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Research Article

Study on determination of caffeine and theobromine in beverages, beverage ingredients, and pharmaceuticals by high-performance liquid chromatography with diode array detection (HPLC-DAD)

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Abstract

A simple and reliable analytical method for the determination of caffeine and theobromine in beverages (e.g., cola drinks, bottled tea drinks, energy drinks), beverage ingredients (e.g., cocoa powder, green coffee beans, roasted coffee, matcha powder, bagged tea), and pharmaceuticals (e.g., analgesic and antipyretic drugs) was developed and validated by using high-performance liquid chromatography with diode array detection (HPLC-DAD). The cola and energy drinks were degassed, diluted, and filtered before analysis, while a clean-up step utilizing magnesium oxide (MgO) was applied to the bottled tea drinks. The beverage ingredient samples were extracted with hot water and incorporated with MgO clean-up. The pharmaceuticals were ultrasonicated with a mixture of water/methanol (70:30, v/v). Caffeine and theobromine were completely separated on a C18 column (15 cm × 4.6 mm × 5 µm) with isocratic elution using mobile phase as water/methanol (70:30, v/v) mixture at a flow rate of 0.7 mL/min. Analytical signals of the two analytes were recorded at a wavelength of 272 nm. The instrumental method exhibited good precision (RSD < 5%), linearity (R^2 > 0.999 over concentration range of 5–500 mg/L), and adequate detection limits (LOD = 1 mg/L). The entire analytical methods for different sample matrices showed acceptable recovery (89–106%) and repeatability (RSD < 5%). The validated methods were applied to analyze caffeine and theobromine in 40 real samples. In most drink and drug samples, measured concentrations of caffeine were in good agreement with label claims (bias < 10%).

Keywords: *Caffeine, theobromine, beverage, pharmaceutical, HPLC-DAD.*

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1. INTRODUCTION

Caffeine and theobromine (Figure 1) are two xanthine alkaloids naturally existed in plants such as tea, coffee, and cocoa, resulting in their widespread occurrence in food and beverages [1]. In pharmaceutical industry, caffeine is mainly used as a eugeroic or as a mild cognitive enhancer, while theobromine is s used as a bronchodilator and as a vasodilator [2]. As a result, several studies have been conducted to determine concentrations of these compounds in beverage, good, and pharmaceutical samples by using different methods such as spectrophotometry [3], electrochemical analysis [4], capillary electrophoresis [5], and liquid chromatography [6-9]. Among these methods, high-performance liquid chromatography with spectrophotometric detection (i.e., ultraviolet-visible detector or diode array detector) has been frequently used to analyze caffeine and/or theobromine in food, beverage, and pharmaceutical samples [6-9].

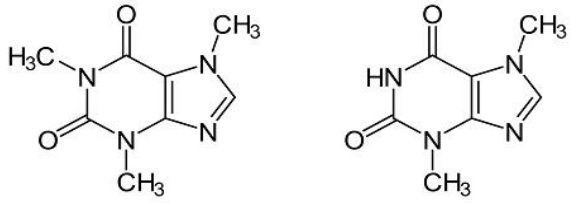

Figure 1. *Chemical and structural formulas of caffeine and theobromine*

There are some studies that investigated analytical methods for the determination of caffeine [10-13] or simultaneous analysis of caffeine and theobromine [14,15] in tea and coffee samples in Vietnam. To the best of our knowledge, no comprehensive study has been conducted to quantify caffeine and theobromine in different types of beverages, beverage ingredients, and pharmaceuticals. In the present study, analytical methods for the determination of caffeine and theobromine were developed, validated, and applied to three beverage types (i.e., cola drinks, bottled tea drinks, and energy drinks), five types of beverage ingredients (i.e., cocoa powder, green coffee beans, roasted coffee, matcha powder, bagged tea), and antipyretic-analgesic tablets. The samples were subjected to dilution (for liquid samples) or solid-liquid extraction (for solid samples), followed by quantification analysis on a reverse phase high-performance liquid chromatography with diode array detection system (RP-HPLC-DAD). Our results can provide simple and reliable analytical procedures for food and drug control purposes regarding caffeine and theobromine.

