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DEPARTMENT OF ANALYTICAL CHEMISTRY

Separation of selected inorganic ions using the sequential injection chromatography

Diploma thesis

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Hradec Králové 2014 Patrícia Jarošová

„Prohlašuji, že tato práce je mým původním autorským dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Tato práce nebyla použita k získání jiného či stejného titulu“.

V Hradci Králové:

In the beginning, I would like to thank my supervisors Dr. Burkhard Horstkotte, Ph.D., M.Sc. and Doc. PharmDr. Hana Sklenářová, Ph.D. for their guidance, support and patience. Also I would like to thank my family and friends.

ABSTRAKT

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Názov diplomovej práce: **Separace vybraných anorganických iontů pomocí sekvenční injekční chromatografie**

Bola skúmaná schopnosť predkolóny Dionex Ionpac® CG5A (2 x 50 mm, P/N 046104) separovať niektoré katióny prechodných kovov vo vzorke vody, za použitia systému sekvenčnej injekčnej chromatografie (SIC). Použitá separácia bola iónovo výmenná, kolónu tvorili častice pokryté dvojvrstvou funkčného latexu schopného vymieňať katióny aj anióny. Ako chelatačné činidlo v mobilnej fáze bola použitá pyridín-2,6-dikarboxylová kyselina (PDCA) a reakčné činidlo obsahovalo 4-(2-pyridylazo) resorcinol (PAR). Detekcia prebiehala spektrofotometricky.

Pri optimalizácií boli použité dve metódy: simplex a univariantná metóda. Boli nájdené ideálne podmienky pre separáciu. Mobilná fáza pozostávala z PDCA v koncentrácií 4 mmol/l, kyseliny mravčej v koncentrácií 40 mmol/l, síranu sodného v koncentrácií 4 mmol/l a hydroxidu sodného v koncentrácií 2 mmol/l. Reakčné činidlo bolo zložené z PAR (0,3 mmol/l), hydroxidu amónneho (150 mmol/l), hydrogenuhličitanu sodného (60 mmol/l) a hydroxidu sodného (45 mmol/l).

Vzorka o objeme 90 µl bola, pre dosiahnutie lepšej symetrie píku, umiestnená medzi dve zóny vody a následne dávkovaná do kolóny prietokom 8 µl/s. Bola dosiahnutá separácia troch katiónov a to Cu (II), Zn (II) a Fe (II) v čase kratšom ako 4 minúty.

ABSTRACT

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Title of Diploma Thesis: **Separation of selected inorganic ions using the sequential injection chromatography**

The ability of the guard column Dionex Ionpac® CG5A (2 x 50 mm, P/N 046104) to separate some transition metal cations from water samples, using Sequential injection chromatography (SIC) system was demonstrated. The type of separation was based on ion exchange interaction and the column was filled with particles functionalized by a bilayer of anion-exchange and cation-exchange latex. The chelating agent used in eluent was pyridine-2,6-dicarboxylic acid (PDCA). A post-column reagent with 4-(2-pyridylazo) resorcinol (PAR) was used for spectrophotometric detection.

For optimization, two methods were used: Simplex and univariant studies. Ideal conditions of the separation were found. The mobile phase consisted of PDCA, formic acid, sodium sulphate, and sodium hydroxide in the concentrations 4 mmol/l, 40 mmol/l, 4 mmol/l and 2 mmol/l, respectively. The concentrations of the post-column reagent components were 0.3 mmol/l of PAR, 150 mmol/l of the ammonium hydroxide, 60 mmol/l of the sodium hydrogencarbonate and 45 mmol/l of the sodium hydroxide.

The volume of the sample was 90 µl and it was placed between two zones of water to get better peak symmetry. An eluent flow rate of 8 µl/s was used. Determination of three cations - Cu (II), Zn (II) and Fe (II) – in water samples was performed. The separation time was shorter than 4 minutes.

CONTENT

ABBREVIATIONS

5-Br-PADAP – 2-[(5-bromo-2-pyridyl)azo]-5-diethylaminophenol

CDA – chelidamic acid

DAD – diode array detector

FIA – flow injection analysis

id – inner diameter

IDA – iminodiacetic acid

HPLC – high performance liquid chromatography

MSA – methane sulphonic acid

PAR – 4-(2-pyridylazo)resorcinol

PDCA – 2,6-pyridine dicarboxylic acid

PEEK – polyetheretherketone

PP – piston pump

PTFE – polytetrafluorethylen

R_S – resolution

RSD – relative standard deviation

SIA – sequential injection analysis

SIC – sequential injection chromatography

SP – syringe pump

TEA – trietanolamine

TMAOH – tetramethylammonium hydroxide

SDS – sodium dodecylsulphate

SV – selection valve

VIS – visible range

UV – ultra violet range

1. INTRODUCTION

The determination of transition metals in various matrices is in the center of attention in the last years because of the environmental pollution as well as their biological function in enzymes and their role as micronutrients. The most commonly used methods to analyze a sample are atomic absorption spectroscopy and inductively coupled plasma – mass spectroscopy [1]. However, no matter how universal they are, the questions of purchase and maintenance cost, the time for one analysis and some spectral interferences are causing limitations in some practical applications. As a conclusion, other methods that would be cheaper and easy to use are studied.

Firstly HPLC, which is the most flexible tool for analyte separation, can be coupled with different detectors and used for different sample matrices [2]. Up to eight transition metals can be analyzed in this technique under 15 minutes [3]. A lot of works has been dealing with separation of transition metals using HPLC with mainly ion-exchange columns.

Despite of the pros of HPLC, it does not allow quick analysis of water samples and lacks of simplicity. This leads us to the technique of Sequential Injection Chromatography (SIC), where a separation column, similar as in HPLC, is used. The difference in the pressures of both systems – the pumps of a SIC system cannot create a pressure that would allow using typical HPLC columns. So columns used in SIC have to be shorter and suitable for low-pressure separation.

As well as the columns, also the separation conditions are different. Although a lot of experiments were done to optimize the conditions of determination of transition metals in HPLC systems, they cannot be transferred to another system without any changes. After the optimization process, SIC can be an ideal way of determination of selected transition metals in a short time and easy-to-use equipment.

2. OBJECTIVES AND THE AIM OF THE WORK

The aim of the work was to construct a sequential injection chromatography system for the separation of transition metal cations, using a Dionex IonPac CG 5A guard column with post-column colorimetric reaction.

In detail, the following objectives were:

- To test the suitability of the guard column for the low pressure separation of transition metal cations.
- To optimize the operational parameters i. e. flow-rates and injection volumes, the conditions of separation using PDCA as a chelating reagent and of the post-column reaction using PAR as reagent by both, Simplex optimization method and univariant method.
- To optimize the method to be, easy-to-use, rapid and economic.
- To demonstrate a separations of transition metal cations, to evaluate the methods and system´s performance considering peak resolution, sensitivity, calibration range, signal-to-noise ratio and time of analysis.

3. THEORY

3.1. HPLC

3.1.1. Principle of HPLC

HPLC is an abbreviation for high performance liquid chromatography. Chromatography is a separation method, which means that the compounds of interest from sample are being separated during the analytical process. It is a qualitative and quantitative method, so the nature of compounds as well as their concentration can be determined. The analytes from the sample interact with two phases: the stationary and the mobile phase, which are immiscible in each other. The stationary phase is generally a particle bed (resin) or a porous foam-like cylinder (monolith) packed into a steel or polymer tube (column).

The sample is injected into, and carried forward by the mobile phase. The compounds of the sample interact with stationary phase. It depends on the type and strength of the interaction, which component will pass the stationary phase fastest and will be detected first, which next, and which last. So the components of the sample are separated according to their interaction strength. This principle is typical for an adsorption chromatography, it can differ for other types [4].

The column is the part of the system responsible for the separation. It is a tube of generally 5 to 25 cm long with inner diameters of typically 2 to 8 mm. In the case of resins, particles are nowadays spherical with diameter ranging from 1.7 to 5 μm [5]. These can be based on a silica, zircon oxide, other metal´s oxide, porous graphitic carbon, polymer or a hybrid [6]. Certain ligands can be attached to this basic structure. The nature of ligand dictates a nature of the stationary phase and also the separation. Polar ligands (– OH, $-NO_2$) are used in a classic adsorption separation. Nonpolar ligands (– C8. – C18, fenyl) are used in the reverse mode of the separation. Ligands with a middle strength of polarity (– CN, $-NH_2$) are used in the both modes. Special ligands are used in the chiral chromatography and the ion-exchange chromatography [5].

Different ways of separation divide chromatography to:

a) Adsorption chromatography – the principle was described above.
b) Partition chromatography – mobile and stationary phase are in liquid state, they are immiscible in each other and elements are soluble in both phases. Separation depends on the partition coefficient of the compounds between both phases. The compound, which is the most soluble in the mobile phase will elute first, the least soluble compound will elute last.
c) Size-exclusion chromatography – the separation is based on the size of the molecules. Usually large organic molecules such as proteins are separated this way. The stationary phase is made of a gel, which has pores. Larger molecules cannot fit into the pores, so they are not retained while smaller molecules are slowed down by the pores.
d) Affinity chromatography – is used for separating specific molecules. The stationary phase is modified with a ligand that binds only a specific molecule from the sample. This method is used for separating antigenes, antibodies, hormones etc.
e) Ion-exchange chromatography – is described below.
f) Chiral chromatography – is used for separating different chiral forms of one molecule from each other by using a chiral ligand [5].

The detection is the final step in chromatography. In the ideal case, the detector is very sensitive to distinguish the slightest change in the concentration of the detected substance, signal stability and reproducibility is given, it has a quick response, a small inner volume and a change of the outer environment does not have a significant influence on the detection [5].

3.1.2. Detection techniques

Typical detection techniques can be divided into several groups: e.g. optical detection (spectro-photometry, fluorometry, refractive index detection) electrochemical detection (e.g. conductometry, amperometry, potentiometry, coulometry), aero-based detection (charged aerosol detection, evaporative light scattering), and mass spectrometry. Some detection techniques are universal such as refractive index detection while others are highly selective such as fluorometry.

The mostly used detection technique in HPLC is spectrophotometry (UV–VIS). It can be used for substances which absorb in ultraviolet (UV) range of 190 to 400 nm or visible light and near infrared (VIS) between 400-800 nm. UV–VIS detection presents about 70% of all HPLC applications. The principle is based on the interaction of electromagnetic rays with the analytes inside the detection cell by absorbance. The light intensity after interaction and before are put in relation (transmittance) and used for data evaluation [4, 5]. In practice, the negative logarithm of the transition is used (absorbance, see Formula 1, in section number 8 Formulas), which is proportional to the concentration of analytes given by Lambert-Beers Law. Generally, a blank solution is measured to evaluate the light intensity in absence of the absorbing compounds. The final unit, which is depicted in the chromatogram, is absorbance unit.

According to the construction, four types of spectrophotometric detectors can be mentioned: detectors with a pre-set wavelength, detectors with a wavelength that can be set before the measurement in the respective range of wavelengths, detectors scanning several different wavelengths at the same time (commonly 4) and diode array detectors (DAD).

DADs are mostly used in spectrophotometry. A DAD scans the whole spectrum of wavelengths (e.g. 190 – 800 nm) in the real time. After leaving the detection cell, the light is divided according to the wavelengths and each one is transferred to the respective photodiode [6]. The resulted chromatogram is available in three-dimensional form (elution time, signal height, wavelength), so it is easy to decide, which wavelength is the best for the detection of a given substance. DAD is, together with MS detector, one of the modern and the most effective detectors with respect to sensitivity and selectivity of the detection step.

3.1.3. Evaluation of the chromatogram

A chromatogram is a graph with two axes. The x-axis represents the registration or separation time, usually in minutes, the y-axis represents the signal intensity, e.g. the absorbance. When a compound is detected, it is registered in the chromatogram as a peak. An ideal peak is of gaussian form, symmetric, without tailing or fronting, narrow and high. To describe the quality of the separation and chromatogram, the following parameters are used:

The retention time is a parameter used for the identification of a certain compound for qualitative analysis. It is the time between the injection of the sample to the maximum of the corresponding peak signal. It depends on factors like mobile or stationary phase and it needs to be compared with a standard.

For a quantitative analysis, the peak heights or more typical, the peak areas are used. Again, it is essential to compare a result with a standard either using a separated standard solution (external standard) or by addition of the standard to the sample (internal standard).

The resolution is a quantity that can be calculated from the widths and retention times of two neighbouring peak. In fact it indicates if two peaks are separated enough to be detected as two peaks (Formula 2).

The factor of symmetry shows how symmetric a peak is (Formula 3).

A signal to noise ratio is calculated from the height of the peak and a height of the baseline noise (Formula 4). Depending on this value an elevation in the chromatogram is defined as a peak or noise.

The repeatability is a quality that estimates the value of the relative standard deviation (Formula 5) in subsequent measurements of the comparing solution.

