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High throughput method for determination of caffeine in coffee drinks

Diploma thesis

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Hradec Králové 2016 Lýdia Mihalčíková

Prehlasujem, že táto práca je mojím pôvodným dielom. Všetky zdroje, z ktorých som čerpala sú zahrnuté v zozname literatúry a použité úryvky sú náležite citované. Moja práca nebola použitá na získanie iného titulu.

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Abstract

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Work title: High throughput method for determination of caffeine in coffee drinks

Caffeine is a xanthine alkaloid acting like a stimulant of heart and central nervous system. Quantification of caffeine in coffee drinks is significant to show how much of caffeine was in each cup which has been taken per day prior to prevent a caffeine overdose. The development of high-throughput sequential injection analysis (SIA) spectrophotometric assay for the determination of caffeine in coffee drinks was performed. Sample was treated with carrez reagent for matrix suppression followed by filtration thereafter analyte was isolated from organic acids by a short C18 monolithic column (10x4.6mm). The flow rate of the separation step was 10 μL s^{-1} with 10 % *v/v* of methanol as the mobile phase. Caffeine was detected at 274 nm. The influence of main parameters affecting the quantification of caffeine was optimized. Duration of one sample analysis was 15 minutes. During one hour it was possible to analyze 4 samples. Linear range was 1 - 15 mg L^{-1} and determination coefficient (r^2) was 0.9969. The limit of detection (LOD) and limit of quantification (LOQ) were 0.128 and 0.425 mg L^{-1}, respectively. The relative standard deviation (RSD) was 3.58 % (n = 12, 10 mg L^{-1}). Under optimal conditions, the method was successfully applied to determine caffeine in different real samples including the soluble coffee, coffee from espresso machine and brewed-coffee drinks.

Abstrakt

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Názov práce: Rychlá metoda stanovení kofeinu v kávových nápojích

Kofeín je látka s výrazným stimulačným účinkom. Stimuluje nielen činnosť srdca ale aj centrálneho nervového systému, tým zvyšuje pozornosť a znižuje únavu. Z hľadiska chemickej štruktúry ho zaraďujeme do skupiny xanthinových alkaloidov. Vzhľadom k jeho častej konzumácií najmä v podobe kávy, je nutné sledovať hladiny kofeínu v obľúbenom nápoji, aby sme boli schopní predchádzať stavom predávkovania a návyku. Z tohto dôvodu bola navrhnutá metóda na stanovenie kofeínu s využitím sekvenčnej injekčnej analýzy a detekciou pomocou UV-VIS spektrofotometra. K úprave vzoriek boli použité zrážacie činidlá a následne bol analyt izolovaný od organických kyselín pomocou monolitickej kolóny. V separačnom kroku bola zvolená rýchlosť 10 $\mu L\ s^{-1}$ a mobilnou fázou bol 10% roztok metanolu. Kofeín bol stanovovaný pri vlnovej dĺžke 274 nm. Hlavné parametre stanovenia boli optimalizované. Analýza jednej vzorky trvala približne 15 minút, pričom meranie u každej bolo prevedené celkom 3-krát. Bola zmeraná kalibračná závislosť s determinačným koeficientom $r^2 = 0,9969$ v rozsahu koncentrácii 1 – 15 mg L^{-1} . Limit detekcie (LOD) bol 0,128 mg L^{-1} a limit kvantifikácie (LOQ) 0,425 mg L^{-1}. Relatívna smerodatná odchýlka bola 3,58% (n = 12, 10 mg L^{-1}). Metóda bola úspešne aplikovaná pri stanovení kofeínu v rôzne pripravených vzorkách kávy.

Content

Abbreviations

HPLC- high- performance liquid chromatography

LOD- limit of detection

LOQ- limit of quantification

LOV- Lab on valve

RSD- relative standard deviation

SD- standard deviation

SIA- sequential injection analysis

SPE- solid phase extraction

UV- ultraviolet radiation

VIS- visible radiation

1 Introduction

Caffeine is daily consumed in coffee, tea, cocoa, chocolate or medicaments. It is xanthine alkaloid with its chemical structure of 1,3,7- trimethylxanthine. Caffeine effects heart, stimulates central nervous system and also increases the blood pressure. According to high consumption of caffeine worldwide and its potential undesirable effects or potential withdrawal symptoms, it is important to monitor the levels of caffeine in coffee and other caffeine-containing beverages (black tea, energy drinks etc.). Therefore, the purpose of my work is to develop new, simple, fast, and cheap analytical method for determination and quantification of caffeine in coffee drinks.

2 Aim of the work

The aim of the thesis was to optimize the method for determination of caffeine in coffee beverages using short monolithic pre-column to separate the analyte from the matrix with application of sequential injection analysis and UV detection. The main assignment was to optimize the parameters of experiment. It was important to select appropriate conditions for the measurement such as sample volume, sample flow rate, right selection of mobile phase. Under the optimal conditions, it was required to measure the calibration curve, express the limit of detection, limit of quantification and verify the repeatability. Finally, the method has been applied for analysis of real samples.

3 Theoretical part

3.1 Sequential injection analysis

Sequential Injection (SI) is one of the most advanced and efficient analytical methods based on flow programming. At first time, this technique was presented by Růžička and Marshall in 1990. Sequential injection technique is a tool for automation of laboratory procedures including sample metering, reaction timing, mixing of reagents etc. Automation allows to achieve good repeatability, higher sample throughput, miniaturization, e.g. reduction of reagent consumption [1].

The main parts of the SIA system are:

- Syringe pump
- Holding coil
- Multiposition selection valve
- Detector
- Computer

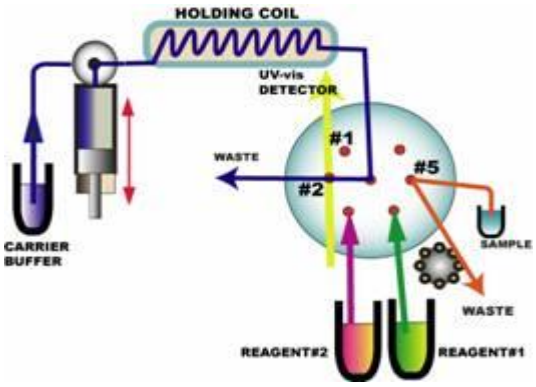


Fig.1: Schematic presentation of the SIA system

Sequential injection uses programmable, bi-directional flow, precisely monitored by a computer. SI is based on injection of sample and reagent into a stream of a carrier solution.

During this process, sample and reagent zones disperse between each other and the reaction product is formed on their interface. A flow through the detector changes measured physical parameters when the product reaches the flow cell [1].

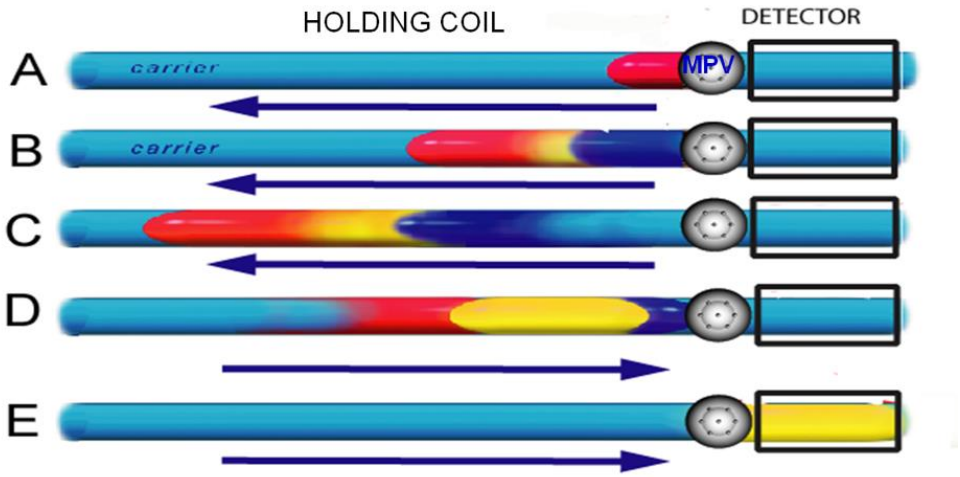


Fig.2: Principle of SIA

Sample and reagent are injected sequentially by multiposition valve into the carrier stream. Each port of valve is used for different purpose and provide the appropriate arrangement of reagents, sample and detector according to experimental requests. When the reaction product starts to be formed at the interface of reagent zones, the reversal flow is applied to provide the mixing and transport of product into the detector. At the end of measurement cycle, residues in the system are washed out with carrier and disposed to waste. The time interval between sample injection and analyte detection is reproducible and the analysis of all samples is provided in the same way. Therefore, SIA allows the comparison of standard with unknown content of the same substance [1].