2. MATERIALS AND METHOD

2.1. Chemicals

Standard materials of caffeine and theobromine (≥ 98%) were supplied by the National Institute for Food Control (Vietnam) and Sigma-Aldrich (USA), respectively. Methanol Chrom AR® HPLC Super Gradient (≥ 99.9%) was purchased from Macron Fine Chemicals™ (Germany). Magnesium oxide (> 98.5%) was obtained from Xilong Scientific Co., Ltd. (China). Deionized water used in this study was purified by a WaterPro RO System (Labconco Cooperation, USA).

The stock standard solutions of caffeine (C-1000) and theobromine (T-1000) were prepared by dissolving 100 mg of each standard material described above in 100.0 mL methanol. These two stock standard solutions were used to prepare mixed working standard solutions at concentrations of caffeine and theobromine varying from 0.1 to 500 mg/L (i.e., TC-0.1, TC-0.2, TC-0.5, TC-1, TC-2, TC-5, TC-10, TC-20, TC-50, TC-100, TC-200, and TC-500). These solutions were stored in polypropylene tubes at 4°C.

2.2. Sample information

The samples were purchased from supermarkets, convenience stores, and pharmacies in Hanoi, Vietnam during February and May, 2024. A total of 40 samples were collected, including antipyretic-analgesic tablets (n = 11, abbreviated as D-1 to D-11), cola drinks (n = 5, CD-1 to CD-5), bottled tea drinks (n = 5, TD-1 to TD-5), energy drinks (n = 10, ED-1 to ED-10), cocoa powder (n = 2, CC-1 and CC-2), cocoa bean (n = 1, CC-3), green coffee bean (n = 1, CF-1), roasted coffee powder (n = 2, CF-2 and CF-3), bagged green tea (n = 1, T-1), matcha powder (n = 1, T2), and bagged black tea (n = 1, T-3). Label claims with caffeine concentrations were reported for drugs and some drinks (e.g., cola and energy drinks), which were described in Table 2. All the samples were stored at room temperature (about 25°C) until analysis. Some representative samples of different categories were selected for method optimization and validation purposes.

2.3. Instrumental conditions

A HPLC-DAD system (HPLC-10AD VP) from Shimadzu Corporation (Japan) was used to analyze caffeine and theobromine. A reverse phase column Inertsil® ODS-3 (15 cm length × 4.6 mm inner diameter × 5 µm; GL Sciences, Inc., USA) was used to perform chromatographic separation. Mobile phase consisted of water and methanol was operated at isocratic elution mode. Ultraviolet (UV) spectrum of caffeine and theobromine were recorded by using a photoLab 7600 UV-Vis spectrophotometer (Xylem Analytics, Germany. Investigations of different mobile phase compositions (ratios of water/methanol ranging from 80:20 to 50:50) and flow rates (ranging from 0.6 to 0.8 mL/min) were conducted to select proper HPLC conditions.

2.4. Instrument validation

With analytical parameters obtained from Section 2.3, the HPLC-DAD instrument was validated for several factors, including: signal stability, limits of detection (LOD), limits of quantification (LOQ), linearity, and calibration curves.

2.5. Sample preparation

The tablet drug samples were treated according to previous studies with some modifications [9,16]. Ten tablets of each type were weighed and average weight of one tablet was calculated. Ten tablets were then ground by using mortar and pestle. One hundred mg portions of the tablet powder were taken for extraction with 10-mL portions of three solvent types (methanol, water/methanol 70/30, and water) at different extraction times (10 min, 20 min, and 30 min) by using an ultrasonic water bath (WUC-32; 40 kHz; Shenzhen Jiayuanda Technology Co., Ltd., China). After ultrasonication, the sample tubes were centrifuged at 3500 rpm for 10 min. The extracts were diluted with deionized water before analysis.