3.1.4. Ion-exchange chromatography

This type of chromatography is used for the separation of the charged molecules. The stationary phase is an ion-exchanger. It is formed by a macromolecule, to which functional groups are attached. A part of each group is during the separation exchanged for ion of same charge from the sample. The ion-exchanger can have either alkaline functional groups and exchange anions or acidic functional groups and exchange cations, which is depicted in the formulas below [4]:

$-\mathbf{RY} + \mathbf{X}^- <=> -\mathbf{RX} + \mathbf{Y}^-$ for anion exchanger

$-\mathbf{RY} + \mathbf{X}^+ <=> -\mathbf{RX} + \mathbf{Y}^+$ for cation exchanger

Y^- and Y^+ - exchangeble ions, which are bonded to the functional groups of ion-exchanger

The ion-exchanger can be formed by a weak or a strong functional group. Strong ion-exchangers separate weak ions from sample and vice-versa. To achieve the separation, it is essential for the groups to be dissociated. The strong groups are always dissociated, while the dissociation of the weak groups depends on the pH value of the eluent. Weak acids or bases have given pK_A/pK_B values, which reflect their acido-basic properties i.e. if they are dissociated, i.e. present as ions in the solution at a certain pH.

The elution can be performed in two ways, either the eluent has components, which bind to the stationary phase and replace the sample ions, or the pH is being changed, so that weak ions are neutralized and eluted from the column. Furthermore, weak ion-exchangers work similarly. They are used to separate strong ions, which show a charge over a wide pH range. To elute ions from the column, the pH needs to be changed, so that functional groups will be neutralized or ions that create stronger ion-pairs to the functional groups have to be present in the eluent.

The elution can be isocratic, so only one unchanged eluent is used, or gradient type, i.e. properties like the ion strength or the pH value are changed during the separation [6].

The strength of interaction varies with the type of the ions. Generally, it increases with the diameter of an ion and its charge. Larger ions are not surrounded by molecules of water that much as smaller ones, so they easily create interactions [4].

3.2. Flow techniques

3.2.1. Sequential injection analysis

Sequential injection analysis (SIA) is a technique of automated analysis (Figure 1). It is effective, relatively cheap, easy to manipulate, and due to small volumes of reagent and samples used for analysis, also environmentally friendly.

It is a technique based on programmable flow. It belongs to the second generation of flow techniques, while the former technique flow injection analysis is counted as the first generation. The most important part of the system is a bi-directional pump that makes all solutions to be moved by the flow as programmed. Another part is a multi-position valve, which enables to aspirate different solutions and samples. All the aspiration and dispensing steps are done with the pump, which is connected to the common port of the valve by a tube called holding coil. The respective ports of the valve are then connected to solutions such as sample and reagents or waste. The pump can make each solution in the system flow, and the valve chooses, which port will be used [7].

The system works according to a program, so it is very flexible. In each step of the program parameters, such as the volume of the sample or the eluent and the flow-rate, i.e. a speed of the pump, can be changed and also should be, in order to optimize the method. Solutions used in the here-used system are eluent, reagent, and sample or standard. The eluent or mobile phase (in SIA also "carrier") washes the whole system, carries the sample and almost every tube is filled with it. The reagent interacts with an analyte and changes its properties, so it can be detected. The sample/standard is a solution that should be analyzed. A detector serves for the detection of the products from the previous step where analytes interact with the reagent. For the high and also low-pressured systems similar detectors mentioned in the chapter HPLC are used.

The holding coil is a coiled tube, which serves for mixing the solution. This is a place, where the direction of flow is changed by flow reversal. SIA is designed to use as small volumes of the solutions as possible, so each tube is as short as possible in most of applications. The holding coil is together with a reaction coil the only tube long enough to mix solutions.

A sample, which is about to be analyzed, is injected before or after the reagent and they are together carried by the eluent or carrier. There is an improvement from flow injection analysis (FIA), where the reagent is a component of the carrier and the sample is injected into this mixture and thus the reagent consumption is higher. Also SIA has a programmable flow and FIA has continuous flow. Because in FIA there is no reverse flow in the system, solutions are mixed in the mixing points and are detected at the same line. Although, in SIA when the direction of the flow changes, the solutions are mixed more efficiently. When a solution is mixed better, the product zone has a homogeneous color (in the case of the color reactions) and its detection could be carried out quickly [7].

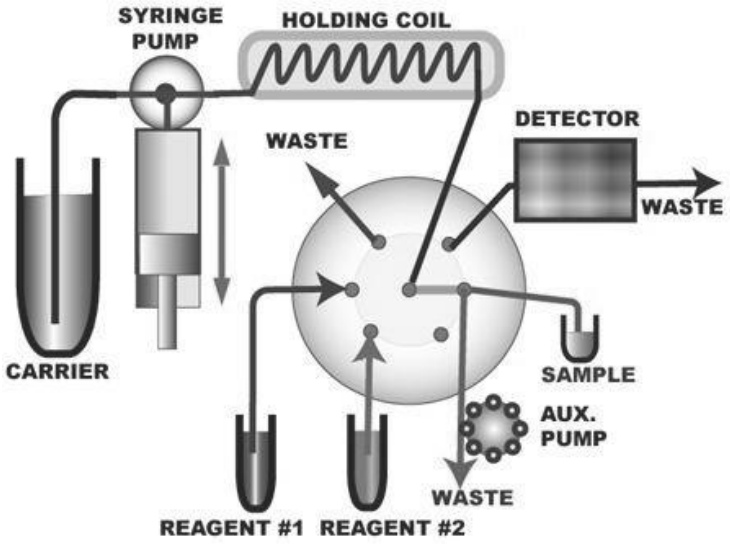


Figure 1: A scheme of sequential injection analysis system [7]

3.2.2. Sequential injection chromatography

Sequential injection chromatography (SIC) is a combination of two techniques: chromatography and SIA (Figure 2). All instrumentation and the programmable flow are taken over from SIA, the use of a column comes from chromatography, but without using high-pressure equipment. SIC can be performed in various formats: reversed chromatography, ion-exchange chromatography or affinity chromatography. Mainly used columns are monolithic or core-shell columns, while the use of particle columns is limited, because they generate high back-pressures in the flow system.

Using SIA instrumentations with chromatography principles enables quick changes of eluent, keeping precise aspirated volumes, flow-rates, using not so expensive machines and lower production of waste. As it was said before, also here, the multiposition valve is a very important component in the system. It allows switching very quickly from one eluent to another eluent (used in gradient chromatography) or to the sample or reagent, whatever needed. It is also connected to the column. The whole system is in the beginning filled up with eluent. Then sample is injected onto the column, separated and after mixing up with reagent (if post-column derivatization is needed), detected in the detection flow cell.

It was not easy to add a separation column into a flow system. Sorbents used for HPLC were made of small particles, which put up too high resistance to pressure for the pump. But with the invention of monolithic columns this problem was solved. These columns are not filled with small particles. It is one big, connected particle with pores inside. To be detailed, it contains macropores (1 – 2 μm), which lower the flow resistance and mesopores (12 nm), which enlarge the active surface for separation [6].

Columns in SIC cannot be very long neither. For this reason the guard columns, which are not appropriate for separation in HPLC systems, usually 25 or 50 mm long, are used. That is the reason, why also the number of components separated in one analysis is limited.

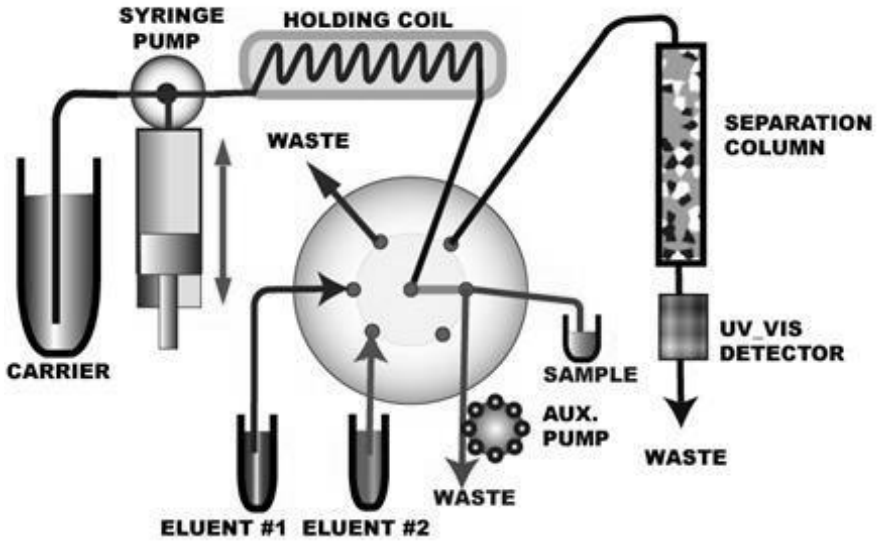


Figure 2: A scheme of sequential injection chromatography system [7]

3.3. Analysis of transition metals

3.3.1. The analytes

Pollution of water is an environmental problem of recent years and because of that the determination of transition metals has been studied more intensively. Transition metals are important parts of the metaloenzymes in human body and they work properly as long as they are in certain concentrations. A lack of Cu can cause anaemia or problems with the blood circulation, but in high concentration, Cu accumulates in the kidneys and liver and can cause their damage or anaemia as well. A typical disease caused by high level of Cu is Wilson´s disease. Fe is a part of a dye in the erythrocytes and is essential for their function, therefore deficiency of Fe leads to anaemia. Overexposure to Zn may cause acute renal failure, anaemia, pancreatitis or muscle pain. High level of Ni can lead to heart and liver damage, skin irritation and decreased body weight. Cd in high levels can inactivate enzymes by replacing Zn. And finally Pb is one of the most poisoning heavy metals and its target organs are brain (affecting young children), bones, kidneys, reproductive and cardiovascular systems, and thyroid gland [8].

3.3.2. Overview of separation and determination of transition metals

In this section, the overview of the works, studying the determination of transition metals is shown. The idea of this kind of separation and detection was studied intensively over the years. The aim was to find an analytical method, which would be sensitive, quick, easy to use, and cheap. The closest to this aim was HPLC with its high sensitivity and speed [11]. The works vary in the stationary phases, chelating agents, ways of detection, times of analysis and also the number of analytes. The summary of these parameters are in Table 1, determined cations are in Table 2, below.

Table 1: The summary of different determinations of transition metals

Reference	Sample	Mobile phase	Stationary phase	Chelating agent	Detection (wavelength)	Analysis time (min)	Preconcent-ration
Pobozy [10]	water	100 mM tartrate acid 0.06 mM SDS 3% methanol	C18 + SDS	tartrate acid	PAR (510 nm)	15 (+15-25)	+ Cellex P
Lasheen [11]	water	80 mM pottasium oxalate pH 4.5 using formic acid	IonPac CS5	pottasium oxalate	PAR (520 nm)	12	+ guard CG5
Cardellicchio [12]	water	7 mM PDCA 66 mM KOH 5.6 mM K_2SO_4 74 mM HCOOH	IonPac CS5A	PDCA	PAR (530 nm)	20	+ guard CG5A
Murgia [13]	water	64 mM oxalic acid 80 mM TMAOH[I] 40 mM KOH	IonPac CS5A	oxalic acid	PAR 530 nm	15	+ guard CG5A
Zeng [14]	water, beer	0.8 mM oxalic acid 2.5 mM MSA	IonPac SCS1	oxalic acid + MSA	conductor	25	+ guard SCG1
Lu [15]	biochemical samples	100 mM oxalic acid 0.5 M NaCl gradient elution	IonPac CS5A	oxalic acid + NaCl	5-Br-PADAP[III] (560 nm)	selected 12, all ions 22	+ guard CG5A
Dias [16]	fuel ethanol	2 mM CDA 3 mM TEA[II] 12 mM HCl 50 % v/v of methanol	IDA - silica column (two of them)	chelidamic acid (CDA)	PAR (510 nm)	24	-
Bashir [17]	freshwater	35 m*M* KCl 65 m*M* KNO pH 2.5 using HNO_3	IDA - silica column	-	PAR (495 nm)	20	-

[I]TMAOH - tetramethylammonium hydroxide
[II]TEA - trietanolamine
[III]5-Br-PADAP - 2-[(5-bromo-2-pyridyl)azo]-5-diethylaminophenol

Table 2: Cations - determined in given study

Reference	Ions								
	Fe^{III}	Pb^{II}	Cu^{II}	Ni^{II}	Zn^{II}	Co^{II}	Cd^{II}	Mn^{II}	Fe^{II}
Pobozy [10]	-	-	-	+	+	+	+	+	-
Lasheen [11]	-	+	+	+	+	+	+	-	-
Cardellicchio [12]	+	-	+	+	+	+	+	+	+
Murgia [13]	-	+	+	+	+	+	+	-	-
Zeng [14]	-	-	+	+	+	+	-	-	-
Lu [15]	+	+	+	+	+	+	+	+	+
Dias [16]	+	+	+	-	+	+	+	+	+
Bashir[17]	-	-	-	-	+	+	+	+	-

The columns, used in the majority of the studies are from Dionex® (IonPac CS5, CS5A and SCS1) and serves for ion-exchange chromatography. The first two mentioned columns function as the cation-exchangers (sulphonate functional groups) as well as the anion-exchangers (quaternary ammonium groups). The third one, used by Zeng et al [14] works only as the weak cation exchanger functionalized with carboxylic acid groups. The columns, used in the rest of the works, were C 18 (modified with sodium dodecyl-sulphate) and iminodiacetic acid functionalized silica columns.

