sciendo Acta Marisiensis - Seria Medica 2024;70(3):194-199 DOI: 10.2478/amma-2024-0026

RESEARCH ARTICLE

Determination of caffeine by micellar electrokinetic chromatography in different beverages

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A fast, simple and cost-effective capillary electrophoresis (CE) method was developed for caffeine determination in different beverages (energy drinks, soft drinks). Because caffeine is neutral from electrophoretic point of view and migrates with the electroosmotic flow (EOF), micellar electrokinetic chromatography (MEKC) was chosen as the separation method. The optimum separation conditions consisted of 25 mM sodium tetraborate, 100 mM sodium dodecyl sulphate, pH 9.30, 20°C temperature, 20 kV voltage, 50 mbar/sec hydrodynamic injection, UV detection at 270 nm. Employing the optimized conditions caffeine was quantified in less than 3 minutes. The analytical performances of the method were verified in terms of accuracy, linearity, limit of detection and quantification, precision and robustness. The method was applied also to detect caffeine in coffee and tea. The advantage of MEKC over other analytical methods, particularly compared with the more frequently used HPLC methods, lies in its lower operating costs and higher environmental friendliness.

Keywords: caffeine, beverages, energy drinks, soft drinks, coffee, capillary electrophoresis, micellar electrokinetic chromatography

Received 21 July 2024 / Accepted 28 August 2024

Introduction

Caffeine (1,3,7-trimethylxanthine) is a purine alkaloid that occurs naturally in the beans of coffee plants (*Coffea arabica* and *Coffea canephora*), cocoa plant (*Theobroma cacao*), tea plant leaves (*Camellia sinensis*). Other caffeine sources include guarana seeds, kola nuts, yerba mate or guayasa and more than other 30 plants. The caffeine amount present in these sources varies, the highest concentration is found in guarana (4-7%), followed by tea leaves (3-4%), mate leaves (2-3%), coffee beans (1-2%), kola nuts (1.5%) and cocoa beans (0.05%) [1,2].

Caffeine appears in the form of white, silky, acicular crystals or as a bitter-tasting crystalline powder. It is efflorescent and sublimes when heated. Caffeine is easily soluble in boiling water and chloroform, slightly soluble in water (1:60), and very slightly soluble in alcohol [3]. The chemical structure of caffeine is presented in **Figure 1**.

Caffeine is a central nervous system (CNS) stimulant and acts primarily as an adenosine receptor antagonist (particularly on A1 and A2A receptors). This prevents adenosine mediated downregulation CNS activity, and stimulates the medullary, respiratory, vagal, and vasomotor centers in the brain. It is also a nonselective competitive phosphodiesterase (PDE) inhibitor, raising the intracellular concentration of cyclic AMP (cAMP), activating protein kinase A (PKA), which then phosphorylates various target proteins, leading to enhanced cellular responses and increased neurotransmitter release. Caffeine is the most used psychostimulant substance in the world, consumed usually for recreative purposes, primarily in the form of coffee and tea [4,5].

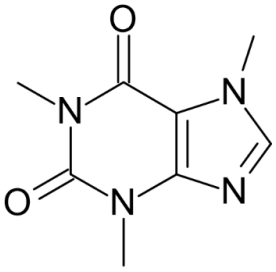

Fig. 1. Caffeine (1,3,7-trimethylxanthine) chemical structure

People often use caffeine recreationally to increase energy or enhance alertness, helping them stay awake for longer periods; however, while low doses can provide a pleasant stimulant effect, higher doses can lead to psychological symptoms like anxiety [4].

Caffeine can be found in coffee and tea, but also in energy drinks, many soft drinks, foods or certain medications, and dietary supplements. Because the drinking of soft and energy drinks and other beverages is a common habit all over the world, such food products are seen as having significant economic and social relevance [3,6].

The first beverage containing caffeine was prepared in the late 19th century in Atlanta (Georgia, USA) by John Pemberton and was named Coca-Cola® based on the two main ingredients of the original recipe coca leaves and kola nuts (source of caffeine). Coca-Cola® emerged as one of the world's most recognizable brands, maintaining its dominance in the global soft drinks market throughout the 20th and 21st centuries. [7].

Caffeine contributes to the overall appeal of soft drinks, enhancing their refreshment, flavor, and hydration qualities that consumers enjoy. Energy drinks often include

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caffeine as a key ingredient to enhance their stimulating effects; these beverages are popular for their ability to boost energy levels, improve focus, and increase alertness. The caffeine content in soft drinks differs among various brands and is monitored by the US Food and Drug Administration (FDA), which sets a maximum limit of 200 mg/L [7]. Additionally, the European Food Safety Authority has stipulated that energy drinks containing more than 150 mg/L of caffeine must be labeled as having „high caffeine content," along with indicating the exact amount of caffeine [7,8].

