Simultaneous HPLC Determination of Caffeine, Theobromine, and Theophylline in Food, Drinks, and Herbal Products

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Abstract

A rapid and selective high-performance liquid chromatographic (HPLC) method is developed for the separation and determination of caffeine, theobromine, and theophylline. The chromatography is performed on a Zorbax Eclipse XDB-C8 column (4.6 × 150 mm i.d., 5-µm particle size) at 25°C, with a mobile phase of water–THF (0.1% THF in water, pH 8)–acetonitrile (90:10, v/v). The flow rate is 0.8 mL/min, and detection is by UV at 273 nm. This method permits the simultaneous determination of caffeine, theobromine, and theophylline in food, drinks, and herbal products with detection limits of 0.07–0.2 mg/L and recoveries of 100.20–100.42%. Correlation coefficients, for the calibration curves in the linear range of 0.2–100 mg/L, are greater than 0.9999 for all compounds. The within- and between-day precision is determined for both retention times and peak area. The data suggests that the proposed HPLC method can be used for routine quality control of food, drinks, and herbal products.

Introduction

Xanthine derivates caffeine (CF), 1,3,7-trimethylxanthine, theobromine (TB), 3,7-dimethylxanthine, theophylline (TF), and 1,3 -dimethylxanthine are widely found in the human diet. These compounds naturally occur in food products such as tea, coffee, and cocoa beans, with TB and CF being the two most abundant xanthines in chocolate. In recent years, xanthine derivatives have received an increased attention in the food and nutrition industry because they can cause various physiological effects. CF is used as a central nervous system, cardiac, and respiratory stimulant. TF and TB are widely used as smooth muscle relaxants. All three of these compounds can cause diuresis. The lack of reference procedures and well-characterized food-based products makes it difficult to accurately evaluate the dietary intake and resulting biological effects of these compounds on humans (1). Recently published methods reporting the determination of CF, TF, and TB in various sample mixtures cover a broad spectrum of the instrumental analysis. The most popular techniques for the determination of CF in different mixtures, especially in the recent reports, consist of high-performance liquid chromatography (HPLC) and its variants (1–9). Other methods include batch UV–vis spectrophotometry (10,11), thin-layer chromatography and its variants (1,12–14), ion chromatography (15), Fourier transform-Raman spectrometry (16), Fourier transform-infrared spectrophotometry (17), etc.

In most cases, however, these methods involve tedious and laborious pre-treatment steps before the chromatographic determination, long analyses time, or tedious or limited applications (only CF or only TB). All of the HPLC methods previously mentioned used C18 columns (1–9,18) to determinate xanthine derivates.

The main objective of this study is to produce a quick and reproducible method for the routine, simultaneous analyses of CF, TB, and TF in food, drinks, and herbal products.

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Materials and Methods

Materials

All solvents and reagents were of analytical grade unless indicated otherwise. The solutions were prepared with deionized water (Milli-Q-quality). The standards of CF, TB, and TF were obtained from Sigma (Deisenhofen, Germany). Acetonitrile and methanol (HPLC grade) were obtained from Sigma. Chloroform and tetrahydrofurane (THF) were HPLC grade, obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ).

Instrumentation and chromatographic conditions

The HPLC–diode array detection (DAD) model Agilent HP 1100 system equipped with an autosampler (Waldbronn, Germany) was used. The analytical column was the Zorbax Eclipse XDB-C8 (4.6 × 150 mm, i.d., 5-µm particle size). The mobile phase used was water–THF (0.1% THF in water, pH 8)–acetonitrile (90:10, v/v). The pH was adjusted with 0.1 M NaOH. The mobile phase was filtered (0.45-µm nylon filter), and the run time was 8 min, with a flow rate of 0.8 mL/min, and the

column temperature of 25°C. The analytes were detected at 273 nm.

The Supelclean LC-18 SPE cartridges 6 mL (0.5 g) used for solid-phase extraction (SPE) were obtained from Supelco (Bellefonte, PA). The SPE was performed in a 12-position Vacuum Manifold (Supelco).