The chelating agents were weak acids: PDCA, oxalic acid and its salt, tartrate acid and chelidamic acid. The sample matrix was usually water, but also biochemical samples or ethanol.

Most of the works used HPLC while one combined HPLC with FIA. The detection times were all above 12 minutes, but at least four metals were determined throughout.

In comparison with these works, in the present work, the analysis was shorter and the number of detected cations was lower.

3.3.3. Principle of applied measurement

SIC was the technique used in this work, as mentioned before, and the principle of the separation will be explained in the following text.

The ion-exchange column is the part, which serves for the separation. In the used column, the surfaces of the particle are covered by cation as well as anion exchanger groups. The transition metals can be separated either as cations alone or as anions in a complex with a ligand. For example, oxalic acid is a moderate complexing agent, so cations are separated in a form of the complex on an anion exchanger or as free cations on a cation exchanger, depends on the respective cation [2, 9].

On the other hand, at set pH, PDCA forms with cations neutral complexes, which differs in stability. As long as a cation does not form a strong complex with PDCA, it can exist in a free form and be retained by the column. So the cations forming the most stable complexes with PDCA are leaving the column first.

After leaving the column, the mobile phase is mixed with a post-column reagent containing PAR, which displaces PDCA and creates colored complexes with the cations. However, PAR does not need to be dissociated to form complexes with metal cations, the pH value is important, because if the reagent is too acidic it looses sensitivity and if it is too alkaline the baseline noise increases.

4. EXPERIMENTAL

4.1. Materials and methods

4.1.1. Materials

4.1.1.1. Instrumentation

All experiments were carried out using a Sequential Injection Chromatography system (SIChrom™) from FIAlab company (Bellevue, WA, USA). For the whole system two pumps were used. One pump, which for the eluent, sample and water, is considered as the main pump and is in following assigned as piston pump (PP). It consisted of a medium pressure, biocompatible pump from Sapphire Engineering (IDEX Corporation, Oak Harbor, WA, USA) of 4 ml total dispense volume. The second, additional pump, assigned as syringe pump, (SP, type Cavro XL) from Tecan (San Jose, CA, USA) was used for the post-column addition of the PAR reagent.

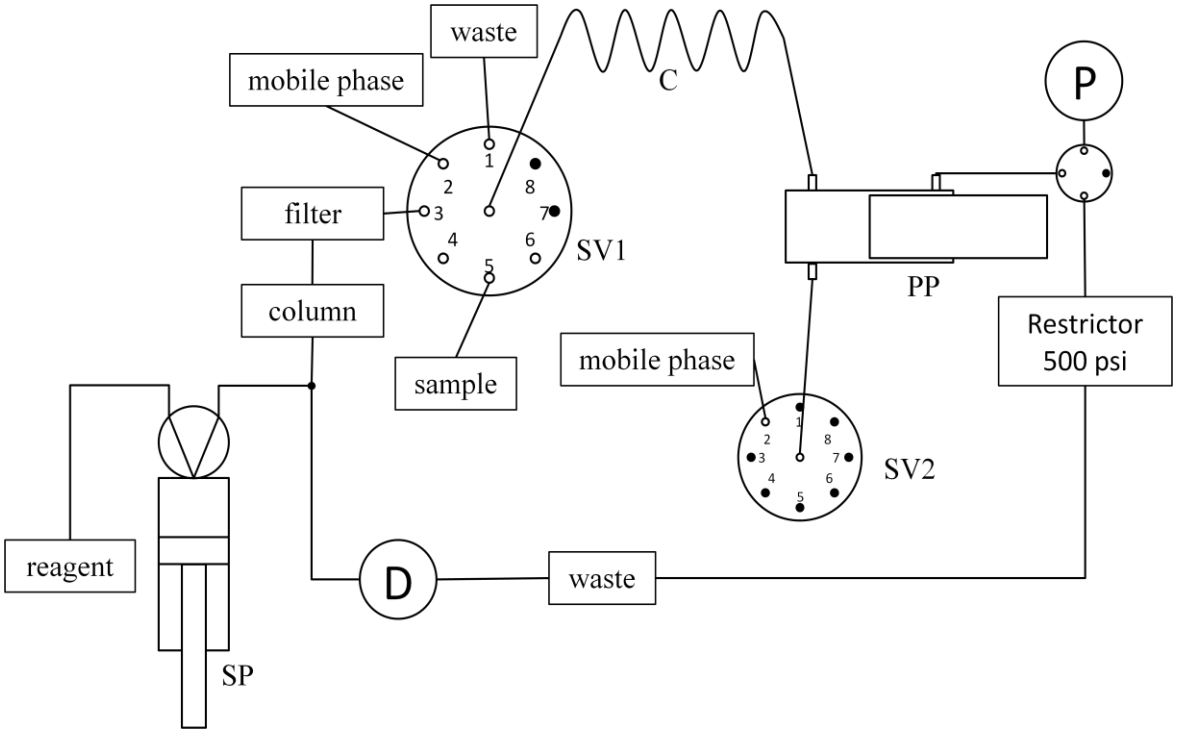
Further components were two eight-port selection valves (SV1, SV2 type Cheminert, 12U-0484H) from VICI (Valco Instruments Co. Inc., Houston, TX, USA), both connected to PP through central ports. SV1 was then connected to the eluent reservoir, waste, the column and the samples. SV2 was connected to the respective eluents and used, especially for the optimization of the eluent composition.

The SP had a rotary computer-controlled three-way head-valve. A manual four-port valve was used for the connection of PP either to a barometer or a pressure restrictor of 500 psi to protect the pump, in case that the column back pressure would increase above this limit. All tubing connections were made of PTFE tubing of 0.8 mm id used for solutions aspiration and high pressure connections or connections that needed reduced diameter were made of PEEK.

A miniature spectrophotometer was used as detector. In the SIC system a miniature fiber-optic spectrophotometer from OceanOptics Inc. (Dunedin, FL, USA), type USB4000, was used. The light source was a bright white LED, mounted onto an optical fiber SMA connector (FIAlab). Connections between the light source and the spectrophotometer to

a micro-volume detection flow cell, made of Ultem® polymer with 1 cm optical path lenght were made of 0.6 mm i.d. optical fibers (all from FIAlab®).

The most important piece of the separation system is the column. A Dionex Ionpac® CG5A guard column (2 x 50 mm, P/N 052836) from Thermo Fischer Scientific company (Waltham, Massachusetts, USA) was used. The stationary phase consists of 9 μm cross-linked DVB (55 %) beads, with a bilayer of anion-exchange and cation-exchange functionalized latex. The column was stored in 0.5 mol/l solution of NaOH if not used for more than one day.



Scheme 1: The construction of the used analyser system

SP: Syringe pump, PP: Piston pump, C: Holding coil, D: Detector, SV1: Rotary selection valve SV2: Rotary selection valve, P: barometer

4.1.1.2. Solutions

All solutions were prepared with Milli-Q water and reagents were purchased from Sigma Aldrich (Prague, Czech Republic).

The eluent as well as the reagent were made fresh for each measurement from the concentrated stock solutions (Table 3 and Table 4), which were kept frozen until needed. PDCA was dissolved in NaOH solution (750 mmol/l) and a final concentration of PDCA stock solution was 250 mmol/l. PAR was also dissolved in NaOH solution (100 mmol/l) and its final concentration was 50 mmol/l.

Other reagents and samples had stock solutions in a liquid state and were also prepared fresh every experimental day.

Table 3: Stock concentrations of compounds of eluent and reagent

Eluent´s compounds	Concentration [mmol/l]
Formic acid	2000
Sodium hydroxide	2000
Sodium sulphate	500
Reagent´s compounds	
Ammonium hydroxide	4000
Sodium hydrogencarbonate	1000
Sodium hydroxide	2000

Table 4: Stock concentrations of analytes

Samples	
Original compound	Concentration [mmol/l]
$Fe(NO_3)_3$	1.049
$FeSO_4$	1.022
$Co(NO_3)_2$	1.010
$CuSO_4.5H_2O$	0.038
$Zn(NO_3)_2$	0.988
$Ni(NO_3)_2$	1.018
$Cd(NO_3)_2.4H_2O$	1.050

4.1.2. Methods

4.1.2.1. Simplex

The simplex method or Simplex algorithm is a mathematical method of linear programming, developed by George Dantzig in 1947. A simplex is a term that originated from geometry and describes a polytope – an object which exists in *n* dimensions having *n+1* corners, or "vertices". For two dimensions it is a triangle [18].

A simplex optimization is supposed to find a vertex in this *n* dimensional space where a "desirability function" will have the best value, e.g. the resolution should be the largest.

To create a theoretical polytope, vertices have to be set. Each vertex is made by combination of *n* variables, e.g. concentrations of compounds in solution. In the beginning *n+1* combination sets – vertices are tested. In these initial experiments each variable is tested at three levels, e.g. low, moderate and high concentrations of a compound. These are initial vertices and form a simplex. In the next step, the vertex with the worst value of the "desirability function" is replaced by a better combination being a reflection in the *n*-dimensional space on the surface formed by all other vertices. In the case that a better result is achieved, the new vertex is accepted and the old worst vertex is eliminated, thus forming a new simplex, which is now closer to the ideal parameter set. In this new simplex, the new worst vertex is intended to be replaced and so on.

In practice, simplex is run by a computer program. Different modifications and improvements can be made, e.g. in this work variables were limited in the pre-set range. More information can be found in literature [19].

4.1.2.2. Running of the program

When not used for more than a day, the column was stored in an alkaline solution as recommended by the producer. So the first thing to do, before experiments started, was to clean the column and whole system (SP, PP, holding coil, sample channel) with eluent. After that a loop of three measurements was carried out. Each measurement consisted of the aspiration of the eluent into the PP (through SV1 or, if more eluents were tested through SV2) and the reagent into the SP followed by the aspiration of the sample into the holding coil, then dispensing sample and eluent to the column and reagent to the tube, where it was mixed with eluent flowing out of the column. The final mixture flowed to the detection cell. The detection wavelengths were 510, 520 and 530 nm, measured simultaneously against a reference wavelength 560 nm. After each aspiration or dispension step a delay of a few seconds was added. This was done to release the pressure.

In the final version of the program, aspirating of water was added into the process. The first solution aspirated into the holding coil, was the eluent, then 300 µl of water, half of which was pushed into the column. Then another 150 µl of water were aspirated, followed by the sample. By this, the sample was sandwiched between two volumes of water to improve analyte stacking on the stationary phase. Finally, the stacked solutions were pushed towards the column.

4.2. The progression of the optimization process

The aim of the work was to separate at least three cations of the transition metals using sequential injection analysis in the combination with a short, guard column in the short analysis time.

In the technical note from the producer of the chosen ion-exchange column [3], concentrations of the components of the eluent and the reagent are set for the HPLC separation with the assembly of guard column and separation column. Because here, only the guard column could be used due to the limited pressure provided by the PP, for the new system the composition of the eluent and the reagent has to be optimized, which was the main part of the work. The resolution and the peak height were in the centre of attention in this part.

In the next parts, the separation of ions was optimized – the symmetry of the peaks, baseline noise in comparison with peak height etc.

This was just a brief summary, in next few pages these steps will be described in detail.

4.2.1. The optimization of the mobile phase

4.2.1.1. Simplex method

The first step in the optimization of the eluent was using the Simplex program, which was described above. Variables were the concentrations of the eluent´s constituents: PDCA, formic acid, sodium sulphate and the pH value. The concentration of the sodium hydroxide was then calculated according to the aimed pH. The reason, why the pH value was used as variable instead of the concentration of sodium hydroxide, is that PDCA, as an essential compound for separation, is a weak acid, so it is either dissociated and able to bound free ions or undissociated and so nonfunctional.

According to the concentrations from the Simplex optimization the eluent was prepared. As a model sample a mixture of Cu(II) and Cd(II) was used, both in a concentration of 10 μmol/l. The amount of the sample used for the optimization was 30 μl, the flow-rate of the eluent provided by PP was 8 μl/s and the flow-rate of the SP was 5μl/s.

To measure the separation efficiency of each eluent, a value of a resolution (R_S) - calculated from the retention times of two peaks and their widths (see Formula 2) - was used. This number was evaluated as a desirability function to each measurement, which means that the priority was to achieve the R_S as high as possible. The Simplex optimization was carried out to look for such a combination of the components´ concentrations and the pH value, that would achieve the best R_S.

4.2.1.2. Univariant study

To find out, if any other combination of concentrations shows better result, also univariant study was proceeded. In the study one compound is chosen and its concentration is changed.