Sample preparation procedures for solid beverage ingredient samples (i.e., cocoa, coffee, and tea) were referred from Vietnam Standards (i.e., TCVN 9723:2013 and TCVN 9744:2013) with some minor modifications. The solid sample was ground in a blender and 100 mg of sample was transferred into a 50-mL falcon tube with 500 mg MgO and 25.0 mL water. After mixing, the tube was heated at 90°C (using a constant temperature bath SB-1200, EYELA, Japan) for 20 min with regular mixing. The sample tube was then cooled to room temperature and the upper solution was taken for filtration (using filter paper and 0.22 µm nylon membrane) before analysis.

For tea drink samples, 5 mL of each sample were transferred into a 15-mL falcon tube with 1 g MgO. The tube was mixed well and heated at 90°C for 20 min. After cooling to room temperature, the sample solution was filtered through a filter paper and a 0.22 µm nylon membrane before analysis.

For cola and energy drink samples, about 40 mL of each sample was transferred into a 50-mL falcon tube and degassed for 10 min in the ultrasonic bath. The degassed sample solution was diluted ten times with water before injection to the HPLC-DAD system.

2.6. Method validation and application

The analytical methods were validated for specificity, recovery, and repeatability by analyzing blank, matrix-spike, and replicate samples for a solid matrix (green tea) and a liquid matrix (cola drink). The procedural blanks were prepared by using deionized water with equivalent amounts as real samples and treated by the same procedures. The matrix-spike samples were fortified with standard solution containing caffeine and theobromine before extraction. Recoveries were calculated by using the following equation: $R\% = (C_2 – C_0) \times 100\% \div C_1$. In which, C_0 and C_2 were measured concentrations of target compounds in non-spike and spiked samples, respectively, and C_1 was theoretical spiking level. The validated methods were then applied to analyze caffeine and theobromine in 40 real samples. Concentrations of target compounds in the samples were calculated by external standard method using calibration curves. Each sample was analyzed in triplicate to obtain average concentration.

3. RESULTS AND DISCUSSION

3.1. Instrumental conditions

The asorbances of single standard solutions of caffeine and theobromine at a concentration of 50 mg/L were measured over a wavelength range of 200 to 400 nm (Figure 2). As both compounds exhibited absoprtion peaks at 272 nm, this wavelength was selected to measure analytical signals of caffeine and theobromine in the HPLC-DAD system.

Chromatograms of theobromine and caffeine obtained by different mobile phase compositions (ratios of water and methanol of 80:20, 70:30, 60:40, and 50:50) and flow rates (0.6, 0.7, and 0.8 mL/min) are presented in Figure 3. Increasing methanol proportion may reduce retention times of both compounds and then reduce resolution. With the water/methanol ratio of 60:40, theobromine peak overlapped with vacant peak, while overlapping of vacant peak/theobromine peak and of theobromine peak/caffeine peak was

observed with the ratio of 50:50. Although peaks of theobromine and caffeine were well separated with two ratios (water/methanol 80:20 and 70:30), the water/methanol ratio of 70:30 was selected due to reasonable retention times and good peak shapes. The flow rate of 0.7 mL/min was selected because peak broadening occurred with 0.6 mL/min flow rate and decreasing of peak areas was observed with 0.8 mL/min flow rate. Therefore, the mobile phase composing water and methanol with a ratio of 70:30 and a flow rate of 0.7 mL/min was selected for further experiments.