Real samples

The natural products Indian tea, Green tea, Mate tea, Barcaffe Classic, Barcaffe Dekote, Barcaffe Light, Nescafe classic, and Cocoa Powder were obtained from Macval Tea D.O.O. (Novi Sad, Serbia), Droga (Portoroz, Slovenia), Nestle (Belgrade, Serbia), and Aleva (Novi Knezevac, Serbia), respectively. Different energy drinks and beverages such as Nestea Lemon, Cockta, Coca Cola, Coca Cola Light, Red Bull, Shark, Pepsi, Fast, Energis, Booster, and chocolate milk were obtained from Coca Cola Co. (Ljubljana, Slovenia), Palanacki Kiseljak (Smederevska Palanka, Serbia), Coca Cola HBC (Belgrade, Serbia), Red Bull GmbH (Vienna, Austria), Shark GmbH (Vienna, Austria), Pepsi-Cola Co. (Ljubljana, Slovenia), Max Co. (Novi Sad, Serbia), Nectar (Backa Palanka, Serbia), and Ljubljanske Mlekarne (Ljubljana, Slovenia), respectively. The baking chocolate Ideal and Milka chocolate were obtained from Pionir (Subotica, Serbia) and Kraft Foods (Bremen, Germany), respectively.

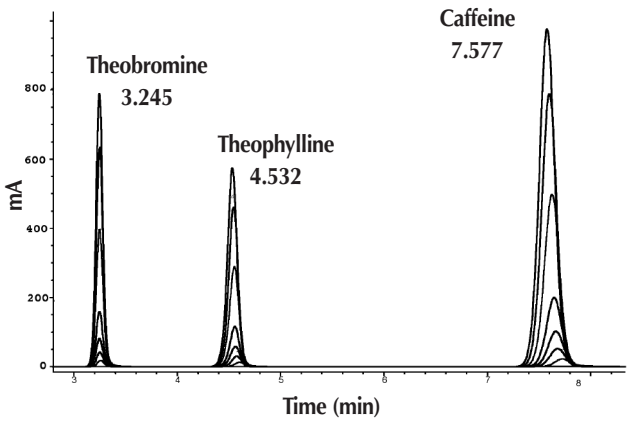

Figure 1. Chromatograms of working stock solution (0.2–10.0 µL injection volume) under the optimized conditions.

Preparation of standard stock solution

The standard stock solution of CF, TB, and TF was prepared by weighing 30 mg, 10 mg, and 10 mg of the standard substances, respectively, and dissolving in 10 mL water, pH 8 adjusted with 0.1 M NaOH. The solution was stable approximately 3 days under refrigeration (4°C).

Preparation of working standard solution

A working solution was prepared by diluting 100 µL of the stock solution to 1.00 mL with water, pH 8 adjusted with 0.1 M NaOH to give concentrations of CF, TB, and TF at 0.3, 0.1, and 0.1 µg/µL, respectively.

Calibration

The working standard solutions (0.2–10.0 µL) of CF, TB, and TF were injected into the HPLC, and the peak area responses were obtained. A method of the external standard calibration was used. The separation of the standard mixture of CF, TB, and TF using the method described is shown in Figure 1. The linear standard curves for CF, TB, and TF were obtained separately by plotting concentration versus area.

Sample preparation and extraction

The water extracts of an Indian tea (5.00 g), green tea (5.00 g), and mate tea (5.00 g) were made by mixing for 30 min in hot water (200 mL, first boiled) in a thermal flask on the magnetic stirrer. The extracts were then filtered through a filter paper to remove the particulate matter. Ten milliliters of filtrate, adjusted to pH 8 with 0.1 M NaOH, were subjected to the cleanup procedure as described later.

The coffee powder samples were weighed (5 g) and extracted with boiling hot water (200 mL) by mixing in the thermal flask for 5 min on the magnetic stirrer. The extracts were then filtered through the filter paper to remove the particulate matter. Ten milliliters of filtrate, adjusted to pH 8 with 0.1M NaOH, were subjected to the cleanup procedure as described later.

