GRIGORE T. POPA UNIVERSITY OF
MEDICINE AND PHARMACY IASI

HABILITATION THESIS

RESEARCH AND CONTRIBUTIONS IN THE FIELD OF ANALYTICAL CHEMISTRY AND PHARMACEUTICAL ANALYSIS

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CONTENTS

Rezumat

Teza de abilitare intitulată "*Cercetări și contribuții în domeniul chimiei analitice și analizei farmaceutice*" este o sinteză a celor mai reprezentative direcții de cercetare abordate în perioada postdoctorală.

Lucarea își propune prezentarea activității mele științifice după susținerea tezei de doctorat, intitulată "*Cercetări analitice privind realizarea de noi metode de determinare cantitativă a unor substanțe medicamentoase inhibitoare ale secreției gastrice (Antihistaminice H_2)*", incluzând deasemenea și principalele perspective de dezvoltare a carierei academice, profesionale și de cercetare.

Cercetarea și dezvoltarea compușilor farmaceutici a condus la o revoluție în domeniul sănătății umane. Pentru ca produsele farmaceutice să serveasca scopurilor pentru care au fost introduse în terapie, au fost dezvoltate metode de analiză pentru estimarea concentrației în substanțe active, excipienți și impurități.

Studiile de biodisponibilitate și farmacocinetică furnizează informații legate de comportamentul compusului medicamentos, inclusiv în medii biologice, confirmând mecanismul de acțiune propus, doza și schema de administrare, eficacitatea, siguranța și asocierea între beneficiul și riscul total al medicamentului.

Tehnicile instrumentale de detecție și cuantificare au un rol decisiv în controlul de calitate al medicamentelor și în studiul profilului biologic al acestora.

Validarea metodei este o problemă importantă în analiza substanțelor medicamentoase, în conformitate cu reglementările convenţionale, cum ar fi Food and Drug Administration (FDA), European Medicines Agency (EMEA) şi International Conference of Harmonization (ICH).

Procesul confirmă că procedura de analiză utilizată este adecvată pentru utilizarea sa prestabilită şi demonstrează fiabilitatea rezultatelor obţinute. Metodologia de validare are rolul de a demonstra că metoda propusă corespunde utilizării pentru care a fost prevăzută. Prelucrarea statistică a rezultatelor unei analize oferă posibilitatea de a obţine valoarea cea mai probabilă ce se află cât mai aproape de valoarea reală.

Principalele direcții pe care le-am abordat în activitatea de cercetare ştiinţifică desfășurată dupa obținerea în anul 2004 a titlui de *Doctor în domeniul Farmacie* vizează elaborarea și validarea de noi metode de analiză pentru substanțe medicamentoase, forme farmaceutice și principii active din plante medicinale precum și introducerea de noi reactivi analitici și senzori electrochimici pentru determinarea unor ioni și a unor compuși farmaceutici.

Teza de abilitare este structurată pe secțiuni care sintetizează aceste direcții de cercetare, respectând criteriile recomandate de CNATDCU.

Astfel, prima direcție de cercetare prezentată este intitulată "*Senzori electrochimici cu aplicații farmaceutice"*. Electrozii cu membrană selectivă ocupa un loc important în domeniul chimiei analitice datorită avantajelor pe care aceștia le oferă: construcție simplă și utilizare

facilă; indică rezultatele direct în activitate ionică; selectivitate şi sensibilitate (pot fi utilizați pentru analize rapide pe probe complexe, care conțin cantități mici de analit); se pot determina specii importante, care nu pot fi măsurate prin alte metode.

Senzorii electrochimici sunt utilizați în analiza și controlul de calitate al substanţelor medicamentoase ca atare sau din forme farmaceutice, a materiilor prime şi produşilor intermediari obţinuţi în procesele industriale sau a metaboliţilor în cazul analizelor biomedicale şi al monitorizării terapiei.

Sunt prezentate rezultatele obținute în domeniul senzorilor electrochimici cu membrană bazată pe matrici de incluziune, care conțin diverşi ionofori, utilizați pentru determinarea cantitativă a ranitidinei, nizatidinei, famotidinei, scopolaminei, lisinoprilului sau a unor metale grele.

Analiza metalelor grele reprezintă a altă direcție de cercetare descrisă în secțiunea "*Tehnici instrumentale avansate pentru analiza metalelor grele*". Studiile noastre au urmărit elaborarea şi validarea unor metode de determinare cantitativă prin spectroscopie de absorbție atomică (AAS) sau potenţiometrie, după caz, pentru fiecare metal luat în studiu. A fost determinat conținutul în metale grele din unele sorturi de tutun și o serie de produse alimentare de origine vegetală, stabilind și influența tipului de sol asupra conținutului de metale grele din plante.

Cercetările au continuat cu evaluarea capacității reale a unor extracte vegetale apoase de a lega şi favoriza eliminarea metalelor grele din organism ca alternativă la terapia clasică cu agenţi de chelare.

Spectrul terapeutic și cel al aplicațiilor analitice pentru bazele Schiff a fost abordat în secțiunea "*Contribuții la dezvoltarea unor noi baze Schiff cu aplicații farmaceutice*".

Bazele Schiff constituie o clasă importantă de compuși organici care prezintă o gamă largă de activități biologice, inclusiv antibacteriene, antifungice, antivirale, antimalarie, antiproliferative, anti-inflamatoare, anti-cancer, anti-HIV, antihelmintice și antipiretice. Sunt folosite ca liganzi chelativi în domeniul chimiei compușilor coordinativi, complecșii lor metalici prezentând deasemenea interes medical sau analitic.

În studiile prezentate au fost sintetizate o serie de baze Schiff care au fost caracterizate prin analiză elementală, solubilitate, puncte de topire, spectre UV-VIS, spectre IR sau RMN.

Datorită capacității foarte bune de coordinare unele baze Schiff au fost folosite ca reactivi de complexare pentru instituirea de noi metodele de analiză cantitativă spectrofotometrică a unor cationi metalici de interes bio-medical.

După sinteză, unii dintre liganzii Schiff liberi și complecșii lor metalici au fost supuși unui screening al activității lor biologice *in vitro* asupra bacteriilor, fungilor sau al efectului antiinflamator.

Doua secțiuni distincte descriu cercetările întreprinse pentru completarea profilului analitic al fumaratului de bisoprolol și al lisinoprilului.

Secțiunea "*Contribuții la profilul analitic al fumaratului de bisoprolol*" este alocată unuia dintre cele mai utilizate beta-blocante în tratamentul bolilor cardiovasculare. Studiile noastre au urmărit dezvoltarea unor noi metode de determinare cantitativă a fumaratului de bisoprolol prin cromatografie de lichide de înalta performanță (HPLC) sau spectrofotometrie în

VIS. Metodele au fost validate și aplicate la estimarea cantitativă a bisoprololului din forme farmaceutice dozate sau lichide biologice.

Lisinoprilul este un medicament din clasa inhibitorilor enzimei de conversie a angiotensinei (ACE), utilizat în tratamentul hipertensiunii arteriale, insuficienței cardiace congestive, infarctului miocardic dar și în prevenirea complicațiilor renale ale diabetului.

Secțiunea "*Contribuții la profilul analitic al lisinoprilului*" include studiile care au urmărit dezvoltarea unor noi metode de determinare cantitativă a lisinoprilului prin cromatografie de lichide de înalta performanță (HPLC) sau spectrofotometrie în VIS. Și aceste metode au fost validate și aplicate la estimarea cantitativă a lisinoprilului din forme farmaceutice dozate.

În secțiunea "*Metode analitice moderne utilizate pentru cuantificarea unor substanțe medicamentoase*" sunt descrise în sinteza câteva metode de determinare prin HPLC a unor compuși medicamentoși din medii biologice (alprazolam, clopidogrel) sau forme farmaceutice dozate (proxicam). Am studiat deasemenea și separarea prin cromatografie de schimb ionic a unor hidrolizate de hemoglobină.

Metode spectrofotometrice au fost aplicate și la determinare cantitativă a Fe(II) din forme farmaceutice sau a hidroxiureei din lichide biologice. Cedarea nitratului de miconazol din produse (filme) cu aplicare dermică a fost cuantificată prin spectrofotometrie în UV.

Rezultatele obținute în urma acestor cercetări s-au materializat prin publicarea unor articole în reviste de specialitate și participarea la manifestări științifice.

Ultima secțiune a tezei de abilitare "*Evoluția și dezvoltarea carierei profesionale, științifice și academice*" este dedicată unei sinteze a realizărilor științifice, didactice și academice în perioada postdoctorală și prezentării planurilor de evoluție și dezvoltare a carierei științifice și profesionale.

Abstract

The habilitation thesis entitled "*Research and contributions in the field of analytical chemistry and pharmaceutical analysis*" is a synthesis of the most representative research directions approached in the postdoctoral period.

The paper aims to present my scientific activity, after the presentation of my PhD thesis entitled "*Analytical research regarding new methods of quantitative determination of inhibitory medical substances of gastric secretion (Antihistamines H_2)*", also including the main development perspectives of the academic, professional and research career.

Research and development of pharmaceutical compounds has led to a revolution in the field of human health. Methods of analysis were developed for the estimation of the concentration in active substances, excipients and impurities, in order for the pharmaceutical products to serve the reason they were introduced into therapy.

Studies of bioavailability and pharmacokinetics offer information regarding the behaviour of drug compound, including in biological mediums, confirming the proposed action mechanism, the dosage and administration, the efficiency, safety and the association between the total benefit and risk of the medicine.

The instrumental techniques of detection and quantification play a decisive role in the quality control of drugs and in their biological profile study.

The method validation is an important problem in the analysis of medicinal substances, according to conventional regulations, such as Food and Drug Administration (FDA), European Medicines Agency (EMEA) and International Conference of Harmonization (ICH).

The process confirms that the used analysis procedure is adequate for its pre-established use and demonstrates the reliability of the obtained results. The validation methodology has the role of demonstrating that the proposed method corresponds to the usage for which it has been prescribed. The statistic processing of the results of an analysis offers the possibility of obtaining the most probable value closest to the real value.

The main directions I have approached in the scientific research activity after obtaining in 2004 the title of *Doctor of Pharmacy*, aims the elaboration and validation of new analysis methods for medicinal substances, pharmaceutical forms, active principles from medicinal plants, as well as the insertion of new analytical reagents and electrochemical sensors in order to determine certain ions and pharmaceutical compounds.

The habilitation thesis is structured into sections which synthesise these research directions, respecting the criteria recommended by CNATDCU.

Thus, the first presented research direction is called "*Electrochemical sensors with pharmaceutical applications*". Selective membrane electrodes play an important role in the field of analytical chemistry due to their advantages: simple frame and easy use; shows the results directly in the ionic activity; selectivity and sensitivity (they can be used in case of rapid

analysis on complex samples which contain small quantities of analyte); one can determine important species which cannot be measured through other methods.

Electrochemical sensors are used in the analysis and quality control of medicinal substances as such or from pharmaceutical forms, of raw materials and intermediate products obtained in industrial processes or of metabolites in case of biomedical analyses and therapy monitoring.

One can see the results obtained in the field of electrochemical sensors with a membrane based on inclusion matrices, which contain various ionophores used in the quantitative determination of ranitidine, nizatidine, famotidine, scopolamine, lisinopril or some heavy metals.

The analysis of heavy metals represents another research direction described in the section "*Advanced instrumental techniques for the analysis of heavy metals*". Our studies have followed the elaboration and validation of some methods of quantitative determination through atomic absorption spectroscopy (AAS) or potentiometry, as needed, for each metal in the study. The heavy metal content of some types of tobacco was also studied, as well as a series of foods of vegetable origin, also establishing the influence of the type of soil on the content of heavy metals from the plants.

The research continued with the evaluation of the real capacity of some vegetable watery extracts to bind and favour the removal of heavy metals from the body, as an alternative to the classic chelating agents therapy.

The therapeutic spectre and the one of the analytical applications for the Schiff bases were approached in the section "*Contributions to the development of novel Schiff bases with pharmaceutical applications*".

Schiff bases represent an important class of organic compounds which present a wide range of biological activities, including antibacterial, antifungal, antiviral, antimalarial, antiproliferative, anti inflammatory, anti-cancer, anti-HIV, antihelmintic and antipyretic. They are used as chelating ligands in the area of coordination compound chemistry, their metal complexes also presenting medical or analytical interest.

In the presented studies a series of Schiff bases, which were characterised through elemental analysis, solubility, melting point, UV-VIS spectres, IR spectres or NMR, were synthesised.

Due to their good capacity of coordination, some Schiff bases have been used as complexing reagents for the establishment of new methods of spectrophotometric quantitative analysis of some metal cations of bio-medical interest.

After synthesis, some of the free Schiff ligands and their metal complexes have been subjected to a screening of their biological *in vitro* activity on bacteria, fungi or the anti inflammatory effect.

Two different sections describe the research made for the completion of the analytical profile of bisoprolol fumarate and lisinopril.

The section "*Contributions to the analytical profile of the bisoprolol fumarate*" is dedicated to one of the most used beta-blockers in the treatment of cardiovascular diseases. Our studies have aimed to develop new methods of quantitative determination of bisoprolol

fumarate through high-performance liquid chromatography (HPLC) or VIS spectrophotometry. The methods were validated and applied at the quantitative estimation of bisoprolol in pharmaceutical dosage forms or biological liquids.

Lisinopril is an angiotensin-converting enzyme (ACE) inhibitor used in the treatment of high blood pressure, congestive heart failure, heart attack, but also in the prevention of kidney complications of diabetes.