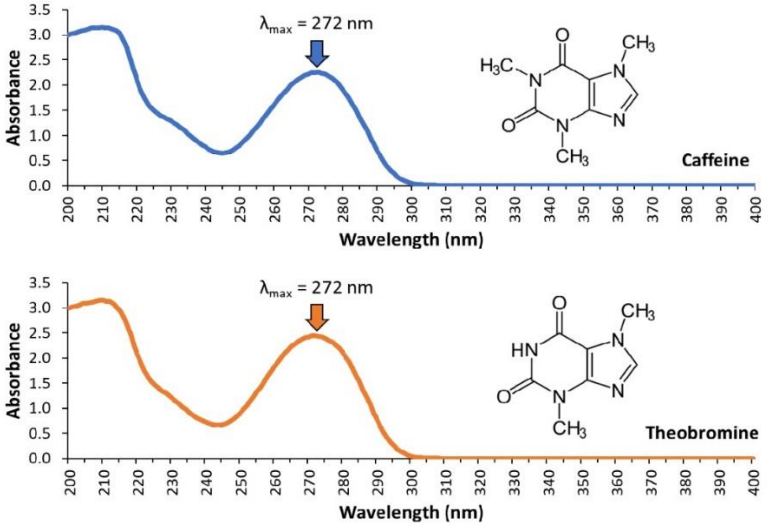

Figure 2. *UV spectrum of caffeine and theobromine at 50 mg/L*

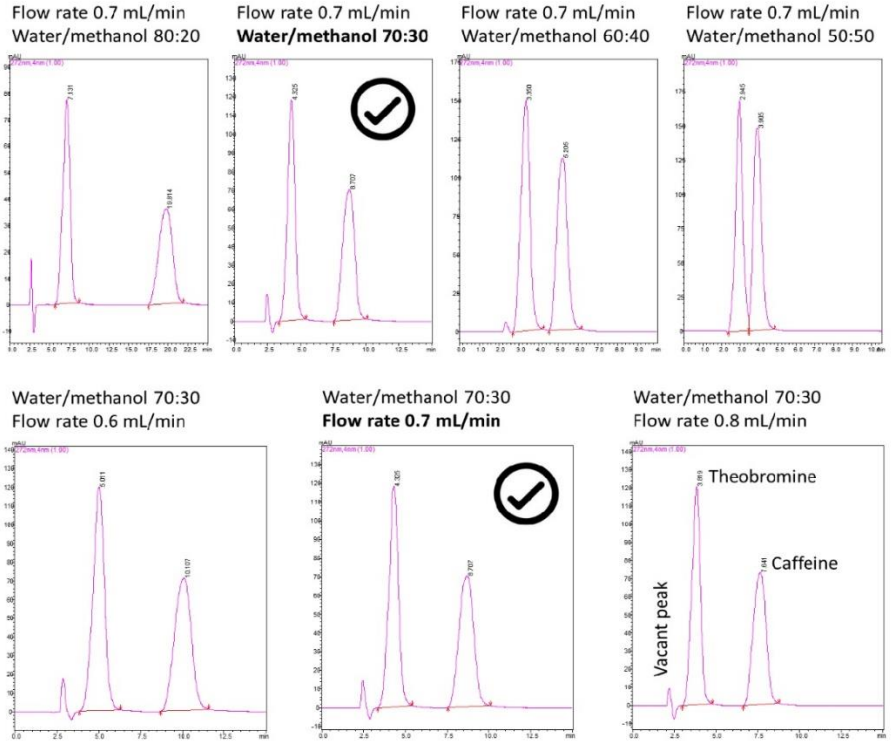

Figure 3. *Chromatograms of theobromine and caffeine obtained by different mobile phase compositions and flow rates*

3.2. Instrument validation results

The analytical conditions for theobromine and caffeine were assigned as follows: (1) injection volume 20 µL; (2) isocratic elution with water/methanol (70:30) at 0.7 mL/min; (3) detection wavelength 272 nm; (4) analysis time 15 min; (5) column temperature 40°C. This instrumental method was validated by analyzing mixed standard solutions of theobromine and caffeine with validation results shown in Table 1. These results indicated that the selected conditions are suitable and can be applied to analyze theobromine and caffeine in real samples.