The cocoa powder (5 g), baking chocolate (8.85 g of crude

Table I. Study of Chromatographic Variables

Chromatographic conditions	t_R (min)			W_{50} (min)			N		
	TB	TF	CF	TB	TF	CF	TB	TF	CF
Effect of flow rate (mL/min) of the mobile phase water–THF (0.1% THF in water, pH 8)–acetonitrile (90:10, v/v)									
0.5	4.050	5.358	8.469	0.1264	0.689	0.6974	5689.55	335.02	816.97
0.8	3.249	4.552	7.626	0.0759	0.151	0.1671	10151.39	5034.55	11538.53
1.2	2.458	3.458	6.854	0.0608	0.103	0.1511	9054.54	6244.32	11399.10
Effect of mobile phase water–THF (0.1% THF in water, pH 8)–acetonitrile (0.8 mL/min)									
85:15	3.025	4.235	7.349	0.0719	0.139	0.1652	9806.25	5142.65	10963.42
90:10	3.249	4.552	7.626	0.0759	0.151	0.1671	10151.39	5034.55	11538.53
95:5	3.455	4.901	8.021	0.0854	0.181	0.1987	9067.55	4061.83	9027.57

powder), chocolate milk (25 mL), and Milka chocolate (8.22 g) were filled up to 200 mL with water in a plastic container and extracted for 30 min at 60°C in the ultrasonic bath. The extracts were then filtered through the filter paper to remove the particulate matter. Ten milliliters of filtrate, adjusted to pH 8 with 0.1M NaOH, were subjected to the clean-up procedure as described later.

The samples of fizzy drinks were degassed for 15 min in an ultrasonic bath to release the CO_2. Prior to the analysis, the samples were adjusted to pH 8 with 0.1M NaOH, filtered through a 0.22-µm nylon filter, and injected directly into the HPLC.

Table II. Statistical Parameters of the Calibration Curve for Each Compound (Linear Regression), with LODs and LOQs

	Theobromine	Theophylline	Caffeine
Linear range (mg/L)	0.50–100.00	0.40–100.00	0.2–100.00
Slope	3658.8	3806.0	3499.5
Intercept	15.988	13.271	48.733
Correlation coefficients (R^2)	0.9999	0.9999	0.9999
LOD (mg/L)	0.20	0.10	0.07
LOQ (mg/L)	0.50	0.40	0.20

Table III. Application Results for Natural Products, Beverages, and Food Samples*

Beverage and food samples	Found values			Declared values		
	TB	CF	TF	TB	CF	TF
Nestea lemon (mg/L)	< LOD	38.8	< LOD	n.d	n.d.	n.d.
Cockta (caffeine free) (mg/L)	< LOD	0.0	< LOD	n.d	0.0	n.d.
Coca cola (mg/L)	< LOD	106.7	< LOD	n.d	≤ 150	n.d.
Coca cola light (mg/L)	< LOD	118.0	< LOD	n.d	≤ 150	n.d.
Red bull (mg/L)	< LOD	307.9	< LOD	n.d	≤ 320	n.d.
Guarana (mg/L)	< LOD	237.0	< LOD	n.d	≤ 250	n.d.
Booster (mg/L)	< LOD	313.2	< LOD	n.d	≤ 320	n.d.
Energis (mg/L)	< LOD	238.4	< LOD	n.d	≤ 250	n.d.
Pepsi (mg/L)	< LOD	119.1	< LOD	n.d	≤ 150	n.d.
Shark (mg/L)	< LOD	348.7	< LOD	n.d	≤ 350	n.d.
Choco milk (mg/L)	225.8	< LOD	14.8	n.d.	n.d.	n.d.
Indian tea (mg/100 g of sample)	39.5	1013.0	< LOD	n.d.	n.d.	n.d.
Green tea (mg/100 g of sample)	32.0	1263.1	< LOD	n.d.	n.d.	n.d.
Mate tea (mg/100 g of sample)	98.3	1116.7	< LOD	n.d.	n.d.	n.d.
Barcaffe light (≤ 0.8%) (mg/100g of sample)	< LOD	719.8	< LOD	n.d	≤ 800	n.d.
Barcaffe dekofe (≤ 0.1%) (mg/100g of sample)	< LOD	71.2	< LOD	n.d.	≤ 100	n.d.
Barcaffe classic (≤ 2%) (mg/100g of sample)	< LOD	1328.5	< LOD	n.d	≤ 2000	n.d.
Nescaffe (mg/100 g of sample)	< LOD	3594.7	< LOD	n.d.	n.d.	n.d.
Cocoa (mg/100g of sample)	462.1	48.9	< LOD	n.d.	n.d.	n.d.
Baking chocolate (mg/100 g of sample)	1004.1	158.0	< LOD	n.d.	n.d.	n.d.
Milka chocolate (mg/100 g of sample)	100.4	5.6	< LOD	n.d.	n.d.	n.d.