The section "*Contributions to the analytical profile of the lisinopril*" includes the studies which aimed to develop new methods of quantitative determination of lisinopril through high-performance liquid chromatography (HPLC) or VIS spectrophotometry. The methods were validated and applied at the quantitative estimation of lisinopril in pharmaceutical dosage forms.

In the section "*Modern analytical methods used for the quantification of some pharmaceutical substances*" one can find the synthesized description of a few HPLC determination methods of some drug compounds in biological mediums (alprazolam, clopidogrel) or pharmaceutical dosage forms (piroxicam). We have also studied the separation through ion exchange chromatography of haemoglobin hydrolysed.

Spectrophotometric methods were also applied in the quantitative determination of Fe (II) from pharmaceutical products or of hydroxyurea from biological liquids. The disposal of miconazole nitrate from products (films) with dermal application was quantified through a UV spectrophotometric method.

The results obtained after these researches have materialised by publishing some articles in specialty publications and participating in scientific events.

The final section "*The evolution and development of the professional, scientific and academic career*" is dedicated to a synthesis of the scientific, didactic and academic accomplishments from the postdoctoral period and to the presentation of the evolution and development plans of the scientific and professional career.

Scientific achievements

I. Electrochemical sensors with pharmaceutical applications

I.1. Introduction

Selective membrane sensors are a system in which a special membrane separates two electrolyte solutions containing the same chemical species in different concentrations, and between which there is a potential difference. As the potential changes because of the variation in concentration of one of the chemical species it comes into contact with, it is considered that such a membrane, specific to a certain chemical species, functions as a transducer whose potential is a measure of the activity of that particular chemical species (Kristensen et al., 2017; Shujahadeen et al., 2018).

Research on selective membrane electrodes began to be published at the end of the 1960s and the beginning of the 1970s. Knowledge in this area has noted a continuous growth, stimulated by more and more varied samples, multi-components and often from very different matrices which include numerous interferences.

The complexity of the samples or the matrices in which one can find the analyte or the interest analytes, often impose elaborate pre-treatments in order to achieve their separation and purification, followed by their pre-concentration in order to bring the analyte to a detectable and quantifiable concentration.

Selective membrane electrodes play an important role in the field of analytical chemistry due to their advantages: simple frame and easy use; shows the results directly in the ionic activity; selectivity and sensitivity (they can be used in case of rapid analysis on complex samples which contain small quantities of analyte); one can determine important species which cannot be measured through other methods.

Electrochemical sensors are used in the analysis and quality control of medicinal substances as such or from pharmaceuticals, of raw materials and intermediate products obtained in industrial processes or of metabolites in case of biomedical analyses and therapy monitoring.

The literature on the use of specific electrochemical sensors in the analysis of pharmaceutical products is very up-to-date (Cosofret et al., 2017; Almeida et al., 2018). Selective membrane sensors based on electroactive material (ionophore) incorporated in vinyl polychloride (PVC) matrix have the advantage of design, long life and saves active material.

They have been used to determine azithromycin (Abu-Dalo et al., 2015), oxomemazine (Yousry et al., 2016), phenobarbital (Alrabiah et al., 2016), oxalate (Hoseini et al., 2016), umeclidinium (Yehia et al., 2017), tetracaine (Hassan et al., 2017), galantamine (Abdel-Haleem et al., 2018) and so on.

Selective membrane sensors based on electro-active material embedded in vinyl polychloride (PVC) matrix have the advantage of design, economy of active material and long service life. The studies were materialized in the following publications:

The studies were materialized in the following publications:

- Apostu M, Bibire N, Ţântaru G. Construction and characterisation of a membrane selective for ranitidine hydrochloride. *Farmacia* 2008; LVI(2): 147-153.
- Vieriu M, Bibire N, Ţântaru G, Apostu M, Mariana Mandrescu M, Dorneanu V. Construcţia şi caracterizarea unui electrod membrană selectiv pentru determinarea Lisinoprilului. *Rev Med Chir Soc Med Nat Iaşi* 2010; 114(4): 1227-1231.
- Apostu M, Bibire N, Vieriu M, Panainte AD, Ţântaru G. Preparation and potentiometric study of ranitidine hydrochloride selective electrodes and applications. *Rev Chim (Bucharest)* 2013; 64(7): 781-784.
- Apostu M, Bibire N, Ţântaru G, Vieriu M, Panainte AD, Agoroaei L. Ion-selective membrane electrodes for the determination of heavy metals. Construction characterization and applications. *Rev Chim (Bucharest)* 2015; 66(5): 657-659.
- Apostu M, Monica Hăncianu M, Ţântaru G, Vieriu M, Nela Bibire N, Panainte AD. Electrochemical sensors with pharmaceutical applications based on polymer inclusion membranes containing phosphomolybdic acid complexes. *Farmacia* 2018; 66(4): 587-491.

I.2. Design and functional characteristics of selective membrane electrodes

↳ *Construction and preparation of membrane electrodes*

Selective membranes were obtained by solubilizing the required amount of ionophore, PVC, plasticizer and additive in THF. The solution was poured into a Petri dish (3 cm in diameter) covered with filter paper and the solvent was allowed to evaporate at room temperature for 24 hours.

A disc cut out from the membrane was attached to the end of a PVC tube, 8 mm in diameter, using a PVC/THF mixture (Fig.1). A 10^{-3}M solution of analyte dissolved in saturated AgCl solution was used as internal reference solution in which the internal Ag/AgCl reference electrode was submerged.

The Ag/AgCl electrode was obtained by electrolysis using a Ag wire as anode in combination with a Pt cathode immersed in a saturated AgCl solution and connected to a 9V/10μA DC source.

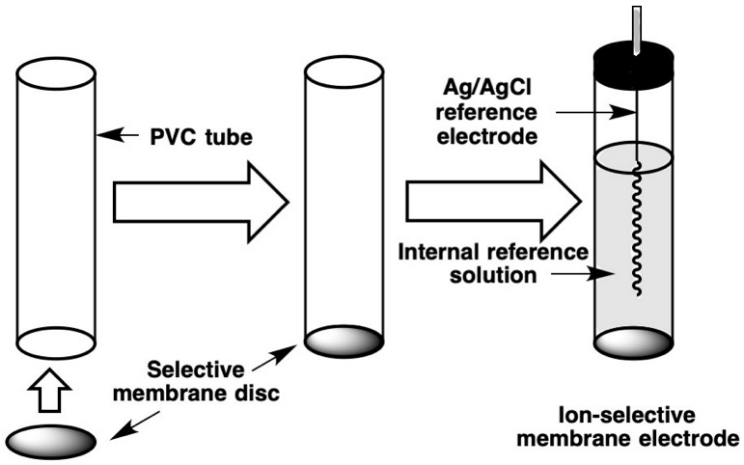


Fig. 1. Construction of electrodes

Prior to use, the selective membrane electrode had been pre-conditioned by immersion in a 10^{-5}M analyte salt solution for 120 minutes. All potentiometric determinations were performed using the electrochemical cell shown in figure 2.

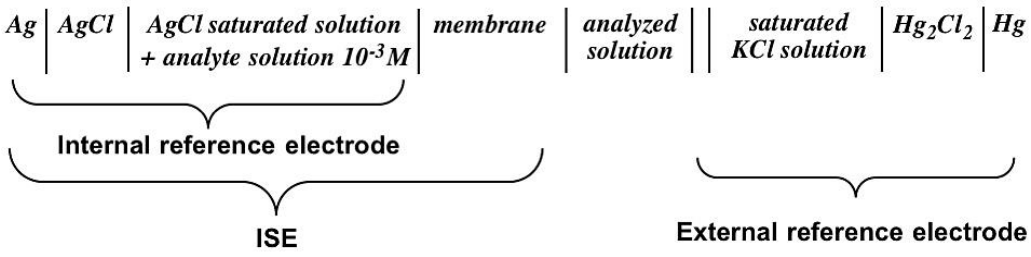


Fig. 2. Electrochemical cell

Potentiometric measurements were carried out using a digital pH/millivoltmeter. The ion-selective membrane electrode was used as indicator electrode in conjunction with a saturated calomel electrode (SCE) as reference electrode.

↳ *Optimization of membrane sensor composition*

The optimal composition of the selective membrane was achieved by varying the mass ratio of the ionophore, PVC and plasticizer to the proportion that exhibited the best performance characteristics.

↳ *Effect of pH*

The selective membrane electrode response could be affected by the hydrogen or hydroxyl ions activity or by the interaction of the ion to be analyzed with them. Adjusting the pH of the solution, using buffer solutions, to an experimentally determined value, can significantly reduce measurement errors. The effect of pH on electrode response was examined by measuring the variation of potential of the cell for three different solutions (10^{-4}, 10^{-3} and 10^{-2}M).

↳ *Total ionic strength*

Because the electrodes responded to ionic activity, and the sought result was the concentration of the analyte, it was important to keep the activity coefficient constant for all solutions. It was necessary to achieve a constant and relatively high concentration of a high purity electrolyte to which the selective membrane did not respond.

A 0.1 value of the ionic strength (μ) was found to be optimum for samples with concentration below 10^{-2}M and it was obtained through dilution using KNO_3 1M. The measured potential of 10^{-2} and 10^{-1}M solutions was not influenced by the ionic strength.

↳ *Response time*

The response time of a pair of electrodes is the time it takes for the potential to reach a stable value, with the current 1 to 6 mV range being used. Most selective membrane electrodes respond faster to concentration increases than decreases and are slower at low concentrations.

Ideally, electrode potential readings should be made when a constant value is obtained. In practice, the potential moves asymptomatically to a limit value and we need to decide when it is close enough to this value, because the errors caused by reading after a certain time are negligible.

↳ *Validation parameters of potentiometric determination methods*

Experimental data obtained were subjected to statistical processing, establishing for each constructed electrode its linear response range, limit of quantification, precision, accuracy, selectivity and robustness of the method.

• Linearity

For each electrode the concentration range of linear relationship between the measured potential and the concentration of the analyzed ion was determined.

• Precision

The precision of the method was studied in terms of repeatability and reproducibility. Two series of measurements have been done in different days for three different concentration levels (10^{-4}, 10^{-3} and 10^{-2}M) of the analyte. For each concentration level three determinations series were carried out.

• Accuracy

The accuracy of the electrodes was assessed by analyzing three standard solutions with analyte concentration of 10^{-4}, 10^{-3} and 10^{-2}M, respectively. The correspondence between the real and the analytical result obtained from measurements was evaluated by calculating the relative error - Xd(%), using the the following equation:

$$Xd(\%) = \frac{|Xr - Xa|}{Xa} \cdot 100$$

where Xr was the value calculated from the calibration curve for the theoretical value Xa.

• Robustness

Robustness was tested by investigating the ability of the proposed methods to remain unaffected by small but deliberate changes in the working parameters, thus providing an indication of their reliability during normal use. The degree of reproducibility of the results was investigated by analyzing the same samples under various conditions, such as different analysts and tools.

• Electrode selectivity

The selectivity of electrodes was investigated using the separate solution method (Srinivasan et al., 1969) and the potentiometric selective coefficients (K), were calculated using the following equations:

$$\log K = \frac{E_{(II)} - E_{(I)}}{P} + \log[A^{y+}] - \log[I^{z+}]$$

$$K = 10^{\frac{\Delta E}{P}} \cdot \frac{[A^{y+}]}{[I^{z+}]}$$

Two separate solutions of the same concentration (10^{-3}M) were prepared for the primary ion (A^{y+}) and the interfering secondary ion (I^{z+}). Their potentials E_I (for A^{y+}) and E_{II} (for I^{z+}) were measured (P - slope of the calibration curve). The separate solution method is one of the methods recommended by IUPAC (Umezawa et al., 2000).

I.3. Electrochemical sensors based on polymer inclusion membranes containing polyoxometalates

Polyoxometalates (POM) are a class of inorganic compounds that have attracted the interest of researchers since the nineteenth century due to their diversity and their properties. In 1826, Berzelius described their first representative, ammonium phosphomolybdate (Berzelius, 1826). In 1933, Keggin determined through X-ray diffraction, the structure of phosphotungstic anion - $[PW_{12}O_{40}]^{3-}$ - that bears his name (Keggin, 1933).

Polyoxometalates are aggregates, in general anionic, of transition metals (mainly W, Mo, Nb and V) with oxygen, structured as clusters with metal-oxygen and metal-oxygen-metal bonds. Those groups contain at least three atoms of the above-mentioned group, at their highest oxidation state, in combination with heteroatoms in heteropoloxymetalates or without heteroatoms such as Si, Ge or P in isopolyoxometalates.

Almost any element can be incorporated into POM, leading to an overwhelming diversity of structures that can function as catalysts or that have antibacterial, antiviral, electrical, optical or magnetic properties (Kamiya et al., 2013; Van Eldik et al., 2017).

Heteropolioxometalates with high molecular weight forms with many organic cations, ion-pair complexes with high lipophilicity that can be used as ionophores for PVC matrices with active recognition function.

I.3.1. Electrochemical sensors based on polymer inclusion membranes containing phosphomolybdic acid complexes

Heteropolyacids precipitate with some organic substances with basic groups, forming ion-pair complexes, that are crystallized substances, with a set composition, which can be used to obtain polyvinyl chloride (PVC) matrix membranes.

The study describes the construction and characterization of ion selective membrane sensors (ion selective electrodes - ISE) with PVC matrix for the determination of ranitidine (ISE-RP), famotidine (ISE-FP), and nizatidine (ISE-NP) using as electroactive material their respective complexes with phosphomolybdic acid.