4.2.1.2.1. Formic acid concentration optimization

The first parameter to be optimized was the formic acid concentration. This component was chosen as first, because it has one of the lowest effects on the resolution.

The concentrations of formic acid were 30, 40, 50, 60, 70 and 80 mmol/l. For the other variables, medium values from the simplex optimization were chosen: sodium sulphate 3 mmol/l, PDCA 5 mmol/l and pH 2.5. To accomplish constant pH value, sodium hydroxide was added in concentrations 1.5, 2, 2.5, 3.1, 3.6 and 4.1 mmol/l, respectively. In these and following measurements the sample consisted of Cu(II) (10 μmol/l), Co(II) (10 μmol/l) and Fe(II) (20 μmol/l). The volume of the sample was 30 μl, flow-rate of the PP was 8 μl/s and the SP´s flow-rate was 6 μl/s.

4.2.1.2.2. Sodium sulphate concentration optimization

The salt content in the eluent strongly influences the separation, the higher the concentration of the salt, the faster elution of the analytes. This is because the sodium cation competes with the analyte cations for the negatively charged sulphonate groups on the stationary phase while as counterion, an anion is chosen, which does not form strong complexes with the analyte cations. Instead, a chelating anion is added to the eluent, here PDCA, which forms complexes with the transition metal cations of significantly different stabilities, was used. Therefore, without PDCA as chelating compound the separation would not be possible. As a consequence, the both concentrations, of the salt and the chelating compound, have to be carefully adjusted.

As recommended by the column producer, sodium sulphate as neutral and well-soluble salt was used. According to the Simplex study, used concentrations were 0, 1, 2, 4, 7 and 10 mmol/l using 40 mmol/l formic acid, 5 mmol/l PDCA, and 2 mmol/l sodium hydroxide. The same standard solution and flow-rates were used as in the previous experiment.

4.2.1.2.3. PDCA concentration optimization

PDCA is an abbreviation for a pyridine-2,6-dicarboxylic acid. It is a compound of the eluent essential for the separation. It forms a complex with divalent and trivalent ions of the transition metals. The complexes with different metal cations have different strength according to which they can be separated. The concentrations of PDCA used in the third univariant study were 1, 2, 3, 4, 5, 6 and 7 mmol/l. For the other components, the following concentrations were chosen: formic acid 40 mmol/l and sodium sulphate 4 mmol/l. The pH value was set to 2.5, which was equal to 2 mmol/l of sodium hydroxide in each measurement, because the concentration of PDCA was not involved in the calculation of the pH value. The amount of the sample and flow-rates stayed the same as in the previous measurements.

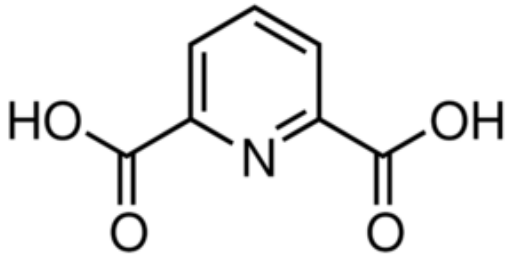


Figure 3: The structure of non-dissociated PDCA [20]

4.2.2. The optimization of the post-column reagent

The next step in the work was to optimize the reagent. In the technical note [3] the concentrations of the reagent´s components were given with: 0.5 mmol/l 4-(2-pyridylazo) resorcinol (PAR), 1.0 mol/l 2-dimethylaminoethanol, 0.50 mol/l ammonium hydroxide and 0.30 mol/l sodium bicarbonate.

Dimethylaminoethanol is flammable, corrosive liquid with a bad smell, so it is not very pleasant to work with. By test it was proven, that this compound is not needed in the reagent and, the resultant chromatograms were not significantly different.

At the beginning of the first part of the work, meaning optimization of the eluent, the reagent was made of PAR (0.5 mmol/l), ammonium hydroxide (100 mmol/l), sodium hydrogencarbonate (75 mmol/l) and sodium hydroxide (50 mmol/l). Used concentrations were modified from those recommended in technical note [3]. After the Simplex optimization of eluent was done, the reagent was diluted to half concentration, just to see the effect. Peaks were approximately the same and less concentrated variation was chosen.

4.2.2.1. Simplex method

The optimizing of the reagent started with the Simplex method using three variables being the concentrations of ammonium hydroxide, the sodium hydrogencarbonate and the sodium hydroxide. The volume of sample was 30 µl and flow-rates of both pumps were 8 µl/s.

The results from the first Simplex optimization (see Table 7) were not satisfying, i.e. the initial vertices yielded similar results and there was no increase in signal height with later optimization. The reason was probably that the concentrations of the components were very high, so that no significant change was caused by their variation, or that there was a lack of PAR as the variable, so it was held constant in this first Simplex optimization.

These imperfections were removed and the second Simplex optimization was carried out. PAR was added as variable and the rest of the compounds were used as less concentrated solutions (see Table 8). Also a double-concentrated sample was used and the

injection volume was raised to 50 µl to facilitate the posterior peak height evaluation. This experiment led to better results, i.e. the calculated vertices surpassed the worst results from the two worst initial vertices but did yet not overcome the results from the three best. Therefore another study was tried.

4.2.2.2. Further optimization

The final step in optimizing the reagent composition was not a univariant but a bivariant study, because two variables were changed simultaneously: the sodium hydroxide concentration and the sodium hydrogencarbonate concentration. They were set in levels to enable the pH of the reagent would stepwise increase while the buffer capacity would not differ greatly. The concentration of ammonium hydroxide was kept constant and the remaining values were partly chosen according to previous outcomes (Table 9) but also taken into account the given concentrations of eluent and reagent and their flow-rates from the technical note [3] and the altered values in this work.

In detail, because an equal flow of eluent and reagent was used, and the formic acid was nearly halved than in the original method, the reagent could be by factor of about three-times less concentrated without any expectable affection of the methods sensitivity.

The injection volume was set to 30 µl and the flow-rates of the both pumps were 8 µl/s.

4.2.2.3. PAR univariant optimization

4-(2-pyridylazo)resorcinol alias PAR is the most important compound of the reagent. It forms red-colored complexes with ions, which are measured by spectrophotometry. The recommended concentration in the technical note was 0.5 mmol/l. Firstly, a reagent with this concentration was prepared and it was lowered to 0.25 mmol/l. In this univariant optimization the concentration of PAR was moved from 0.05 up to 0.4 mmol/l.

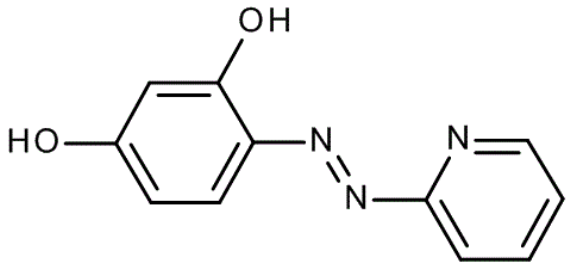


Figure 4: The structure of non-dissociated PAR [21]

4.2.3. Separation of other metal cations

During the previous phase of optimizing the eluent and the reagent composition, just two or three transition metals were used to facilitate peak detection and peak resolution. However, the objective of the work is to try to separate as many metals as possible. Therefore, the next step was to use standard solutions containing more metal cations and see how the column would cope with that. From this point on the eluent and the reagent had the same following optimized composition:

Eluent:	PDCA	4 mmol/l
	formic acid	40 mmol/l
	sodium sulphate	4 mmol/l
	sodium hydroxide	2 mmol/l
	flow rate	8 µl/s

Reagent:	PAR	300 μmol/l
	ammonium hydroxide	150 mmol/l
	sodium hydrogencarbonate	60 mmol/l
	sodium hydroxide	45 mmol/l
	flow rate	8 μl/s

The standard solutions of the metal cations were added to injected analyte stepwise. Firstly only standard with Co(II) and Cu(II), both in concentration 10 μmol/l, was measured. Then experiments continued according to Table 5.

Table 5: The presence or absence of certain ions in experiments

Experiment	Metals [μmol/l]					
	Cu(II)	Co(II)	Zn(II)	Ni(II)	Mn(II)	Fe(II)
1	10	10	-	-	-	-
2	10	10	-	10	-	-
3	10	10	10	-	-	-
4	10	10	-	20	-	-
5	10	10	-	-	10	-
6	10	10	-	-	20	-
7	10	10	-	-	-	20

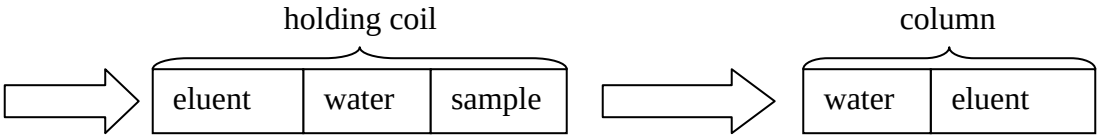
4.2.4. Stacking on the column conditioned with water

Since the separation of more analytes did not show the expected results, another approach was tried to improve separation and the chromatogram (peak shapes and the resolution).

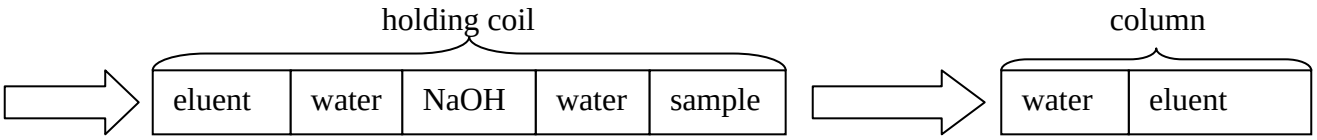
This experiment included injecting water prior to and after the sample. Until this point, the program consisted of following steps: PP aspirates at first the eluent, then the sample and then all was dispensed to the column. By adding water better results should be achieved.

Table 6: Brief summary of experiments with adding water

Experiment	Conditions
1	Sample 30 µl no water
2	Sample 80 µl no water
3	Sample 80 µl with water aspirated in piston pump before and after sample
4	Sample 80 µl with water like in Scheme 2
5	Sample 80 µl with NaOH like in Scheme 3



Scheme 2: Sequence of solutions in pump and column no. 1



Scheme 3: Sequence of solutions in pump and column no. 2

Five different procedures were tried, shortly characterized in Table 6 and Schemes 2 and 3. In the first two experiments sample volumes of 30 and 80 µl were measured. Then the measurements were repeated but the aspiration of water was added before and after the sample. So the difference between experiments number 3 and 4 is following. In the first case, sample is surrounded by water in the holding coil, and the column is filled just with eluent. The second case is depicted in the Scheme 2. The experiment number 5 is depicted in the Scheme 3.

The flow-rates of the pumps were 8 µl/s and the concentrations of the elements in the sample were 10 µmol/l.

4.2.4.1. Injection volume with added water

As next step, different volumes of sample were measured using the previous procedure. To put it in the words, at first the eluent was aspirated into the holding coil by the pump, then water (300 µl), after that the half of the aspirated water (150 µl) was dispensed into the column, (which was before filled with eluent from the previous separation or cleaning process). After that water was again aspirated into the holding coil and then the sample (120 µl) the volume of which was about to change.

Again the objective of this experiment was to improve the chromatogram. So, the symmetry of the peaks and resolution were considered.

5. RESULTS AND DISCUSSION

5.1. The optimization of eluent

5.1.1. Simplex method

Table 7: Simplex optimization of the eluent composition

Experiment	PDCA [mmol/l]	Formic acid [mmol/l]	pH	Sodium sulphate [mmol/l]	NaOH [mmol/l]	Result = R_S
Initial vertices						
1	4.00	50.00	3.50	4.00	17.5	1.38
2	5.85	55.46	3.88	4.44	31.3	1.19
3	4.44	73.14	3.88	4.44	41.3	1.24
4	4.44	55.46	5.12	4.44	53.1	0.60
5	4.44	55.46	3.88	5.90	31.1	0.95
Calculated vertices						
6	4.88	61.33	2.50	4.74	3.1	2.11
7	**5.12**	**64.51**	**2.72**	**3.04**	**5.3**	**2.52**
8	**5.24**	**66.21**	**2.51**	**1.38**	**3.4**	**2.52**
9	4.28	66.21	2.50	3.47	3.4	1.59
10	4.76	60.21	2.50	3.47	3.1	2.29
11	4.84	63.39	2.50	3.50	3.2	1.59
12	4.28	66.21	2.50	3.47	3.4	1.59

In the Table 7, the results of the simplex for optimization of the eluent composition are given. The improvement of the resolution connected with one variable was significant and that was the pH value. In the last five measurements this variable had the same value 2.5. This so-called loss of the dimension of the pH value was caused by the boundaries set in the beginning, which means that the pH value tended to decrease even further, but was limited to do so. This was done to avoid high acidity in order not to damage the column.