* n.d. = not declared.

Cleanup procedure

The Supelclean LC-18 SPE cartridges were conditioned with 2 × 6 mL methanol, followed by 2 × 6 mL HPLC-grade water. The sample extracts were then passed through the SPE cartridges, washed with 6 mL HPLC grade water, air dried under a vacuum for 10 min, and the elutes were rejected. CF, TB, and TF were eluted from the SPE cartridges with 10 mL of chloroform into an evaporating flask. The solution was evaporated to dryness under nitrogen. The residue of all samples was reconstituted in 1 mL of water pH 8, except for the cocoa powder, chocolate, and chocolate milk, which were reconstituted in 2 mL. Prior to the analysis, the samples were filtered through a 0.22-µm nylon filter and injected on the HPLC.

Recovery study for the cleanup procedure

The solution for this study was prepared by diluting 100 µL of the stock solution to 10.00 mL with water, pH 8, and subjected to the cleanup procedure as described previously. The recovery study was carried out in five replicates. The recovery for CF, TB, and TF after the SPE extraction procedure was calculated.

Results and Discussion

Study of chromatographic variables

The development of the method was based on the experience obtained from the methods previously developed for the analysis of CF and some other compounds of interest (1–9). Of the columns tested (Hypersil ODS C18 100 × 4.6 mm, Cosmosil C18 150 × 4.6 mm, Cosmosil C18 250 × 4.6 mm, Phenomenex Luna 5 µm C8 150 × 4.6 mm, Phenomenex Luna 5 µm C8 250 × 4.6 mm, and Zorbax Eclipse XDB-5 µm C8 column 150 × 4.6 mm), it was only by using the Zorbax Eclipse XDB-5 µm C8 column 150 × 4.6 mm that a good separation of TB and TF was achieved, from each other as well as from CF.

The effect of the flow rate and the composition of the mobile phase on the retention time (t_R), the peak width (W_{50}), and a number of theoretical plates (N) for TB, TF, and CF were studied using the working standard solution. The injection volume of the working standard

solution was 5 µL, and the column temperature was at 25°C. The results (mean values of three injections) are shown in Table I. According to the results from Table I, the combination of 0.8 mL/min flow rate and water–THF (0.1 % THF in water, pH 8)–acetonitrile (90:10, v/v) as the mobile phase is selected as a compromise between the analyte retention time (sampling rate), separation efficiency (number of theoretical plates), and the consumption of solvents.

Three factors were considered when the pH of the mobile phase was chosen. Firstly, xanthines have to be in a stable form; secondly, the lifetime of the column stationary phase is reduced at the low pH; and finally, because of the similar chemical properties of all three compounds, pH is very important for a good separation. In view of these considerations, a pH value of 8 was chosen.

Validation of the HPLC method

The characteristics and procedures used for validation were those described in USP 24 (19) and in the International Conference of Harmonization Guidelines (Q2A, Q2B) (20,21). In addition, some other literature data were used (22).