Capillary electrophoresis (CE) is a method in which the separation takes place inside a thin fused silica capillary filled with an electrolyte solution; analytes migrate in electrolyte solutions through the capillary, under the influence of an electric field, separation occurring based on the differences between the electrophoretic mobilities of the analytes and on the amplitude of electroosmotic flow (EOF). Among the CE techniques most used in drug analysis are capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MEKC). The advantages of using CE in the analysis of medicinal substances are related to the rapid method development, relatively low cost of consumables, low consumption of analytes, reagents and samples, and the possibility of using different systems of detection. Currently, in the analysis of pharmaceutical compounds, CE is a feasible alternative and complementary method to the more extensively used high performance liquid chromatography (HPLC) techniques [9,10].

A large variety of analytical methods have been proposed for the separation and/or determination of caffeine in various foods and beverages. These methods include UV spectrophotometry [11,12], high performance liquid chromatography with diode array detection (HPLC-DAD) [13,14], liquid chromatography – mass spectrometry [15,16], gas chromatography – mass spectrometry (GC-MS) [17]. Traditionally chromatographic methods have been used for caffeine determination in foods and beverages, while electrophoretic methods like CE have received less attention.

Injac et al. developed a MEKC method to quantify several compounds, including caffeine and theobromine, in foods, beverages, natural products, pharmaceuticals, and cosmetics, using specific optimized conditions and UV detection [18].

Meinhart et al. developed a robust and efficient MEKC method for quantifying caffeine traces in decaffeinated coffee using central composite design optimization and statistical analysis of multiple responses [19].

Bizzotto et al. compared CE and HPLC for measuring residual caffeine in decaffeinated coffee, finding CE to be faster, more cost-effective, and environmentally friendly, while HPLC offers a lower detection limit [20].

Elbashir et al. developed a quick and simple CE method to simultaneously determine caffeine, vanillin, and ethyl vanillin in beverages, achieving separation in under 3 minutes with good accuracy and precision; the method offers a cost-effective and efficient alternative for the food industry, particularly for analyzing these compounds in energy drinks [21].

Asensio- Ramos & D'Orazio published in 2023 an interesting review regarding the application of capillary electromigration techniques to coffee analysis [22].

The purpose of this study was the development of a rapid, simple and cost-effective CE method to quantify caffeine in various commercially available beverages from the Romanian market.

Methods

Instrument and analytes

The electrophoretic determinations were carried out on an Agilent 1600 CE system equipped with a DAD detector (Agilent Technologies, Waldbronn, Germany). Electropherograms were registered using Chemstation 7.01 software (Agilent Technologies).

The determinations were carried out using fused silica capillaries (Agilent Technologies, Waldbronn, Germany) with a 50 μm diameter, a total length of 40 cm, and an effective length of 32 cm.

To determine the pH of the buffer solutions, we used a Terminal 740 pH-meter (Inolab, Germany).

Caffeine of pharmaceutical grade was acquired from Sigma Aldrich (Germany). Substances of analytical grade were used in the determinations: sodium phosphate dibasic heptahydrate, sodium tetraborate decahydrate, sodium dodecyl sulfate (Merck, Darmstadt, Germany), methanol, sodium hydroxide (Lach Ner, Neratovice, Czech Republic). Throughout the experiments, double-distilled deionized water (Millipore) was used.

Different beverages (soft drinks and energy drinks) were acquired from local supermarkets.

Electrophoretic conditions

Capillaries were conditioned with 1 N NaOH for 30 minutes, followed by 0.1 N NaOH for 15 minutes, and then rinsed with water for 15 minutes. Before each determination, capillaries were preconditioned with 0.1 N NaOH for 2 minutes, water for 1 minute, and then with the background electrolyte (BGE) for 1 minute.

The preparation of the BGE solution consisted of dissolving the appropriate amounts of components in purified water, adjusting the pH with 1 N NaOH if necessary.

Standard solutions of caffeine were prepared in a methanol:water (1:1) mixture; a stock solution of 10 mg/mL was prepared and subsequently diluted to the appropriate concentrations with the BGE before use to obtain the required concentration.

All samples and BGEs were homogenized in the ultrasonic bath for 3 minutes, and then filtered through a 0.45 μm PTFE Millipore (Millipore, USA) filter membrane.

