

Automated Sequential Injection Method for Determination of Caffeine in Coffee Drinks

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Abstract An automated sequential injection analysis spectrophotometric assay for the determination of purine alkaloids in coffee drinks was developed. The sample was treated with a carrez reagent for matrix suppression followed by filtration; subsequently, alkaloids were separated from organic acids using a short C18 monolithic column (10 × 4.6 mm). The flow rate of the separation step was 10 μL s⁻¹ with 10% v/v of methanol as the mobile phase. The sum of alkaloids evaluated as caffeine was detected at 274 nm. The influence of the main parameters affecting the quantification of purine alkaloids was optimized. One sample analysis lasted 15 min when aspirated in triplicate. The linear range was 1–15 mg L⁻¹, and the determination coefficient (*r*²) was 0.9969. The limit of detection and limit of quantitation were 0.128 and 0.425 mg L⁻¹, respectively. The repeatability evaluated as the relative standard deviation (RSD) was 3.58% (*n* = 12, 10 mg L⁻¹). Under optimal conditions, the method was successfully applied to determine purine alkaloids in different real samples including soluble coffee, coffee from an espresso machine, and brewed coffee drinks.

Keywords Automation · Coffee drinks · Monolithic column · Sequential injection analysis · Purine alkaloids

Introduction

Caffeine, as a main purine alkaloid, is consumed daily in coffee, tea, cocoa, chocolate, and pharmaceuticals. It is a xanthine alkaloid with a chemical structure of 1,3,7-trimethylxanthine. Caffeine affects the heart, stimulates the central nervous system, and increases the blood pressure. Due to the high consumption of caffeine worldwide and its potential undesirable effects or potential withdrawal symptoms, it is noteworthy to monitor the levels of caffeine in coffee and other caffeine-containing beverages (black tea, energy drinks, etc.). In respect of the high consumption of caffeine, many articles dealing with the determination of caffeine in different samples have been recently published. In addition to a variety of analytical procedures being developed, many analytical techniques have been used to determine caffeine, including electroanalytical and optical detections and separation methods for a determination of caffeine with other active substances in complex matrices.

Caffeine and paracetamol were determined by square wave voltammetry using a modified glassy carbon electrode (Tefera et al. 2016). The limit of detection for caffeine was 0.79 μmol L⁻¹, and the voltammetric method was successfully utilized to analyze caffeine in cola beverages and teas. The development of a sensitive and selective voltammetric method for caffeine determination in tea, coffee, energy drinks, and pharmaceuticals applying a Nafion-covered lead film electrode as an alternative to spectroscopic and chromatographic methods was also demonstrated (Tyszcuk-Rotko and Beczkowska 2015). The obtained detection limits for caffeine after 120 s of accumulation were 1.7 × 10⁻⁸ mol L⁻¹ (peak 1) and 2.2 × 10⁻⁷ mol L⁻¹ (peak 2). Caffeine determination based on the fluorescent emission of bovine serum albumin was tested (Alesso and Fernández 2016). The sensitivity and selectivity of the suggested method allowed its application to a

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variety of samples. Therefore, this method was even successfully applied for caffeine determination in dietary supplements and energy drinks.

A novel LC-MS-based procedure for the determination of caffeine and its major metabolite paraxanthine in hair has been published (De Kesel et al. 2015). The experiment was mainly focused on the effects of a hair decontamination procedure. Caffeine was found in all wash solvents in different concentrations, while caffeine metabolite was not detected. The suitability of the method was demonstrated by its application to hair samples from 10 healthy volunteers. A green analytical method based on dispersive liquid-liquid micro-extraction and GC determination of caffeine in teas and coffees was used (Sereshti and Samadi 2014). Under optimal conditions, the method was applied for caffeine determination in various beverages. The limit of detection (LOD) and the limit of quantitation (LOQ) were 0.02 and 0.05 $\mu\text{g mL}^{-1}$, respectively.

A simple and reliable HPLC-DAD method was presented to analyze complex multi-drug formulations consisting of caffeine and other active substances (Dewani et al. 2015). An effective chromatographic separation of all formulation components was achieved using a C18 reverse-phase column and a mobile phase composed of phosphate buffer and acetonitrile. The total run time was 20 min.

