mL) was then passed through the cartridge and the elute was rejected. The column was then washed with 5 mL of water. Again, air was passed to expel any remaining water. Theobromine and caffeine were eluted from the cartridge with 10 mL of chloroform and collected in an evaporation flask (50 mL). The chloroform was removed on a water bath under vacuum. The residue in the flask was dissolved in water and made up to 4 mL. An aliquot (5−10 μL) of this solution was injected onto the HPLC column.

Recondition of Sep-pak C_{18} Cartridge. After use, the cartridge was washed with 80% (v/v) methanol in water (5 mL) and then with 2 mL of methanol for further use.

Recovery Studies. To verify the accuracy and precision of the sample cleaning procedure, the recovery studies were carried out for individual compounds and for their mixture.

Caffeine Recovery. Caffeine stock solution (0.5, 1.0, 1.5, and 2.0 mL) was passed though a preconditioned Sep-pak C_{18} cartridge. The aqueous elute was rejected. Caffeine was eluted from the cartridge with 10 mL of chloroform. The chloroform was removed on a water bath under vacuum. The residue in the flask was dissolved in 2 mL of water and made up to 4 mL. An aliquot (5−20 μL) of this solution was injected onto the HPLC column. The percentage of caffeine was calculated by comparing the values with the calibration curve. Eight replicate estimations were done to express the data as range, mean, and standard deviation.

Theobromine Recovery. Theobromine stock solution (0.5, 1.0, 1.5, and 2.0 mL) was passed through a preconditioned Sep-pak C_{18} cartridge. The aqueous elute was rejected and the theobromine was eluted with 10 mL of chloroform. The chloroform was removed on a water bath under vacuum. The residue in the flask was dissolved in 2 mL of water and made up to 4 mL. An aliquot (5−20 μL) of this solution was injected onto the HPLC column. The percentage of theobromine was calculated by comparing the values with the calibration curve. Eight replicate estimations were done to express the data as range, mean, and standard deviation.

Theobromine and Caffeine Recovery from their Mixture. Theobromine and caffeine stock solutions, 80 and 10 mL, respectively, were taken in to 100-mL volumetric flask and made up to volume to get a concentration of each 0.08 mg/mL. This solution (0.5, 1.0, 1.5, and 2.0 mL) was passed through a preconditioned Sep-pakC_{18} cartridge. The aqueous elute was rejected and the cartridge was eluted with 10 mL of chloroform. The chloroform was removed on a water

Table 1. Content of Theobromine and Caffeine in Fermented and Dried Cocoa Beans Samples[a]

	theobromine (%)				caffeine (%)		
sample	without Sep-pak C_{18} treatment	after Sep-pak C_{18} treatment	Koer and Gerritsma method	± SE (15 df)	without Sep-pak C_{18} treatment	after Sep-pak C_{18} treatment	*t* value (10 df)
i	2.10^b	1.82^a	1.80^a	0.0365	0.21^x	0.21^x	0.00NS
ii	1.75^b	1.51^a	1.55^a	0.0365	0.20^x	0.24^y	8.33**
iii	1.90^b	1.70^a	1.65^a	0.0365	0.25^x	0.26^y	16.67**
iv	1.55^b	1.40^a	1.40^a	0.0365	0.20^x	0.25^y	13.89**
v	2.30^b	2.00^a	2.10^a	0.0365	0.21^y	0.20^x	2.78*
vi	2.00^b	1.85^a	1.80^a	0.0365	0.29^y	0.28^x	2.78*
vii	2.40^b	2.02^a	2.17^a	0.0403	0.22^y	2.17^x	2.78*
viii	2.20^b	2.00^a	1.95^a	0.0365	0.21^y	1.95^x	2.78*

[a] SE, standard error of means; df, degrees of freedom. *$p \leq 0.05$; ** $0.05 < p \leq 0.01$; NS, not significant. Means carrying different superscripts (a, b, or c in rows for theobromine and x or y in rows for caffeine) differ significantly ($0 < p \leq 0.05$).

bath under vacuum. The residue in the flask was dissolved in 0.5, 1.0, 1.5, and 2.0 mL of water, respectively. An aliquot (5, 10, 15, and 20 μL) of this solution, as well as a mixture of these standards before treatment, was injected onto the HPLC coloumn. The percent of theobromine and caffeine recovered in three replicate estimations of the above, before and after the Sep-pak C_{18} treatment, was calculated by comparing the values with the standard calibration curves of theobromine and caffeine, respectively. These twelve sets of values were analyzed by *t* test for difference.