I.3.1.1. Materials and methods

↳ *Reagents*

All chemicals were of analytical-reagent grade. Reagents used while preparing the membranes were produced by Fluka or Aldrich: polyvinyl chloride (PVC), phosphomolybdic acid (PMA), o-nitrophenyloctyleter (o-NPOE), di(butyl)butylphosphonate (DBBP), dioctylphthalate (DOP), sodium tetraphenylborate (NaTPB) and tetrahydrofuran (THF).

↳ *Synthesis of complexes*

Ranitidine, famotidine, and nizatidine (Fig. 3) produced during the reaction with phosphomolybdic acid (Fig. 3), crystalline yellowish-green crystals.

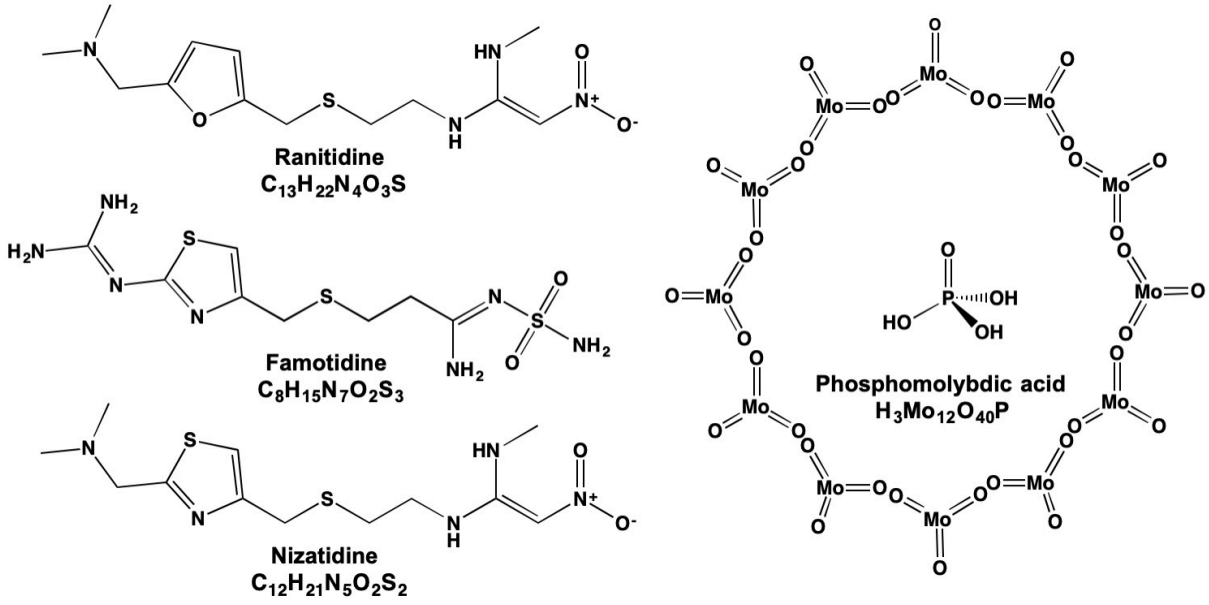


Fig. 3. The chemical structures of ranitidine, famotidine, nizatidine and phosphomolybdic acid

Precipitation took place at pH 1.0 and ~50°C using 1% phosphomolybdic acid solution. After 60 minutes of rest at room temperature, the complexes were separated through filtration, washed with a saturated solution of the precipitate and then with distilled water and dried to constant weight at room temperature in a vacuum desiccator.

The insoluble complexes were characterized by UV spectra (*HP 8453 - diode-array spectrophotometer*), specific absorbance ($\mathbf{A_{cm}^{1\%}}$), solubility (**S**), and melting points (*MEL-TEMP II*) (Table 1).

Table 1. Specific absorbance, solubility and melting points of the complexes

Complex	$A_{cm}^{1\%}$	S (g/L)	Melting point
R-PMA (*ranitidine phosphomolybdate*)	136.11 (λ = 312 nm)	$5.3392 \cdot 10^{-3}$	> 350°C with decomposition
F-PMA (*famotidine phosphomolybdate*)	147.84 (λ = 209 nm)	$8.1495 \cdot 10^{-2}$	> 350°C with decomposition
N-PMA (*nizatidine phosphomolybdate*)	157.25 (λ = 309 nm)	$5.0999 \cdot 10^{-3}$	> 350°C with decomposition

Elemental analysis (*CE 440 Elemental Analyzer*) confirmed the formation 3:1 complexes of R-PMA or F-PMA or N-PMA, respectively, as shown in table 2.

Table 2. Elemental analysis of complexes

%	R-PMA $[C_{13}H_{23}N_4O_3S]_3 \cdot [PMo_{12}O_{40}]$		F-PMA $[C_8H_{16}N_7O_2S_3]_3 \cdot [PMo_{12}O_{40}]$		N-PMA $[C_{12}H_{22}N_5O_2S_2]_3 \cdot [PMo_{12}O_{40}]$	
	Found	**Calculated**	**Found**	**Calculated**	**Found**	**Calculated**
C	16.88	16.92	10.19	10.16	15.41	15.33
H	2.54	2.51	1.73	1.70	2.39	2.36
N	6.12	6.07	10.30	10.37	7.62	7.45

↳ *Apparatus*

Potentiometric measurements were carried out using a 301 digital Hanna pH/millivoltmeter. The ion-selective membrane electrode was used as indicator electrode in conjunction with a OP-0830P Radelkis saturated calomel electrode (SCE) as reference electrode.

I.3.1.2. Results

↳ *Optimization of PVC membrane sensor composition*

Table 3 shows the slope of the electrodes (mV/decade) depending on the plasticizer used and its proportion (weight percentage - %wt).

The optimal composition for obtaining homogeneous, thin, elastic, mechanically resistant and best-response membranes is shown in table 4.

Table 3. Optimization of membranes composition - slope (mV/decade)

Plasticizer (%wt)	DOP			DBBF			o-NPOE		
	ISE-RP	ISE-FP	ISE-NP	ISE-RP	ISE-FP	ISE-NP	ISE-RP	ISE-FP	ISE-NP
63	53.81	50.14	49.77	52.88	49.89	48.65	47.70	44.02	42.66
64	54.90	51.30	50.82	53.01	50.77	49.13	48.55	44.81	43.55
65	55.30	52.40	51.80	54.85	51.69	50.01	49.12	46.21	44.87
66	55.01	52.00	51.23	54.17	51.01	49.72	48.70	45.78	44.11
67	54.11	51.68	50.71	53.69	50.13	49.00	48.11	45.00	43.17

Table 4. Composition of PVC matrix selective membranes (%wt)

Electrode	Ionophore (%wt)	Plasticizer (%wt)	Additive (%wt)	Matrix (%wt)
ISE-RP	R-PMA (3)	DOP (65)	NaTPB (1)	PVC (31)
ISE-FP	F-PMA (3)	DOP (65)	NaTPB (1)	PVC (31)
ISE-NP	N-PMA (3)	DOP (65)	NaTPB (1)	PVC (31)

↳ *Effect of pH*

The effect of pH of the solution on the performance of sensors was studied by varying the pH in the range 1.0-10.0. The results are illustrated in figure 4. Electrode response according to pH had the same profile for three concentration levels, and the optimal pH range was found to be in between 2.0 and 6.0. The potential measurements were conducted at pH 4.0 maintained using acetate buffer solution.

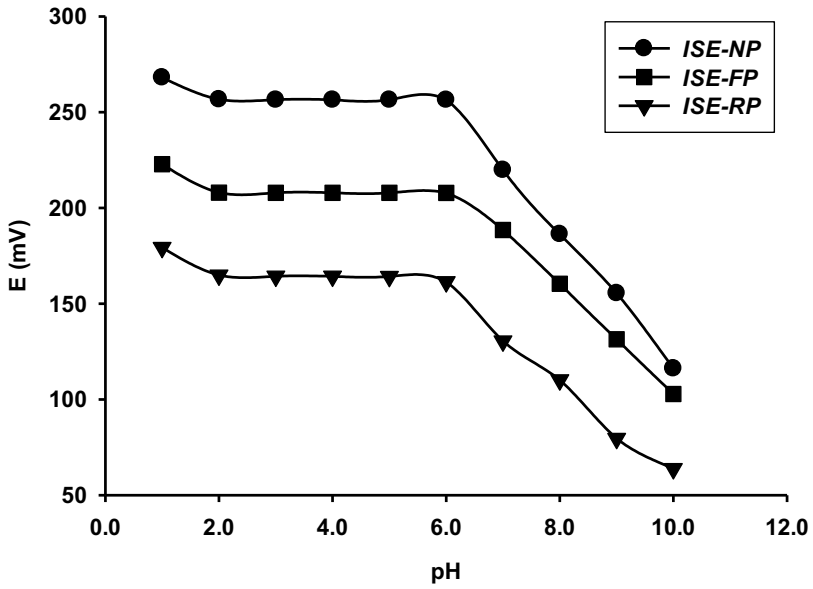


Fig 4. The effect of pH on the potential responses of the membrane electrodes

↳ *Response time*

For lower concentrations the response time was within 55 seconds while the electrodes response to higher concentrations was virtually instantaneous in all cases.

↳ *Linearity*

The response of the electrodes was studied in the 10^{-7}-10^{-1}M concentration range at pH 4.0 and at 0.1 ionic strength (Table V).

A graphical method was applied for calculating the limit of quantification (LOQ) defined as the intersection of the regression line for the linear domain with the range when the electrode response is relatively constant. An example is shown in figure 5 for ISE-RP (E = mV, C = mol/L, pC = - log C).

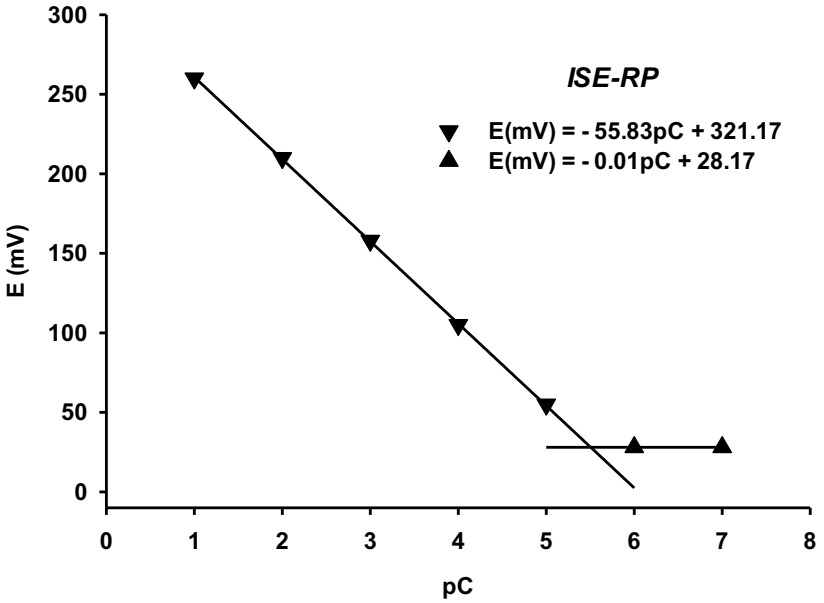


Fig 5. LOQ and calibration curves for ISE-RP

↳ *Validation paramaters of potentiometric determination methods*

The statistical processing of the esperimental data is shown in the table 5.

Table 5. Validation parameters of potentiometric determination methods

Electrode		**ISE-RP**	**ISE-FP**	**ISE-NP**
Linearity range		10^{-1}-10^{-5}M	10^{-1}-10^{-5}M	10^{-1}-10^{-5}M
Regression		E = -55.3·pC+321.7	E = -52.4·pC+324.7	E = -51.8·pC+382.2
Correlation coefficient (r^2)		0.9986	0.9981	0.9973
Slope		55.3 mV/decade	52.4 mV/decade	51.8 mV/decade
Standard deviation (σ)		0.6239	0.5300	0.4393
LOQ		$5.76 \cdot 10^{-6}$M	$2.65 \cdot 10^{-6}$M	$3.32 \cdot 10^{-6}$M
Repeatability Ist Series	**SD**	2.04	1.71	1.05
	RSD	2.05%	1.75%	1.06%
Repeatability IInd Series	**SD**	2.07	1.57	0.76
	RSD	2.08%	1.60%	0.77%
Reproductibility	**SD**	2.00	1.62	0.90
	RSD	2.01%	1.66%	0.92%
Accuracy	$\overline{Xd}$	1.91%	2.17%	1.57%

↳ *Electrode selectivity*

The table 6 presents interferences against which selectivity of the selective membrane electrodes was tested.

Table 6. Selectivity coefficient (K)

Interferer	ISE-RP	ISE-FP	ISE-NP
NH_4^+	$1.27 \cdot 10^{-3}$	$5.30 \cdot 10^{-3}$	$1.12 \cdot 10^{-3}$
Na^+	$3.70 \cdot 10^{-3}$	$4.30 \cdot 10^{-3}$	$1.26 \cdot 10^{-3}$
Ca^{+2}	$1.90 \cdot 10^{-3}$	$3.44 \cdot 10^{-3}$	$4.19 \cdot 10^{-3}$
Mg^{+2}	$2.54 \cdot 10^{-3}$	$9.21 \cdot 10^{-4}$	$2.36 \cdot 10^{-4}$
Al^{+3}	$8.96 \cdot 10^{-4}$	$8.19 \cdot 10^{-4}$	$3.35 \cdot 10^{-4}$
Ranitidine	-	$1.11 \cdot 10^{-1}$	$1.60 \cdot 10^{-1}$
Famotidine	$1.23 \cdot 10^{-1}$	-	$1.32 \cdot 10^{-1}$
Nizatidine	$1.78 \cdot 10^{-1}$	$1.90 \cdot 10^{-1}$	-

↳ *Analytical applications*

The constructed electrodes have been used to quantify through direct potentiometry the analytes from some pharmaceutical products such as injectable solutions, tablets, and capsules (Table 7).