HPLC coupled to UV-VIS detection for the determination of caffeine and phenolic compounds in different coffee brands has been developed (Belguidoum et al. 2014). Many aspects were taken into account (e.g., packaging and roasting degree) during the measurement. The obtained results pointed out that these procedures have a strong effect on the concentration of caffeine in coffee beverages. LOD ranged from 0.75 to 14.79 mg L^{-1} , while LOQ ranged from 2.26 to 44.44 mg L^{-1} . The separation of all compounds was achieved within 13 min.

A new simple spectrophotometric method for the determination of caffeine and other substances (e.g., caramel and riboflavin) contained in cola-type beverages and energy drinks has also been described (Žiak et al. 2014). Caffeine shows native fluorescence in aqueous solutions, and thus, fluorescence detection was coupled with a standard addition method and successfully applied for its determination. The developed procedure provided comparable results with those of an HPLC reference method.

A flow potentiometric method for the determination of caffeine in saliva was performed (Timofeeva et al. 2016), including a stepwise injection analysis with potentiometric detection and single-drop liquid micro-extraction (SDLME). SDLME was used to eliminate the interfering effects of saliva and caffeine metabolism products. A linear range of 10^{-5} – 10^{-2} mol L^{-1} was found for caffeine with a detection limit of 6×10^{-6} mol L^{-1} . The sample throughput was six samples per hour. The method was applied to real samples, and the

obtained results agreed with the results from the reference HPLC method.

A novel method based on solid phase extraction (SPE) combined with flow injection (FI) for the determination of caffeine in coffee beans has been described (Salinas-Vargas and Canizares-Macias 2014). Caffeine was extracted from coffee beans in hot water. The C18 lab-made minicolumn was coupled to an FI system that consisted of a peristaltic pump, an injection valve, two selection valves, and a UV spectrophotometric detector. This configuration allowed for the direct injection of aqueous extracts into the system without any previous dilution or increased the sample throughput. The total analysis time was 7 min with an LOD of 128 $\mu\text{g mL}^{-1}$. The suggested method could be used as an alternative for caffeine determination because it is cheap, precise, and fast. But the flow system coupling flow and sequential injection principles together with the lab-made C18 column suffer from low adaptability and applicability in other laboratories. Additionally, a greater amount of eluent must be used in case of its continuous flow by a peristaltic pump.

Another simple method used for the preparation of a flower-like silver nanostructure, which could allow for an effective surface enhance Raman scattering (SERS) detection of caffeine, was published (Zheng et al. 2016). The SERS measurement pointed out that silver nanostructures have high SERS activity. Therefore, these nanostructures have the potential to serve as effective SERS substrates for the detection of caffeine. A detection sensitivity of 10^{-8} mol L^{-1} was reached.

Fast analysis of caffeine in beverages and drugs by paper spray tandem mass spectrometry PS-MS (Taverna et al. 2016) was applied to cola, espresso, energy drinks, tea, and drugs containing caffeine. The overall analysis time was app. 6 min including centrifugation prior to the analysis. The detection and quantification limits ranged from 1.2 to 1.6 $\mu\text{g mL}^{-1}$. The use of a labeled internal standard was needed for quantitative analysis by PS-MS.

Simultaneous determination of saccharine, caffeine, salicylic acid, and benzoic acid in different matrices by salt and air-assisted homogeneous liquid-liquid extraction and HPLC was also developed (Heydari and Mousavi 2016). Caffeine and benzoic acid were determined in soft drinks using the mentioned method, but the analysis of all analytes were accomplished in 30 min while a 250-mm-long reversed phase C18 column was applied for the separation step.

The purpose of the presented work was to develop a new, simple, fast, and cheap analytical method for the determination and quantitation of purine alkaloids evaluated as caffeine in coffee drinks. The aim was to optimize the determination of purine alkaloids in coffee beverages using a short monolithic pre-column that is commercially available (compared to the previously applied C18 lab-made minicolumn) to separate the alkaloids from the matrix through the application of a

sequential injection analysis and determination by a UV detection. The developed method was used to analyze real samples including soluble coffee, coffee from an espresso machine, and brewed coffee drinks.

