Determination of Caffeine and Associated Compounds in Food, Beverages, Natural Products, Pharmaceuticals, and Cosmetics by Micellar Electrokinetic Capillary Chromatography

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Abstract

A method is described for quantitating caffeine, theobromine, theophylline, paracetamol, propyphenazone, acetylsalicylic acid, salicylic acid, and codeine phosphate in corresponding real samples of food, beverages, natural products, pharmaceuticals, and cosmetic preparations by micellar electrokinetic capillary chromatography. The separation is carried out at 25°C and 25 kV, using a 20mM phosphate buffer (pH 9.0), 80mM sodium dodecyl sulfate, and 7.5% (v/v) acetonitrile. UV detection is at 210 nm. The method is shown to be specific, accurate (recoveries over the range 98.9–101.2%), linear over the tested range (correlation coefficients ≥ 0.9993), and precise (relative standard deviation below 2.1%). The method is applied for the quantitative analysis of these compounds in different foods, beverages, natural products, pharmaceuticals, and cosmetic products.

Introduction

Caffeine (CF), 1,3,7-trimethylxantine, is a well-studied compound because it has been found as a major alkaloid in approximately 60 herbs, including *Thea sinesis* (tea leaves), *Cola nitida* (cola nuts), and *Theobroma cacao* (coffee beans) (1). Today, CF is believed to be one of the most popular drugs in the world because we consume it on daily basis in various foods and beverages. Due to its pharmacological effects, CF can also be found as a common ingredient in pharmaceuticals. It is used as a central nervous system, cardiac, and respiratory stimulant. In modern painkillers and antimigraine pharmaceutical preparations, CF is associated with other compounds such as paracetamol (PA), propyphenazone (PR), acetylsalicylic acid (AS), codein phosphate (CP), and others (1,2).

Recently published methods reporting the determination of CF in various sample mixtures cover a broad spectrum of instrumental analyses. The most popular techniques for the determination of CF in different mixtures, especially in recent reports, comprise of high-performance liquid chromatography (HPLC) and its variants (2–10). Other methods include batch UV–vis spectrophotometry (11–13), thin-layer chromatography (TLC) and its variants (1,14–17), ion chromatography (18), Fourier transform-Raman spectrometry (19), Fourier transform infrared (FTIR) spectrophotometry (20,21), etc.

In most cases, however, these methods involve tedious and laborious pre-treatment steps before the chromatographic determination. Micellar electrokinetic capillary chromatography (MEKC) has been proven to be a more efficient, rapid, and simple method for these analyses, and has become an efficient technique for the analysis of drug mixtures and other samples. MEKC is a mode of capillary electrophoresis in which ionic micelles are used as pseudo-stationary phase. The addition of micelles in buffer solution, where the electrophoretic process takes place, allows the determination of neutral and charged analytes in a single injection.

Many applications of MEKC separations have now been reported, including on CF analysis and its determination in different mixtures comprising food, beverages, and pharmaceuticals. Denz et al. (22) performed a simultaneous determination of CF, PA, and PR by MEKC, whereas Kölhead at al. (23) successfully coupled MEKC to FTIR detection in order to separate and quantitate CF, PA, and other associated compounds. Gotti et al. (24,25) performed MEKC analysis of methylxantines (CF, theophylline [TF], theobromine [TB]) in chocolate, cacao, and

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Theobroma cacao beans. MECK analysis of catehines in green tea extract dietary supplements was carried out by Weiss et al. (26). Some other reports have also been published concerning the determination of CF in different complex mixtures by MEKC (27,28).

In this paper a novel, simple, and environmentally friendly method was developed for the simultaneous determination of CF and other associated compounds such as TB, TF, PA, PR, AS, salicylic acid (SA), and CP found in different food, beverages, pharmaceuticals, and cosmetic products. An optimization study of the technique variables such as buffer type, pH, buffer concentration, organic modifier, surfactant, capillary temperature, injection time, and running voltage was carried out. The validated method was applied to the analysis of different products containing different amounts and combinations of the associated compounds previously mentioned.

The aim of the work is to develop unique, robust, and acceptable MEKC method with a short preparation (pre-analysis) time for the routine analysis of CF and associated compounds from different real samples such as food, beverages, natural products, pharmaceuticals, and cosmetics.