Table 1. *Instrument validation results for HPLC-DAD analysis of theobromine and caffeine*

Parameter	Theobromine	Caffeine
Retention time (min)	4.258 ± 0.024 (RSD = 1%)	8.407 ± 0.063 (RSD = 1%)
Peak area variation (%)	RSD = 1% (at 50 mg/L)	RSD = 2% (at 50 mg/L)
LOD (mg/L)	1	1
LOQ (mg/L)	3	3
Calibration curve (5–500 mg/L)	Area = 49506 × C – 21258	Area = 44171 × C – 23998
R^2	0.9999	0.9999
Calibration curve bias (%)	< 7	< 5

3.3. Sample preparation conditions

The tablet samples were extracted with different conditions and recovery of caffeine (compared to label claim) was presented in Figure 4. Generally, there is no significant difference in caffeine recovery between these conditions, suggesting that caffeine can readily be dissolved in these solvents with assisted ultrasonication. The selected condition comprised water/methanol (70:30) mixture (same as HPLC mobile phase composition) with an extraction time of 20 min. The ratio of drug sample weight and solvent volume was assigned as 100 mg/10 mL. Extraction of drug samples with HPLC mobile phase has also been applied by previous studies on the determination of caffeine in pharmaceuticals [9,16].

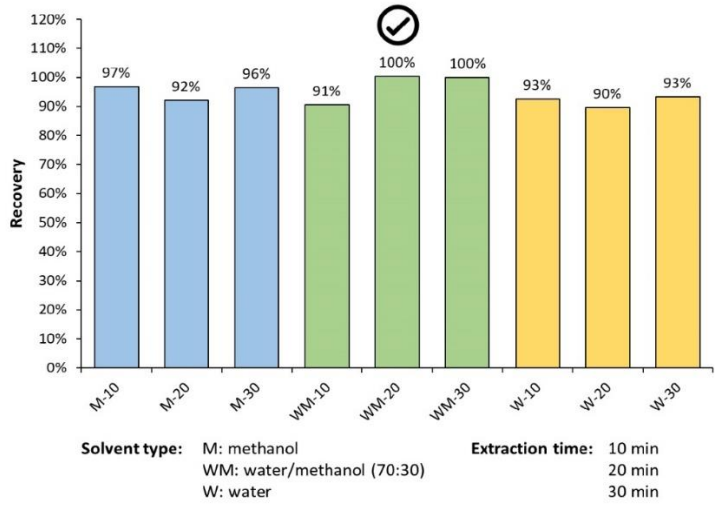

Figure 4. *Recovery of caffeine in tablet sample obtained by different extraction conditions*

The sample preparation conditions for tea, coffee, and cocoa mainly followed the procedures proposed by the Vietnam Standards (i.e., TCVN 9723:2013 and TCVN 9744:2013) except for a reduction of sample weight and solvent volume (from 1 g/250 mL to 0.1 g/25 mL). We also tested two conditions for preparation of bottled tea drinks: (1) direct analysis without clean-up with MgO; and (2) clean-up with MgO (1 g MgO for 5 mL sample) before analysis. In the earlier case, unbalance peak shapes of caffeine and high baseline levels were observed, which may prevent correct peak assignment and concentration calculation (Figure 5). Therefore, a clean-up step with MgO is necessary for both beverage ingredients and botted tea drinks. Meanwhile, degassing and dilution steps are only required for cola drinks and energy drinks.