Statistical Analysis. Theobromine and caffeine contents in the cocoa beans and cocoa products were determined before and after cleaning through Sep-pak C_{18} cartridge. Also, the total theobromine content was determined by Geritsama and Koers' method (*6*). Six replicate estimations were done for each sample, and the data were analyzed by *t* test to compare two samples (caffeine) and by one-way analysis of variance followed by Duncan's new multiple range test (*20*) to compare three samples for statistical significance.

RESULTS AND DISCUSSION

The recovery of caffeine and theobromine by HPLC after passing through a Sep-pak C_{18} cartridge was in the range of 98.00−100.10 and 97.8−100%, with mean recoveries of 99.30 ± 0.72 and 98.94 ± 0.81%, respectively. The recovery of caffeine before and after passing the caffeine−theobromine mixture through a Sep-pak C_{18} cartridge was in the range of 97.78−100.59 and 95.83−100.04%, with mean recoveries of 99.14 ± 0.81 and 98.38 ± 0.1.41, respectively. The recovery of theobromine before and after passing through the Sep-pak C_{18} cartridge was in the range of 96.92−99.90 and 96.11−100.19%, with mean recoveries of 98.50 ± 0.94 and 98.65 ± 2.06, respectively. The twelve sets of values for percent recovery before and after passing through a Sep-pak C_{18} cartridge analyzed by *t* test showed $t = 0.9$ (10 df) for theobromine and $t = 0.72$ (10 df) for caffeine were statistically not significant; confirming that the recoveries were significantly comparable ($0 < p \leq 0.05$). The chromatograms of standards of caffeine (a) and theobromine (b) and their mixture (c) are given in Figure 1. Considering the physiological effects of the quantity of caffeine and theobromine consumed, it is important to estimate the caffeine and theobromine content of cocoa and cocoa products. At present, HPLC is the best tool for the determination of purine alkaloids in cocoa extract on a C_{18} reverse-phase column using a UV detector. The solubility of caffeine and theobromine is very much related to temperature (*21*). The pigments present in the filtrate reduce the life of the HPLC column and interfere in the analysis if they are not removed prior to injection onto the HPLC (*18*). It was observed that achievement of good resolution of the caffeine and theobromine peak was difficult because of the presence of interfering cocoa pigments in the extract when nonpurified cocoa extract was injected onto the HPLC column (Figure 2a). This problem was solved by standardizing a sample preparation procedure described earlier. This modified method gave good resolution and sharp peaks for caffeine and theobromine without affecting the resolution or accuracy of the determination (Figure 2b). The caffeine and theobromine retention times of 3.193 and 5.091 min obtained by using a C_{18} reverse-phase 250 × 4.6 mm size column and a mobile phase of acetonitrile/water (20:80) demonstrate the