Table 7. Direct potentiometric quantitative determination

Active substance	Tablets/capsules			Injectable solution		
	Labeled	Quantified	FRX	Labeled	Quantified	FRX
Ranitidine (n = 6)	75 mg	74.70 ± 0.24 mg	75 ± 5.62 mg	50 mg/2 mL	49.79 ± 0.13 mg/2 mL	50 ± 2.50 mg
Famotidine (n = 6)	40 mg	39.52 ± 0.12 mg	40 ± 3.00 mg	20 mg/5 mL	19.56 ± 0.08 mg/5 mL	20 ± 1.00 mg
Nizatidine (n = 6)	300 mg	298.60 ± 0.27 mg	300 ± 15.0 mg	100 mg/4 mL	98.54 ± 0.17 mg/4 mL	100 ± 5.00 mg

I.3.1.3. Discussions

Synthesised complexes (ranitidine phosphomolybdate, famotidine phosphomolybdate, nizatidine phosphomolybdate) show a high stability and a low solubility, proprieties which favour their use as ionophores in preparing the selective membranes based on polymer inclusion matrices. Phosphomolybdic acid complexes with similar proprieties have been the basis of the design of electrochemical sensors for midodrine (Elzanfaly et al., 2013), amitriptyline (Rahman et al., 2016), oseltamivir phosphate (Hamza et al., 2017) or promethazine (Sarma et al., 2017).

The ionophore had to have suitable solubility in the membrane matrix, rapid exchange kinetics, high stability, and sufficient lipophilicity to prevent membrane transfer in the sample solution. The results of the potentiometric determinations showed that although changes were

made regarding the ionophore ratio (1-5%), there was no significant leap in potential. According to literature data, the plasticizer solubilized the ion pairs complex and its proportion regulated both membrane permeability and ion mobility in order to obtain the most selective and sensitive response (Zareh et al., 2012). The best results were obtained for a membrane containing a mixture of ionophores / plasticizer / additives / matrix in the following mass ratios (%wt) 3/65/1/31.

Ion selective electrodes respond to activity of the ion. Activity of the ion is moderated by its concentration, and the ionic strength of the solution. For the same concentration the activity will decrease with an increase in the ionic strength. For a correct determination a constant and high ionic strength (0.1) is required by using an inert salt, KNO_3, which does not affect the system undergoing measurement (Skoog et al., 2003).

Due to the importance of pH on electrodes response, after the optimization of the electrode's components, the effect of pH solution on the response of electrodes was investigated. The reason of increasing electrode potential at pH = 1 is maybe as a result of hydronium ion interference in the performance of ionophore that it makes ionophore to respond concurrently to H^+ ions (Gupta et al, 2006; Rezaei et al., 2008), while in alkaline media, the membrane potential decreases due to the gradual precipitation of ranitidine, famotidine or nizatidine as free bases (Soleymanpour et al., 2006).

An electrode response time is an important factor in analytical applications and it relates to concentration variations (Erden et al., 2006). For lower concentrations the response time was within 55 seconds while the electrodes response to higher concentrations was virtually instantaneous in all cases.

One of the most important features of the ion-selective membrane is their selective behaviour in investigating the level of reliable measurement capability for the sample (Gupta et al, 2006). Because of their similar structures, molecular weights and molecule dimensions, ranitidine, famotidine, and nizatidine interfered with each other slightly as far as the response of electrodes. Since there are no pharmaceutical products containing any association of those three active ingredients, it is unlikely that these interferences will actually occur. The cations usually present in the excipients used in the formulation of tablets/capsules did not exhibit any interference.

The linear response and the quantification limit for each electrode are set to the values specified in the literature for electrodes of a similar construction (Huang et al., 2000; Issa et al., 2005; Rahman et al., 2016).

Electrodes were used for the quantitative determination of ranitidine, famotidine, and nizatidine from pharmaceutical products. The results obtained were within the limits set by the Romanian Pharmacopoeia X^{th} edition (FRX) regarding the accepted variation of content for the active substance when compared to the labelled value.

I.3.1.4. Conclusions

A series of selective membrane potentiometric sensors have been constructed based on electro-active material embedded in the PVC matrix. The sensors were evaluated as far as their functional characteristics and then they were used for the quantitative determination of ranitidine, famotidine, and nizatidine within 10^{-1}-10^{-5}M concentration range. Determinations

are non-destructive, and they can be done even by direct analysis of turbid or viscous solutions with a sensitivity superior to classic titrimetric and spectrophotometric methods, but inferior to that of high performance liquid chromatography. The main disadvantages are the short life of the sensor membrane and the need for its periodic renewal. The proposed methods can be used for routine analysis in drug quality control laboratories providing a quick, simple, accurate and inexpensive solution considering the necessary equipment involved and the consumption of reagents.

I.3.2. Electrochemical sensors based on polymer inclusion membranes containing silicotungstic acid complexes

Silicotungstic anion (STA) is a heteropolioxometalate with high molecular weight that forms with many organic cations, ion-pair complexes with high lipophilicity.

The study presents the construction and characterization of selective membrane sensors for the determination of ranitidine (ISE-RS) and nizatidine (ISE-NS) using their complexes with silicotungstic acid as ionophores.

I.3.2.1. Materials and methods

↳ *Reagents*

All reagents used while preparing the membranes were analytical reagent grade, produced by either Fluka or Sigma-Aldrich: polyvinyl chloride (PVC), silicotungstic acid (STA), o-nitrophenyloctyleter (o-NPOE), di(butyl)butylphosphonate (DBBP), dioctylphthalate (DOP), sodium tetraphenylborate (NaTPB) and tetrahydrofuran (THF).

↳ *Synthesis of complexes*

Ranitidine and nizatidine (Fig. 6) form amorphous light yellowish precipitates with silicotungstic acid (Fig. 6) in acid medium.

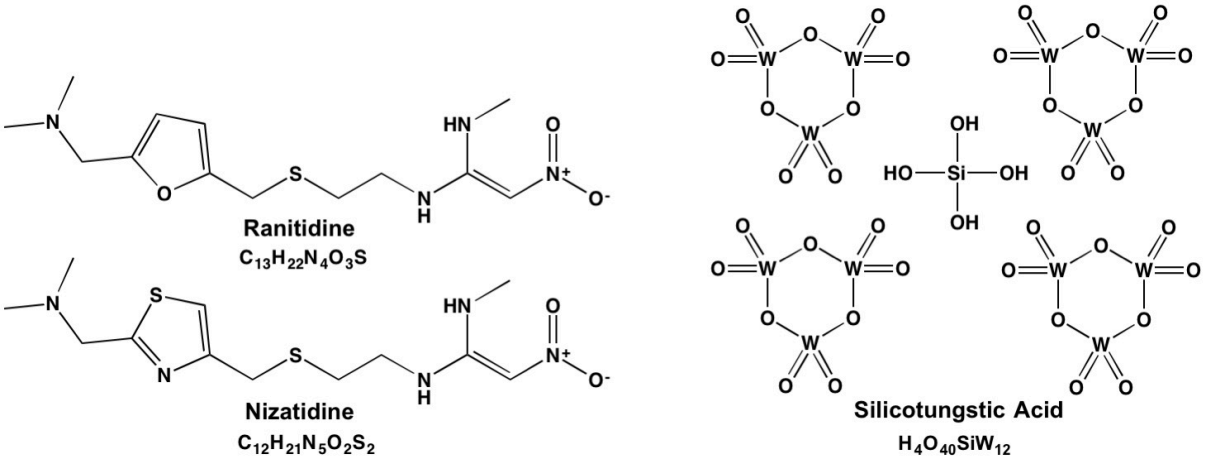


Fig. 6. Structures of ranitidine, nizatidine and silicotungstic acid

Precipitation is achieved at pH 1.0 and 50°C using 5% silicotungstic acid solution. After 60 minutes of rest at room temperature, the complexes were separated through filtration, washed with a saturated solution of the precipitate and then with distilled water, and dried to constant weight at room temperature in a vacuum desiccator.

The insoluble complexes were characterized by UV spectra (*HP 8453 - diode-array spectrophotometer*), specific absorbance ($\mathbf{A_{cm}^{1\%}}$), solubility (**S**), and melting points (*MEL-TEMP II*) (Table 8).

Table 8. Specific absorbance, solubility and melting point

Complex	$A_{cm}^{1\%}$	Solubility (g/L)	Melting point
R-STA (*ranitidine silicotungstate*)	63.01 (λ = 261 nm)	$8.1427 \cdot 10^{-3}$	> 350°C with decomposition
N-STA (*nizatidine silicotungstate*)	31.67 (λ = 264 nm)	$1.4928 \cdot 10^{-2}$	> 350°C with decomposition

Elemental analysis (*CE 440 Elemental Analyzer*) confirmed the formation of 4:1 complexes of R- STA or N-STA respectively as shown in table 9.

Table 9. Elemental analysis of complexes

Element %	R-STA $[C_{13}H_{23}N_4O_3S]_4 \cdot [SiW_{12}O_{40}]$		N-STA $[C_{12}H_{22}N_5O_2S_2]_4 \cdot [SiW_{12}O_{40}]$	
	Found	**Calculated**	**Found**	**Calculated**
C	15.07	15.10	13.82	13.71
H	2.16	2.14	2.10	2.01
N	5.50	5.42	6.81	6.66

↳ *Apparatus*

Potentiometric measurements were carried out using a 301 digital Hanna pH/millivoltmeter. The ion-selective membrane electrode was used as indicator electrode in conjunction with a OP-0830P Radelkis saturated calomel electrode (SCE) as reference electrode.

I.3.2.2. Results

↳ *Optimization of membrane sensor composition*

Table 10 shows the slope of the electrodes (mV/decade) depending on the plasticizer used and its proportion.

The optimal composition for obtaining the most homogeneous, thin, elastic, mechanically resistant and best-response membranes is shown in table 11.

Table 10. Optimization of membranes composition - slope (mV/decade)

Plasticizer	DOP		DBBF		o-NPOE	
	ISE-RS	ISE-NS	ISE-RS	ISE-NS	ISE-RS	ISE-NS
63%	36.81	42.77	37.28	42.65	37.70	44.66
64%	37.90	43.82	38.01	44.13	38.55	45.55
65%	38.22	44.70	39.03	45.51	39.81	46.92
66%	38.03	43.23	38.17	43.72	38.70	45.11
67%	37.31	42.71	37.69	43.00	37.71	44.17

Table 11. Mass percentage composition of PVC matrix ion-selective membranes

Electrode	Ionophore	Plasticizer	Additive	Matrix
ISE-RS	R-STA (2.5)	o-NPOE (65)	NaTPB (1)	PVC (31.5)
ISE-NS	N-STA (2.5)	o-NPOE (65)	NaTPB (1)	PVC (31.5)

↳ *Effect of pH*

Figure 7 shows the electrode response in the 1.0-10.0 pH range.

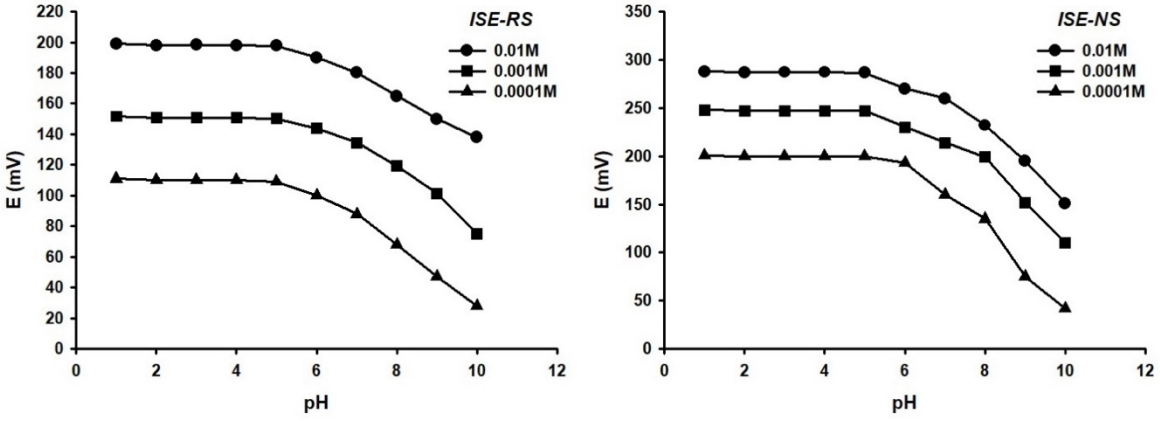


Fig. 7. Effect of pH on electrode response

↳ *Linearity*

The electrodes response was studied in the concentration range between 10^{-7}-10^{-1}M at pH 3.0 and 0.1 ionic strength (E = mV, C = mol/L, pC = - log C) (Table 5).

A graphical method was applied for calculating the limit of quantification (LOQ) defined as the intersection of the regression line for the linear domain with the range when the electrode response was relatively constant (Fig. 8).