Materials and Methods

Chemicals

All chemicals were of analytical grade quality and were purchased from Sigma-Aldrich, Czech Republic (caffeine anhydrous, potassium hexacyanoferrate(II) trihydrate, zinc sulfate heptahydrate, and gradient grade methanol). Ultra-pure water prepared by a Milli-Q (Millipore, USA) was used for solution preparation and dilution.

Sequential Injection System

A FIALab 3000 (eight-port selection valve, 5-mL piston pump), FIALab Instruments, USA, was used for taking all measurements in the flow system using a UV source D1000 CE, optical fibers (300 μm I.D.), and a USB4000 detector, Ocean Optics, USA, for detection at 275 nm. For separation, a C18 monolithic pre-column (10 \times 4.6 mm), Merck, Czech Republic, was incorporated into the flow system between the selection valve and the Z-flow cell (10 mm optical path length) of the detector.

Flow System Optimization

To ensure the proper conditions for measurements in the SIA system aspiration, the separation of sample components and final detection of the analyte was optimized. It was especially important to select the relevant sample volume and flow rate of the separation of purine alkaloids from other matrix components, mainly phenolic acids. Separation on the short monolithic pre-column was selected to eliminate all other residues which could affect the final results by potential carryover. Therefore, the residues were washed out from the column with 10% methanol. Each measurement was repeated in triplicate and compared with a blank (distilled water). To maintain the accuracy of aspirated volumes, an aspiration flow rate of 50 $\mu\text{L s}^{-1}$ was used.

The flow rates of the separation and detection steps should be the same, and thus, a compromise between separation efficiency, signal height, repeatability, and analysis time had to be found. Four different elution rates were tested during optimization: 5, 10, 15, and 20 $\mu\text{L s}^{-1}$. A standard solution of caffeine with a concentration of 10 mg L^{-1} was used for flow rate optimization, and 10% methanol was used as the eluent. Great effort was expended to provide enough time for separation of the matrix component and caffeine and for the detection of the

analyte in real time. Therefore, it was necessary to determine the relation between the flow rate of elution of the analyte retained on the short monolithic pre-column sorbent and its absorbance.

Different sample volumes of caffeine standard solutions were tested to guarantee appropriate conditions for sample analysis: 20, 40, 50, 60, 80, 100, and 150 μL . It was necessary to take into account the sensitivity and robustness of the detector, and thus, the aim was to find a volume that was small enough for separation, but still sufficient for sensitive detection. The tested sample volume passed through the column at a flow rate of 10 $\mu\text{L s}^{-1}$.

Calibration and Repeatability

The calibration curve was measured under optimal conditions. The absorbance of the eluate was determined in a series of calibration solutions with concentrations of 1, 2.5, 5, 10, 15, 20, and 25 mg L^{-1} against a blank. The equation of regression line and determination coefficient was expressed from the obtained results. LOD and LOQ were determined by the following equations: $\text{LOD} = 3 \sigma S$; $\text{LOQ} = 10 \sigma S$, where σ is the standard deviation and S corresponds to the slope of the calibration curve.

The repeatability (expressed as relative standard deviation, %RSD) was determined under optimal conditions to guarantee the reliability of the obtained results for a 10 mg L^{-1} standard solution, and the measurement was performed 12 times.

Off-line Sample Pretreatment

Coffee is a quite complex sample matrix, and filtration was needed for safe aspiration of the samples into the flow system. To eliminate the matrix, precipitants were used during an off-line sample pre-treatment process. Two reagents were tested: zinc sulfate and potassium hexacyanoferrate(II). Reagents were added at a certain ratio to the sample, and then the precipitate was manually filtrated out using a 0.45- μm nylon syringe filter. To achieve the most effective interference removal, it was necessary to find the appropriate ratio of precipitants. Therefore, five different ratios of reagents were examined during the optimization step.

Analysis of Real Samples

Three different methods of coffee preparation were performed for the respective types of coffee drinks including brewed coffee, instant coffee, and espresso from a coffee machine. Different brands of coffee were used to make an objective comparison of the caffeine content in various types. All the real samples were diluted to get the final expected concentrations close to 10 mg L^{-1} . After the elimination of matrix effects by precipitation, the samples were prepared for analysis

under optimal conditions in the flow system. The absorbance was monitored, and the efficacy of the method was tested and evaluated.