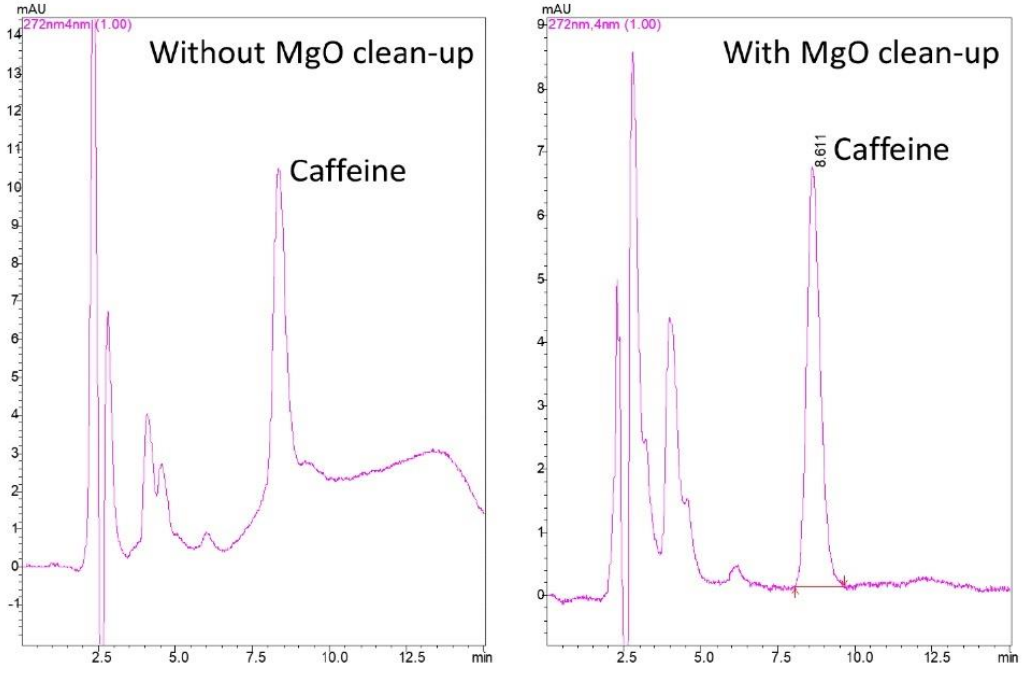

Figure 5. *Chromatograms of bottled tea sample with and without MgO clean-up*

3.4. Method validation results

The method validity was demonstrated by specificity, recovery, repeatability, and working range, which were demonstrated by analytical results of blank, matrix-spike, and replicate samples (Table 2). No peak of theobromine and caffeine was found in the chromatograms of procedural blank samples, confirming the method specificity and indicating that there is no potential contamination source of target compounds during analytical processes. Recoveries of theobromine and caffeine in matrix-spike samples ranged from 89% to 106% and relative standard deviations of repeated analysis were less than 5%. These results satisfied the Guidelines for Standard Method Performance Requirements specified by the AOAC International for method recovery (80–110% over concentration levels of 100 ppb to 10 ppm) and repeatability (RSD = 7.3% for concentration level of 10 ppm) [17]. The validated methods therefore can be applied to determine theobromine and caffeine at concentrations from ppm to percent levels in real samples.

Table 2. *Method validation results for HPLC-DAD analysis of theobromine and caffeine*

Parameter	Theobromine	Caffeine
Procedural blank	No peak found	No peak found
Green tea (n = 3) spiked with standards at level 10 mg/L	Recovery: 92–96% RSD = 2%	Recovery: 89–94% RSD = 3%
Cola drink (n = 3) spiked with standards at level 10 mg/L	Recovery: 95–103% RSD = 4%	Recovery: 101–106% RSD = 2%
Working range (LOQ–upper quantification level)	Tablet: 0.50–50 mg/g Solid: 1.0–125 mg/g Liquid: 5–500 mg/L	Tablet: 0.50–50 mg/g Solid: 1.0–125 mg/g Liquid: 5–500 mg/L

3.5. Analytical results of real samples

The analytical results of caffeine and theobromine in real samples of this study are tabulated in Table 3. Caffeine was measured in all the samples, while theobromine was found in 7 out of 40 samples (including three cocoa samples, one coffee sample, and three tea samples).

Concentrations of caffeine in tablet samples ranged from 9.1 ± 0.1 to 67.8 ± 1.5 mg/tablet, which were in accordance with the label claims (10 to 65 mg/tablet). Except for a tablet sample (D-7) with a bias of –12%, the remaining samples exhibited good agreement between measured and reference concentrations of caffeine, which satisfied generally accepted bias within ±10% for pharmaceutical analysis [18].