Materials and Methods

Materials

All solvents and reagents were of analytical grade unless indicated otherwise. Solutions were prepared with deionized water (Milli-Q-quality). CF, TB, TF, PA, PR, AS, SA, and CP (USP quality) were obtained from Sigma-Aldrich Corp. (St. Louis, MO). Sulfacetamide sodium (SUL) was used as internal standard (IS) and was obtained from Vetprom A.D. Wholesale pharmacy (Belgrade, Serbia; EU5 quality)

Acetonitrile and methanol (HPLC grade) were obtained from Sigma-Aldrich Corp. Buffer solutions were prepared by dissolving the appropriate amount of NaH_2PO_4 and/or $Na_2B_4O_7$ in deionized water and the pH was adjusted with NaOH or HCl. $NaH_2PO_4 \times H_2O$ and $Na_2B_4O_7 \times 10H_2O$ were from Kemika d.d. (Zagreb, Croatia). Sodium dodecylsulphate (SDS) was from Riedel-de Haën AG (Seelze, Germany).

Equipment

The HP^{3D} Capillary Electrophoresis system (Hewlett Packard, Waldbronn, Germany) with a diode-array detector and controlled by HP ChemStation software was used to perform the reproducibility of the method. Compounds were determined on a 56 cm (50 cm to the detector) × 50 µm i.d. fused silica capillary (with bubble cell, 150 µm) (Agilent). A Consort C-831 pH meter (Turnhout, Belgium) was used for pH measurement.

Operating conditions

The capillary was conditioned prior to first use by flushing with 0.1M NaOH for 20 min and then with water for 10 min. The capillary was conditioned at the beginning of each day by flushing with methanol for 3 min, water for 1 min, and then rinsed for 2 min with 0.1M NaOH and 3 min with background electrolyte (BGE). This was followed by hydrodynamic sample injection at 250 mbars (injection time was 5 s; pressure was 50 mbar). Separations were performed at 25°C. UV detection was evaluated at 210 nm (TB, CF, TF, PA, AS), 215 nm (CF, PA, CP, AS, TF), 220 nm (TB, CF, TF, CP), 254 nm (CF, PA, PR), and 275 nm (TB, CF, TF). Detection at 210 nm was selected as suitable for all compounds.

The BGE was 20mM phosphate buffer, pH 9.0, containing 80mM SDS and 7.5% (v/v) acetonitrile.

Real samples

The natural products Indian tea, Green tea, Mate tea, Barcaffe classic, Barcaffe dekote, Barcaffe light, Nescafe gold, and Cacao powder were obtained from Droga Kolinska d.d. Food Industry (Ljubljana, Slovenia), Teekanne GesmbH (Salzburg, Austria), Aviva (Sandpoint, USA), Droga Portoroz Food Industry d.d. (Portoroz, Slovenia), Nestle Oesterreich GmbH (Vienna, Austria), and Kras d.d. Food Industry (Zagreb, Croatia), respectively. Different energy drinks and beverages such as Nestea lemon, Cockta, Coca Cola, Coca Cola light, Red Bull, Shark, Pepsi, and chocolate milk were obtained from Coca-Cola Beverages GmbH (Vienna, Austria), Droga Kolinska d.d. Food Industry (Ljubljana, Slovenia), Red Bull Gmbh (Vienna, Austria), Shark Gmbh (Vienna, Austria), Radenska d.d. (Radenci, Slovenia), and Ljubljanske mlekarne d.d. (Ljubljana, Slovenia), respectively. Baking chocolate was obtained from Mercator d.d. (Ljubljana, Slovenia).

The pharmaceutical formulations Caffetin tablet (PA 250 mg, PR 210 mg, CF 50 mg, CP 10 mg), Caffebol tablet (CP 10 mg, PA 460 mg, CF 50 mg), Oldon tablet (CF 50 mg, PA 200 mg, AS 300 mg), Teolin tablet (TF 125 mg), and Kombikaf (CF 50 mg, PA 400 mg—powder form in paper bag) were obtained from Alkaloid AD (Skopje, FYR Macedonia), ZorkaPharm-Hemofarm STADA group (Sabac, Serbia), Zdravlje Actavis company (Leskovac, Serbia), Lek d.d. a Sandoz company (Ljubljana, Slovenia), and Ivancic&Sinovi, pharmacy (Belgrade, Serbia), respectively. Perfect body firming gel produced by Oriflame (Dublin, Ireland) and magistral anti-cellulite gel with CF and SA obtained from Pharmacy Institution (Apotekarska ustanova in Serbian; Belgrade, Serbia) were used for analysis of cosmetics.