Results and Discussion

Flow System Optimization

At first, the flow rates of sample delivery to the column (separation step) and analyte detection were optimized. The aim was to find the appropriate flow rate that guaranteed the efficient elution of the analyte retained on the monolithic pre-column and at the same time allow for its separation from the sample matrix. Additionally, if the flow rate is too high, the detector is not able to accurately scan the analyte signal. Conversely, if the flow rate is too slow, the experiment would take a very long time and have poor sensitivity. During the optimization process, a constant sample volume of 50 μL was used. The main criterion for its selection was the height of the detector response balanced by the analysis time, the number of points scanned by the detector, and the effective separation of caffeine from the sample matrix.

In view of the obtained results, the best detector response according to analysis duration and standard deviation was obtained at a flow rate of 10 $\mu\text{L s}^{-1}$. A flow rate of 5 $\mu\text{L s}^{-1}$ also provided sufficient detector response. However, this flow rate significantly prolonged the experiment. The shortest duration of measurement was performed with a flow rate of 20 $\mu\text{L s}^{-1}$. Unfortunately, the repeatability of the obtained values was inadequate. Therefore, the flow rate of 10 $\mu\text{L s}^{-1}$ was selected as optimal for the elution and detection of the analyte. With this flow rate, sufficient separation of caffeine from the matrix was achieved as demonstrated in Fig. 1.

Next, the sample volume that was optimal for the transport of analytes onto the column, the retention of caffeine on the monolithic sorbent, and its precise detection was tested. The evaluation criterion was analysis time and intensity of the detector response. Ten microliters per second was used as the appropriate flow rate for aspiration of the sample to the monolithic pre-column to decrease the back pressure in the flow system while ensuring the separation of phenolic acids and caffeine. The same flow rate was selected for elution and detection of the analyte.

The obtained results documented increased absorbance with increasing sample volume as was expected. Also, the increased volume extended the duration of one measurement cycle, but not significantly. Therefore, 150 μL was selected as the optimal sample volume. According to the repeatability of the results and duration of the measurement, the selected volume guaranteed proper detection of the analyte with sufficient sensitivity and repeatability.

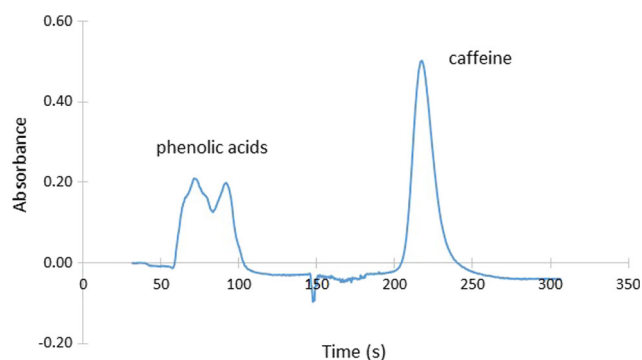


Fig. 1 Separation of phenolic acids (chlorogenic and gallic) from caffeine; C18 monolithic pre-column (10 \times 4.6 mm), mobile phase 10% methanol, flow rate of 10 $\mu\text{L s}^{-1}$

The control program was modified in response to the previous observations. The final optimal parameters were a sample volume of 150 μL and a 10 $\mu\text{L s}^{-1}$ flow rate for separation and detection. The sample aspiration flow rate was 50 $\mu\text{L s}^{-1}$. This parameter was not optimized, and the selected flow rate value was considered appropriate according to previous testing measurements.

Calibration and Repeatability

The calibration curve was measured with a caffeine concentration range of 1–15 mg L^{-1} and was corrected using the blank measurement. The regression line was expressed with a determination coefficient of 0.9969. The limit of detection (LOD) and the limit of quantitation (LOQ) were 0.128 and 0.425 mg L^{-1} , respectively.