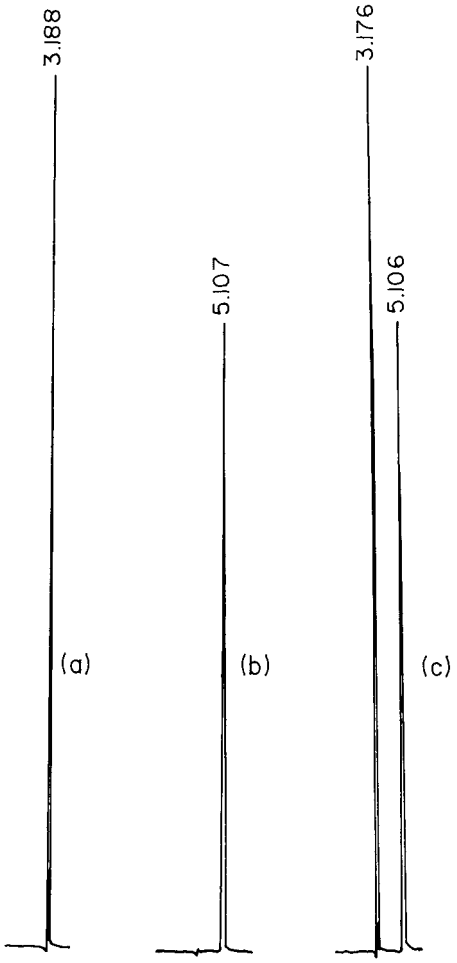

Figure 1. Chromatograms of (a) theobromine standard solution of 8 mg/100 mL (RT 3.188 min); (b) caffeine standard solution of 8 mg/100 mL (RT 5.107 min); and (c) theobramine and caffeine mixture solution of 8 mg/100 mL each (RT 3.176 and 5.106 min).

Table 2. Content of Theobromine and Caffeine in Cocoa Products[a]

	theobromine (%)				caffeine (%)		
sample	without Sep-pak C_{18} treatment	after Sep-pak C_{18} treatment	Koer and Gerritsma method	± SE (15 df)	without Sep-pak C_{18} treatment	after Sep-pak C_{18} treatment	*t* value (10 df)
LFCP							
i	2.33^{b}	2.03^{a}	2.00^{a}	0.0351	0.25^{y}	0.23^{x}	5.56**
ii	2.25^{c}	2.00^{b}	1.85^{a}	0.0365	0.20^{y}	0.18^{y}	5.56**
HFCP							
i	2.46^{b}	2.00^{a}	1.98^{a}	0.0365	0.17^{x}	0.20^{y}	8.33**
ii	2.50^{c}	2.30^{a}	2.35b	0.00365	0.15^{y}	0.14^{x}	2.78*
chocos							
i	0.12^{a}	0.11^{a}	0.11^{a}	0.0036	0.01^{x}	0.011^{x}	2.78*
ii	0.13^{b}	0.12^{ab}	0.11^{a}	0.0036	0.01^{y}	0.008^{x}	5.56*

[a] SE, standard error of means; df, degrees of freedom. * $p \leq 0.05$. ** $0.05 < p \leq 0.01$. Means carrying different superscripts (a, b, or c in rows for theobromine and x or y in rows for caffeine) differ significantly ($0 < p \leq 0.05$).

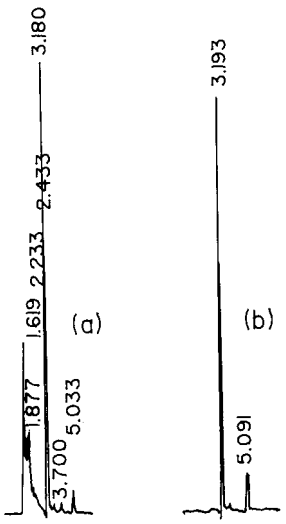

Figure 2. Chromatograms of (a) cocoa sample before Sep-pak C_{18} cartridge treatment (RT 3.180 and 5.033 min) and (b) cocoa sample after Sep-pak C_{18} cartridge treatment (RT 3.193 and 5.091 min).

rapid analysis time of this method. The aqueous extracts before and after passing through the Sep-pak C_{18} cartridge were screened for their resolution and accuracy for the determination of caffeine and theobromine. Caffeine and theobromine results from both the methods of analysis in fermented and dried cocoa beans and its products are presented in Tables 1 and 2, respectively. The theobromine values obtained by Gerritsma and Koers (*6*) method and in the improved method compare well, but significantly higher values were obtained when the cleanup procedure was not followed, perhaps because of interfering pigments and unstable baseline. Caffeine values were comparable as there was no clear-cut pattern of difference with Sep-pak C_{18} cartridge treatment.

CONCLUSION

This modified method is ideally suited to the rapid routine analysis of a large number of cocoa samples. This study demonstrates the potential of using a Sep-pak C_{18} cartridge for the purification of cocoa extract before injection onto a HPLC C_{18} reverse-phase column. With this method good reproducibility of the results is established. Also, the modified method increases the column life and reduces the analysis time. More than 25 samples can be prepared using a single Sep-pak C_{18} cartridge by following the cleanup procedure carefully.

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