Sample preparation from energy and soft drinks involved degassing in an ultrasonic bath for 5 minutes, filtering through a 0.45 μm Millipore filter, and diluting 1.0 mL of the product to a final volume of 10.0 mL in a volumetric flask without preliminary extraction.

For coffee and tea sample preparation, 1 g of accurately weighed tea leaves or roasted coffee was transferred into a 100.0 mL conical flask, and 70°C hot water was added up to a final volume of 100.0 mL. The solution was placed in a boiling water bath for an hour, cooled to room temperature, then filtered through filter paper to eliminate particulate particles. The filtrate was then sonicated for 3 minutes and filtered again using a 0.45 μm Millipore filter. Further dilutions with the BGE were carried out just before the analysis.

In the preliminary analysis the following electrophoretic conditions were used: 25 mM BGE concentration, temperature 25° C, voltage +20kV, hydrodynamic injection with 50 mbar/sec. at anode. UV detection took place at the cathode at a wavelength of 210 nm (control wavelength) and 270 nm (specific wavelength for caffeine), respectively.

Results

Preliminary analysis

Caffeine a weak basic (essentially neutral) compound, has an extremely low electrophoretic mobility, and cannot be quantified by CZE, method in which migration is based on the own electrophoretic mobility of the analyte, because it migrates together with EOF. pH is one of the critical factors in CZE analysis because it affects the charge of the analytes and the ionization of silanol groups on the capillary wall, which has a significant impact on the analyte electrophoretic mobility and on the EOF. The pH value of the BGE (phosphate, borate) was examined within the range of 7.0–11.0 at a fixed BGE concentration of 25 mM. In all situations caffeine migrated alongside the EOF.

For caffeine determination a MEKC method was applied, by adding an anionic surfactant, sodium dodecyl sulfate (SDS), to the BGE, to promote micelle formations. MEKC extends the applicability of CE techniques to neutral analytes, like caffeine. Because micellar structures are present, MEKC not only incorporates the electric field and EOF interaction but also makes use of the partition chromatography mechanism [23,24].

Preliminary studies were conducted using different concentrations of standard stock solutions, to test the influence of BGE pH, organic modifier (methanol concentrations 0 - 20%), type and concentration of BGE (borate and phosphate BGE), SDS concentration (25-100 mM).

Method optimization

The influence of analytical parameters on the determination was assessed using a One factor at a time (OFAT) strategy, by varying each parameter within a certain interval while keeping all other parameters constant.

The introduction of the surfactant, SDS, into the BGE led to the delimitation of caffeine from the EOF. Increasing the concentration of SDS (25-100 mM) improved the separation as well as the shape and amplitude of the peaks. However, concentrations higher than 100 mM led to the generation of a current of more than 100 μA in the capillary, which can generate instability in the electrophoretic system. An attempt was made to decrease the concentration of SDS and to compensate for this change by adding organic solvent (methanol) to the BGE solution, which would reduce the hydrophobic interactions between caffeine and micelles. But the addition of methanol did not improve the separation resolution, on the contrary, an addition of 20% methanol makes the differentiation between caffeine and EOF inefficient.

Voltage was varied in the 15-25 kV range, and a slight increase in migration times was observed with the decrease of the applied voltage. The limiting factor in the case of voltage is the generation of a high current and Joule effect, which makes it difficult to dissipate the generated heat.

Capillary temperature was varied in the range of 15°C - 25°C, and it was observed that a decrease in temperature leads to a slight increase in migration times.

The influence of the injection parameters (injection pressure, injection time) was also monitored over the interval 30-50 mbar, respectively 1-2 sec.; the injection settings influence the magnitude and form of the peaks, higher pressure and shorter time resulting in better results.

The optimal separation conditions that were subsequently used to verify the analytical performance of the method were the following: 25 mM sodium tetraborate BGE, 100 mM SDS, pH 9.30, voltage + 20 kV, temperature 20°C, hydrodynamic injection 50 mbar/sec., UV detection at 270 nm. Figure 2 presents an example of typical electropherogram obtained with optimized analytical conditions.

Analytical performance verification

The validation of the optimized CE method was evaluated in terms of accuracy, linearity, limit of detection (LOD), limit of quantification (LOQ), intra- and interday precision, and robustness.

Intra-day precision was determined by injecting caffeine samples at three different concentrations (0.05, 0.15, 0.5 mg/mL) six times on the same day (n = 6), while inter-day precision was determined by injecting samples of the same concentration level six times on three consecutive days (n = 18). Relative standard deviations (RSDs) (%) were calculated for the migration time and peak area.