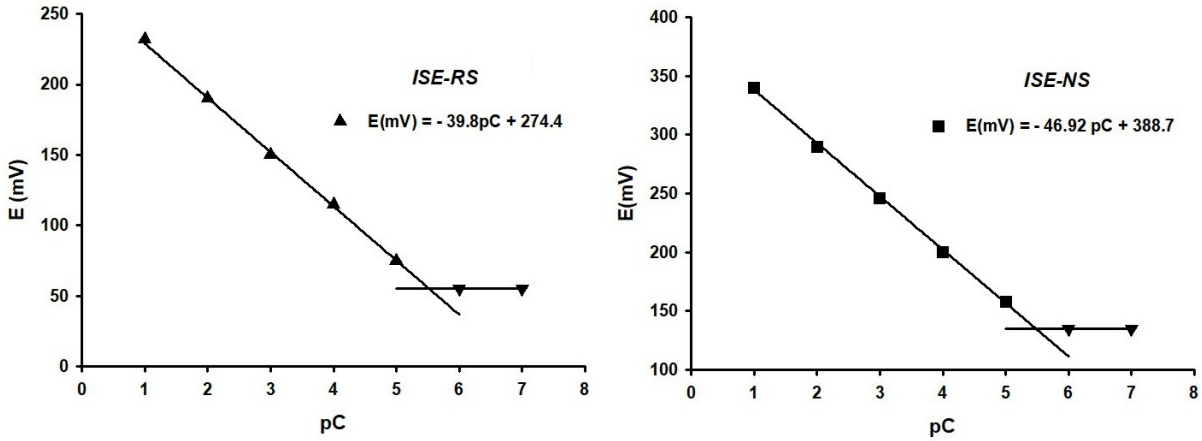


Fig. 8. LOQ and calibration curves for ISE-RS and ISE-NS

↳ *Validation parameters of potentiometric determination methods*

The statistical processing of the esperimental data is shown in the table 12.

Table 12. Validation parameters of the potentiometric methods

Electrode		**ISE-RS**	**ISE-NS**
Linearity range		10^{-1}-10^{-5}M	10^{-1}-10^{-5}M
Regression equation		E = - 39.81·pC + 277.4	E = - 46.92·pC + 388.7
Regression coefficient (r^2)		0.9954	0.9939
Slope (P)		39.81mV/decade	46.92mV/decade
Standard deviation (σ)		0.4676	0.5403
LOQ		$2.83 \cdot 10^{-6}$M	$3.43 \cdot 10^{-6}$M
Repeatability Ist Series	**SD**	3.11	2.09
	RSD	3.17%	2.14%
Repeatability IInd Series	**SD**	3.31	1.97
	RSD	3.36%	2.01%
Reproductibility	**SD**	3.20	1.98
	RSD	3.25%	2.04%
Accuracy	$\overline{Xd}$	3.26%	2.19%

↳ *Electrode selectivity*

Table 13 presents interferences against which selectivity of the selective membrane electrodes was tested.

Table 13. Selectivity coefficient (K)

Interferer	**ISE-RS**	**ISE-NS**	**Interferer**	**ISE-R**	**ISE-N**
NH_4^+	$2.10 \cdot 10^{-4}$	$9.34 \cdot 10^{-5}$	**Ranitidine**	-	$1.60 \cdot 10^{-1}$
Na^+	$1.30 \cdot 10^{-4}$	$2.76 \cdot 10^{-4}$	**Famotidine**	$1.76 \cdot 10^{-4}$	$3.32 \cdot 10^{-4}$
Ca^{+2}	$3.50 \cdot 10^{-5}$	$8.95 \cdot 10^{-5}$	**Nizatidine**	$2.46 \cdot 10^{-1}$	-
Mg^{+2}	$6.54 \cdot 10^{-4}$	$7.36 \cdot 10^{-4}$	**Zn^{+3}**	$3.24 \cdot 10^{-4}$	$5.16 \cdot 10^{-4}$
Al^{+3}	$1.35 \cdot 10^{-4}$	$3.35 \cdot 10^{-4}$	**Fe^{+3}**	$1.62 \cdot 10^{-4}$	$4.05 \cdot 10^{-4}$

↳ *Pharmaceutical applications*

The constructed and characterized sensors were used to determine by direct potentiometry ranitidine and nizatidine from pharmaceutical products (Table 14)

Table 14. Direct potentiometric determinations from pharmaceutical products

Drug	Tablets/capsules			Injectable solution		
	Labeled	Found	RPX	Labeled	Found	RPX
Ranitidine (n = 6)	75 mg	74.46 ± 0.29 mg	75 ± 5.62 mg	50 mg/2mL	49.37 ± 0.22 mg/2 mL	50 ± 2.50 mg
Nizatidine (n = 6)	300 mg	300.40 ± 0.27 mg	300 ±15.0 mg	100 mg/4 mL	100.48 ± 0.26 mg/4 mL	100 ± 5.00 mg

I.3.2.3. Discussions

H_2 receptor blockers prevent the excito-secretory gastric effect of histamine, autacoid which represents an essential link in the control of parietal secretion (Feldman et al., 1990; Nash et al., 1994).

The frequent use of ranitidine and nizatidine in the treatment for gastric and duodenal ulcer (Mattos et al., 2017; MacFarlane, 2018) imposes the necessity of establishing some new methods of their analysis.

Ion pair complexes (4:1) of ranitidine and nizatidine with silicotungstic acid as ionophores have been used for constructed PVC matrices with active recognition function (Shanmugam et al., 2006; El-Tohamy et al., 2010; Fernandes et al., 2011; Abu Shawish et al., 2011; Khater et al., 2015).

X-ray diffraction analysis of ranitidine (Toshimasa et al., 1990; Huq et al., 2003) showed that the N atom in the dimethylamino group gets protonated (pK_a = 2.19). Nizatidine has an extra N atom in the protonated thiazole nucleus (pK_{a1} = 2.1, pK_{a2} = 6.8) similar to famotidine (Basavaiah et al., 2011). The fact that famotidine (pK_a = 7.93) did not precipitate with silicotungstic acid led to the conclusion that the N atom of the thiazole nucleus was not involved in the complexation reaction. These data confirm our results on the combining ratio by elemental analysis.

Inclusion polymer membranes are distinguished by mechanical strength, ease of obtaining the desired shape with a reduced thickness, and the ability to achieve good electrolytic contact.

The characteristics of sensors were significantly affected by the relative proportions of the membrane components and the type of plasticizer (Bakker, 2015; Mikhelson, 2016). Each component plays a special role in the membrane function and electrode response. For example, the weight ratio of plasticizer: PVC may be 1:1 (Cieplak et al., 2017), or usually 2:1 (Gupta et al., 2007) as in this study. Increasing the amount of plasticizer improves to a large extent adhesive properties of the membrane but it facilitates the membrane deterioration (Bobacka et al., 2008).

The potential response of optimized electrodes is pH-independent in the range from 1.0 to 5.0. Therefore this pH range can be taken as the working pH range. There were slight interferences from the hydrogen ions at lower pH levels. In alkaline media, the membrane potential decreased due to the gradual precipitation of ranitidine or nizatidine in the measuring solution. Potential measurements were carried out after adjusting pH to 3.0 with buffer solution.

The degree of reproducibility of the results was investigated by analyzing the same samples under various conditions, such as different analysts and tools. The recorded potentials were very close, thus confirming the validity of the method (ICH, 2005).

Ranitidine and famotidine interfere with each other's analysis on a reduced level in terms of electrode response. The cations present in the excipients commonly used in the formulation of the tablets/capsules did not interfere (Gupta et al, 2006).

The main disadvantages are the short life of the sensor membrane and the need for its periodic renewal. Preconditioning the electrodes by immersion in the analyte solution or their use for large series of determinations had a negative effect on the electrode response. The ionophore regeneration from the outer membrane gel layer (produced by hydration) was attempted by immersing the exhausted electrode for 24 hours in a 5% precipitating reagent solution followed by immersion for 2-3 hours in a 10^{-1}M analyte solution. The service life of the regenerated electrode did not exceed 10 determinations due to the ease of ionophore transfer from the gel layer of the outer surface of the membrane into the sample solution, process that occurred slowly to the ionophore set in the PVC matrix in the initial membrane construction process.

The results obtained for determination of ranitidine and nizatidine in pharmaceutical products fell within the limits set by the Romanian Pharmacopoeia X^{th} edition (FRX), regarding the accepted content variation of active substance depending on the labelled dose.

I.3.2.4. Conclusions

Two potentiometric sensors have been constructed based on ion pair complexes of ranitidine and nizatidine with silicotungstic acid as ionophores. The electroactive compound was dispersed in a PVC matrix using o-nitrophenyloctyl ether as plasticizer. The main functional characteristics of the sensors were studied and they were used for the assay of ranitidine and nizatidine in the 10^{-1}-10^{-5}M concentration range, with a response time lower than 50 seconds. The methods proposed for the determination of ranitidine and nizatidine in pharmaceutical forms were fast, simple, accurate and inexpensive.

I.4. Electrochemical sensors based on polymer inclusion membranes for the determination of heavy metals

Heavy metal toxicity is a clinically significant medical condition that improperly treated may result in significant morbidity and mortality. Review of the literature discloses the use with good results of membrane ion selective electrodes for the determination of heavy metals from various media (Sadeghi et al., 2002; Gupta et al., 2006; Singh et al., 2006; Gupta et al., 2007; Bakhtiarzadeh et al., 2008).

This study presents the construction and characterization of some ion-selective membrane electrodes with PVC matrix for the determination of the following cations Cu^{+2}, Cd^{+2}, Ni^{+2}, Pb^{+2}, and Hg^{+2}.

I.4.1. Materials and methods

↳ *Reagent*

All reagents used while preparing the membranes were produced by Fluka or Aldrich: polyvinyl chloride (PVC), acetoacetanilide (AAA), dicyclohexane-24-crown-8 (DCH24C8), poly-(4-vinyl pyridine) (P4VP), dibenzo-18-crown-6 (DB18C6), dicyclohexyl-18-crown-6 (DC18C6), o-nitrophenyloctyleter (o-NPOE), di(butyl)butylphosphonate (DBBP), dioctylphthalate (DOP), sodium tetraphenylborate (NaTPB) and tetrahydrofuran (THF).

↳ *Apparatus*

Potentiometric measurements were carried out using a 301 digital Hanna pH/millivoltmeter and a saturated calomel electrode (SCE) as reference electrode.

I.4.2. Results

↳ *Optimization of membrane sensor composition*

Optimal proportions used to obtain homogeneous, thin, elastic and with good mechanical strength membranes (Amemiya et al., 2000; Bakker et al., 1997; Moody et al., 1970; Lima et al., 1986) are shown in table 15.

Table 15. Mass percentage composition of PVC matrix ion-selective membranes

Electrode	Electroactive compound	Plasticizer	Additive	Matrix
Cu^{+2} ISE	AAA (1)	*o*-NPOE (67)	NaTPB (1)	PVC (31)
Cd^{+2} ISE	DCH24C8 (1)	DBBP (67)	NaTPB (1)	PVC (31)
Ni^{+2} ISE	DB18C6 (1)	DOP (67)	NaTPB (1)	PVC (31)
Pb^{+2} ISE	DC18C6 (1)	*o*-NPOE (67)	NaTPB (1)	PVC (31)
Hg^{+2} ISE	P4VP (1)	DOP (67)	NaTPB (1)	PVC (31)

↳ *Effect of pH*

The effect of pH on electrode response was examined by measuring the potential variation of the electrochemical cell with three solutions of various concentrations (10^{-4}, 10^{-3} and 10^{-2}mol/L). The response of the electrodes to 10^{-3}mol/L solution in the 1.0-9.0 pH range is shown in figure 9.

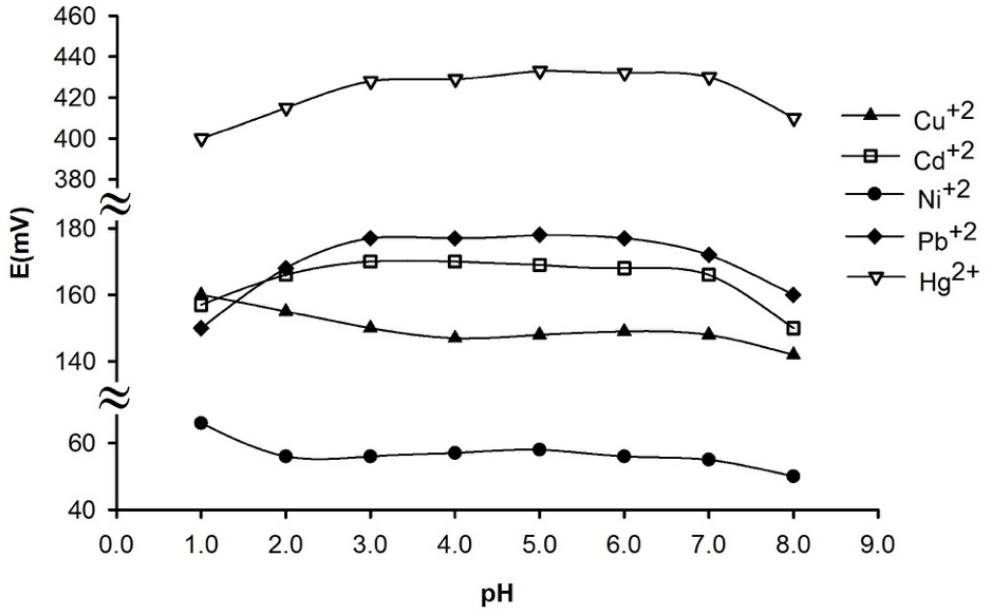


Fig. 9. The effect of pH on electrode response

↳ *Linearity*

The electrodes response was studied in the concentration range between 10^{-7}-10^{-1}mol/L at pH 7.0 and 0.1 ionic strength (E = mV, C = mol/L, pC = - log C). A graphical method was applied to calculate the limit of quantification (LOQ) defined as the intersection of the regression line for the linear domain with the range when the electrode response was relatively constant. An example is shown in figure 10 for Cd^{+2} ISE.