The selection of appropriate type of detector highly depends on the type of the analytical reaction. Spectrophotometric, fluorescent or electrochemical detectors are available in case of SIA systems. The whole analysis as well as the collection and storage of data are monitored by computer with appropriate control software [1].

3.2 UV- VIS detection

UV/UV-VIS detectors are most frequently used in flow systems to measure components using an absorption spectrum in the ultraviolet or visible region. Electromagnetic field with higher frequency interact with electrons, cause their excitation and transfer them onto the higher energetical level. Beam of the electromagnetic radiation passed through the detector flow-cell will change its intensity according to the interaction. The intensity of the beam will decrease while it´s passing through the flow cell. Wavelength range of 200-400 nm corresponding to UV region. In these areas, interact molecules which contain double bonds in their structure, so called chromophores. The majority of organic compounds is possible to be analyzed by UV/VIS detectors.

In case of flow systems, components detected by UV/VIS spectrophotometry are preferably analyzed, and thus this type of detector is most widely used. Online measurement of absorbance is enabled by flow cell that is directly connected to optical fibers. Flow cell and optical fibers are connected with light source and detector. It is always preferable to carry out the spectrophotometry in two wavelengths, the secondary serving as a reference to stabilize the signal. Flow through cell for spectrophotometry could be designed in different ways. Nowadays the technology allowed us to change the position of flow cell and place it in the best position in flow channel. This is the key component of miniaturized LOV design [1, 8, 9].

3.3 Solid phase extraction (SPE)

The SPE is extraction method developed for sample pretreatment. Analyte and interfering substances in liquid phase are retained or washed out from a solid sorbent in special SPE column. Solid phase extraction is used for isolation of the analytes from variety of matrices that could potentially cause measurement failure. In many cases the concentration of analyte is very low. The SPE also provides pre-concentration of analyte and increases the sensitivity of assay. Therefore, analytical laboratories often use SPE to pre-concentrate and purify samples for analysis [2].

Advantages of SPE method:

- Fast analysis
- Effectivity (using small volumes of solutions)
- Automation
- Easy manipulation

A typical solid phase extraction involves four basic steps.

- Conditioning
- Sample loading
- Washing
- Elution

Firstly, the column is activated with an appropriate solvent, which wets the surface and penetrates in to the sorbent. Then commonly water (as a sample solvent) is washed through the column. After that the sample is aspirated to the column. As the sample passed through the sorbent, the analytes in the sample interact and retain on the sorbent while the solvent and other impurities pass through the column. Then washing of residues from the sample matrix is needed. Finally the analyte is eluted with appropriate eluent.
The SPE separates the analytes due to different interactions between chemical compounds. According to the properties of analyte, it is necessary to select the appropriate type of sorbent [2].

Principal separation modes in SPE

- **Normal phase SPE**

Normal phase SPE procedures usually involve a polar analyte, non-polar matrix and a polar stationary phase. Polar bonded silicas with -CN,-NH_2 or diol are typically used under normal phase conditions. Retention of an analyte is due to interactions between polar functional groups of analyte and polar groups on the sorbent surface. These include dipol- dipol interactions, hydrogen bonding, or others. A compound adsorbed by these mechanisms is eluted by a solvent that disrupts the bindings. Usually, solvent that is more polar than the sample´s matrix is applied for elution [3, 4].

- **Reversed phase SPE**

This procedure involve a polar mobile phase and a nonpolar stationary phase. The analyte is typically nonpolar. In this case, the hydrophilic silanol groups at the surface have been chemically modified with hydrophobic alkyl or aryl groups. C-18 and C-8 are standard monomerically bonded silicas. Polymerically bonded materials provide more complete coverage of silica surface. The following materials are also utilized for reverse phase SPE: phenyl bonded silica or hydrophobic C18-like bonded silica coated with hydrophilic polymer. Retention of organic analytes from polar solutions is due to the attractive forces between the carbon-hydrogen bonds in the analyte and functional groups on the silica surface. These attractive forces are called van der Waals forces. Afterwards, the analyte is collected by nonpolar solvent [3, 4].

- **Ion exchange SPE**

Ion exchange SPE can be used for compounds that are charged in solution. Anionic (negatively charged) compounds can be isolated by aliphatic quaternary amine groups which are bonded to silica. A quaternary amine as positively charged cation attracts anionic species in solution. While, cationic (positively charged) compounds are isolated using silica with aliphatic sulfonic acid groups. The sulfonic groups attract cationic species from a solution. The retention mechanism is based on the electrostatic attraction of the charged functional group on compound to the charged group bonded to the silica surface. When one of these functional group is neutralized, the electrostatic force is disrupted and the compound is eluted [3, 4].

Highly selective sorbents

Molecularly imprinted polymer (MIP) sorbents consists of crossed linked polymers which have a selectivity for a single analyte or a group of structurally related analytes. High selectivity of these sorbents allow to provide experiments with lower detection limits [5].

Restricted access materials (RAM) sorbents have been developed to allow direct injection of biological matrices into on-line SPE systems. RAMs are mainly used for the analysis of substances with low molecular mass. A hydrophilic barrier enables the small molecules to permeate through the hydrophobic stationary phase. Therefore, this method allow to separate macromolecules from small ones with low molecular mass [6, 7].

Other ways for separation of the analyte from the matrix

To separate the analyte from the matrix components not only SPE step is needed but also short chromatographic column could be applied for this reason. Monolithic pre-column is one possibility how to automate simple and quick separation in low-pressure flow systems. In this case elution of the matrix and the analyte is carried out in one step and the selection of the elution solvent/mobile phase should reach compromise between washing the matrix and separation of the analyte at the same time.

3.4 LOV technique

Miniaturized SI-LOV instruments are the small automated analyzers. Miniaturized SI systems use only small volumes of samples and reagents to perform the assays at high speed. The LOV module is transparent, therefore it allows the visual control of all sample manipulations. The dispersion of sample and reagent zones in the system is visualized and controlled by injecting a dye into the colorless carrier stream. The system is designed to make the distance between sample aspiration and detection as short as possible [1].

The characteristic features of LOV module are the short channel between the central port and detector and the long holding coil, situated upstream from the multiposition valve. With this design of LOV module, dispersion is controlled by the flowrate and by selecting appropriate volumes of samples and reagents injected into the holding coil. By using LOV is also possible to aspirate small amount of SPE sorbents and provide solid phase extraction in this miniaturized configuration [1].

During the experiment 10 - 20 μL of samples is typically used for analysis and the waste is usually about 0.1 - 0.2 mL. This configuration is advantageous, economical and decreases the volumes of samples and reagents. For detection several types of detectors can be used. The most commonly used are spectrophotometric and fluorimetric [1].

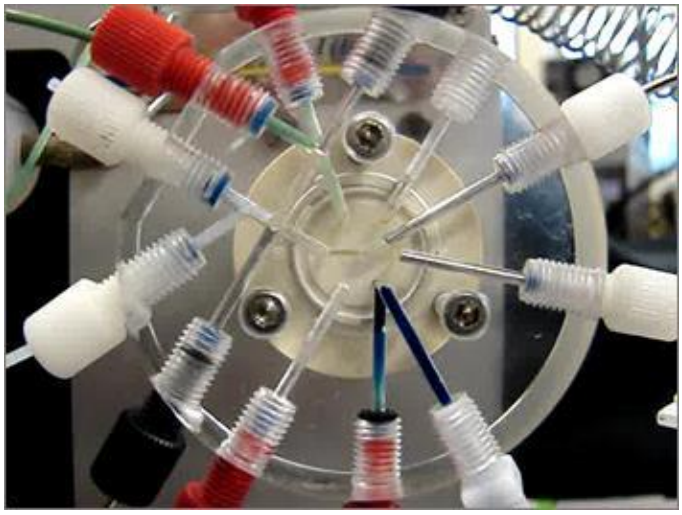


Fig.3: Lab on valve configuration

3.5 Caffeine

- *Caffeine in beverages*

Coffee and tea are two of the most widely consumed beverages in the world. Coffee and various types of tea such as green tea or black tea contain a lot of bioactive substances. The coffee beverages are rich in polyphenols, tannic acid, pyrogallic acid also chlorogenic acids such as caffeic, p-coumaric acids which play role in quality and flavors of coffee. However, the main active secondary metabolites are alkaloids, especially caffeine [14,22].