Linearity was verified by plotting calibration curves and calculating regression equations and correlation coefficients. Eight solutions of different concentrations (concentration range 0.01–1 mg/mL) and three replicates per concentration were injected.

The LOD and LOQ were calculated as follows, the standard deviation of the regression equation was divided

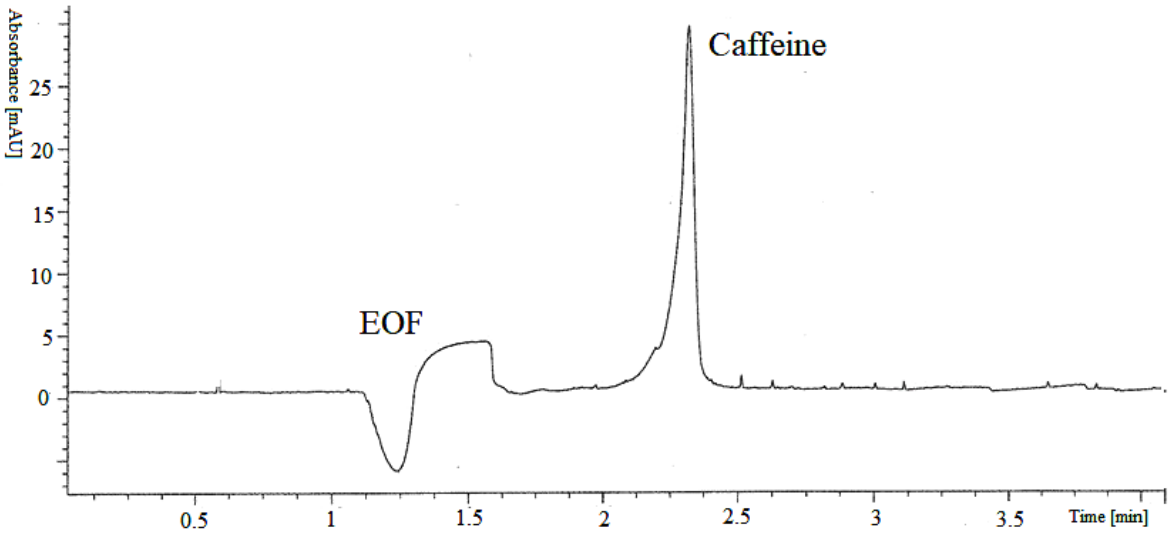

Fig. 2. Caffeine determination by MEKC (electrophoretic conditions: 25 mM sodium tetraborate BGE, 100 mM SDS, pH 9.30, voltage + 20 kV, temperature 20ºC, hydrodynamic injection 50 mbar/sec., UV detection at 270 nm, analyte concentration: 0,25 mg/mL)

by the slope of the regression equation, which was multiplied by 3.3 for the LOD and 10 for the LOQ.

The recovery study was determined by spiking caffeine at 0.05, 0.1 and 0.25 mg/mL concentration level into a sample of 1 mL Coca-Cola®. The solutions were filtered and injected into the CE system, with three replicates injected for each sample.

Validation data are presented in **Table I**.

The robustness of the method was verified by performing small deliberate variations in the experimental conditions, such as changes in BGE concentration (± 5 mM), SDS concentration (± 5 mM), voltage (± 2 kV), and temperature (± 2ºC), and observing the effects on the migration times and peak area. RSD (%) for both responses was below 5% in all circumstances, demonstrating the robustness of the method.

Table I. Analytical performance of the optimized method

Intra-day precision (n = 6)		
Concentration (mg/mL)	**RSD (%) migration time**	**RSD (%) peak area**
0.05	0.03	0.26
0.15	0.03	0.20
0.5	0.02	0.24
Inter-day precision (n = 18)		
Concentration (mg/mL)	**RSD (%) migration time**	**RSD (%) peak area**
0.05	0.06	0.39
0.15	0.05	0.35
0.5	0.04	0.37
Accuracy		
Concentration (mg/mL)	**Recovery (%)**	
0.05	98.25	
0.15	99.18	
0.5	100.36	
Linearity		
Regression equation (0.01 - 1 mg/mL)	y= 65.071x + 0.6012	
Coefficient of correlation	0.9993	
LOD (mg/mL)	0.030	
LOQ (mg/mL)	0.094	

Caffeine determination in beverage matrices

The identification of caffeine in beverages was made based on migration time, UV spectra and confirmed by spiking. The quantification of caffeine in beverage samples was performed using a calibration curve made in the range of 0.01 -1 mg/mL (see linearity study). The MEKC results showed no interference from the matrix ingredients in any of the tested samples, demonstrating that the procedure is selective.