Preparation of standard stock solution

Standard stock solutions of all standards (including IS) were prepared by weighing 10 mg of the drugs and dissolving in 10 mL water–methanol (1:1, v/v). All solutions were stable approximately 3 days under refrigeration (4°C). The stock solutions were diluted in the concentration range of 0.5–300 mg/L with running buffer before use to obtain the concentration ranges required.

Sample preparation and extraction

Tea water extracts of Indian tea (1.75 g), green tea (1.75 g), and mate tea (2 g) were made by shaking them for 30 min in hot water (200 mL, first boiled) in a thermal flask. The extracts were then filtered though filter paper to remove particulate matter. Tea samples were then injected directly without pre-analysis dilution.

Coffee powder samples were weighed (5 g) and extracted with boiling hot water (100 mL) in a thermal flask for 5 min. The

extracts were then filtered though filter paper and injected directly without dilution.

The carbonated drink samples were degassed for 15 min in an ultrasonic bath. Ice tea and decarbonized drinks were filtered through a 0.22-µm nylon filter and injected directly, with the exception of Red Bull and Shark samples, which were firstly diluted with running buffer (1:1, v/v).

Cocoa powder (5 g), baking chocolate (5 g of crude powder), and chocolate milk (25 mL) were filled up to 50 mL with water–methanol (1:1, v/v) in a plastic thermos and extracted for 30 min at 60°C in an ultrasonic bath. The extracts were first filtered though filter paper and then though 0.22-µm nylon filter. Extracts were diluted (1:1, v/v) with running buffer before analysis.

First, 20 tablets or powder (for each product) were accurately weighed, finely ground to fine powder, and thoroughly mixed. Different aliquots of these powders (each) were weighed and transferred into a volumetric flask. They were extracted with 10 mL water–methanol (1:1, v/v), first by shaking, then in an ultrasonic bath for 15 min at room temperature. The extracts from each powder (tablets and powder form) were filtered (0.22 µm nylon filter), transferred to volumetric flasks, and filled up to 10 mL with water–methanol (1:1, v/v). Extracts were diluted (1:9, v/v) with running buffer before analysis.

Five grams of each cosmetic were filled up to 50 mL with water–methanol (1:1, v/v) in a plastic thermos and extracted for 30 min at 40°C in an ultrasonic bath. The extracts were first filtered though filter paper, then though 0.22 µm nylon filter, and diluted (1:4, v/v) with running buffer before analysis.

All samples were spiked before extraction with a different volume of the IS (for beverages and chocolate milk) or with a different amount of the solid form of IS (for tea, coffee, cocoa powder, baking chocolate, pharmaceuticals, and cosmetics) to give a final concentration of 50 mg/L.

Results and Discussion

Method optimization

To optimize the method, preliminary studies were carried out using different concentrations of standard stock solutions, for testing the influence of the pH (over the range of 6–10), organic modifier (methanol and acetonitrile were tested in concentrations from 0 to 20%), type and concentration of buffer (the borate and/or phosphate buffer molarity was varied from 5 to 50 mM), concentration of SDS (over the range of 10–100 mM), running voltage (5–30 kV), and capillary temperature (20–35°C).

The results show that the best separation was at pH 9.0 with BGE containing SDS (most common surfactant used in MEKC) and phosphate buffer. The results demonstrate that SDS concentration has an influence on the mobility of all compounds. A concentration of 80 mM was selected for the experiment to give the best shape, width, and symmetry of the peaks. Using BGE with borate buffer and a combination of borate and phosphate was difficult, because of very similar migration times of CF, PA, TB, and TF. A concentration of 20 mM of phosphate buffer (pH 9.0) was considered to give a suitable peak shapes and resolution.

The addition of some kind of organic modifier can be crucial for the optimization peak purity. Methanol was tested first, but separation was not providing good results for CF and TB. Then acetonitrile was selected as a potential modifier, and the presence of 7.5% (v/v) of acetonitrile in the BGE resulted in better separation and symmetry, and shoulders disappeared.

The effects of running voltages and capillary temperature were tested simultaneously. The best results were obtained at 25 kV, and an acceptable level of baseline noise was achieved by performing experiments at 25°C.