- *Caffeine as a compound*

Caffeine is a xanthine alkaloid acting like a stimulant of heart and central nervous system. The first consumers of caffeine were from Ethiopia at about 1000 years ago. Caffeine was extracted from the bean of the coffee plant and leaves of the tea bush, also we can naturally find it in seeds and fruits of plants such as guarana or cola. Pure caffeine is an odorless alkaloid with a bitter taste. As a pure compound was isolated for the first time in 1819. Caffeine with chemical structure of 1,3,7- trimethylxanthine has a lot of applications in different fields [13,14].

- *Effects of caffeine (positives vs. negatives)*

Caffeine effects heart, stimulates central nervous system and also increases blood pressure. Many of effects of caffeine may be related to the action of methylxanthine on neurons. Caffeine positively effects memory, coordination, learning, vigilance. It´s decreasing the fatigue and increasing attention. Clinically it can be used for example at the recovery of coma. However, children in general do not appear more sensitive to these effects than adults. The central nervous system does not develop a great tolerance to the effects of caffeine although the withdrawal symptoms are reported. A lot of experiments showed that polyphenols and chlorogenic acids (such as caffeic and ferulic acids) reduce cholesterol levels [14, 15, 22].

Coffee polyphenols have antioxidant activity. These bioactive substances modulate the substrate oxidation in human body and also decrease high concentration of glucose in blood circulation. Polyphenols content is also associated with the reduction of body fat. Despite of these benefits of caffeine and other substances, we have to be careful with consumption of coffee beverages. It could cause undesirable effects like anxiety and nervousness. Caffeine is considered as a marker for heart disease, asthma or kidney dis-function. The fatal dose of caffeine is usually more than 10 grams (170 mg/kg). It has been connected with coma and death in case of caffeine overdose (more than 200 mg/kg per day) [11, 14, 22].

- *Application of caffeine*

Caffeine is consumed daily in the form of coffee, tea, cocoa, chocolate. It can be used as an additive in many carbonated drinks. About 120,000 tons of caffeine is consumed worldwide every year. Caffeine is found in pharmacological preparations such as cold or flu remedies, painkillers (analgesics) and anti-migraine drugs [14].

Because of the high consumption of caffeine worldwide and its potential undesirable effects, it is important to monitor the levels of caffeine in coffee and other caffeine-containing beverages (tea, energy drinks). Therefore, it is important to involve simple, fast, reliable and not expensive analytical method to follow the effects of caffeine on the human body [15].

3.6 Published studies related to determination of caffeine in different samples

Regarding to high consumption of caffeine worldwide, many articles dealing with determination of caffeine in different samples have been published. As well as variety of analytical procedures has been developed.

M. Tefera et al. presented a new method for caffeine and paracetamol determination by square wave voltammetry using modified glassy carbon electrode. Method investigated the effect of pH and scan rate on the voltametric response of caffeine and paracetamol to provide the determination under optimal conditions. The calculated detection limit for caffeine was 0.79 μmol L^{-1}. The modified glassy carbon electrode was successfully utilized for analysis of caffeine and in cola beverages and teas [16].

K. Tyszczuk-Rotko and I. Beczkowska presented a development of new, sensitive and selective voltametric method for caffeine determination in tea, coffee, energy drinks and pharmaceuticals by applying a Nafion covered lead film electrode. The utility of suggested method was successfully tested and voltametric method was evaluated by UV-VIS spectrophotometry. Therefore, this method can be used as an alternative to more expensive spectroscopic and chromatographic methods for caffeine determination in real samples. The obtained detection limits for caffeine following 120 s of accumulation time were equal to 1.7 x 10^{-8} mol L^{-1}(peak 1) and 2.2 x10^{-7}mol L^{-1}(peak 2) [17].

M. Alesso and L.P. Fernándes developed a new method for caffeine determination based on fluorescent emission of Bovine Serum Albumine (BSA). Sensitivity and selectivity of suggested method allowed variety sample analyses. Therefore this method was successfully applied for caffeine determination even in dietary supplements or energy drinks [18].

P. De Kesel et al. presented a novel LC-MS based procedure for determination of caffeine and its major metabolite paraxantine in hair. The experiment focused mainly on effect of hair decontamination procedure. Caffeine was found in all of wash solvents in different concentration. While caffeine metabolite was not detected in any of wash solvents. The suitability of method was demonstrated by applying it to hair samples of 10 healthy volunteers [19].

Sareshti and Samadi developed novel green analytical method based on dispersive liquid-liquid microextraction and GC-nitrogen phosphorus detection of caffeine in teas and coffees. Under optimal conditions the method was applied for caffeine determination in various beverages. LOD and LOQ were 0.02 and 0.05 μg mL^{-1}, respectively [20].

P. Dewani et al. presented a simple and reliable HPLC-DAD method to analyze a complex multi-drug formulations consisting of caffeine and other drug substances. Effective chromatographic separation of all formulation components was achieved by using C18 reverse-phase column and the mobile phase composed of phosphate buffer and acetonitrile. The total run time was 20 min. The suggested HPLC method was validated and successfully applied for analysis of all substances in tablet formulation [21].

Belguidoum et al. presented analytical method based on HPLC coupled to UV-VIS detection for determination of caffeine and phenolic compounds in different coffee brands. Many aspects has been taken in to account (e.g. packaging or roasting degree) during the measurement. The obtained results pointed out that these procedures have effect on concentration of caffeine in coffee beverages. The limit of detection (LOD) ranged from 0.75 to 14.79 mg L^{-1}, while the limit of quantification (LOQ) ranged from 2.26 to 44.44 mg L^{-1}. The separation of all compounds was achieved within 13 min [22].

Žiak et al. developed new simple spectrophotometric method for determination of caffeine and other substances (e.g. caramel or riboflavin) contained in cola-type beverages and energy drinks. Caffeine shows native fluorescence in aqueous solutions. Fluorescent detection was coupled with standard addition method and successfully applied for caffeine determination. Developed procedure provided comparable results with those from HPLC reference method [23].

Timofeeva et al. developed a flow potentiometric method for determination of caffeine in saliva. Stepwise injection analysis with potentiometric detection and single-drop liquid microextraction (SDLME) was performed. SDLME was utilized to eliminate interfering effects of saliva and caffeine metabolism products. A linear range of 10^{-5}–10^{-2} mol L^{-1} was established for caffeine with detection limit at $6x10^{-6}$ mol L^{-1}. The sample throughput was 6 samples per hour. The method was applied to real samples and the obtained results agreed with results of reference HPLC method [24].

M. E. Salinas-Vargas and M. D. Canizares-Marcias presented novel method based on SPE combined with flow injection (FI) for determination of caffeine in coffee beans. Caffeine was extracted from coffee beans by hot water. The C18 minicolumn was coupled to FI system. This configuration allowed direct injection of aqueous extracts into the system without previous dilution and increased the sample throughput. The total analysis time was 6 min and LOD was 128 μg mL^{-1}. Suggested method could be an alternative for caffeine determination because it is cheap, precise and fast [25].

H. Zheng et al. developed a simple method for preparation of flower-like silver nanostructure which could allow effective SERS (surface enhance Raman scattering) detection of caffeine. The SERS measurement pointed out that silver nanostructures have a high SERS activity. Therefore, these nanostructures have a potential as effective SERS substrates for detection of caffeine. The detection sensitivity of 10^{-8} mol L^{-1} based on flower-like silver nanostructures can be reached [26].

4 Experimental part

4.1 Materials

4.1.1 Chemical substances

Caffeine anhydrous, Sigma-Aldrich, Prague, Czech republic

Methanol, Sigma-Aldrich, Prague, Czech republic

Potassium hexacyanoferrate(II) trihydrate, Sigma Aldrich, Prague

Zinc sulfate heptahydrate, Sigma Aldrich, Prague

Ultra-pure water prepared by Milli-Q (Millipore, USA)

4.1.2 Instrumental equipment

FIAlab 3000 (8-port selection valve), FIAlab Instruments, USA

Piston pump (volume 5ml), FIAlab Instruments, USA

UV source D1000 CE

Detector USB4000, Ocean optics, USA

Optical fibers (300µm)

C18 monolithic pre-column (10x 4.6mm)

4.2 Experimental procedure

4.2.1 Preparation of stock solutions

Caffeine stock solution 1 was prepared by dissolving 50 mg of caffeine in 50 mL volumetric flask and filled with distilled water to the mark. Concentration of stock solution 1 was 1000 ppm (1000 mg L^{-1}).

Caffeine stock solution 2 was prepared by diluting 5 mL of stock solution 1 in 50 mL volumetric flask. Concentration of stock solution 2 was 100 ppm (100 mg L^{-1}).

4.2.2 Preparation of standard solutions

Standard solutions of caffeine were prepared as calibration solutions for quantification of caffeine in different types of coffee drinks.