5.1.2. Univariant optimization of eluent

Since the pH values generated by the Simplex program were the same in the last five measurements, it would not be effective to continue this way. Therefore, according to the Simplex results, the pH variable was preserved on the value of 2.5. The other variables were then changed, one by one, that is why it is called univariant studies. For the further understanding, the variables were formic acid, sodium sulphate and PDCA, and the values connected to them were their concentrations in the used eluent. To each variable a set of values was matched and each value was measured three times and the results, such as the R_S or the peak height, were the average values from these three measurements.

5.1.2.1. Formic acid concentration optimization

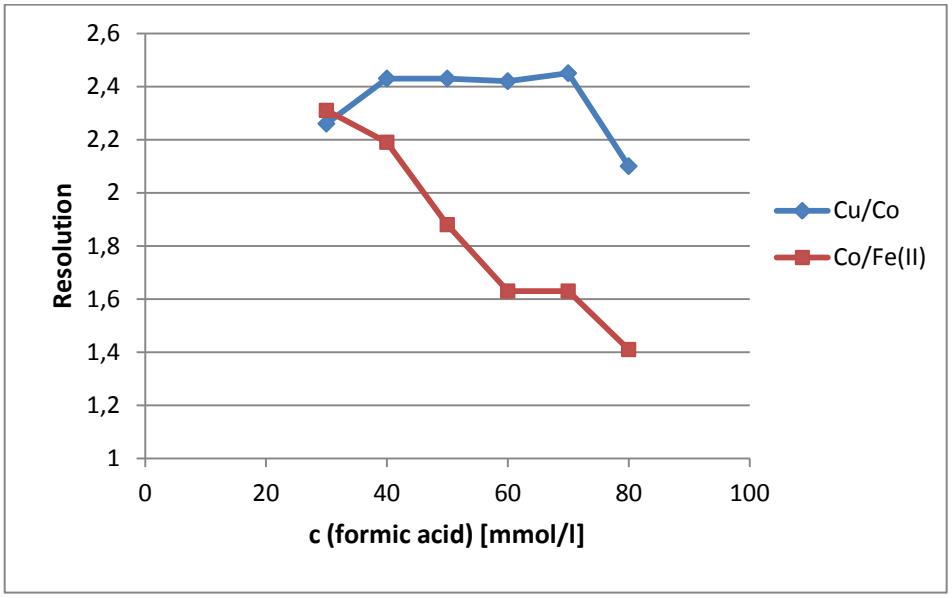


Figure 5: Resolution between ions according to different concentration of formic acid in eluent

As it can be seen from the results shown in Figure 5 with an increasing concentration of formic acid the resolution of Fe(II) and Co(II) decreased and the resolution of Cu(II) and Co(II) did not change significantly, with only exception being the lowest concentration. As the best result a concentration of 40 mmol/l was chosen and also used in the following univariant studies.

5.1.2.2. Sodium sulphate concentration optimization

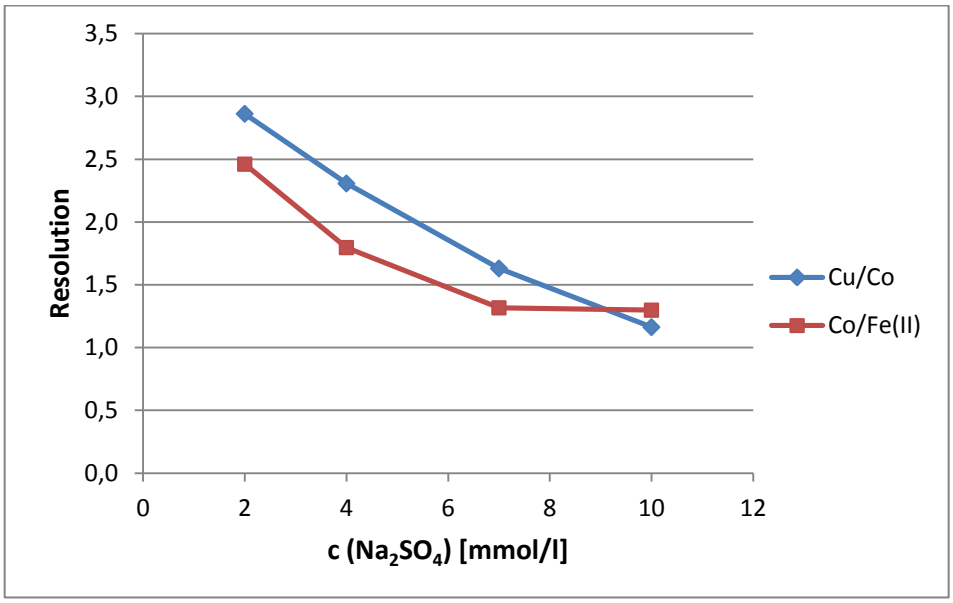


Figure 6: Resolution between ions according to different concentration of sodium sulphate in eluent

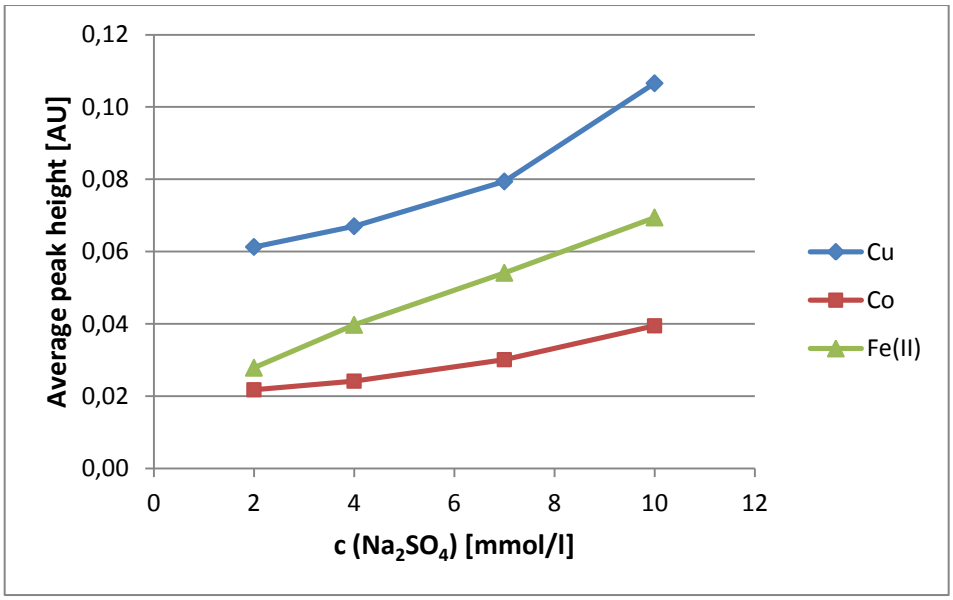


Figure 7: Average peak height according to changing concentration of sodium sulphate in eluent

In the Figure 6 is depicted, how the resolution decreased with increasing concentration, while likely due to a shorter time and so less peak broadening, the peak

heights increased (see Figure 7). Taking R_S as well as peak height into the consideration, an intermediate value - 4 mmol/l - was chosen for further work.

5.1.2.3. PDCA concentration optimization

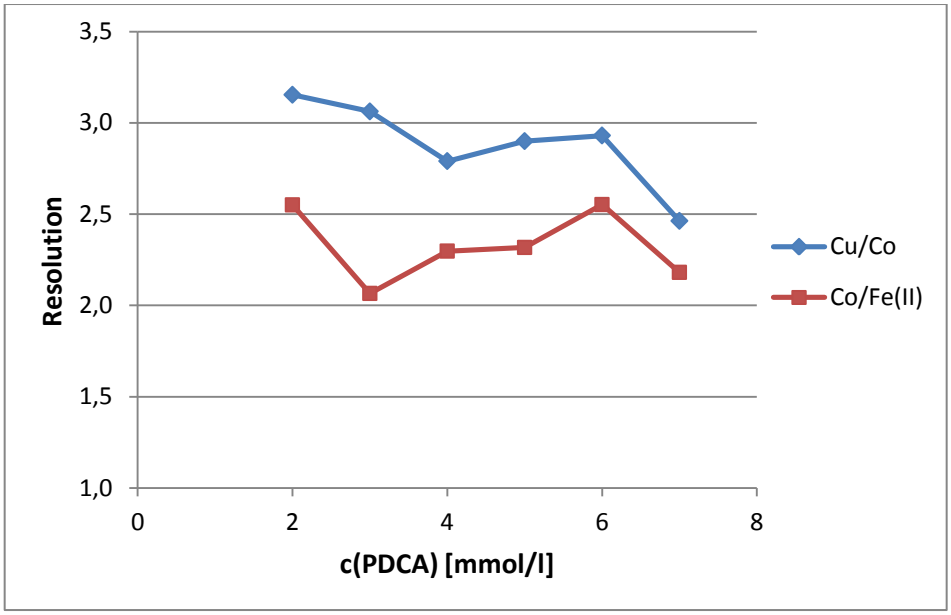


Figure 8: Resolution between ions according to different concentrations of PDCA in eluent

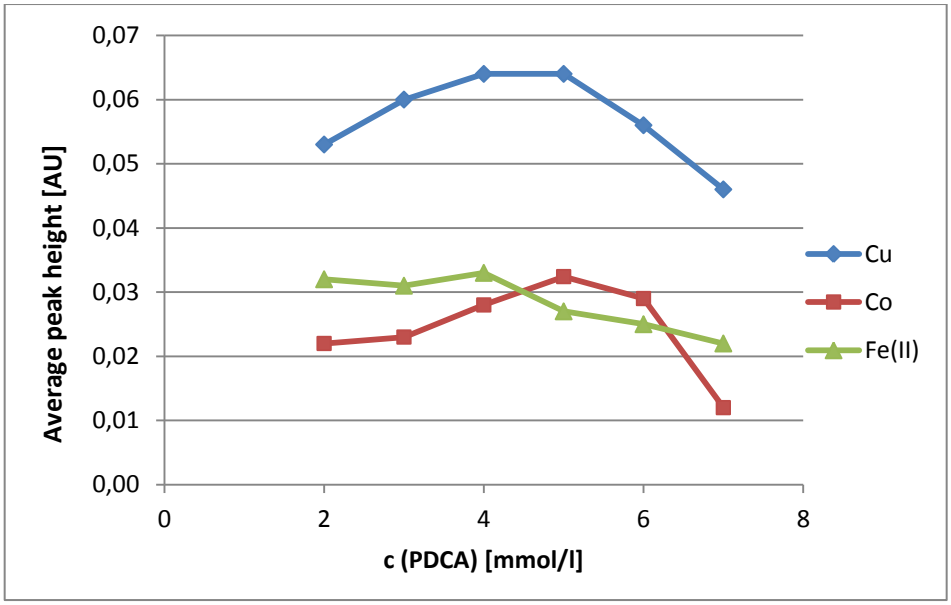


Figure 9: Average peak height according to different concentrations of PDCA in eluent

As in the previous univariant study not only the peak R_S (Figure 8) but also the peak heights (Figure 9) were taken into consideration when deciding about the best result. The best results with respect to the peak resolution were obtained with concentrations 2 and 6 mmol/l, but under these conditions, the peak height was low. Finally, a PDCA concentration of 4 mmol/l was chosen as a compromise between peak resolution and peak heights.

5.2. The optimization of post-column reagent composition

5.2.1. Simplex method

Table 8: Simplex optimization of the reagent composition number 1

Experiment	NH_4OH [mmol/l]	$NaHCO_3$ [mmol/l]	NaOH [mmol/l]	Result = peak height [AU]
Initial vertices				
1	200.0	90.0	90.0	0.081
2	385.1	149.0	99.8	0.074
3	243.7	339.9	99.8	0.070
4	243.7	149.0	131.7	0.074
Calculated vertices				
5	290.8	10.0	110.5	0.062
6	258.8	243.0	103.2	0.062
7	305.3	225.3	60.8	0.062

Table 9: Simplex optimization of the reagent composition number 2

Experiment	NH_4OH [mmol/l]	$NaHCO_3$ [mmol/l]	NaOH [mmol/l]	PAR [mmol/l]	Result = peak height [AU]
Initial vertices					
1	50.00	60.90	60.90	0.18	0.132
2	96.30	50.00	60.90	0.18	0.179
3	60.90	96.30	50.00	0.18	0.127
4	**60.90**	**60.90**	**96.30**	**0.13**	**0.192**
5	60.90	60.90	60.90	0.26	0.177
Calculated vertices					
6	75.30	19.80	91.80	0.19	0.155
7	96.18	37.21	93.86	0.20	0.132
8	61.55	54.98	69.14	0.18	0.138
9	84.64	43.13	85.62	0.19	0.165

The concentrations of the reagent components and peak heights are mentioned in the Table 8. The initial vertices had the better results than calculated vertices, so another simplex study was made. PAR was added to variables, injection volume was increased – from 30 µl to 50 µl and concentrations of other compounds were changed also (more details in the section Experimental).

In the Table 9 values from the second simplex study are depicted. Despite the changes, no improvement was observed and different approach was chosen.