Fifteen beverages were analyzed, and caffeine content was determined between 0 – 31.72 mg/mL. All analyses were carried out in triplicate, with each replicate representing the mean of three injections. The content of caffeine were in concordance with the content declared by the manufacturers. The results are presented in **Table II**.

Table II. Caffeine content of different types of beverages (energy drinks, soft drinks)

Product name	**Caffeine content (mg/100 mL) ± SD**
Coca-Cola®	9.68 ± 0.24
Coca-Cola zero®	10.41 ± 0.31
Pepsi®	9.71 ± 0.22
Pepsi light®	9.96 ± 0.28
Pepsi max®	19.43 ± 0.54
Pepsi twist®	10.61 ± 0.32
Fanta®	0
Sprite®	0
Schweppes Kinley®	0
Mountain Dew®	14.22 ± 0.41
Adria Cola®	8.48 ± 0.37
American Cola®	8.16 ± 0.39
Red Bull®	31.78 ± 0.19
Red Bull sugarfree®	31.91 ± 0.21
Burn®	31.42 ± 0.29

The results show a wide range of caffeine content across different beverages. As expected, energy drinks have significantly higher caffeine levels compared to cola drinks and soft drinks. This high caffeine content aligns with their marketed purpose of providing an energy boost.

Among the cola drinks, Coca-Cola®, Pepsi-Cola®, and their variations have moderate caffeine content, typically around 9.7 to 10.6 mg/100 mL. Non-cola soft drinks like Fanta®, Sprite®, and Schweppes Kinley® have no caffeine, which is consistent with their branding as caffeine-free beverages.

Mountain Dew® stands out among the soft drinks with a relatively high caffeine content of 13.45 mg/100 mL, higher than that of most cola drinks. Almost twice as much caffeine is found in Pepsi Max® as in regular Pepsi®; which is part of Pepsi Max®'s branding as a „maximum taste, zero sugar", a beverage with a greater caffeine level in order to deliver a more potent energy boost without the added calories.

The method was also applied for the determination of caffeine in different samples of coffee and tea. As expected, caffeine concentration in tea samples were higher than concentration in ground coffee. Caffeine presence was confirmed in all samples, but due to the high variability in caffeine content across different brands, the exact quantification was not performed. For 1 g of ground coffee, we identified between 10 - 20 mg of caffeine, while for 1 gram of tea leaves, we identified between 25 - 40 mg caffeine. This variation highlights the inherent variations in caffeine content brought about by elements including plant species, growing environment, and processing techniques.

The concentrations of caffeine identified in coffee and tea samples should be regarded as estimations, as the preparation methods for tea and coffee can significantly influence the caffeine content. This variability in preparation techniques was not fully assessed in the current study but will be further examined in future research to ensure a more accurate quantification.

Conclusions

We have successfully developed and applied a MEKC method for the determination of caffeine in various beverages from the Romanian market. The method proved to be effective, selective, and robust for identifying and quantifying caffeine in different matrices, including soft and energy drinks. The optimized conditions for the MEKC method included using a 25 mM sodium tetraborate BGE with 100 mM SDS, at pH 9.30, +20 kV voltage, and 20°C temperature. These conditions facilitated the delimitation of caffeine from the EOF and ensured precise and accurate measurements in less than 3 minutes. The procedure demonstrated good linearity, precision, and recoveries

The caffeine content in the tested beverage samples ranged from 0 to 31.91 mg/100 mL, with energy drinks containing significantly higher caffeine levels compared to cola drinks and non-cola soft drinks. The results were consistent with the caffeine content declared by the manufacturers. While caffeine was detected in all tested coffee and tea samples, quantification was not performed due to high variability in caffeine content across different brands.

The developed MEKC method is a viable alternative for caffeine determination in beverages, offering a rapid, cost-effective, and environmentally friendly approach to traditional chromatographic methods. This method is reliable for routine analysis in quality control laboratories, ensuring product conformity and consumer safety.

Authors' contribution

AU (Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing – original draft; Writing – review & editing), DGS (Conceptualization; Data curation; Formal analysis; Visualization; Writing – original draft; Writing – review & editing), GH (Conceptualization; Data curation; Formal analysis; Project administration; Resources; Supervision; Visualization; Writing – original draft; Writing – review & editing)

Conflict of interest

None to declare.

Acknowledgments

The study was supported by the University of Medicine, Pharmacy, Science, and Technology „George Emil Palade" in Târgu Mureș, internal grant contract number 511/3/17.01.2022.

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