Calibration solutions were prepared by diluting stock solution (100 ppm) by distilled water in 10 mL volumetric flask. Concentrations of calibration solutions were 1 ppm, 2.5 ppm, 5 ppm, 10 ppm and 15 ppm.
As a blank distilled water has been used. (*Table 1*)

Table 1: Preparation of standard solutions

Concentration of standard solution (ppm)	Volume of stock solution 2 (µL)	Volume of distilled water (mL)
1	100	9.90
2.5	250	9.75
5	500	9.50
10	1000	9.00
15	1500	8.50

4.3 System optimization

To ensure the proper functioning of the SIA system for analysis, it´s highly important to create an appropriate control program. It is particularly necessary to focus on aspiration, separation of sample components and finally detection of the analyte. Especially, it is important to select the relevant sample volume and flowrate. To eliminate potential interferences, precipitants were used during sample preparation process. Then it was advantageous to apply separation on the short monolithic pre-column to separate other interferences from the analyte. Elimination of all the residues which could affect the final results by potential carryover was important. Therefore, the residues were washed out from the column by 10% methanol. The experiment was performed in the SIA system using UV/VIS spectrophotometer for detection. Detection was performed at wavelength of 274 nm. Each measurement has been repeated in triplicate and compared with a blank (distilled water). To keep the accuracy of aspiration volumes, it was required to select appropriate aspiration flowrate. During experiment the aspiration rate was 50 µL s^{-1}. For all measurements FIAlab 3000 system with single piston pump and 8-port selection valve was utilized. System scheme is demonstrated in *Figure 4*.

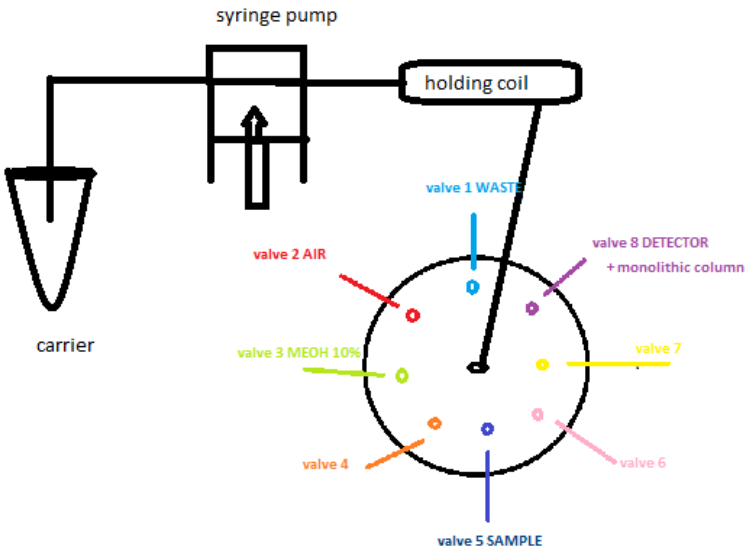


Fig.4: System scheme

4.3.1 Optimization of elution rate

Four different elution rates were tested during measurement: 5, 10, 15 and 20 µL s^{-1}. The eluent was 10% methanol solution. Standard solution of caffeine with concentration of 10 ppm (10 mg L^{-1}) has been used for flowrate optimization. The main effort was to provide enough time for separation of the matrix component and caffeine and detection of the analyte in real time. Therefore, it was required to determine the relation between the flowrate of elution of the analyte retained on the short monolithic pre-column sorbent and its absorbance in the detector. The results are summarized in chapter (5.2).

4.3.2 Optimization of sample volume

Different 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 important to select the relevant volume corresponding to analysis parameters. Therefore, it was necessary to take into account the sensitivity and robustness of the detector. The aim was to find small volume for separation but still sufficient volume for detection. The exact volume was aspirated from the volumetric flask in to the system, afterwards the analyte passed through the column with flowrate of 10 μL s^{-1}. Standard solution of caffeine with concentration of 10 ppm (10 mg L^{-1}) has been used for volume optimization. Relation between sample volume and the absorbance of analyte eluted from the monolithic pre-column has been examined and the results are presented in the chapter (5.1).

4.4 Calibration curve

The calibration curve was measured under optimal conditions. Absorbance of eluate was determined in a series of calibration solutions with concentrations of 1 ppm, 2.5 ppm, 5 ppm, 10 ppm, 15 ppm against the blank (0 ppm). Subsequently, the relation between the absorbance and concentration of calibration solutions was plotted into the graph. The equation of regression line and determination coefficient were expressed from the obtained results. Limit of detection (LOD) and limit of quantification (LOQ) were determined by following equations:

$$LOD = \frac{3*\sigma}{S} \qquad LOQ = \frac{10*\sigma}{S}$$

σ- standard deviation

S- slope of a calibration curve

The results are summarized in chapter (5.4).

4.5 Repeatability

The repeatability was determined under the optimal conditions to guarantee the reliable results. During the measurement, the concentration of standard solution was 10 ppm. The experiment was performed 12 times. The repeatability was expressed as relative standard deviation (RSD, %) and it was calculated from 12 measurements. The results are summarized in chapter (5.5).

4.6 Optimization of precipitation

The application of precipitants was important part of sample pre-treatment according to elimination of potential interferences. 2 reagents were used: zinc sulfate hepta-hydrate and potassium hexacyanoferrate(II) tri-hydrate. Reagents were added in a certain ratio to analyte and then the precipitate was manually filtrated out using 0.45 μm nylon syringe filter. To achieve the most effective interferences removal, it was necessary to find the appropriate ratio of precipitants. Therefore, 5 different ratios of reagents have been examined during experiment and the results are presented in chapter (5.6).

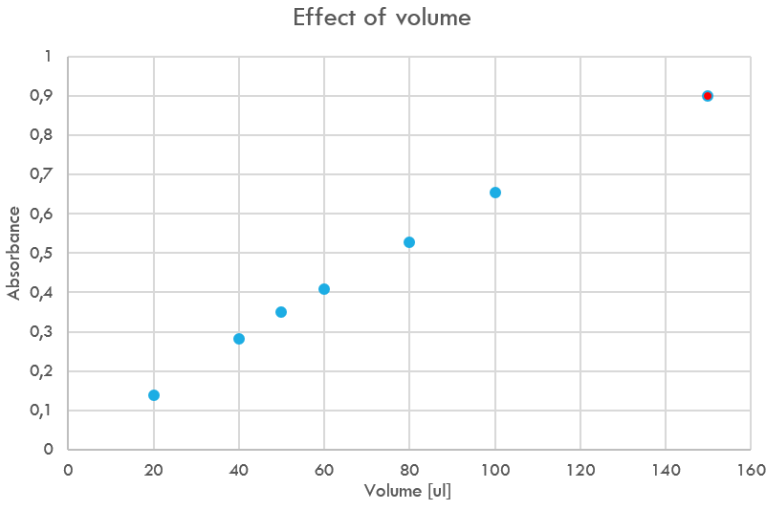
4.7 Analysis of real sample

Three different ways of coffee preparation were performed during experiment. Brewed coffee, instant coffee and espresso from coffee machine were prepared. Different brands of coffee were used for objective comparison of caffeine content in various types. All the real samples were diluted to desired concentration. After the elimination of matrix effects, samples were prepared for analysis under the optimal conditions. The absorbance was monitored and the efficacy of method was tested and evaluated. Final results are summarized in chapter (5.7).

5 Results and discussion

5.1 Optimization of sample volume

The aim was to determine the optimal sample volume needed for the aspiration of sample to the column and retention of the analyte on the monolithic sorbent. The evaluation criterion was duration of one measurement cycle and intensity of the detector response. As appropriate flow rate for aspiration of sample 10 µL s^{-1} has been used. The same flow rate has been selected for elution and detection of the analyte. The results are summarized in Table 2 and on Graph 1.



Graph 1: Optimization of volume sample

Table 2: Optimization of sample volume

Volume sample (μL)	*Absorbance*					
	Measurements					
	1.	2.	3.	Average	SD	RSD (%)
20	0.138	0.141	-	0.139	0.002	1.77
40	0.266	0.296	0.286	0.283	0.015	5.44
50	0.354	0.347	0.347	0.349	0.004	1.26
60	0.415	0.408	0.401	0.408	0.006	1.68
80	0.519	0.522	0.543	0.528	0.013	2.46
100	0.648	0.658	0.655	0.654	0.005	0.79
150	0.902	0.889	0.899	0.897	0.007	0.73

Only two values of absorbance were determined for volume of 20 μL. The last value was not included due to the baseline noise. In view of this fact, the absorbance could not be evaluated.