We studied the selectivity (different samples with different matrices) and linearity in the range of 0.5 to 300 mg/L (0.5, 1.0, 3.0, 5.0, 10.0, 25.0, 50.0, 75.0, 100.0, 125.0, 150.0, 250.0, and 300.0 mg/L). Also studied were the results of the assay, and limit of detection (LOD—signal to noise [S/N] ratio 3:1), and limit of quantitation (LOQ—S/N ratio 10:1) of each compound were determined and shown in Table II. There was no interference in the HPLC results by the matrices ingredients in any of the tested samples, which indicates that the methods are selective (Figure 2).

The accuracy of the method was determined by analyzing the solutions of the known concentrations (the working standard solutions) and comparing the measured and known values. The mean recoveries for all compounds were in the range of 100.20–100.42% (n = 6 for each of the presented concentra-

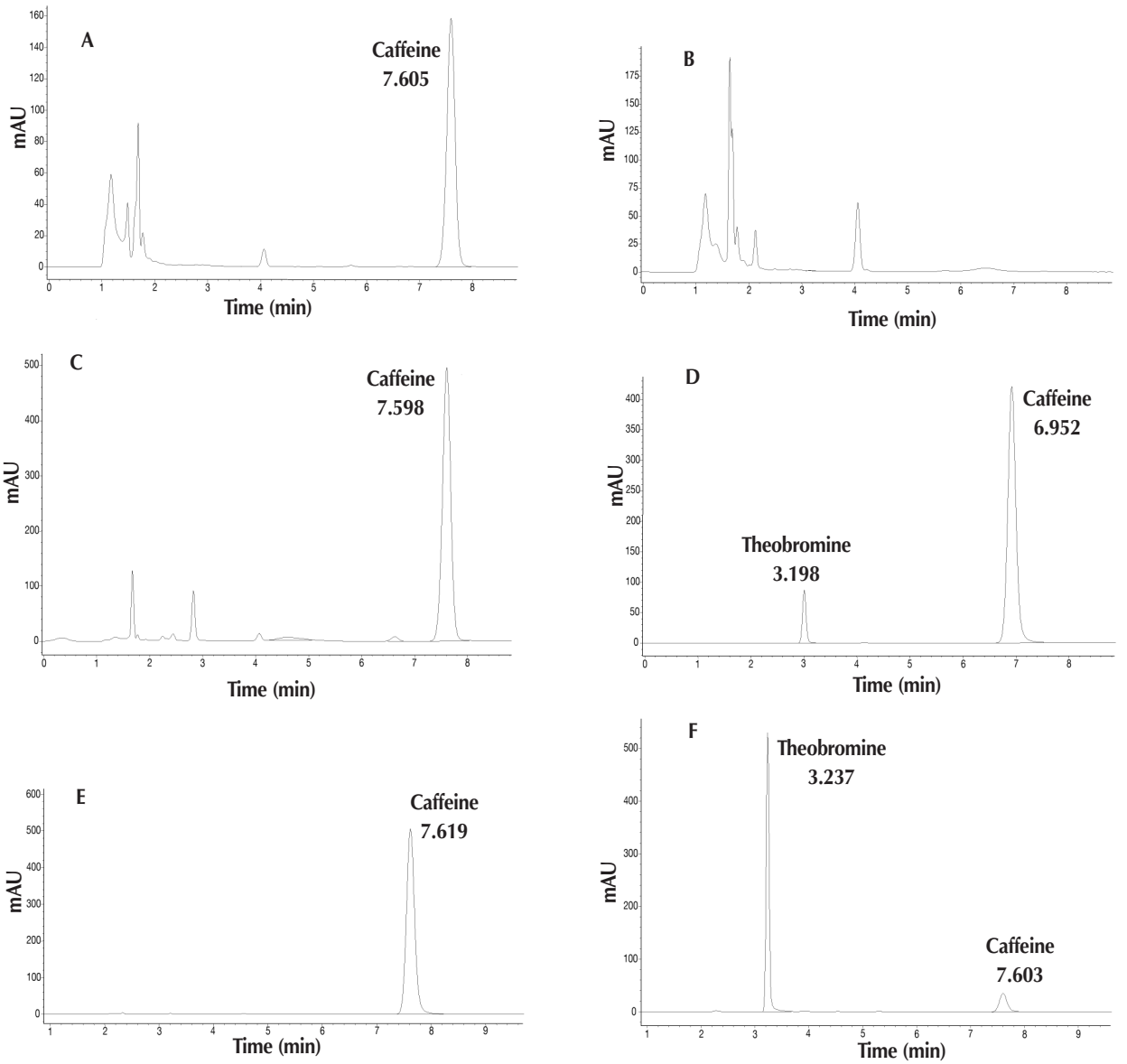

Figure 2. Chromatograms of some real samples under the optimized conditions, at 273 nm. Mobile phase was water–THF (0.1% THF in water, pH 8)–acetonitrile (90:10, v/v), flow rate was 0.8 mL/min, column temperature was 25°C: Coca cola (A), Cockta (caffeine free) (B), Red Bull (C), Mate tea (D), Barcaffe classic (E), Cocoa (F).

tions), proving a good accuracy of the method.

A repeatability test was performed to determine an intra-day variation in the peak's areas and retention times. The highest value for relative standard deviation (RSD) was 0.84% (n = 6), which indicates that repeatability of the method is acceptable.

An intermediate precision was evaluated over three days (inter-day repeatability) using the working solution. This solution (0.2–10.0 µL) was injected daily under the same conditions and the results were used for the repeatability study. The solution was stored at room temperature (25 ± 2°C) in sunlight, decreasing the recovery values approximately from 100.42 to 96.6% for all compounds. When stored in a refrigerator in the dark, the recovery ranged from 100.42% to 98.7% over three days for all compounds. The RSD values (0.11–0.78% for migration time and 0.80–2.06% peak area) indicate that the intermediate precision is acceptable.

The parameters of the optimum HPLC conditions were slightly modified in order to evaluate the robustness (22). The effect of different concentrations of THF (± 0.05%), as well as the effect of pH of the mobile phase (± 0.06), column temperature (± 1ºC), flow rate (± 0.05 mL/min), and detection wavelength (± 3 nm) were determined. No significant variations in specificity, accuracy, and precision were found over the tested ranges, which indicated a good robustness of the method (the RSDs were lower than 2.28% for the retention time and peak area).