The electropherogram obtained in the separation under selected conditions is presented in Figure 1 (concentrations of all standards were 150 mg/L). In all cases (concentration ≤ 120 mg/L), peaks have good symmetry (0.9–1.1) and resolution ($R_s \geq 1.4$).

Some recently published papers have presented novel MEKC methods for the analysis of CF and some other compounds of interest such as PA, TF, and TB. Kolhed et al. (23) and Weiss et al. (26) presented results with longer migration times of each analyte than in our experiments. On the other hand, previously published papers described some very time-consuming and multifarious extraction procedures for the analysis of CF, TB, and TF in cacao powder and chocolate by MEKC (24,25). Chen et al. (27) developed a capillary electrophoretic determination of theanine, CF, and catechins in fresh tea leaves, but without successful separation of CF and TF.

The advantage of the newly presented MEKC method over published papers is the potential for analyzing CF and associated compounds in different matrices without interface and with good resolution of peaks up to concentration of 120 mg/L for

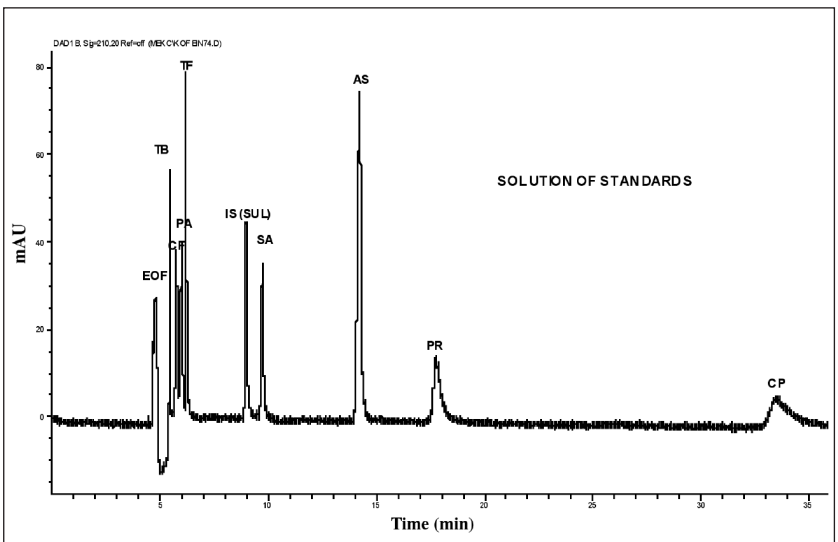

Figure 1. Electropherogram obtained for 150 mg/L of all standards under the optimized conditions, at 210 nm. The BGE was 20 mM phosphate buffer, pH 9.0, containing 80 mM SDS and 7.5% (v/v) acetonitrile; the temperature and voltage were 25°C and 25 kV, respectively.

each compound. With higher concentrations, separation of PA, CF, and TB is not possible because of higher width values, and according to that, R_s values are not acceptable (Figure 1).

Validation of the method

The characteristics and the procedures used for validation were those described in USP 24 (29) and in the International Conference of Harmonization Guidelines (Q2A, Q2B) (30,31). In addition, some other literature data were used (32,33).

We studied selectivity and linearity in range of 0.5 to 300 mg/L. Different samples with different matrices, blank samples such as cockta beverage (CF-free), milk, carbopol excipient gel, and tablet powder matrix (sodium dodecyl sulphate, lactose, magnesium stearate, povidone, microcrystalline cellulose, talc, sodium carboxymethyl starch, colloidal silica, calcium hydrogen phosphate dehydrate, croscarmellose sodium, and glyceryl behenate mixed in equal range) were analyzed. There was no interference in MEKC results by the matrices ingredients in any of the tested samples, which indicates that the methods are selective (Figure 2).