The obtained results pointed out that with the higher sample volume the absorbance of analyte increased as was expected. Also, the increasing volume extended the duration of one measurement cycle, but not significantly. Therefore, 150 μL has been selected as optimal sample volume. According to repeatability of results and duration of measurement selected volume guarantee the proper detection of analyte.

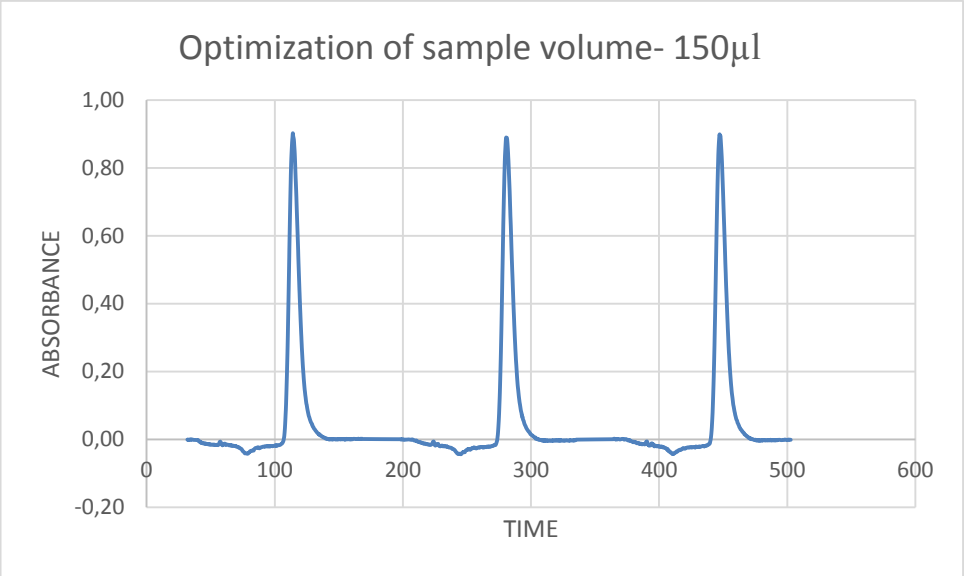
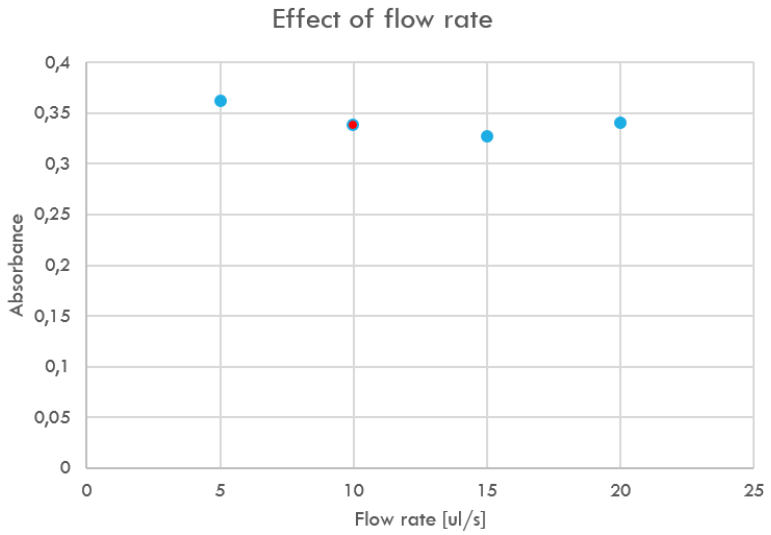


Fig.5: Optimization of sample volume- elution and detection of analyte. (The volume sample was 150 µL. Aspiration and elution flowrate was 10 µL s^{-1}. 10% MeOH has been used as eluent/mobile phase.)

5.2 Optimization of aspiration flow rate to column and elution flow rate

The aim was to find the appropriate flowrate to guarantee the efficient elution of analyte retained on the monolithic pre-column and separation from the sample matrix. Additionally, if the flowrate is very high, the detector could not be able to scan the analyte signal precisely. On the other side if the flow rate is too slow, then the experiment takes unnecessarily long time. During the optimization process, constant sample volume of 50 µL has been used. The main criterion for the selection was the height of the response of the detector in compromise with analysis time, number of points scanned by detector and effective separation of caffeine from sample matrix.



Graph 2: Optimization of aspiration rate on column and elution flowrate

Table 3: Optimization of aspiration rate on column and elution flowrate

Flow rate ($\mu L\ s^{-1}$)	*Absorbance*					
	Measurement					
	1.	2.	3.	Average	SD	RSD (%)
5	0.360	0.354	0.371	0.362	0.008	2.28
10	0.329	0.348	0.338	0.338	0.009	2.77
15	0.317	0.317	0.344	0.327	0.015	4.57
20	0.349	0.323	0.350	0.341	0.015	4.48

In view of the results, the best response of detector according to analysis duration and standard deviation was obtained at the flowrate of 10 µL s^{-1}. The flowrate of 5 µL s^{-1} also provided sufficient response of detector. However, this flowrate prolongs the experiment significantly. The shortest duration of measurement was performed with flowrate of 20µL s^{-1}. Unfortunately, the repeatability of obtained values was inadequate.

Therefore, the flowrate of 10 µL s^{-1} has been selected as optimal for elution and detection of analyte. With this flowrate was also achieved sufficient separation of caffeine from matrix as it is demonstrated on *Fig.7: Separation process- elimination of matrix.*

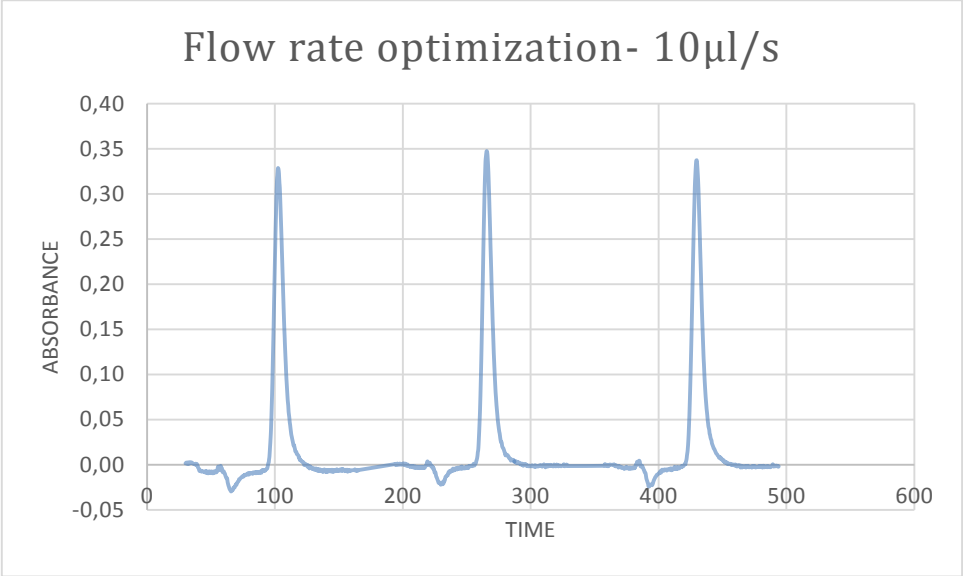


Fig.6: Optimization of flowrate- aspiration, elution and detection of analyte. (The volume sample was 50 µL. As optimal flowrate has been used 10 µL s^{-1}. The eluent was 10% MeOH.)

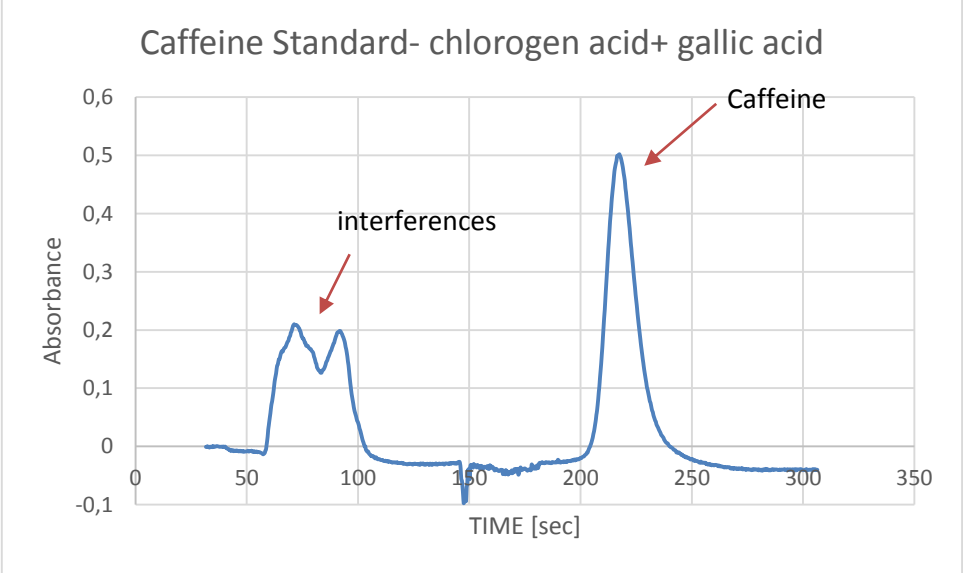


Fig. 7: Separation process- elimination of matrix (Sufficient separation of caffeine from matrix was achieved by flowrate 10 µL s^{-1}.)