5.2.2. Further optimization

Table 10: Bivariate optimization of the reagent composition

Experiment	NH_4OH [mmol/l]	NaOH [mmol/l]	$NaHCO_3$ [mmol/l]	PAR [mmol/l]	Peak height[AU]		RSD %	
					Cu	Co	Cu	Co
1	150	0	150	0.15	0.046	0.011	5.1	2.8
2	150	15	130	0.15	0.054	0.013	2.0	6.1
3	150	30	110	0.15	0.056	0.014	6.7	4.8
4	**150**	**45**	**90**	**0.15**	**0.059**	**0.016**	**0.8**	**1.2**
5	150	60	70	0.15	0.065	0.018	8.0	20.4
6	150	75	50	0.15	0.072	0.018	12.0	17.8

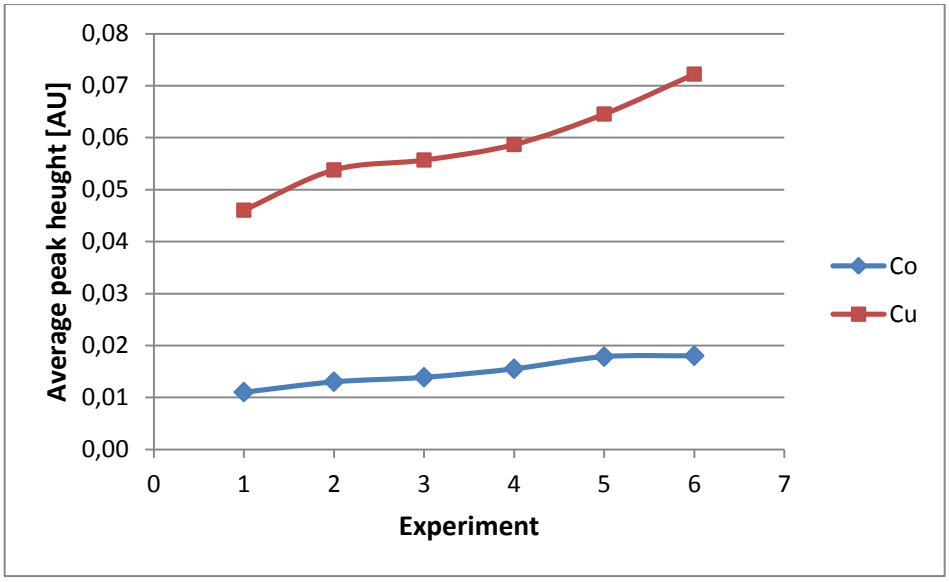


Figure 10: Peak height according to different concentrations of sodium hydroxide and sodium hydrogencarbonate in reagent

The conditions of each measurement, peak heights and RSD are in the Table 10. In the Figure 10 peak heights are depicted. Although the peaks were higher with rising concentration of sodium hydroxide and decreasing concentration of sodium hydrogencarbonate, the baseline noise was more conspicuous due to a spectral shift of the absorbance maximum of PAR with higher pH and larger contribution to the signal background. The final combination was 90 mmol/l of $NaHCO_3$ and 45 mmol/l of NaOH, which was a compromise with relatively high peaks and low baseline noise. Also, this value has the lowest relative standard deviation.

5.2.3. PAR univariant optimization

Table 11: Optimization of the reagent composition

Experiment	PAR [mmol/l]	Peak height [AU]	
		Cu	Co
1	0.05	0.040	0.008
2	0.10	0.048	0.011
3	0.15	0.044	0.010
4	0.20	0.047	0.011
5	0.25	0.054	0.014
6	0.30	0.066	0.021
7	0.40	0.068	0.027

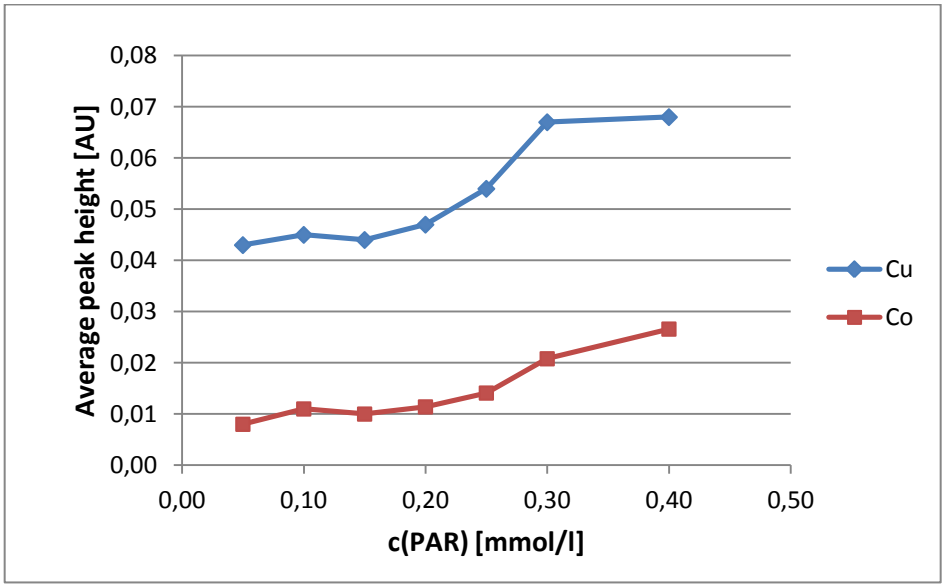


Figure 11: Peak height according to different concentration of PAR in reagent

Another step was based on optimization of the concentration of PAR in the reagent. Tested values together with peak heights are shown in Table 11. The process of deciding which result is the best was based on the peak height (see Figure 11) and baseline noise. It can be seen, that the signal heights increased with increasing concentration of PAR while likewise the baseline noise increased significantly. Since the difference in peak heights between concentrations 0.3 and 0.4 mmol/l were small, 0.3 mmol/l was chosen for further work

5.3. Separation of other metal cations

Table 12: Experiments with more metals

Experiment	Metals [µmol/l]						Result
	Cu	Co	Zn	Ni	Mn	Fe	
1	10	10	-	-	-	-	2 peaks, separated, symmetric
2	10	10	-	10	-	-	2 peaks, separated, no sign of nickel
3	10	10	10	-	-	-	2 peaks, separated, not symmetric, shoulders
4	10	10	-	20	-	-	2 peaks, separated, nickel co-elutes with cobalt
5	10	10	-	-	10	-	2 peaks, separated, no sign of manganese
6	10	10	-	-	20	-	2 peaks, separated, no sign of manganese
7	10	10	-	-	-	20	3 peaks, separated

A summary of the tested standard solutions is given in the Table 12. First of all (No 1 in the Table 12), the original combination – Cu(II) and Co(II) was used, so the results can be compared to other experiments. Both cations were separated in concentrations of 10 µmol/l and according to the expectations, the peaks were completely separated and symmetric.

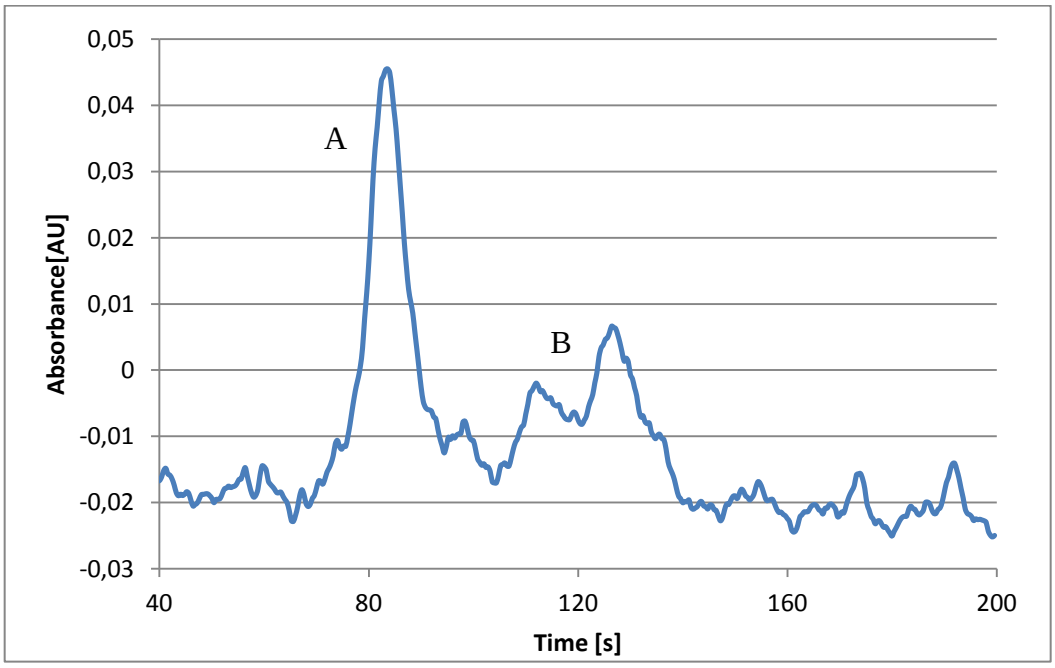


Figure 12: A – the peak of Cu(II) 10 µmol/l, **B** – Co(II) 10 µmol/l and Ni(II) 20 µmol/l merged into one peak

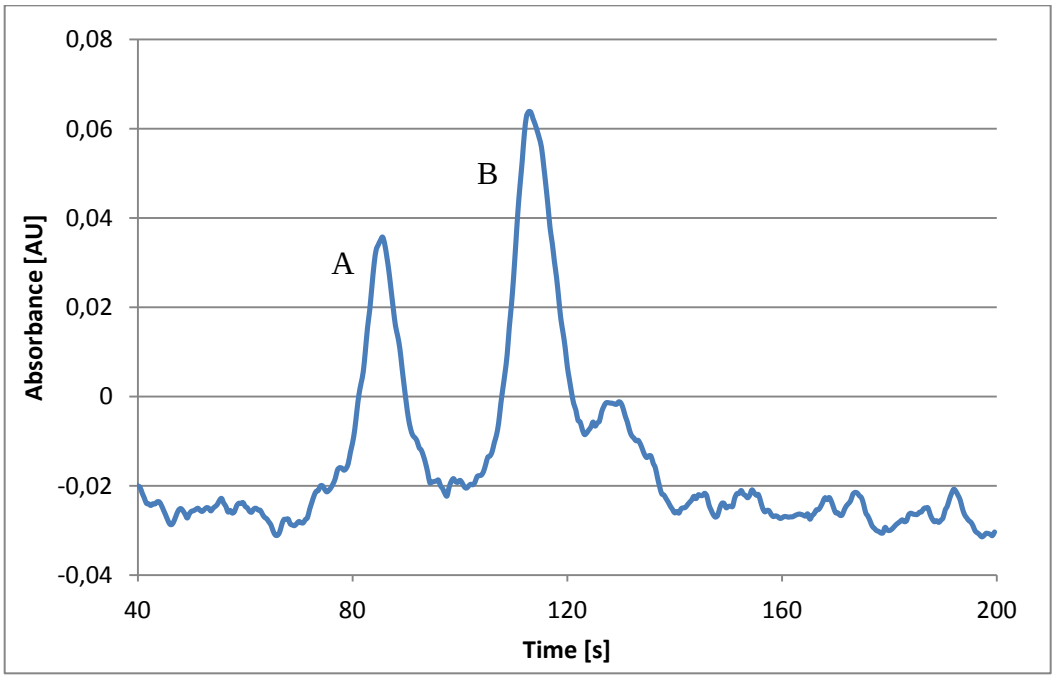


Figure 13: A – the peak of Cu(II) 10 µmol/l, **B -** Co(II) and Zn(II) both 10 µmol/l merged into one peak

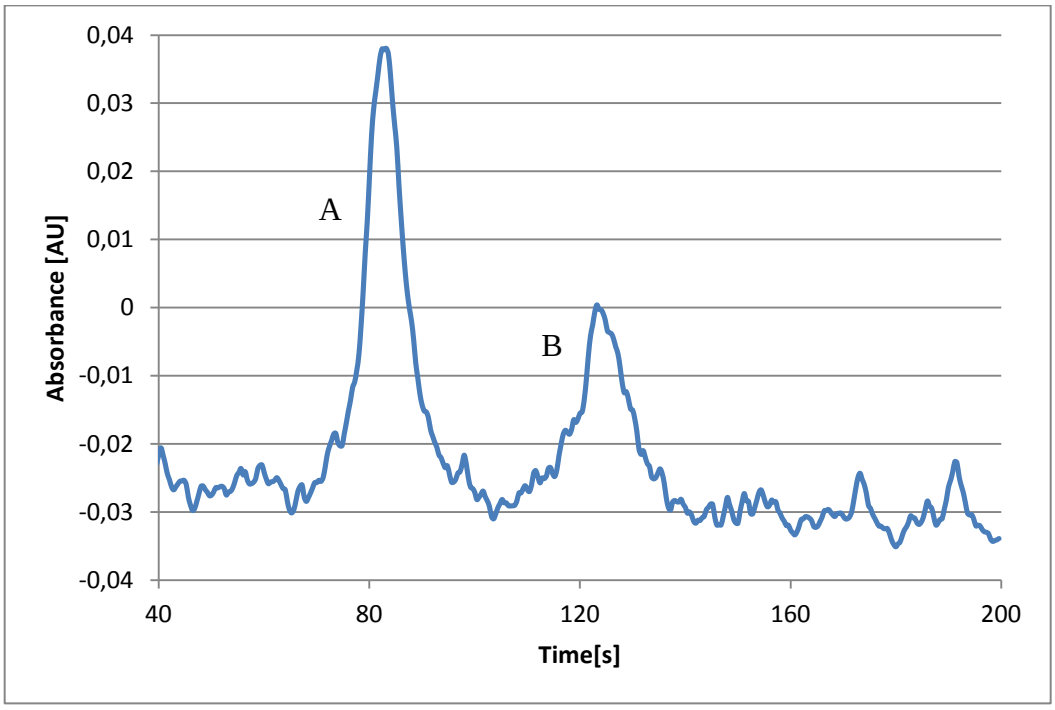


Figure 14: A – the peak of Cu(II) 10 µmol/l, **B –** the peak of Co(II) 10 µmol/l, and no sign of Mn(II) 20 µmol/l