Repeatability was determined under optimal conditions with 10 mg L^{-1} concentration of standard solution. The experiment was performed with and without precipitants being added (including only sample filtration) to compare the effect of reagents used for off-line sample pre-treatment. The aim was to determine the absorbance of the eluent and to express the relative standard deviation for the obtained values ($n = 12$). The relative standard deviation (RSD) using precipitants was 3.58%, whereas without reagents, the RSD increased to 4.68%. The results also pointed out that the absorbance was significantly different

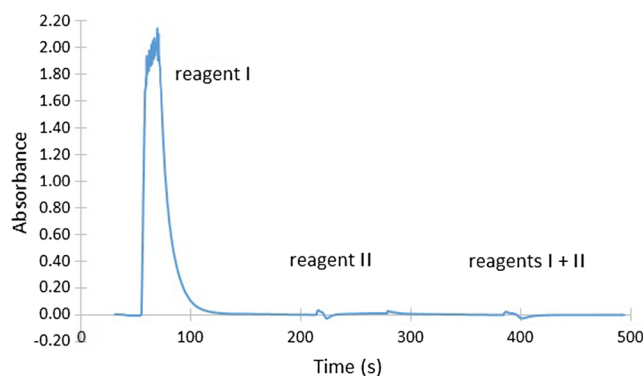


Fig. 2 Interference of Carrez reagents; reagent I: zinc sulfate, reagent II: potassium hexacyanoferrate(II), reagents I + II: ratio 1:1

Table 1 Optimized parameters of the SIA determination of caffeine

Parameter	Tested range	Optimized/found value
Separation/detection flowrate [$\mu\text{L s}^{-1}$]	5–20	10
Sample volume [μL]	20–150	150
Ratio of precipitants	2:1–1:2	1:1
Linear range [mg L^{-1}]	1–25	1–15
LOD [mg L^{-1}]		0.128
LOQ [mg L^{-1}]		0.425
Repeatability ^a [%]		3.58

^a $n = 12$, 10 mg L^{-1} of caffeine

when comparing the presence or absence of reagents. In case of off-line pre-treatment, the measured absorbance was about 10% higher and the detector record was not affected by the matrix content. Therefore, the application of reagents was proven to be important to guarantee the elimination of interferences.

Sample Pre-treatment

To guarantee the elimination of potential interferences, it was required to set the parameters for off-line precipitation. First, it was important to measure the absorbance of the reagents themselves to compare the detector signal. The results are presented in Fig. 2. The detector response was quite significant, especially for reagent I (zinc sulfate). Different ratios and amounts of reagents were tested. A standard solution of caffeine with a concentration of 1 mg L^{-1} was used for these experiments. The data are summarized in Table 1.

It was not reasonable to use precipitants separately, because they do not precipitate. But as was previously mentioned, the

signal of reagents was detected for comparison. It is not efficient to use them at unequal ratios, because this could significantly affect the final results. Therefore, the ratio of 1:1 was selected as optimal. As was expected, the density of the precipitate increased with a greater volume of reagent. This could cause filter blockage and also influence the duration of the sample pre-treatment step. On the other hand, low volume was insufficient for the elimination of all interferences. Therefore, it was important to select the appropriate volume. According to the detector response and acceptable experiment duration, a volume of $100 \mu\text{L}$ of reagents was selected as optimal.

Real Sample Analysis

The experiment was performed with three different types of coffee—brewed coffee, instant coffee, and espresso from a coffee machine. Each way of preparation followed a different procedure of sample preparation.

Table 2 Analysis of real sample—brewed coffee

Brand	Weight [g]	A	c_1 [mg L^{-1}]	c_2 [mg]	c_3 [mg]
Jacobs	0.0264	0.097 ± 0.007	2.24	0.373	25.45 ± 2.03
Bellarom	0.0263	0.093 ± 0.006	2.14	0.357	24.41 ± 1.64
Douwe Egberts	0.0249	0.101 ± 0.007	2.34	0.390	28.19 ± 2.04

c_1 : concentration in $150 \mu\text{L}$ of analyzed sample; c_2 : caffeine content in a sample; c_3 : caffeine content in a cup (1.8 g of coffee)