5.3 Final programme

Programme has been modified due to the previous measurements. The final optimal parameters were sample volume, aspiration flowrate and elution flowrate. The sample volume of 150 μL has been selected as optimal. The aspiration rate of sample was 50 μL s^{-1}. This parameter was not optimized and selected value of flowrate was considered as appropriate according to previous testing measurements. As optimal flowrate for aspiration of sample to column was chosen 10 μL s^{-1}. The same flowrate was selected for elution and detection of analyte.

FINAL PROGRAM: Syringe Pump Valve In	
Syringe Pump Flowrate (microliter/sec) 250 Syringe Pump Aspirate (microliter) 200 Syringe Pump Valve Out	
Valve 1 port 3- MeOH	Aspiration of 10% MeOH
Syringe Pump Flowrate (microliter/sec) 150 Syringe Pump Aspirate (microliter) 700	
Valve 1 port 5 – STANDARD/SAMPLE	Aspiration of sample
Syringe Pump Flowrate (microliter/sec) 50 Syringe Pump Aspirate (microliter) 150	
Analyte New Sample Spectrometer Reference Scan Spectrometer Absorbance Scanning	Analysis settings

Valve 1 port 8	Separation of sample components on the monolithic precolumn
Syringe Pump Flowrate (microliter/sec) 10 Syringe Pump Dispense (microliter) 700 Delay (sec) 20	
Valve 1 port 3 MeOH	Aspiration of 10% MeOH
Syringe Pump Flowrate (microliter/sec) 150 Syringe Pump Aspirate (microliter) 1200	
Valve 1 port 8	Elution
Syringe Pump Flowrate (microliter/sec) 10 Syringe Pump Dispense (microliter) 1200 Delay (sec) 20	
Valve 1 port 1- WASTE	Disposing to the waste
Syringe Pump Flowrate (microliter/sec) 100 Syringe Pump Empty	
Spectrometer Stop Scanning	End of measurement

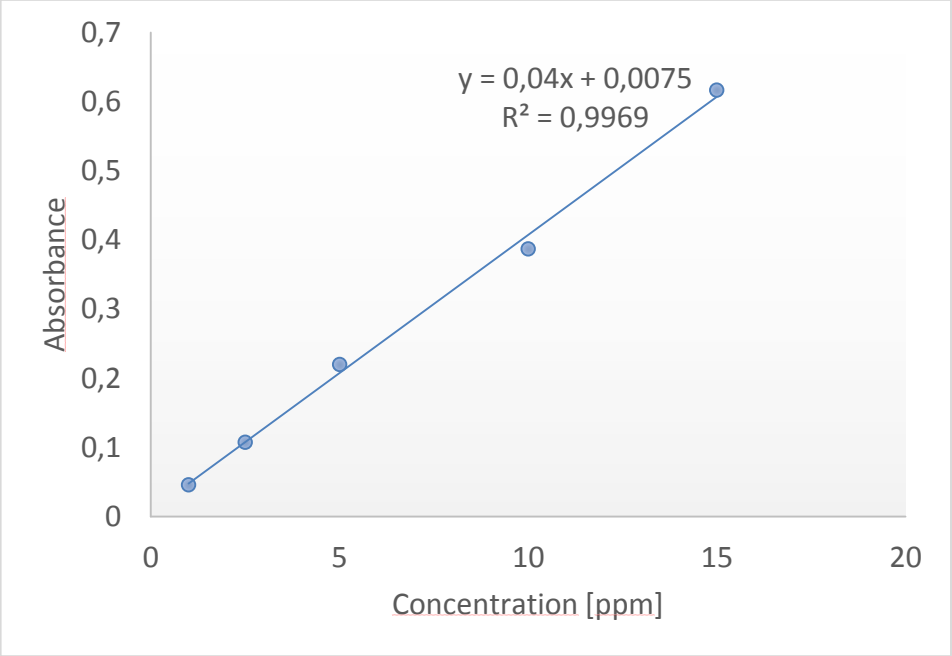
5.4 Calibration curve

Calibration curve was measured in defined range of concentrations. The regression line was expressed from the graph as well as the main parameters, limit of detection (LOD) and limit of quantification (LOQ) have been determined. The results are summarized in *Table 4*.

Table 4 Results of calibration curve measurement

Concentration of calibration solutions (ppm)	Absorbance					
	Measurement					
	1.	2.	3.	Average	SD	RSD (%)
1	0.046	0.049	0.046	0.047	0.002	3.49
2.5	0.106	0.109	0.108	0.108	0.002	1.42
5	0.218	0.214	0.220	0.217	0.003	1.41
10	0.385	0.387	0.357	0.376	0.017	4.46
15	0.615	0.620	0.627	0.621	0.006	0.97

In a view of results, the absorbance increases linearly in a range of concentration from 1 ppm to 15 ppm. Calibration curve has the determination coefficient of 0.9969. The limit of detection (LOD) and limit of quantification (LOQ) were 0.128 and 0.425 ppm, respectively. The mathematical formula for calculation of LOD and LOQ is mentioned in chapter 4.4. The *graph 3* demonstrates calibration curve progress.



Graph 3: Calibration curve progress

The *Figure 7* demonstrates one measurement cycle under optimal conditions. Standard solution with concentration of 15 ppm has been used for demonstration.

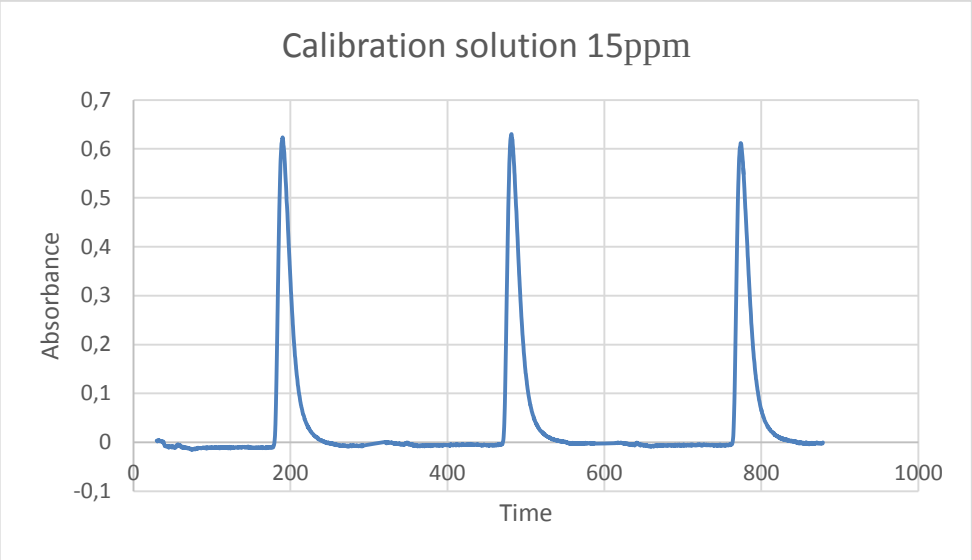


Fig. 8: Calibration curve measurement (one measurement cycle with 15 ppm concentration of calibration solution)

5.5 Repeatability

The repeatability was determined under the optimal conditions with concentration of standard solution of 10 ppm. The measurement was repeated 12 times. The experiment was performed with and without precipitants to compare the effect of reagents for off-line sample pre-treatment. The aim was to determine the absorbance of eluent and express the relative standard deviation from obtained values. The results are presented in *Table 5*.