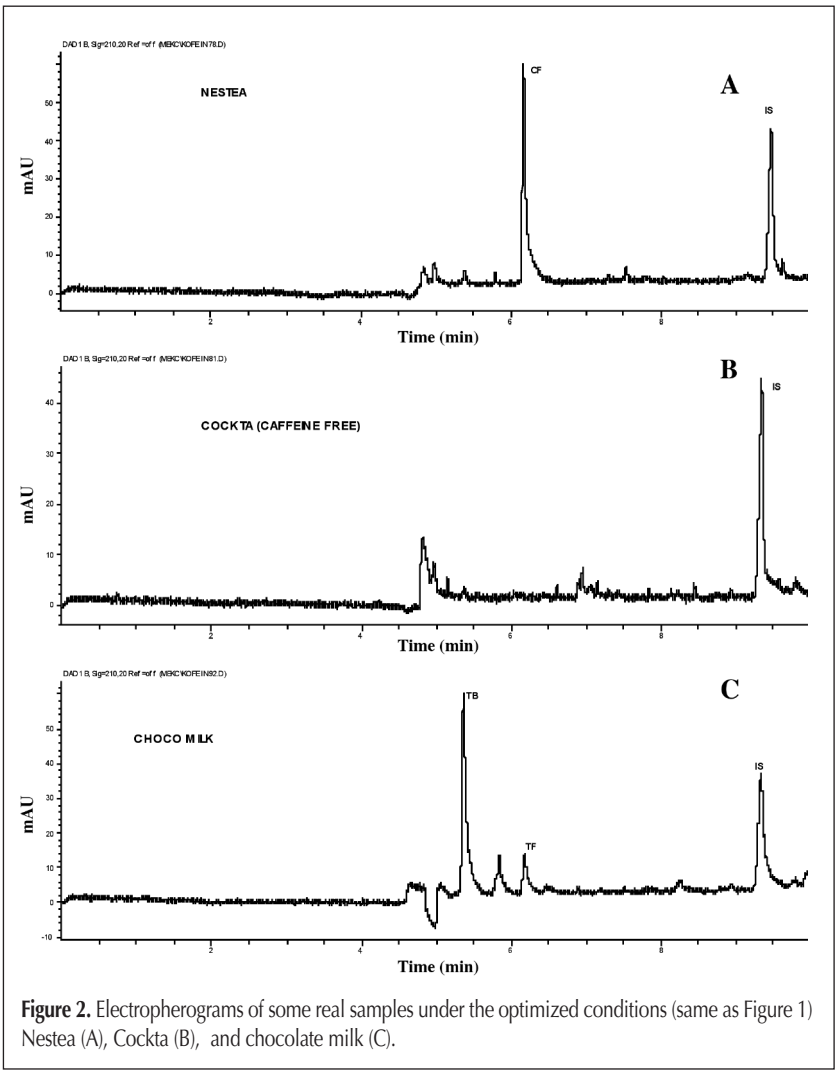

Figure 2. Electropherograms of some real samples under the optimized conditions (same as Figure 1) Nestea (A), Cockta (B), and chocolate milk (C).

The linearity of the assay has to be determined by the analysis of a series of standards at at least five different concentrations (30,31). Linearity was checked in the range 0.5 to 300 mg/L for each investigated compound (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) and acceptable ranges with correlation coefficient higher than 0.999 were chosen (Table I). In addition, the results for assay, 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 are determined and shown in Table I.

Accuracy of the method was determined by analyzing solutions of known concentrations (working standard solutions) and comparing the measured and known values. The mean recoveries for all compounds were in range of 98.9–101.2% (n = 6 for each of presented concentration), proving a good accuracy of the method (Table I).

A repeatability test was performed to determine intra-day variation in peak areas ratios and migration times. RSD below 2.1% (n = 6) indicate that repeatability of the method is acceptable (Table I).

Intermediate precision was evaluated over

Table I. Statistical Parameters of the Calibration Curve for Each Compound (Linear Regression; with LODs and LOQs), Accuracy, and Repeatability Results

	TB	CF	PA	TF	IS	SA	AS	PR	CP
Slope	2.06	3.39	4.31	2.60	4.30	3.54	12.99	5.75	1.91
Intercept	–80.47	–115.55	–70.11	26.62	–111.73	–79.00	–365.31	–159.66	142.86
r	0.9993	0.9995	0.9996	0.9994	0.9998	0.9996	0.9997	0.9993	0.9994
LOD (mg/L)	2.0	1.1	0.7	0.9	0.8	0.9	0.3	0.6	0.7
LOQ (mg/L)	6.7	3.7	2.3	2.9	2.6	3.0	0.9	2.0	2.4
Linear range (mg/L)	6–120	4–120	2–120	3–150	3–200	3–200	1–250	2–200	2–200
Recovery (%) ± SD	98.9 ± 1.2	99.2 ± 0.5	99.7 ± 0.4	99.0 ± 0.8	99.8 ± 0.6	99.3 ± 0.5	99.4 ± 0.4	98.9 ± 1.4	101.2 ± 1.1
RSD (%) migration time	1.3	1.1	1.0	1.4	0.8	0.6	0.7	1.4	1.9
RSD (%) peak area ratio	1.8	1.2	1.1	2.0	1.2	0.9	0.8	2.0	2.1