Table 3 Analysis of real sample—instant coffee

Brand	Weight [g]/volume ^a	A	c_1 [mg L^{-1}]	c_2 [mg]	c_3 [mg]
Nescafe	0.0259/0.5	0.092 ± 0.001	2.11	1.055	73.32 ± 3.89
Yellow E.	0.0251/0.5	0.123 ± 0.004	2.89	1.445	103.63 ± 4.09
GranArom	0.0254/1.0	0.077 ± 0.004	1.74	0.435	30.83 ± 0.92
Jacobs	0.0252/0.5	0.089 ± 0.007	2.04	1.020	72.86 ± 7.20

c_1 : concentration in $150 \mu\text{L}$ of analyzed sample; c_2 : caffeine content in a sample; c_3 : caffeine content in a cup (1.8 g of coffee)

^a Sample volume for precipitation step [mL]

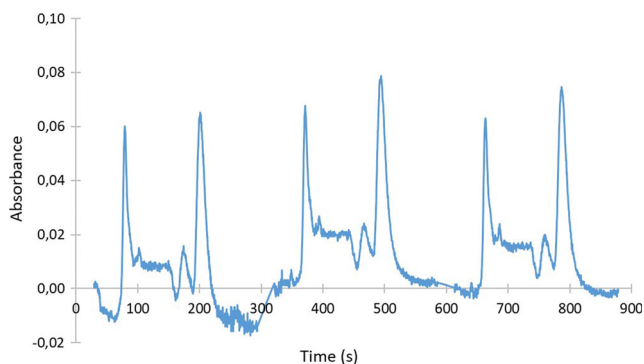


Fig. 3 Triplicate analysis of real coffee sample—instant coffee

Brewed coffee—Three different coffee brands were used for analysis. In the beginning, 0.025 g of sample was weighed into a 50-mL volumetric flask. Then, it was filled with boiling water up to 50 mL. After 15 min, the sample was filtrated. From each sample, 1.5 mL was taken and the precipitation reagents were added. The precipitate was removed by filtration, and the sample was diluted with distilled water in a 5-mL volumetric flask. Finally, a sample volume of 150 μL was analyzed and the caffeine content was determined and recalculated to the original sample weight and volume. The results are summarized in Table 2.

The sample preparation process for instant coffee was very similar to brewed coffee. Four coffee brands were tested. The coffee samples were weighed in 50-mL volumetric flasks, then filled to 50 mL with boiling water. After 15 min, an exact volume of each sample was taken and the precipitants were added. The precipitate was eliminated by filtration, and the samples were diluted in a 5-mL volumetric flask. The samples were analyzed, and the caffeine content was determined. The results are summarized in Table 3, and the real record of the instant coffee analysis is documented in Fig. 3.

Espresso from a coffee machine—Two coffee brands and different coffee preparation programs were utilized. The coffee machine prepared five types of beverages with different volumes. From each sample, 100 μL was taken and the reagents were added. The precipitate was carefully removed by filtration, and the sample was diluted with water in a 50-mL volumetric flask. Then, 150 μL of each sample was analyzed

and the caffeine content was determined. The results are summarized in Table 4, and the actual espresso analysis record is shown in Fig. 4.

Comparing the obtained results, the highest amount of caffeine was determined in coffee beverages prepared in the coffee machine. On the other hand, the lowest amount was determined in brewed coffee. For instant coffee, the caffeine content depends greatly on the coffee brand.

Comparison with Previously Published Articles

A simple automated method for purine alkaloid (caffeine) determination in coffee beverages was developed. The main measurement parameters, including sample volume, separation, and detection flow rates as well as the appropriate ratio of precipitants for off-line sample pre-treatment, were optimized. A 10% methanol solution was used as the mobile phase for the separation of purine alkaloids from phenolic acids.