Table 5 Results of repeatability test

Measurement	10 ppm-with reagents	10 ppm-without reagents
1.	0.289	0.257
2.	0.299	0.257
3.	0.302	0.260
4.	0.293	0.260
5.	0.304	0.275
6.	0.304	0.284
7.	0.323	0.284
8.	0.311	0.285
9.	0.315	0.289
10.	0.322	0.284
11.	0.316	0.285
12.	0.316	0.286
Average	0.308	0.276
SD	0.011	0.013
RSD (%)	3.58	4.68

The relative standard deviation (RSD) was 3.58% using precipitants, whereas without reagents the RSD was 4.68%. The results also pointed out that the absorbance is significantly different in comparison of the presence or absence of reagents. Therefore, the application of reagent is important to guarantee elimination of interferences.

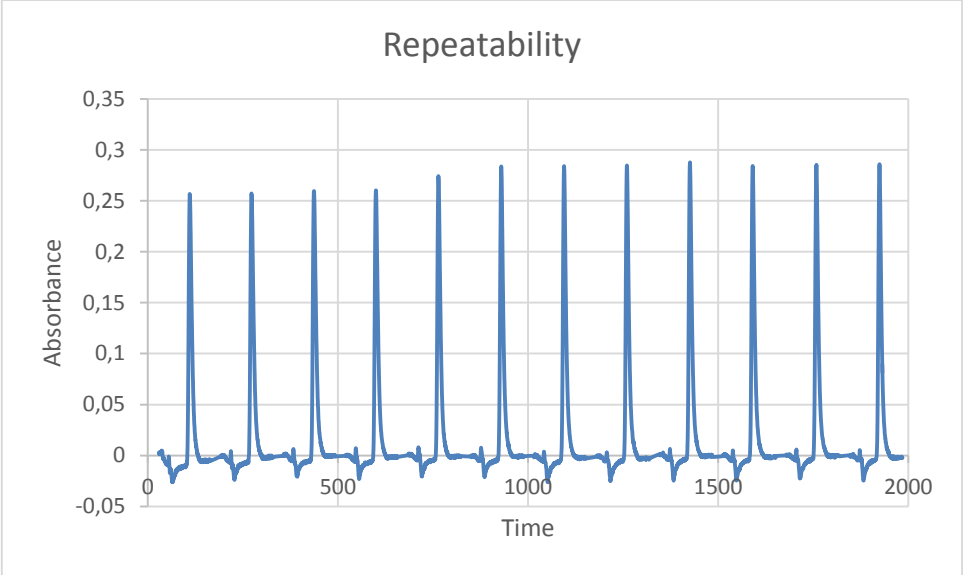
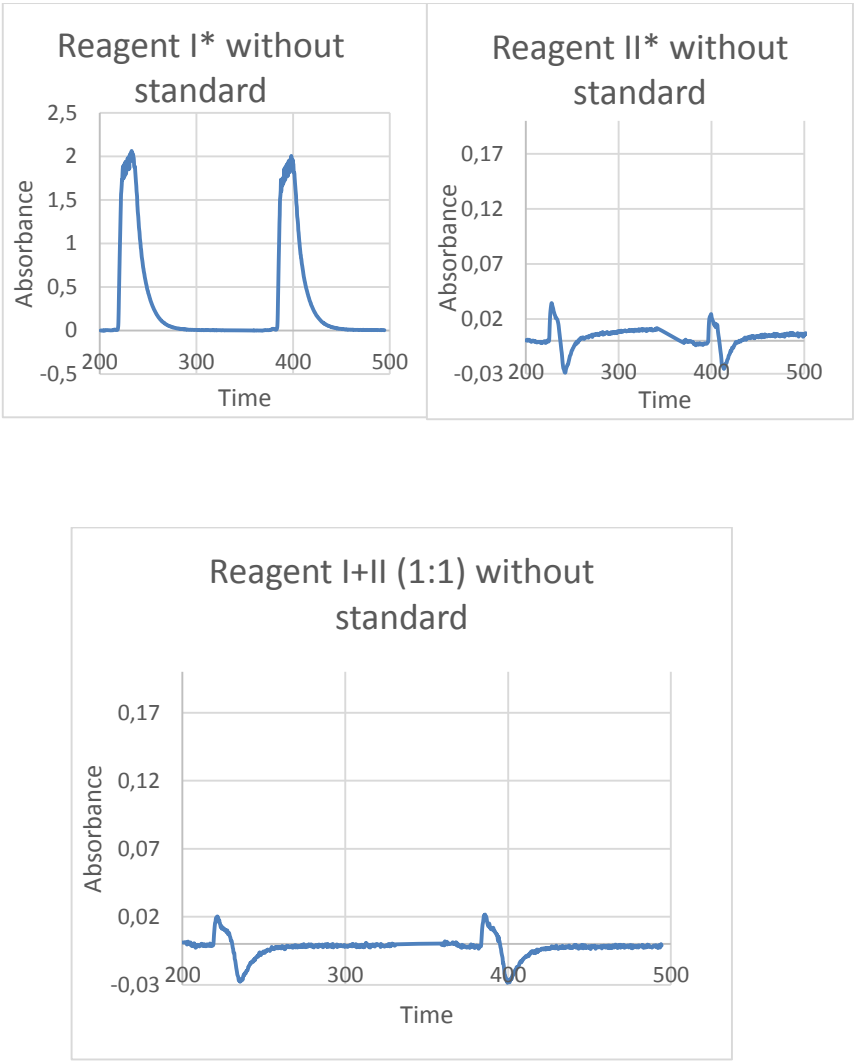


Fig.9: The repeatability measuring process

5.6 Optimization of precipitation

To guarantee the elimination of potential interferences, it was required to set the parameters of precipitation. The measurement was performed under optimal conditions. Firstly, it was important to measure the absorbance of reagents itself to compare the signal of the detector. The results are presented on *Graph 4.*



Graph 4: Effect of reagents without standard solution- comparison of detected signal

*I reagent- zinc sulfate hepta-hydrate *II reagent- potassium hexacyanoferrate tri-hydrate

The response of detector was quite significant, especially in case of reagent I. However, the signal was low if reagents were used both, in ratio 1:1.

Different ratios and amounts of reagents were used for optimization. Standard solution of caffeine with concentration of 1 ppm has been used during experiment. The data are summarized in *Table 6*.

Table 6: Optimization of precipitation

	Ratio of reagents		Volume of reagent (μL)	Precipitation
	Reagent I	Reagent II		
	Zn	Fe		
1.	0	1	100	NO
2.	1	1	100	YES
3.	1	1	200	YES
4.	1	0	100	NO
5.	1	2	100	YES
6.	2	1	100	YES

According to the best ratio of reagents, it was necessary to monitor the response of the detector. It was not reasonable to use precipitants separately, because they do not precipitate. But as it was mentioned before, the signal of reagents has been detected. It is not efficient to use them in unequal ratio because it could affect the final results significantly. Therefore ratio 1:1 has been selected as optimal.

As it was expected, higher volume of reagent increases the density of precipitate. It could cause filter blockage and also influence the duration of experiment. On the other hand, small volume does not have to be sufficient for elimination of all interferences. Therefore, it was important to select the appropriate volume.

According to the response of detector and acceptable experiment duration, volume of 100 μL of reagents was selected as optimal.

5.7 Analysis of real sample

The experiment was performed with three different types of coffee - brewed coffee, instant coffee and espresso from coffee machine. Each way of preparation has its own procedure.

Brewed coffee - Three different coffee brands has been used for sample preparation. In the beginning, approximately 0.025 g of sample was weighed in 50 mL volumetric flask. Then, it was filled up with hot water up to 50 mL. After 15-20 min, the sample was filtrated. From each sample 1.5 mL has been taken and the precipitation reagents were added. The precipitate was removed and the sample was diluted with water in 5 mL volumetric flask. Finally, sample volume of 150 µL has been analyzed and caffeine amount was determined. The results are summarized in *Table 7: Analysis of real sample 1- brewed coffee*

Table 7: Analysis of real sample 1 – brewed coffee

Brewed coffee	Amount (g)	Analysis sample volume (mL)	Abs. of 0.15 ml sample	Conc. in 0.15 ml sample (mg L^{-1})	Conc. in 5 ml (mg L^{-1})	Conc. in 1.8 g coffee (1 cup) (mg L^{-1})
Jacobs	0.0264	1.5	0.097	2.24	7.46	13.43
Bellarom	0.0263	1.5	0.093	2.14	7.13	12.83
Douwe Egberts	0.0249	1.5	0.101	2.34	7.79	14.03

Instant coffee - The sample preparation process was very similar to brewed coffee. Four coffee brands were used during the measurement. The coffee samples were weighed in 50 mL volumetric flasks, then filled up to 50 mL with hot water. After 15 min, exact volume of each sample was taken and the precipitants were added. The precipitate was eliminated by filtration and the samples were diluted in 5 mL volumetric flask. Samples were analyzed and the caffeine content was determined. The results are summarized in *Table 8*: *Analysis of real sample 2- instant coffee.*