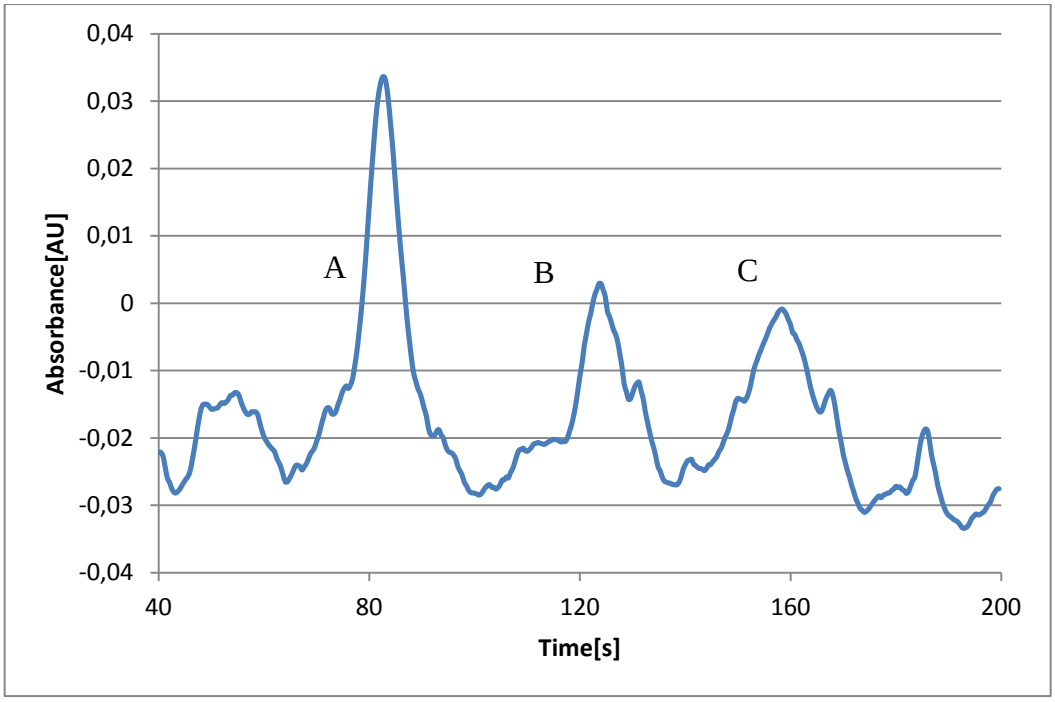


Figure 15: A – the peak of Cu(II) 10 µmol/l, **B –** the peak of Co(II) 10 µmol/l, **C –** the peak of Fe(II) 20 µmol/l

Then a Ni(II) standard was added to the sample (No 2 in the Table 12). Unfortunately in the chromatogram was no signal of it. The nickel peak should have appeared right after copper, so it is possible, that they merged into one peak. When the concentration of Ni(II) was doubled (No 4 in the Table 12), also no new peak appeared. The cupper peak heights in the measurements 1 and 2 were similar, in the No 4, the peak is higher by 7 %. Also in the measurement 4 cobalt´s peak had shoulder, which could be a sign of nickel (see Figure 12).

When instead of Ni(II), Zn(II) standard was added to the sample, the peaks of Cu(II) and Co(II) were closer to each other, because peak of cobalt had a shorter retention time than in the measurement 1. Furthermore, this peak was approximately three times higher than in the first measurement and had a shoulder, which indicated that the peaks of Zn(II) and Co(II) merged into one (Figure 13). After adding Mn(II) to the sample there was no additional peak in the chromatogram, can be caused by low sensitivity of Mn(II) (Figure 14). On the contrary the standard solution including Fe(II) showed a fully separated peak (Figure 15).

5.4. Stacking on the column conditioned with water

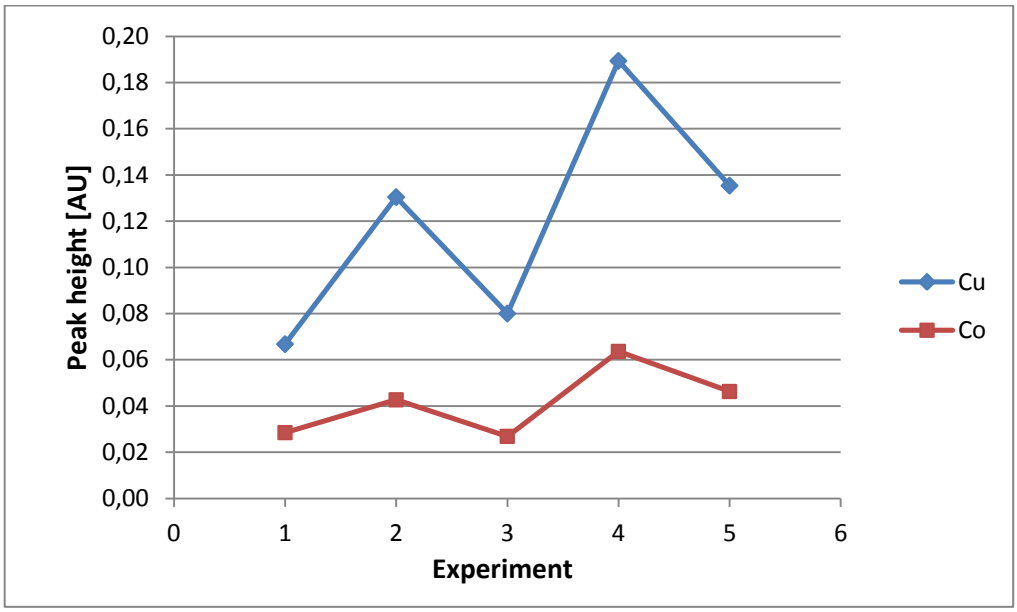


Figure 16: Peak heights according to presence or absence of water and sequence of solutions

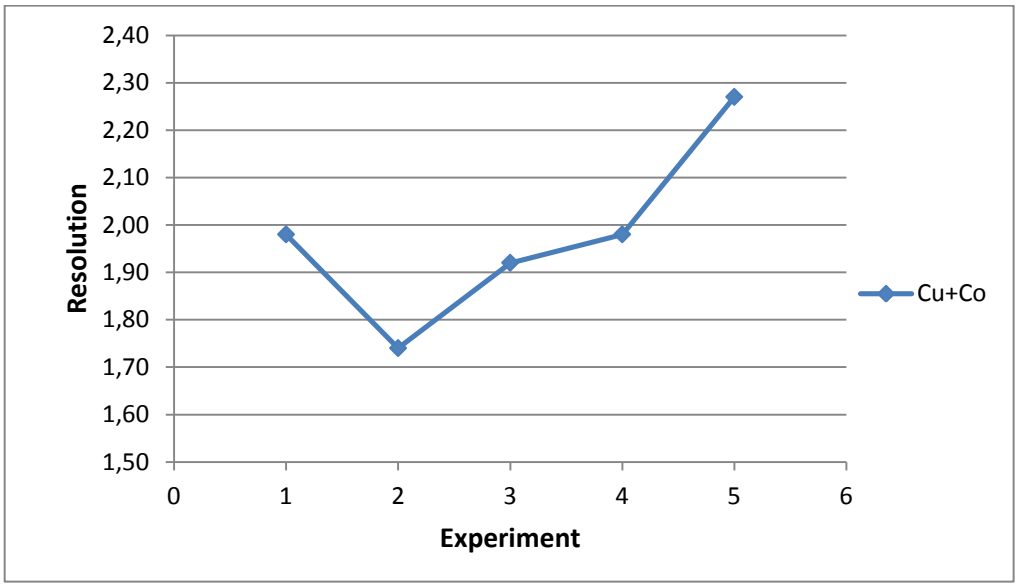


Figure 17: Resolution between peaks of Cu(II) and Co(II) according to presence or absence of water and sequence of solutions

Peak heights under described conditions are depicted in Figure 16, resolutions are shown in Figure 17. The highest peaks, so the best result and also high resolution is the experiment number 4 so the next experiment was also designed according to Scheme 2.

5.4.1. Injection volume with added water

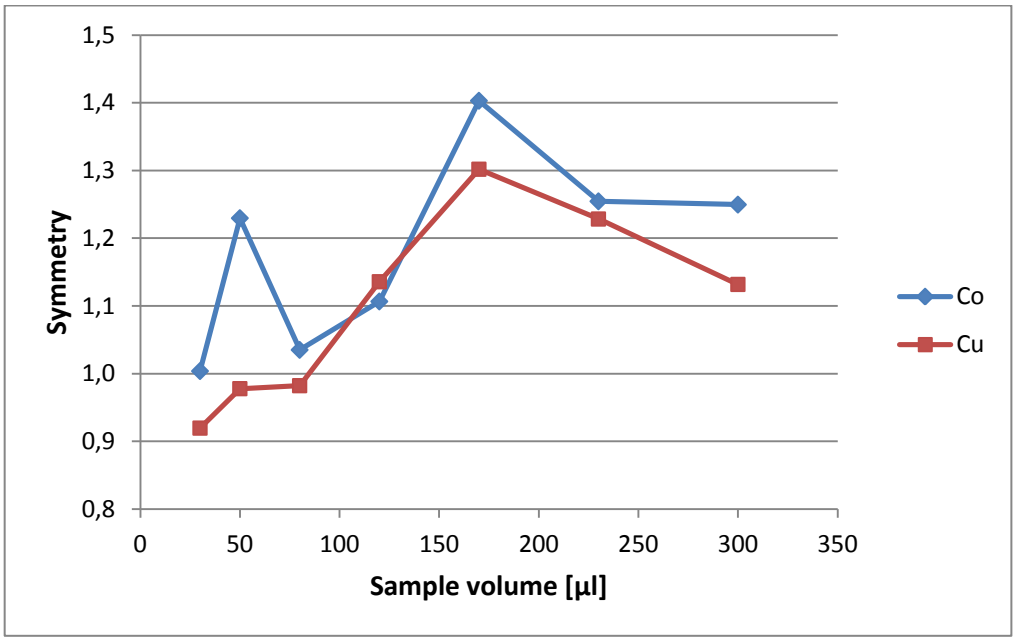


Figure 18: Symmetry of the peaks with increasing sample volume

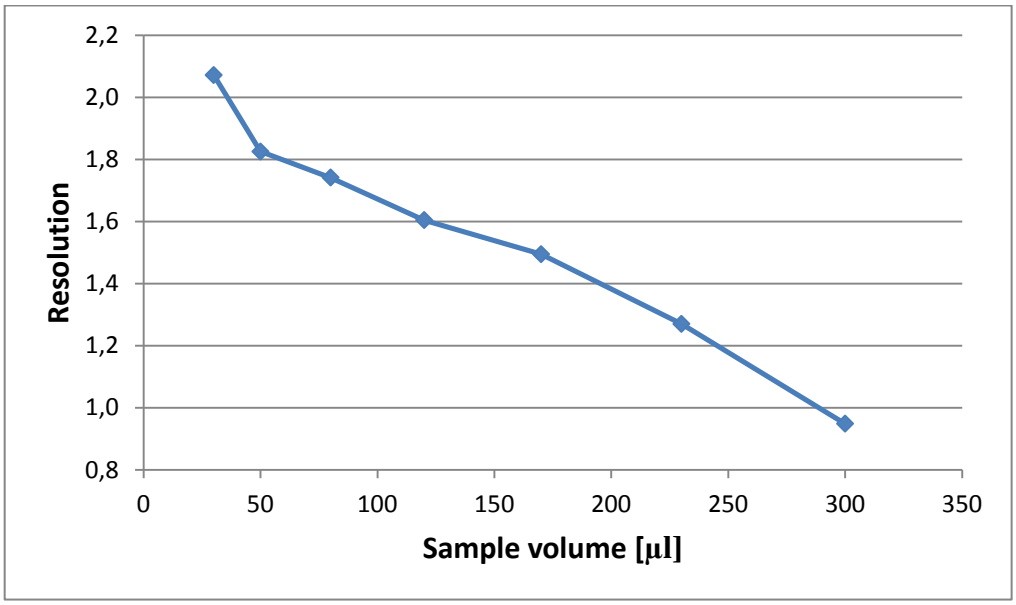


Figure 19: Resolution between Co(II) and Cu(II) with increasing sample volume

In Figures 18 and 19 the symmetry of the peaks and the resolution are shown, respectively. The symmetry of the peaks was calculated according to Formula 3. The best

value this quantity could have is 1.0. The closest to this value are results from the sample of volume 30 μl and as the volume increased, symmetry got worse. The resolution decreased with the increasing volume of the sample, because the peaks became higher and wider, but the retention times stayed the same.