three days (inter-day repeatability) using working solutions (concentrations 10–100 mg/L). These solutions were injected daily under the same conditions and the results were used for the repeatability study. The solutions were stored at room temperature (25 ± 2°C) in sunlight, decreasing recovery values approximately from 101.2 to 96.6% for all compounds in water–methanol 1:1 (v/v). When stored in the refrigerator in the dark, the recovery ranged from 101.1 to 98.7% over three days for all compounds. The RSD values (0.7–2.0% for migration time and 0.9–2.4% peak area ratio) indicate that the intermediate precision is acceptable.

The parameters of the optimum MEKC conditions were slightly modified in order to evaluate the robustness (33). The effects of different concentrations of organic modifier (± 0.5%) and SDS (± 1 mM) in the BGE, as well as the effects of buffer pH (± 0.06), capillary temperature (± 5°C), running voltage (± 1 kV), and detection wavelength (± 3 nm), were determined. The design applied was the fractional factorial design. No significant variations in specificity, accuracy, and precision were found over the tested ranges, which indicated good robustness of the method (RSDs were lower then 2.3% for migration time and peak area ratio).

Application

The present method was tested to determine the previously mentioned compounds in food, beverages, natural products, pharmaceuticals, and cosmetic preparations. The results for beverage, natural products, and food samples are shown in Table II, and for pharmaceuticals and cosmetics in Table III. The results presented for pharmaceuticals and cosmetics show strong agreement between the claimed and found values except for Oldon tablet (Table III). When analyzing the commercial products, the concentrations and amounts were calculated by comparing peak areas of IS and CF and known (added; 50 mg/L) concentration of IS. It is well known that AS is not stable in high humidity environments, so there are three potential explanations for very elevated levels of SA in tablets. Firstly, the manufacturer may have used wet granulation as a technological procedure for producing tablets with AS, CF, and PA, which is strongly opposed. Secondly, packing material could be permeable by moisture from the area, and with high humidity, hydrolysis of AS to SA is possible. Finally, the extraction and preparation procedure used by our research group could speed up the decomposition of AS. Because of these doubts, stability testing of standard solution of AS was done. The solution was prepared by dissolving AS in water–methanol (1:1, v/v) and kept at room temperature for 48 h. Check points were after 0.5, 1, 2, 4, 6, 12, 18, 24, 36, and 48 h. The first detectable level of SA, testing under optimal conditions for the developed MEKC method, was found after 12 h. The results were repeated with aspirin tablet samples, and the results were the same. Therefore, the present extraction and analysis procedure is acceptable for the analysis of freshly prepared samples with AS.

The working solution concentration for each real sample has to be determined before extraction and analysis. It is very important to choose suitable concentrations for each compound within the real sample, and the linear range has to be considered. Otherwise, separation will not be suitable and R_s values as well as peak symmetry will not be acceptable. An example for that kind of problem is shown in Figure 3 for the analysis of Oldon tablets, and it can be also seen in Figure 1 for CF, PA, and TB compounds (concentration of analytes was 150 mg/L and linearity is up to 120 mg/mL for all three analytes).

The advantage of the proposed method over the HPLC and TLC methods described in literature for the analysis of CF and associated compounds in different samples is its lower running costs and higher environmental friendliness. In the proposed and developed method, 20–30 analyses with MEKC require 2 mL of phosphate buffer containing SDS and 7.5% (v/v) acetonitrile,

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

Beverage and food samples	TB	CF	TF
Nestea lemon (mg/L)	–	38.8	–
Cockta (caffeine free) (mg/L)	–	0.0	–
Coca cola (mg/L)	–	105.1	–
Coca cola light (mg/L)	–	123.0	–
Red bull (mg/L)	–	321.8	–
Pepsi (mg/L)	–	122.1	–
Shark (mg/L)	–	356.7	–
Choco milk (mg/L)	226.0	–	14.4
Indian tea mg/1.75g/cup (2dcl)	11.9	40.1	–
Green tea mg/1.75g/cup (2dcl)	–	40.8	–
Mate tea mg/2.0g/cup (2dcl)	8.9	26.5	–
Barcaffe light (≤ 0.1%; g/100 g)	–	0.1	–
Barcaffe dekote (≤ 1%; g/100 g)	–	0.9	–
Barcaffe classic (≤ 2%; g/100 g)	–	1.9	–
Nescaffe (g/100 g)	–	4.0	–
Cocoa (%; g/100 g)	0.5	–	0.3
Baking chocolate (%; g/100 g)	0.4	–	0.1