Table 8: Analysis of real sample 2 – instant coffee

Instant coffee	Amount (g)	Analysis sample volume (mL)	Abs. of 0.15 mL sample	Conc. in 0.15 mL sample (mg L^{-1})	Conc. in 5 mL (mg L^{-1})	Conc. in 1.8 g coffee (1 cup) (mg L^{-1})
Nescafe	0.0259	0.5	0.092	2.11	21	38
Yellow E.	0.0251	0.5	0.123	2.89	29	52
GranArom*	0.0254	1.0	0.077	1.74	9	16
Jacobs	0.0252	0.5	0.089	2.04	20	37

* *1 mL of sample volume has been used because during sample preparation process it was noticed that the sample was colored less than the others. Therefore, it was expected that the concentration of caffeine is lower. According to this, bigger amount of sample was used for analysis.*

Espresso from coffee machine - Two coffee brands and different coffee preparation programs has been utilized. Coffee machine prepared 5 types of beverages with different volumes. From each sample 100 µL volume was taken and the reagents were added. The precipitate was carefully removed and the sample was diluted with water in 50 mL volumetric flask. Then, volume of 150 µL of each sample was analyzed and the caffeine amount was determined. The results are summarized in *Table 9: Analysis of real sample 3- espresso from coffee machine*

Table 9: Analysis of real sample 3- espresso from coffee machine

Coffee types	Original volume	Analysis sample volume (mL)	Abs. in 0.15 mL sample	Conc. in 0.15 mL (mg L^{-1})	Caffeine amount in 0.1 mL (mg)	Caffeine amount in orig. vol.(mg)
Normal	50	0.1	0.270	6.56	2188	49.22
Espresso Gimoka	35	0.1	0.336	8.21	2738	43.12
Bigger	80	0.1	0.174	4.16	1388	49.95
Extra Big	110	0.1	0.142	3.36	1121	55.48
Espresso Tchibo	35	0.1	0.434	10.66	3554	55.98

In view of results, the highest amount of caffeine was determined in coffee beverages prepared in coffee machine. On the other hand, the smallest amount was determined in brewed coffee. In case of instant coffee, the caffeine amount strongly depends on coffee brand.

The experiment was performed under optimal conditions and it has been successfully applied for caffeine determination in different coffee beverages.

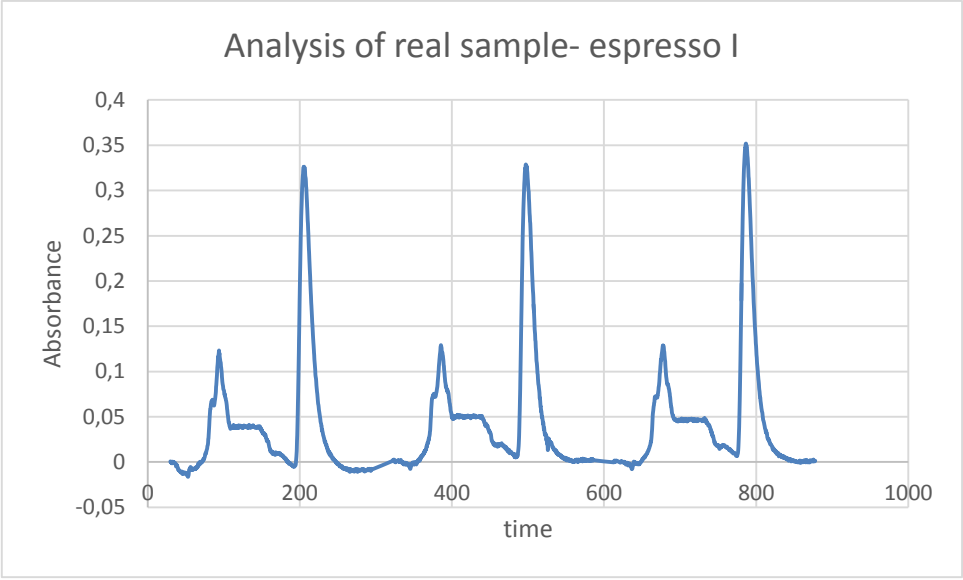


Fig.10: Analysis of real sample- first peak demonstrates elimination of matrix while second peak demonstrates amount of caffeine in standard espresso.

6 Summary

The method for caffeine determination in coffee beverages was performed under optimal conditions. The main parameters of measurement including volume of sample, aspiration and elution flow rates as well as the appropriate ratio of precipitants for off-line sample pre-treatment were optimized. 10% methanol solution has been used as eluent. The resulting parameters are summarized in *Table 10: Optimal parameters of experiment.*

Table 10: Optimal parameters of experiment

Optimal parameters	
Parameter	*Result*
Sample volume	150 μL
Aspiration flowrate of sample to column	10 μL s^{-1}
Elution flowrate	10 μL s^{-1}
Ratio of precipitants	1:1
Eluent	10% MeOH

The calibration curve was measured after optimization process. Limit of detection and limit of quantification were determined from the obtained results. The repeatability measurement was performed with concentration of standard solution of 10 ppm.

Finally, the efficacy of the designed method was tested for real samples. Around 95 mg of caffeine was expected from an average cup of coffee [12]. However, amount of caffeine depends on type of coffee and serving size. In a comparison with other literature sources, satisfying determination of caffeine in coffee beverages has been achieved by applying suggested method.

7 Conclusion

The aim of the thesis was to optimize the method for determination of caffeine using off-line precipitation and separation from the matrix components on the short monolithic pre-column and apply it in the system of sequential injection analysis with UV detection. After setting the optimal parameters, the calibration curve was measured with resulting determination coefficient of 0.9969. The limit of detection (LOD = 0.128 ppm) and limit of quantification (LOQ = 0.425 ppm) were determined. The repeatability measurement was performed and the relative standard deviation (RSD = 3.58%) was expressed. Finally, the effectiveness of method was tested by real sample analyses.

8 Súhrn

Cieľom práce bolo stanovenie kofeínu v rôznych kávových nápojoch s využitím prietokovej analýzy v spojení s UV-VIS detekciou. V úvode bolo potrebné navrhnúť správny postup analýzy a optimalizovať navrhnutý systém tak, aby analýza bola efektívna a presná. Káva akéhokoľvek druhu však neobsahuje len kofeín ale aj ďalšie množstvo substancií, ktoré by mohli potenciálne rušiť stanovenie. Z toho dôvodu bolo nutné eliminovať rušivé látky. Eliminácia bola dosiahnutá jednak činidlami, ktoré po pridaní k vzorke kávy vytvorili precipitát, ktorý bol následne odfiltrovaný. Tým bola manuálne odstránená väčšina interferencií. Zároveň však boli využité i výhody separácie na krátkej monolitnej predkolone. Aplikovaním tejto metódy došlo jednak k oddeleniu matrice od analytu ale zároveň aj k zakoncentrovaniu analytu. Ako elučný roztok/mobilní fáze bol použitý 10% roztok metanolu. Po vhodnej úprave vzorku mohla prebehnúť analýza. Bolo však potrebné optimalizovať požadované parametre merania ako prietokovú rýchlosť či objem analyzovaného vzorku. Ako vhodná prietoková rýchlosť bola zvolená 10 $\mu L\ s^{-1}$ a vhodný objem vzorku pre analýzu bol stanovený na 150 µL. Pod optimálnymi podmienkami bola premeraná kalibračná krivka, ktorej determinačný koeficient bol rovný hodnote 0,9969. Stanovené boli aj limit detekcie (LOD) = 0,128 a limit kvantifikácie (LOQ) = 0,425 mg L^{-1}. Následne bola metóda aplikovaná na analýzu reálnych vzoriek kávy. Analýze boli podrobené celkovo 3 druhy kávy podľa prípravy: instantná, zalievaná a espresso z kávovaru. Z každého druhu boli analyzované vzorky od rôznych výrobcov a výsledky boli následne porovnané. Najvyšší obsah kofeínu bol zaznamenaný v espresse Tchibo pripravenom z kávovaru, a to viac ako 55 mg kofeínu na 1 šálku kávy. Naopak najmenší obsah kofeínu bol stanovený v zalievanej káve a v nekvalitných instantných kávových nápojoch. Obsah kofeínu v nápojoch bol porovnateľný s obsahom uvedeným v literárnych a elektronických prameňoch. Navrhnutá metóda je teda aplikovateľná na stanovenie obsahu kofeínu v káve a navyše je šetrná k životnému prostrediu nakoľko počas experimentu neboli využívané organické extrakčné činidlá.

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