Table 3. *Concentrations of theobromine and caffeine in real samples*

Sample	Theobromine	Caffeine		
	Measured	*Measured*	*Label claim*	*Bias (%)*
Tablets (mg/tablet)				
D-1	Not detected (ND)	9.1 ± 0.1	10	–9.3
D-2	ND	23.2 ± 0.5	25	–7.3
D-3	ND	24.0 ± 1.0	25	–2.4
D-4	ND	25.0 ± 0.5	25	+0.2
D-5	ND	25.9 ± 0.6	25	+3.4
D-6	ND	47.5 ± 1.3	50	–5.0
D-7	ND	57.0 ± 1.0	65	–12
D-8	ND	65.5 ± 0.1	65	+0.7
D-9	ND	66.0 ± 0.6	65	+1.3
D-10	ND	67.0 ± 1.7	65	+3.1
D-11	ND	67.8 ± 1.5	65	+4.4
Cola drinks (mg/L)				
CD-1	ND	104 ± 3	96.97	+7.2
CD-2	ND	107 ± 1	96.97	+10
CD-3	ND	109 ± 2	107.34	+1.5
CD-4	ND	126 ± 1	–	–
CD-5	ND	133 ± 1	127.27	+4.5

Sample	Theobromine	Caffeine		
	Measured	*Measured*	*Label claim*	*Bias (%)*
Bottled tea drinks (mg/L)				
TD-1	ND	49.0 ± 1.0	–	–
TD-2	ND	52.0 ± 0.9	–	–
TD-3	ND	83.0 ± 1.1	–	–
TD-4	ND	98.0 ± 1.6	–	–
TD-5	ND	124 ± 2	–	–
Energy drinks (mg/L)				
ED-1	ND	82.0 ± 1.8	50–80	–
ED-2	ND	217 ± 1	145–255	–
ED-3	ND	245 ± 2	240	+2.0
ED-4	ND	249 ± 6	250	–0.4
ED-5	ND	293 ± 1	300	–2.3
ED-6	ND	298 ± 10	300	–0.6
ED-7	ND	301 ± 10	300	+0.3
ED-8	ND	308 ± 5	300	+2.6
ED-9	ND	319 ± 5	300	+6.3
ED-10	ND	331 ± 4	333.33	–0.6
Cocoa, coffee, and tea (mg/g)				
CC-1	23.0 ± 0.2	1.60 ± 0.07	–	–
CC-2	22.0 ± 0.1	1.91 ± 0.09	–	–
CC-3	13.9 ± 0.1	2.81 ± 0.05	–	–
CF-1	ND	10.9 ± 0.1	–	–
CF-2	ND	15.9 ± 0.2	–	–
CF-3	5.63 ± 0.06	12.4 ± 0.2	–	–
T-1	1.68 ± 0.07	29.1 ± 0.2	–	–
T-2	0.44 ± 0.04	13.4 ± 0.1	–	–
T-3	1.00 ± 0.20	26.7 ± 7.6	–	–

Concentrations of caffeine in drink samples ranged from 49 ± 1.0 to 331 ± 4 mg/L, showing a decreasing concentration order of energy drinks > cola drinks > bottled tea drinks. Good agreement was achieved between measured caffeine concentrations and label claims of energy drinks and cola drinks (bias within ±10%). Concentrations of caffeine in beverage ingredients decreased in the order of tea > coffee > cocoa. Meanwhile, theobromine levels in cocoa samples were markedly higher than those found in tea and coffee samples. Our results were in accordance with values previously reported for similar sample types from Vietnam [10-15] and other countries in the world [7, 8].

4. CONCLUSION

In this study, simple and reliable analytical procedures were developed, validated, and applied to determine caffeine and theobromine in 40 real samples, including cola drinks, bottled tea drinks, energy drinks, coffee, cocoa, matcha, bagged tea, and antipyretic-analgesic tablets. The HPLC-DAD instrumental method exhibited adequate detection limits,

accuracy, and linearity for caffeine and theobromine. The sample preparation protocols were quite simple, time- and chemical-saving, with acceptable recovery and repeatability. Our results indicated good agreement between measured caffeine concentrations and label claims of almost drink and tablet samples. Additional studies on the analysis of caffeine and theobromine should be conducted with a more comprehensive sample list including foods, functional foods, and beverages of unknown origin.

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