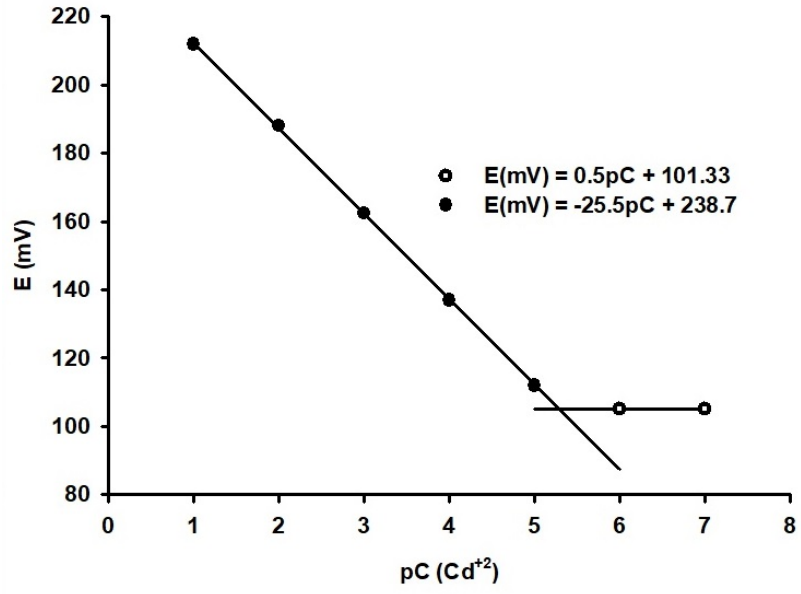


Fig. 10. LOQ and calibration curves for Cd^{+2} ISE

↳ *Validation parameters of potentiometric determination methods*

The statistical processing of the esperimental data is shown in the table 16.

Table 16. Validation parameters of potentiometric determination methods for Cu^{+2}, Cd^{+2}, Ni^{+2}, Pb^{+2} and Hg^{+2} using ISE

Electrode		**Cu^{+2} ISE**	**Cd^{+2} ISE**	**Ni^{+2} ISE**	**Pb^{+2} ISE**	**Hg^{+2} ISE**
Linearity range		10^{-2}-10^{-6}mol/L	10^{-1}-10^{-5}mol/L	10^{-1}-10^{-5}mol/L	10^{-2}-10^{-6}mol/L	10^{-2}-10^{-6}mol/L
Regression		E = 20.3·pC+ 208.4	E = -25.5·pC+ 238.7	E = 23.8·pC+ 13.6	E = -19.1·pC+ 233.6	E = 32.6·pC+ 328.8
Correlation coefficient (r^2)		0.9986	0.9948	0.9982	0.9982	0.9939
Slope		20.3mV/decade	25.5mV/decade	23.8mV/decade	19.1mV/decade	32.6mV/decade
Standard deviation (σ)		0.4676	0.9295	0.4393	0.1516	0.3386
LOQ		$3.63\cdot10^{-7}$mol/L	$5.62\cdot10^{-6}$mol/L	$4.57\cdot10^{-6}$mol/L	$3.16\cdot10^{-7}$mol/L	$7.08\cdot10^{-7}$mol/L
Repeatab. Ist Series	**SD**	3.11	3.89	2.04	2.51	2.07
	RSD	3.17%	3.90%	2.05%	2.48%	2.08%
Repeatab. IInd Series	**SD**	3.31	3.62	2.07	1.58	2.23
	RSD	3.36%	3.63%	2.08%	1.57%	2.24%
Reprod.	**SD**	3.20	3.65	2.00	2.06	2.09
	RSD	3.25%	3.64%	2.01%	2.04%	2.10%
Accuracy	$\overline{Xd}$	3.26%	3.18%	1.91%	1.69%	2.06%

↳ *Electrode selectivity*

Table 17 presents interferences against which selectivity of the selective membrane electrodes was tested.

Table 17. Selectivity coefficient (K)

Interferer	**Cu^{+2} ISE**	**Cd^{+2} ISE**	**Ni^{+2} ISE**	**Pb^{+2} ISE**	**Hg^{+2} ISE**
Cu^{+2}	-	$2.2\cdot10^{-2}$	$2.1\cdot10^{-3}$	$1.4\cdot10^{-3}$	$4.5\cdot10^{-3}$
Cd^{+2}	$1.4\cdot10^{-2}$	-	$3.2\cdot10^{-2}$	$1.0\cdot10^{-3}$	$4.4\cdot10^{-3}$
Ni^{+2}	$3.0\cdot10^{-2}$	$2.0\cdot10^{-2}$	-	$4.8\cdot10^{-4}$	$1.5\cdot10^{-3}$
Pb^{+2}	$3.6\cdot10^{-2}$	$3.1\cdot10^{-2}$	$2.6\cdot10^{-3}$	-	$5.7\cdot10^{-3}$
Hg^{+2}	$3.1\cdot10^{-2}$	$3.5\cdot10^{-2}$	$3.1\cdot10^{-3}$	$7.8\cdot10^{-3}$	-
Zn^{+2}	$7.8\cdot10^{-3}$	$2.7\cdot10^{-2}$	$7.1\cdot10^{-3}$	$3.9\cdot10^{-4}$	$3.5\cdot10^{-3}$
Al^{+3}	$3.9\cdot10^{-3}$	$3.9\cdot10^{-2}$	$2.1\cdot10^{-3}$	$3.1\cdot10^{-3}$	$7.1\cdot10^{-3}$
Co^{+2}	$2.1\cdot10^{-2}$	$2.1\cdot10^{-2}$	$9.1\cdot10^{-3}$	$1.1\cdot10^{-3}$	$4.5\cdot10^{-4}$
Cr^{+3}	$4.2\cdot10^{-3}$	$3.5\cdot10^{-2}$	$1.8\cdot10^{-3}$	$5.1\cdot10^{-3}$	$1.2\cdot10^{-4}$
Fe^{+3}	$1.1\cdot10^{-3}$	$5.7\cdot10^{-2}$	$1.1\cdot10^{-3}$	$6.2\cdot10^{-3}$	$4.5\cdot10^{-4}$
Ca^{+2}	$2.3\cdot10^{-3}$	$6.1\cdot10^{-2}$	$1.0\cdot10^{-2}$	$5.2\cdot10^{-3}$	$2.5\cdot10^{-4}$
Mg^{+2}	$2.2\cdot10^{-3}$	$1.1\cdot10^{-2}$	$9.0\cdot10^{-3}$	$3.0\cdot10^{-3}$	$5.3\cdot10^{-4}$
Na^{+}	$4.4\cdot10^{-2}$	$2.2\cdot10^{-1}$	$6.0\cdot10^{-3}$	$4.0\cdot10^{-3}$	$3.5\cdot10^{-3}$
K^{+}	$3.3\cdot10^{-2}$	$2.4\cdot10^{-1}$	$4.4\cdot10^{-3}$	$3.1\cdot10^{-2}$	$4.5\cdot10^{-3}$

I.4.3. Discussions

A good ionophore in an ionophore-based polymeric membrane with higher selectivity for the primary ion over other ions should be a good donor and able to form a stable complex with the metal ion. The interaction between the good ionophore and metal ions depends on the "hardness" of the ionophore and metal ions (Pearson, 1963).

Presently, membranes used for the analysis of Cu^{+2} and Ni^{+2}, exist in various forms, the most prominent being cyclic ethers such as crown ethers (Singh et al., 2006; Gupta et al., 2007)

Currently, the most efficient method for Cd^{+2} analysis involves the use of membrane technology. The most popular of which makes use of amines and crown ether derivatives (Gupta et al., 2007; Razaei et al. 2008).

Pb^{+2} electrodes incorporate selective membranes include PVC doped with ionophoric systems such as crown ethers, calixarene phosphine oxide derivatives, carbamates, cryptand acyclic amides and oxamides, among others (Barzegar et al., 2005).

Several methodologies have been developed for the detection and quantification of Hg^{+2}. The most popular being cold vapour spectroscopic absorption and membrane separation system which include amines, thiols, Schiff base and calixarene derivatives (Amde et al., 2016).

According to literature data a series of ion-selective PVC matrix membrane electrodes was built using as electro-active material acetoacetanilide (for Cu^{+2}), dicyclohexan-24-crown-8 (for Cd^{+2}), dibenzo-18-crown-6 (for Ni^{+2}), dicyclohexyl-18-crown-6 (for Pb^{+2}) or poly-(4-vinyl pyridine) (for Hg^{+2}).

The best results were obtained for a membrane containing a mixture of ionophores / plasticizer / additives / matrix in the following mass ratios (%wt) 1/67/1/31.

The constructed electrodes were used to quantifying the bioavailability of heavy metals in some aqueous extracts in a simulated digestive system (Shim et al., 2009). The optimal pH range for all electrodes was in between 3.0 and 7.0 and the working technique has imposed the need to do all determinations at pH 7.0.

The response time varied depending on the analyte concentration and it did not depend on whether the potentials were recorded from low to high concentrations or vice versa. For low concentrations, the response time was within 45 seconds while the electrodes response to high concentrations was virtually instantaneous in all cases.

The electrodes were studied from the point of view of main functional characteristics establishing for each constructed electrode its linear response range, limit of quantification, precision, accuracy (ICH, 2005). The robustness of the methods was assessed by comparison of the intra and inter-day assay results measured by two analysts under a variety of conditions such as small changes of laboratory temperature and provenience of chemicals. The percent recoveries were good.

The use of Cu^{+2}, Cd^{+2}, Ni^{+2}, Pb^{+2} and Hg^{+2} specific ionophore reduces the effect of metallic interferences and increases the selectivity of the methods (Gupta et al, 2006).

The electrodes used constantly during the experiment had an average duration of use of approximately 5-6 weeks.

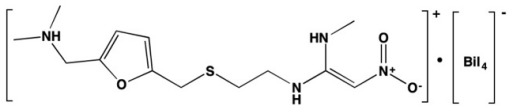
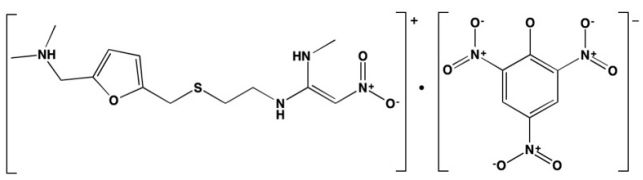
I.4.4. Conclusions

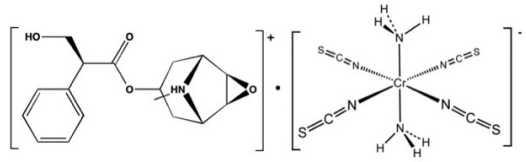
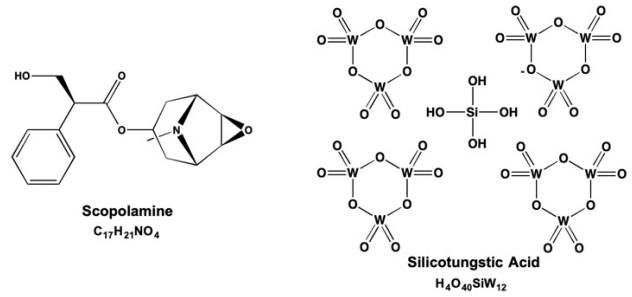
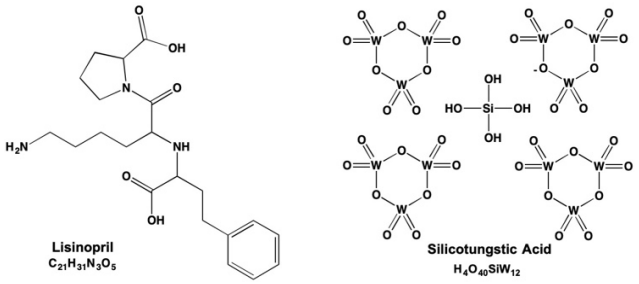
A series of ion-selective PVC matrix membrane electrodes for the determination of Cu^{+2}, Cd^{+2}, Ni^{+2}, Pb^{+2} and Hg^{+2} was built. The electrodes were studied from the point of view of main functional characteristics and they were used for the determination of trace of heavy metals from aqueous extracts. The proposed methods were simple fast and accurate.

I.5. Various studies on other types of electrochemical sensors

The electrochemical behaviour of some compounds in solution in different combinations or in the presence of other compounds with which they can be found in pharmaceutical forms or in biological samples has been investigated by electrochemical sensors (Table 18).