Finally, 90 μl was chosen as the best value for the sample volume. The result was determined by three factors: the resolution, the peak height and the symmetry of the peak.

6. Summary

In this work, the ability of Dionex® guard column was studied. Firstly, according to Technical note from Dionex®, PDCA and PAR were chosen as chelating agents of eluent and post-column reagent, respectively.

To optimize the concentrations of the compounds of the eluent as well as the reagent two studies were used: Simplex optimization, where the concentrations of three or four compounds were changed in one step, and an univariant study, where the concentration of only one compound was changed.

At first the composition of the eluent was optimized. During these studies, the resolution was the observed quality, according to which the best result was chosen. The Simplex study of 12 measurements was provided, followed by univariant studies of formic acid, sodium sulphate, and PDCA concentrations, in this order. In each step, the concentration of optimized compound, which gave the best value of resolution, was chosen: 40 mmol/l, 4 mmol/l, and 4 mmol/l, respectively. The concentration of the last compound of the eluent – sodium hydroxide (2 mmol/l) – was then calculated according to pH value - 2.5.

Next, the composition of the reagent was optimized. The best result was chosen according to the peak height, as long as the post-column reagent does not affect the resolution. This time two simplex studies were provided, because the first one did not bring expected results. One bivariant study, where concentrations of sodium hydroxide and sodium hydrogencarbonate were changed, and one univariant study of PAR followed. The resultant concentrations were: 60 mmol/l of $NaHCO_3$, 45 mmol/l of NaOH, 150 mmol/l of ammonium hydroxide, and 0.3 mmol/l of PAR.

After these optimizations, the number of detected cations was about to be studied. In the previous measurements, the cations Cu(II) and Co(II) were measured. In following measurements, separation of cations Ni(II), Zn(II), Mn(II), and Fe(II) was tested but the outcomes were not satisfying.

So, another improvement, in a form of adding water, was tried. The sample was sandwiched between two volumes of water which significantly improved analyte stacking on the column and thus peak resolution.

Then the volume of the sample was studied in a range of 30 to 300 µl with the result of 90 µl. Finally three transition metal cations were separated: Cu(II), Zn(II), and Fe(II) – see Figure 20, below.

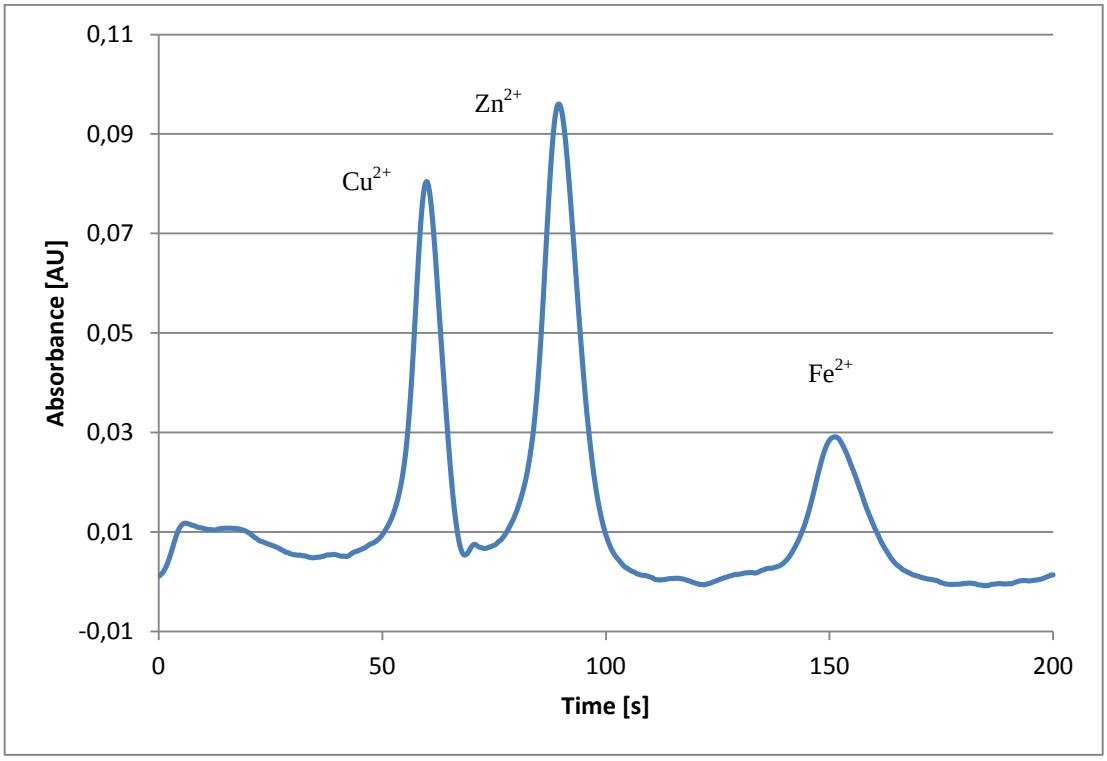


Figure 20: The final sample of volume 90 µl, consisted of Cu(II), Zn(II) both 15 µmol/l, and Fe(II) 28.8 µmol/l

7. Conclusion

The objectives of the work were successfully fulfilled. The Dionex IonPac CG 5A guard column was incorporated into a SIC system with the use of a post-column reagent.

It was proven, that this column alone is suitable for the separation of a small number of transition metal cations. Optimization of the composition of the eluent and the reagent was carried out.

An easy-to-use and rapid method was created, with the addition of the water, which improved the analyte stacking and the resolution.

The separation of the three cations Cu(II), Zn(II), and Fe(II) at an micromolar concentration level was done and the method characteristics were evaluated.

An instrumentation of SIC, used for the separation of transition metals, can differ from one used in this work. Another type of column (i.e. monolithic) can be used, different type of the detection etc. An example of detection is a chemiluminescent variant using sensitive reaction between luminol and hydrogene peroxide, which is catalyzed by some cations of transition metals [22].

8. Formulas

Formula 1: Absorbance

$$A = \log_{10} \frac{I_0}{I}$$

A – absorbance, I_0 – the intensity of the ray before it enters the detection cell, I – the intensity of the ray after it left the cell [24]

Formula 2: Resolution

$$R_S = \frac{2 \cdot (t_{R2} - t_{R1})}{w_{h1} + w_{h2}}$$

R_S – resolution, t_{R1} – retention time of the first neighbouring peak, t_{R2} – retention time of the second peak, w_{h1} – width of the first peak at the baseline, w_{h2} - width of the second peak at the baseline

R_S – the value should be above 1.5 [23]

Formula 3: Symmetry of the peak

$$A_S = \frac{w_{0.05}}{2d}$$

A_S – symmetry of the peak, $w_{0.05}$ – width of the peak 5% above the baseline, d - the first half of $w_{0.05}$ marked by a perpendicular from the highest point of the peak, depicted in the Figure 21

A_S – the value of ideal, symmetric peak is 1.0 [24]

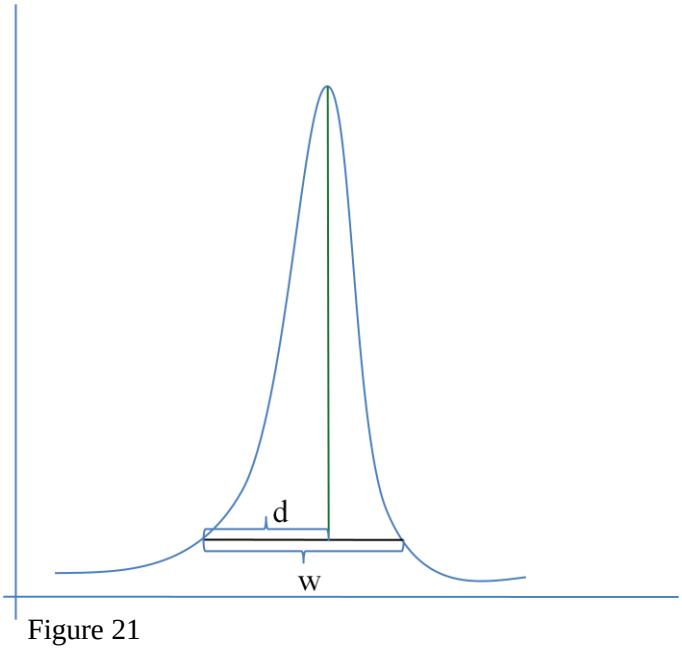


Figure 21

Formula 4: Ratio signal to noise

$$S/N = \frac{2H}{h}$$

H – height of the peak, h – height of the baseline noise [24]

Formula 5: Relative standard deviation

$$RSD_{\%} = \frac{100}{\bar{y}} \sqrt{\frac{\sum(y_i - \bar{y})^2}{n-1}} = \frac{100}{\bar{y}} s$$

RSD – relative standard deviation, $\bar{y}$ – the average of measured values, y_i – single measurement, i is an index, can be from 1 to n, n – number of measurements [24]

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10. SÚHRN

Táto práca sa zaoberala schopnosťou predkolóny IonPac CG 5A od firmy Dionex® separovať katióny prechodných prvkov použitím systému sekvenčnej injekčnej chromatografie (SIC). Jednalo sa o iónovo výmennú chromatografiu, pričom kolóna je schopná vymieňať katióny aj anióny. Najskôr boli vybrané chelatačné činidlá potrebné pre separáciu, a to pyridín-2,6-dikarboxylová kyselina (PDCA) - zložka mobilnej fázy a 4-(2-pyridylazo) resorcinol (PAR) - zložka reakčného činidla.

Optimalizovalo sa zloženie mobilnej fázy a reakčného činidla a to dvomi metódami: Simplexom, kde sa naraz menili koncentrácie troch až štyroch zložiek roztokov a univariantnou metódou, kde sa menila koncentrácia len jednej zložky.

Ako prvé sa optimalizovalo zloženie mobilnej fázy. Každému meraniu v rámci Simplexu je priradená výstupná hodnota, na základe ktorej sa zhodnotí úspešnosť daného merania. Pri optimalizácií zloženia mobilnej fázy bolo tejto hodnote priradené rozlíšenie. V Simplexe sa nameralo 12 experimentov, ale keďže posledných pár meraní bola hodnota pH 2,5, pristúpilo sa na univariantnú metódu a po každej sérií meraní sa pre danú zložku vybrala koncentrácia s najlepším výsledkom: kyselina mravčia 40 mmol/l, síran sodný 4 mmol/l a PDCA 4 mmol/l. Koncentrácia poslednej zložky mobilnej fázy – hydroxidu sodného (2 mmol/l) bola dopočítaná podľa pH hodnoty 2,5.

Ďalej bolo optimalizované zloženie reakčného činidla. Tentoraz bola výstupná hodnota výška píkov, pretože reakčné činidlo nemá vplyv na rozlíšenie. Prebehli dve série simplexových meraní, ale ani jedna nepriniesla požadované výsledky, takže sa prešlo znova na univariantú metódu. Menili sa koncentrácie dvoch zložiek činidla – hydroxidu sodného a hydrogenuhličitanu sodného s výslednými koncentráciami 60 mmol/l a 45 mmol/l v rovnakom poradí. PAR bol optimalizovaný vo vlastnej univariantnej štúdií a finálna koncentrácia bola 0,3 mmol/l.

Po optimalizácií roztokov sa zisťoval počet katiónov, ktoré sa budú dať identifikovať. V predchádzajúcich meraniach boli vo vzorke katióny Cu(II) a Co(II). V nasledujúcich meraniach sa postupne do vzorky pridávali katióny Ni(II), Zn(II), Mn(II) a Fe(II), ale výsledky neboli uspokojivé.

V ďalšom kroku sa vzorka umiestnila medzi dve zóny vody, čo spôsobilo výrazné zlepšenie symetrie píku. Následne bol optimalizovaný objem vzorky a to od 30 do 300 µl, s výsledkom 90 µl. Nakoniec sa podarilo separovať tri katióny prechodných prvkov a to Cu(II), Zn(II) a Fe(II), v koncentráciách rádovo µmol/l a v časovom úseku kratšom ako 4 minúty.