Results of sample measurement

The contents of CF, TB, and TF obtained from the measurement of the numerous different samples of food, beverages, and natural products are shown in Table III. These results show a strong correlation between the declared and determined values of CF, TB, and TF for all analyzed samples, which implies a high efficacy and selectivity of the method used. The use of C8 column packing results in a better resolution, intensity, shape, and symmetry of the obtained peaks compared to C18 (1,2,7,8). The recoveries for CF, TB, and TF were 96.8 ± 0.92%, 93.7 ± 0.87%, and 92.00 ± 0.67%, respectively. The high values for the recovery imply an efficient extraction method and cleanup procedure. The efficiency of the extraction procedure used is also confirmed by the chromatograms, which are clean and without the presence of impurities of the matrix, no matter what type of sample is used. The run time for the analysis is less than 8 min. The lowest concentration that can be quantitated (LOQ) with an acceptable accuracy and precision was 0.5, 0.4, and 0.2 mg/L for TB, TF, and CF respectively. Furthermore, the LOD defined as S/N > 3 was 0.2 mg of TB /L, 0.1 mg TF /L, and 0.07 mg of CF /L. These findings are in a good correlation with literature (8).

Conclusion

The present method was tested to simultaneously measure the CF, TB, and TF in food, beverages, and natural products. In this work a fast, accurate, and sensitive method was developed for the determination of CF, TB, and TF in food, beverages, and natural products. The use of the SPE pretreatment for the samples and results of the recoveries for this procedure confirmed that there is no matrix effect, so the extracts can be assessed with a calibration curve set from the analytes aqueous standard. The use of Zorbax Eclipse XDB-C8 column allowed a simultaneous determination of the xanthine derivates in a short time (5). During the development of the method, approximately 400 sample injections were made, showing no signs of deterioration. Finally, the data for sensitivity, accuracy, reproducibility, and high analysis frequency suggest that the proposed HPLC method could be used for a routine quality control of food, drinks, and herbal products.

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