Table III. Applications Results for Pharmaceuticals and Cosmetics

Pharmaceuticals and cosmetics		CF	PA	TF	SA	AS	PR	CP
Perfect body gel (%)		1.6	–	–	–	–	–	–
Anticellulite gel (%)		1.2	–	–	1.9	–	–	–
Oldon	Expected (mg)	50.0	200.0	–	0.0	300.0	–	–
	Found	48.9	193.4		282.2	15.4		
Caffetin	Expected (mg)	50.0	250.0	–	–	–	210.0	10.0
	Found	49.1	250.2				203.6	7.3
Caffebol	Expected (mg)	50.0	460.0	–	–	–	–	10.0
	Found	48.3	453.9					8.2
Kombikaf	Expected (mg)	50.0	400.0	–	–	–	–	–
		47.1						
	Found	50.0	389.7					
Teolin	Expected (mg)	–	–	125.0	–	–	–	–
	Found	123.4						

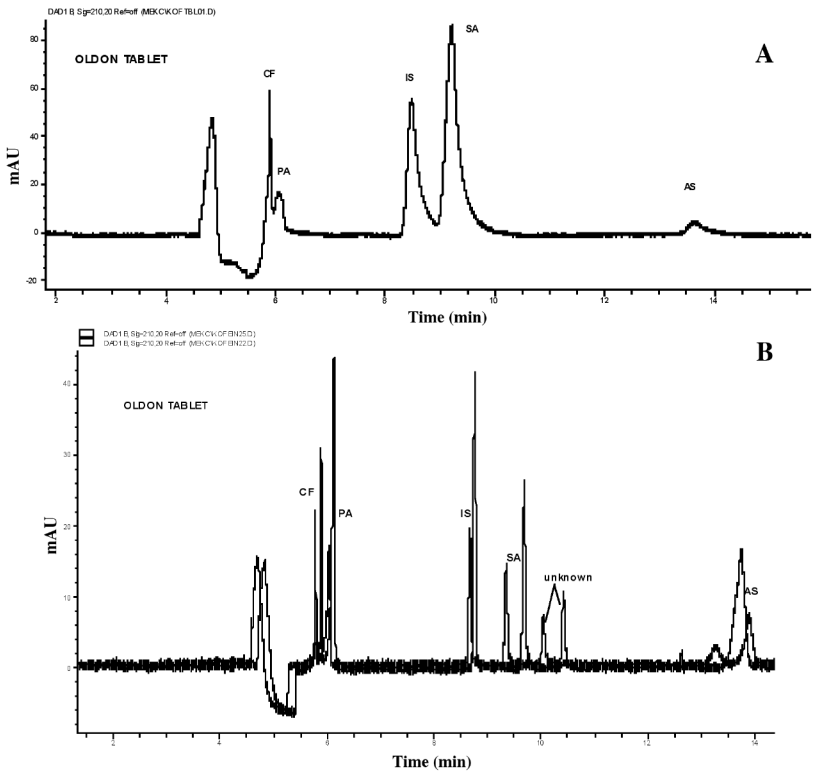

Figure 3. Influence of concentration on separation under the optimized conditions (Oldon tablet); over the linear range (A); –100 mg/L and –50 mg/L (B).

while 20 analyses by HPLC require 300 to 1000 mL of mobile phase with different proportions of the most ordinarily used organic solvents (methanol, acetonitrile, acetone, THF). A disadvantage of the developed MEKC method is lower sensitivity in contrast to some LC methods (34).

Conclusion

The new MEKC method is presented as an efficient technique for determination of CF, TB, TF, PA, PR, AS, SA, and CP in corresponding real samples of food, drinks, pharmaceuticals, and cosmetic preparations using SDS as surfactant (80 mM), 7.5% (v/v) of acetonitrile as organic modifier, and 20 mM phosphate buffer (pH 9.0). The advantage of MEKC over other analytical methods, particularly over HPLC methods, is in its lower running costs and higher environmental acceptability.

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