Table18. Other types of electrochemical sensors

Ionophore	Results/Discussions
 ***Ranitidine - tetraiodo bismuthate (III) complex***	Ranitidine hydrochloride selective electrodes were constructed based on ranitidine-tetraiodo bismuthate (III) ion pair complex in PVC matrix membrane. The used plasticizers were di-butylphthalate (DBPH), di-octylphtalate (DOPH), di-butylphosphate (DBP) and tri-butylphosphate (TBP). The electrodes based on DOPH, DBP and TBP gave the same linear response in between 10^{-5} and 10^{-1}M, while the one based on DBPH ranged between $0.5 \cdot 10^{-5}$ and 10^{-1}M. The slopes of the calibration curves were in between 22.25 and 41.20 mV per decade with correlation coefficients in between 0.9951 and 0.9990. The best detection limit was $6.30 \cdot 10^{-6}$M for the DOPH electrode. This electrode was used for drug determination in pharmaceutical preparations.
 ***Ranitidine - picrate complex***	A ranitidine selective PVC membrane electrode is developed based on an ion-pair compound of ranitidine - picrate as the electroactive substance, and dioctylphthalate as plasticizer. For pH 4.0, the linear response range, slope and quantification limit of the electrode were 10^{-5}-10^{-1}M, 29.98 mv per decade and $3.98 \cdot 10^{-6}$M, respectively, with correlation coefficient, r^2 = 0.9952. The application of the proposed method to the determination of ranitidine hydrochloride in its pure solutions and pharmaceutical preparation is characterized by a high degree of precision and accuracy when compared with the official method.

 ***Scopolamine - reineckate complex***	One membrane selective electrode with PVC matrix for scopolamine is described, with scopolamine - reineckate as electroactive material. This electrode has a linear response in the concentration range 10^{-2}-10^{-5}M scopolamine hydrochloride, with a detection limit of $2.03 \cdot 10^{-6}$M. This electrode was used with good results for quantitative assay by direct potentiometry of scopolamine hydrochloride.
 Scopolamine $C_{17}H_{21}NO_4$ Silicotungstic Acid $H_4O_{40}SiW_{12}$ ***Scopolamine and silicotungstic acid***	The construction and electrochemical response characteristics of a selective electrode with liquid matrix for scopolamine is described, with scopolamine - silicowolframte as electroactive material. This electrode has linear response in the concentration range 10^{-2}-10^{-5}M scopolamine hydrochloride, with a detection limit of $4.4 \cdot 10^{-6}$M. This electrode was used with good results for quantitative assay by direct potentiometry of scopolamine hydrochloride.
 Lisinopril $C_{21}H_{31}N_3O_5$ Silicotungstic Acid $H_4O_{40}SiW_{12}$ ***Lisinopril and silicotungstic acid***	Lisinopril form amorphous light yellowish precipitates with silicotungstic acid in acid medium. In order to analyze lisinopril, the electro-active compound was obtained and included in the selective membrane. The method showed good linearity in the range of 10^{-7} and 10^{-2}M (the correlation coefficient r^2 = 0.9991). The detection limit was $8.66 \cdot 10^{-8}$M and the quantification limit was $7.8 \cdot 10^{-7}$M. There were established the precision (RSD = 1.73%) and the accuracy (mean recovery was 99.92%). The experimental results demonstrated a good sensibility.

II. Advanced instrumental techniques for the analysis of heavy metals

II.1. Introduction

The toxicity of heavy metals represents a rare encountered medical situation, but still clinically significant, which can determine morbidity and important mortality if treated inadequately. Some heavy metals (Zn, Cu, Cr, Fe, Mn) in small quantities are essential for the body in various biochemical processes, but become toxic in large quantities. The toxicity in which heavy metals are involved can be seen most often in cases of occupational exposure, which can lead to a variety of diseases, the metals most encountered in acute or chronic toxicity being Pb, As and Hg. A much less encountered toxicity is the iatrogenic induced toxicity (Au, Li, Ga, Bi and Al) or the one induced through deliberate or not deliberate ingestion as manner of suicide, homicide or accident.

Heavy metals can enter the body through the mouth, inhalation or skin absorption bind themselves to proteins, determining alterations of the enzyme activity. The growth of the synthesis of proteins binding metals (metalloproteins), as a response to the high level of metals, is the body's first defence mechanism. Some heavy metals manifest their toxic effect only after they have accumulated inside a particular organ or tissue in a sufficient quantity, effect encountered in Pb, Hg and Cd (Kostial et al., 1978; Grandjean et al. 2006). Almost all organs are involved, the toxicity of heavy metals manifesting itself at the level of the central and peripheral nervous system, gastrointestinal system, haematopoietic system, renal system, cardiovascular system and to a smaller extent, at a musculoskeletal and reproductive level. The type of organ and the severity of the affection vary according to the involved metal, the manner of penetration, the individual's age and the level of toxicity (Blake at al., 1983). Heavy metals may enter the body orally, through ingestion of contaminated food and water (Kersting et al., 1998; Slob 2006).

Ingested *Cadmium* is accumulated in the kidneys and liver, long exposure being associated to renal and hepatic dysfunctions. It can affect the bone tissue, determining osteomalacia or osteoporosis, artery walls hardening and favouring atherosclerosis and hypertension. *Copper* is an important trace element in the body, component of numerous enzymes with a role in the synthesis of porphyrin and in many metabolic processes. In high concentrations it can cause anaemia, affect the liver and kidneys, irritate the stomach and intestines. *Lead* can cause the disturbance of the haemoglobin synthesis and can have effects on the kidneys, gastrointestinal tract, reproductive system and acute or chronic effects on the nervous system. Recent studies have shown the negative effects on the cognitive function of new-born babies after being exposed to lead. *Mercury* is concentrated in the trophic chain through bioaccumulation (fish and mollusc) and is considered to be a risk factor in cardiovascular diseases, neoplastic diseases, and inhibitor of the immune activity, accelerator of the ageing process. *Nickel* causes affections of the pulmonary tissue with the slow development of malign formations on the respiratory system. The values are high in the cases of people who have suffered a heart attack and low in the cases of acute infections and uraemia (Ellenhorn et al., 1997; Kennedy et al., 1997; Goldfrank et al., 2002; Swaran et al., 2010).

The body can defend itself against toxic metals through their chelating, followed by the biochemical transformation of the complex formed inside a metabolite easy to evacuate through the urinary or biliary tract or faeces.

Therapy with chelating agents consists in the intravenous or oral administration of substances which have heavy metal binding capacities, such as Ethylenediaminetetraacetic acid (EDTA), Dimercaptosuccinic acid (DMSA), Dimercaptopropanesulfonic acid (DMPS) and Para-aminosalicylic acid (PAS). Intravenous administration is an efficient procedure, but with certain risks because, among heavy metals, many essential minerals are eliminated, causing or increasing their deficit. In comparison, the oral chelating treatment does not act immediately, but is absolutely certain. Chelating agents have been successfully used in the treatment of some forms of Parkinson (Zheng et al., 2009) induced by Mn, for removing the toxic effects of heavy metals in cases of children with autism (Tonya et al., 2013) or in the treatment of cardiovascular diseases (Knudtson et al., 2002) caused by calcium deposits.

Our studies aimed at the elaboration and validation of some methods of quantity determination through atomic absorption spectroscopy (AAS) or potentiometrics, according to each metal from the study. The heavy metal content of some types of tobacco was also studied, as well as a series of foods of vegetable origin. The research continued with the evaluation of the real capacity of some vegetable watery extracts to bind and favour the removal of heavy metals from the body, as an alternative to the classic chelating agents therapy.

The studies were materialized in the following publications:

- Agoroaei L, Bibire N, Apostu M, Strugaru M, Grigoriu I, Butnaru E. Content of heavy metals in tobacco of commonly smoked cigarettes in Romania. *Rev Chim (Bucharest)* 2014; 65(9): 1026-1028.
- Apostu M, Țântaru G, Vieriu M, Bibire N, Panainte AD. Study of the presence of lead in a series of foods of plant origin. *Rev Chim (Bucharest)* 2018; 69(5): 1223-1225.
- Apostu M, Țântaru G, Vieriu M, Panainte AD, Bibire N, Agoroaei L. Evaluation of *in vitro* reducing effect of several vegetable extracts on the digestive bioavailability of heavy metals. *Rev Chim (Bucharest)* 2017; 68(4): 683-686.

II.2. Validation of an analytical method for the determination of heavy metals by atomic absorption spectrometry

The analysis through atomic absorption spectroscopy (AAS) has as purpose the determination of the concentration of an element from a sample through the measurement of the absorption of an electromagnetic radiation with a certain wavelength (resonance frequency) in its passing through a homogenous medium which contains the free atoms of the sample to be analysed in a state of vapours uniformly distributed, obtained in an air-acetylene flame.

II.2.1. Materials and methods

↳ *Reagents*

All reagents used were analytical grade, and the glassware was pretreated with 10% HNO_3 and rinsed with double-distilled water to prevent contamination. All necessary solutions

used during determinations were prepared by diluting a 1.0 g/L Cu^{+2}, Cd^{+2}, Ni^{+2} and Pb^{+2} commercial standard solution (Fluka, Germany) with double-distilled water and stored in polypropylene bottles.

↳ *Apparatus*

The study was carried out on an Analytik Jena ContrAA 300 apparatus equipped with an air-acetylene flame and a high-resolution continuum source. The procedure characteristics were optimized according to the manufacturer's recommendations (Table 19).

Table 19. Procedure characteristics

Metal	Cu	Cd	Ni	Pb
λ_{max} (nm)	324.75	228.80	232.00	217.00
Burner (mm)	50	50	50	50
Flame type	C_2H_2/aer	C_2H_2/aer	C_2H_2/aer	C_2H_2/aer
Acetylene/air flow (L/h)	50	50	55	65

II.2.2. Results

↳ *Validation parameters of the AAS methods*

Experimental data obtained were subjected to statistical processing, establishing for each cation its linear response range, limit of quantification, precision, accuracy, selectivity and robustness of the method (IUPAC, 2002; USP, 2017).

The most important validation parameters of the AAS analysis methods of Cu^{+2}, Cd^{+2}, Ni^{+2} and Pb^{+2} are presented in table 20. The calibration curve for Pb^{+2} can be observed in figure 16.

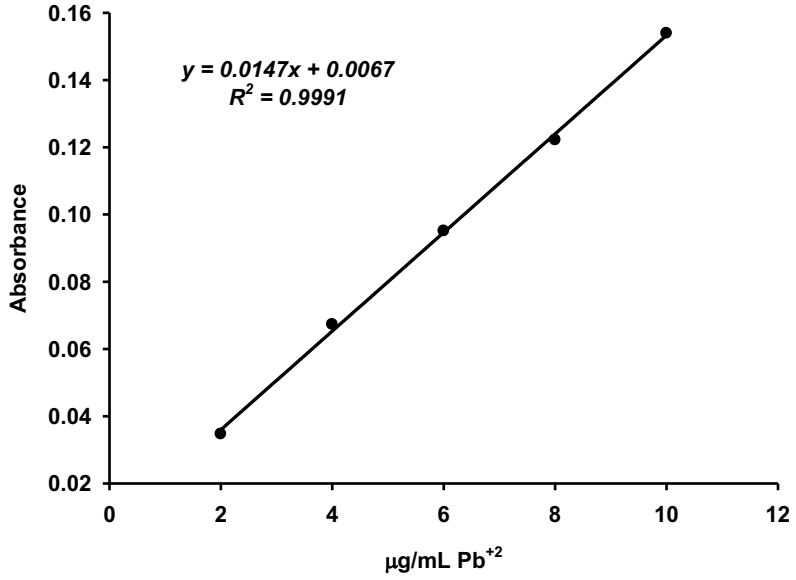


Fig. 16. Calibration curve for Pb^{+2}

Table 20. Validation parameters of the AAS analysis methods of Cu^{+2}, Cd^{+2}, Ni^{+2} and Pb^{+2}

Cation		**Cu^{+2}**	**Cd^{+2}**	**Ni^{+2}**	**Pb^{+2}**
Linearity range		1.0 - 5.0 µg/mL	1.0 - 5.0 µg/mL	2.0 - 10.0 µg/mL	2.0 - 10.0 µg/mL
Regression equation		y = 0.0499x + 0.0062	y = 0.0717x + 0.0364	y = 0.0134x + 0.0221	y = 0.0147x + 0.0067
Correlation coefficient (r^2)		0.9991	0.9953	0.9955	0.9991
Slope		0.0499 µg/mL	0.0717 µg/mL	0.0134 µg/mL	0.0147 µg/mL
Standard deviation (σ)		0.003566	0.007098	0.002173	0.001578
LOD		0.2143 µg/mL	0.2969 µg/mL	0.4864 µg/mL	0.3220 µg/mL
LOQ		0.7146 µg/mL	0.9899 µg/mL	1.6216 µg/mL	1.0734 µg/mL
Repeatability I^{st} Series	**SD**	0.26	0.19	0.61	0.48
	RSD	0.21%	0.18%	1.01%	0.46%
Repeatability II^{nd} Series	**SD**	0.21	0.20	0.60	0.50
	RSD	0.20%	0.21%	1.00%	0.49%
Reproductib.	**SD**	0.23	0.19	0.59	0.49
	RSD	0.22%	0.20%	1.09%	0.48%
Accuracy	$\overline{Xd}$	0.33%	0.80%	0.55%	0.86%

Linearity domain: using calibration solutions, calibration curves y = ax + b were determined (y is the signal intensity, x is the know concentration of the given analyte in the calibration solution).

LOD and LOQ were calculated based on the standard deviation (σ) and the slope (P) of the regression line using the following equations:

$$LOD = \frac{3 \cdot \sigma}{P};\ LOQ = \frac{10 \cdot \sigma}{P}$$

The precision of the method was studied in terms of repeatability and reproducibility. Two series of measurements have been done in different days for three different concentration levels of the analyte. For each concentration level three determinations series were carried out.

The correspondence between the real and the analytical result obtained from measurements was evaluated by calculating the relative error - Xd (%), using the the following equation:

$$Xd(\%) = \frac{|Xr - Xa|}{Xa} \cdot 100$$

where Xr was the value calculated from the calibration curve for the theoretical value Xa.