Compared to previously published articles, the SIA method's main advantages lie in the simplicity of the measurement, quick analysis, and high repeatability. The main drawback is the off-line precipitation that was needed to decrease the content of the matrix components, but the same pre-treatment step is also crucial for other analytical techniques. The main differences were based on the detection and sample pre-treatment needed for removing the matrix from different samples. The quickest (18 h^{-1} for triplicate injection) was fluorescent assay where quenching the emission of bovine serum albumin was used and no sample pre-treatment was included (Alesso and Fernández 2016). On the other hand, stepwise injection combined with single-drop micro-extraction and potentiometric detection required a longer analysis time (Timofeeva et al. 2016), but the extraction used for the elimination of interfering matrix effects was carried out online in the flow system. The described system was applied for caffeine determination in saliva samples, and thus, a different sample pre-treatment, including removal of caffeine metabolites, was needed. The flow system with online SPE using a C18 minicolumn filled with 50- μm particles (Salinas-Vargas and Canizares-Macías 2014) was similar with respect to the application of carrez reagents for off-line precipitation and

Table 4 Analysis of real sample—espresso from coffee machine

Brand	Cup [mL]	A	c_1 [mg L^{-1}]	c_2 [mg]	c_3 [mg]
Normal	50	0.270 ± 0.015	6.56	0.328	164.00 ± 9.07
Espresso Gimoka	35	0.336 ± 0.014	8.21	0.411	143.68 ± 6.09
Bigger	80	0.174 ± 0.011	4.16	0.208	166.40 ± 10.65
Extra Big	110	0.142 ± 0.008	3.36	0.168	184.80 ± 10.11
Espresso Tchibo	35	0.434 ± 0.010	10.66	0.533	186.55 ± 4.09

c_1 : concentration in 150 μL of analyzed sample; c_2 : caffeine content in 100 μL of sample; c_3 : caffeine content in a cup

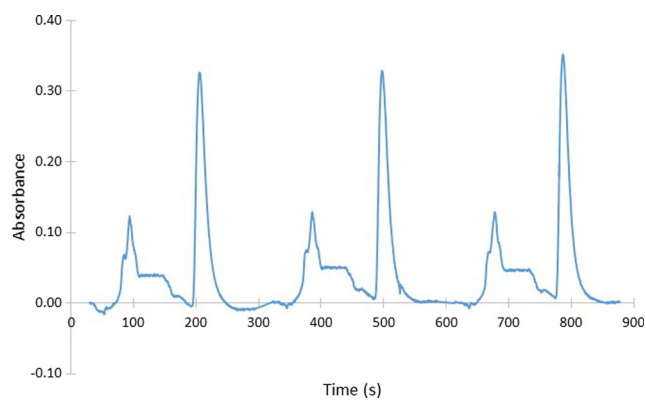


Fig. 4 Triplicate analysis of real coffee sample—espresso

also for the separation of phenolic acids from caffeine. But the analysis throughput was lower while the separation step lasted about 7 min (nine samples were analyzed in 1 h); eluent consumption was higher because of the continuous flow generated by the peristaltic pump and the lab-made minicolumn together with the unique flow system geometry decreases the applicability of this method.

The efficacy of the developed method was tested on real samples, and the determined content of caffeine in different coffee types corresponded with the predicted values found in literature (Bjarnadottir 2016). For brewed coffee, the tested brands contained lower caffeine levels close to 25 mg and only small differences were documented. For instant coffee brands, larger differences (30–100 mg) than were expected were found. And different types of coffee prepared in a coffee machine showed the same content of caffeine (around 150 mg); the only difference was found in the serving size. These results might differ with respect to different types and brands of coffee machines; thus, the information is always relevant for respective users.

Conclusions

The method used for the determination of purine alkaloids evaluated as caffeine using off-line precipitation and simple online separation from the matrix components on a short monolithic pre-column in the system based on the sequential injection principle with UV detection was optimized and applied for analyzing real samples.

The separation using a monolithic pre-column that is commercially available was effective and required less time than previously developed methods based on similar principles. The real sample analyses showed the expected results of caffeine content, and no other interference was observed in the case of brewed coffee, instant coffee, or coffee prepared in a coffee machine. Thus, the developed method revealed its applicability for caffeine determination in coffee drinks, which was the main aim of the presented work. But the applicability

of the method to other sample matrices (biological samples) is limited by the obtained LOD and LOQ limits, which did not reach values comparable with electroanalytical techniques.

Compliance with Ethical Standards

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Conflict of Interest Mihalčíková L declares that she has no conflict of interest. Boonjob W declares that she has no conflict of interest. Sklenářová H declares that she has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

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