II.2.3. Discussions

The linearity of the calibration curve was considered acceptable when the correlation coefficient $r^2 > 0.995$ (European Commission, 2002; Jeevanaraj et al., 2015). The etalon curves

built for the four cations demonstrate a linear dependence between the measured absorbents and the concentrations of the etalon solutions, the correlation coefficient being between 0.9955 si 0.9991.

According to RSD Horwitz function (Gonzaler et al., 2007) the maximum RSD values acceptable is 10% (European Commission, 2002). Therefore, it can be stated that the developed method exhibited a good reproducibility precision based on RSD values obtained.

AAS is a great method of producing accurate results, with a rate of 0.33-0.86%, or an even better rate if appropriate standards are used (Taverniers et al. 2004). In the present study, Xd was found to be 0.33-0.86% suggesting the developed method was accurate for the quantification of Cu, Cd, Ni and Pb.

In atomic absorption spectrometry, the specificity to the reaction takes place in the flame (Rohman et al., 2015). Every element absorbs at a specific wave length. Interferences can results from anions or matrix. The main interference anion is a chloride. The matrix effects can be of two types; mask effects or background effects.

During the determinations, no spectral or chemical interferences were observed in order to alter the measurement result.

II.2.4. Conclusion

In this study, a method of quantitative analysis for the determination of Cu^{+2}, Cd^{+2}, Ni^{+2} and Pb^{+2} by AAS was validated. Several statistical parameters have been taken into account and evaluated for the validation of method: the limit of detection ranged between 0.2143-0.4864 μg/mL for the metals studied ensures the limit of quantification required for quantitative determinations of the concentrations of these elements; good linearity (correlation coefficient $0.9953 \geq r^2 \geq 0.9991$) for each element recommend the method described for determination at trace and ultra-trace level; the established method was found to be precise and accuracy.

The developed methods meet the acceptance criteria of validation parameters according to IUPAC and USP.

II.3. Determination of heavy metals in tobacco of commonly smoked cigarettes in Romania

The composition of tobacco, as well as tobacco smoke, is complex, gathering a large number of substances with major toxicological implications, many incompletely studied. About 4000 chemicals (inorganic and organic) have been identified in tobacco. Among the inorganic components, the following metals have been detected: Cd, Pb, Cr, Ni, As, Hg, Co, Cu, Zn, Fe, Mn, Bi, Ce. Tobacco smoking is an important source of heavy metals in both human body and environment (Rodgman et al., 2008; Golia et al., 2009).

The heavy metals are toxic in low levels, being easily assimilated in the human body during smoking. Exposure to metals through tobacco depends on the amount of metal present in tobacco, a percentage that is transferred to the tobacco smoke and the percentage that is absorbed (Lazarevik et al., 2012) .

Tobacco plant accumulates heavy metals in the following order of leaves > roots > stems. The level of heavy metals in tobacco vary within wide limits and depends on:

- variety and development stage of the plant;
- physico-chemical attributes of soil - influence the mobilization and the transfer of metals from soil into the plant;
- pH - the acidic pH favours a better absorption of metals in plant;
- redox conditions - affects the mobility of oxidised metals;
- interactions between metals in the absorption from soil - absorption of cadmium is stimulated in the presence of lead;
- agricultural practices - the use of fertilizers;
- precipitations;
- technological processing of tobacco (which enriches the metal content in leaves);
- type of product (cigarettes, cigars, chewing tobacco, snuff, etc.).

In general, tobacco plants accumulate heavy metals like Cd, Pb and Zn, preferentially.

Cadmium is the most absorbed heavy metal, so it is the most studied metal in tobacco plant, the toxicity of the metal being an important reason too (Verma et al., 2010).

II.3.1. Materials and methods

↳ *Reagents*

Analytical grade chemicals and reagents were used throughout the analysis. Only double distilled water was used for preparation and dilutions of reagents and samples.

↳ *Apparatus*

The study was carried out on an Analytik Jena ContrAA 300 apparatus equipped with an air-acetylene flame and a high-resolution continuum source.

↳ *Digestion of the tobacco*

Six heavy metals potentially toxic, Cd, Pb, Cr, Ni, Cu and Zn, were determined in tobacco of 15 cigarette varieties (brands) from market, commonly smoked in Romania. After removing filter and paper, 2.5 g of homogenized tobacco was mineralized with 20 mL nitric acid 65% by heating on a sandbath at 150^{o}C until total digestion.

The metals were determined through atomic absorbtion pectrophotometry. The results were expressed in μg/g tobacco dried at 105^{o}C.

II.3.2. Results

In table 21, the concentrations of heavy metals in tobacco of analyzed cigarettes are presented. Table 22 contains the limits and the mean values of these concentrations (SD - standard deviation).

Table 21. Content of heavy metals in tobacco of analyzed cigarettes

Cigarette brand (sample)	µg/g tobacco dried at 105°C					
	Cd	**Pb**	**Cr**	**Ni**	**Cu**	**Zn**
I	1.00	3.44	3.74	0.79	8.77	1.28
II	1.23	4.60	2.81	0.79	9.89	2.23
III	1.39	5.18	6.33	1.04	11.13	2.70
IV	0.80	4.77	6.81	0.96	7.41	1.91
V	1.55	2.73	2.23	0.94	12.43	3.04
VI	1.58	4.71	2.88	0.81	9.56	2.57
VII	0.71	3.97	3.24	1.07	6.96	1.80
VIII	0.80	4.77	4.86	0.82	7.41	1.21
IX	1.11	4.76	5.82	1.09	14.21	5.47
X	0.94	3.51	3.81	0.94	8.94	2.38
XI	1.16	3.72	6.06	1.00	10.66	3.36
XII	0.72	4.30	7.01	0.74	10.27	2.08
XIII	0.76	2.26	1.85	0.78	9.74	2.08
XIV	1.20	4.81	4.90	0.96	6.90	1.68
XV	0.98	4.01	3.27	0.92	7.67	2.07

Table 22. Minima, maxima, median and mean value of the heavy metals concentrations in tobacco

Value	µg/g tobacco dried at 105°C					
	Cd	**Pb**	**Cr**	**Ni**	**Cu**	**Zn**
Minima	0.71	2.26	1.85	0.74	6.90	1.21
Maxima	1.58	5.18	7.01	1.09	14.21	5.47
Median	1.05	4.15	3.81	0.93	9.65	2.15
Mean±SD	1.06±0.28	4.10±0.84	4.37±1.71	0.91±0.11	9.46±2.09	2.39±1.03

II.3.3. Discussions

In table 23, the concentrations of the six metals in tobacco reported in the literature by different country are presented (Verma et al., 2010).

Cadmium is an irritant, thiol depletion inducer and carcinogen toxic. It is considered that 40-60% of cadmium inhaled from tobacco smoke goes directly into the blood stream (it is volatile at 320°C). A cigarette contains 1-2 µg Cd, of this 0.1-0.2 µg are inhaled; smoking more than 20 cigarettes daily can increase Cd concentration in body by ten-folds. The content of cadmium in fat tissue of male smokers can be higher than of non-smokers (Mussalo-Rauhamaa et al., 1986; Nordberg et al., 2007; Verma et al., 2010).

The lowest level of cadmium was recorded in sample VII (0.71 µg/g), followed closely by sample XII (0.72 µg/g) and sample XIII (0.76 µg/g), while the maximum concentration of cadmium was found in tobacco sample VI (1.58 µg/g), followed by tobacco sample V (1.55 µg/g) and sample III (1.39 µg/g). Mean concentrations recorded in the 15 tobacco varieties is 1.06 µg Cd/g tobacco, which is close to the value recorded in Germany in 1998 (1.0 µg/g) (Table 23).

Table 23. Metal concentration levels in tobacco reported by different country

Country	μg/g tobacco					
	Cd	Pb	Cr	Ni	Cu	Zn
USA (1997)	0.98	-	-	-	-	35.1
Russia (1998)	1.11	1.86	0.88	-	-	-
Germany (1998)	1.00	1.62	1.26	-	-	36.3
Finland (1986)					15.6	50
Italy (1989)	-	7.39	-	-	-	-
Yugoslav (1978)					18.9	-
Hungry (1995)	1.89	1.17	-	5.4	-	57.6
Poland (2008)	0.61	0.56	-	-	-	-
Turkey (2001)	-	-	1.63	0.22	-	-
China (2005)	0.18	0.64	-	2.23	4.13	-
India (2010)	0.45	1.94	4.07	8.79	14.0	27.0
Pakistan (2008)	-	14.35	-	-	-	-
Egypt (1999)						76.8
Jordan (2005)					12.90	55.62
Mexico (1991)	4.41	-	-	-	-	-

Smokers have significantly higher blood lead levels than non-smokers (Pavel et al., 2013). In tobacco brand XIII a concentration of 2.26 μg Pb/g was recorded; this minimum value is followed, in ascending order, by the value 2.73 μg Pb/g (V) and 3.44 μg Pb/g (I). The highest concentration in lead, 5.18 μg/g, was determined in sample III; two other samples with high levels were registered for cigarette brands XIV (4.81 μg/g) and IX (4.76 μg/g). Mean concentration of lead, 4.10 μg/g, is close to the value recorded in China; it is a high value compared to the concentrations reported by other countries (Table 23).

Chromium is a methemoglobin inducer, an irritant, allergenic and carcinogen agent (lung, sinonazal cavity), and genotoxic. In 1979 were reported limits from 0.24 to 14.6 μg Cr/g tobacco (Mussalo-Rauhamaa et al., 1986; Rusu, 2013).

The least contaminated with chromium were tobacco samples XIII (1.85 μg/g), V (2.23 μg/g), and II (2.81 μg/g), while the most contaminated were samples XII (7.01 μg/g), IV (6.81 μg/g) and III (6.33 μg/g). The mean value, 4.37 μg Cr/g tobacco, is higher than the dates identified in the literature (Table 23).

Nickel is a toxic of CNS and myocardium, sensitization and carcinogen (in lungs) agent. It is considered that nickel may be combined with carbon monoxide in tobacco smoke of the primary current, with formation of the $Ni(CO)_4$. The smoke of a cigarette may contain up to 50 μg Ni, of which 1.1% is found in the primary current (Klein et al., 2007; Thyssen et al., 2010).

In the analyzed samples, the lowest level of nickel was recorded in tobacco sample XII (0.74 μg/g), while highest concentration in sample IX (1.09 μg/g). Other tobacco samples with high levels of nickel are VII (1.07 μg/g) and III (1.04 μg/g). Mean concentrations of nickel, 0.91 μg/g, is the lowest average recorded in our study. Except the value of 0.22 μg Ni/g tobacco, reported in Turkey, in 2001 (Table 23), the mean determined in our study is less than levels found in literature.

Copper is a hemolytic and methemoglobin inducer toxic (Cotrau et al., 1991). In

analyzed tobacco samples, copper concentrations ranged between 6.90 μg/g (XIV) and 14.21 μg/g (IX), being the highest concentrations recorded in our study. The mean value, 9.46 μg Cu/g tobacco, is close to the concentration level registered in Germany in 1993 (Table 23).

Zinc is a central nervous system, cardiovascular and muscle toxic, but, as in the case of copper, manifests toxicity in high doses; copper and zinc are both oligoelements and, therefore, normally present in the human body in certain limits. The lowest level of zinc was recorded in tobacco of cigarettes brand VIII (1.21 μg/ g), while the highest value was determined in tobacco of sample IX (5.47 μg/g). These values, and the mean value of 2.39 μg Zn/g too, are much smaller than similar concentrations reported in the literature.

Table 24 presents the distribution of the first three elevated concentrations of metals in tobacco samples (the first elevated value of concentration, in descending order: • the maxima value, •• the second higher value, ••• the third higher value).

Table 24. Distribution of the higher concentration levels of heavy metals in tobacco

Cigarette brand (sample)	Cd	Pb	Cr	Ni	Cu	Zn
I						
II						
III	•••	•	•••	•••	•••	
IV			••			
V	••				••	•••
VI	•					
VII				••		
VIII						
IX		•••		•	•	•
X						
XI						••
XII			•			
XIII						
XIV		••				
XV						

Related to this criteria, we estimate that tobacco sample IX is the most contaminated (recording maximum concentrations for nickel, copper and zinc), followed by tobacco samples III and V.

Reported to the mean values of metal concentrations in tobacco of analyzed cigarettes, the highest level was recorded in copper, followed in descending order by chromium, lead, zinc, cadmium, nickel. The study confirms the ability of tobacco plant to retain heavy metals into the leaves.

II.3.4. Conclusions

Six heavy metals potentially toxic have been determined in tobacco of 15 varieties (brands) of cigarettes from market, commonly smoked in Romania.

Reported to the mean values of metal concentrations in tobacco of analyzed cigarettes, the highest level was recorded in copper, followed in descending order by chromium, lead, zinc, cadmium, nickel.

The study confirms the ability of tobacco plant to retain heavy metals into the leaves. Since heavy metals once absorbed, have long biological half-life, their presence in tobacco may result to chronic adverse effects on the individual. It is therefore pertinent that manufacturers of cigarettes check the levels of these metals during processing before final packaging.

II.4. Study of the presence of lead in a series of foods of plant origin

Lead is a heavy metal that produces toxic effects after accumulation of more than 30 μg per deciliter of blood in subjects over age 15 (Merill 2001; Fischbach 2004). Its accumulation may cause disruption of hemoglobin synthesis (Bergdahl et al., 1997; Carlisle et al., 2009), kidney, gastrointestinal tract and reproductive system damage (Walshh et al., 1984; Bernard et al., 1995; Doumouchtsis et al., 2009; Vallverdú-Coll et al., 2016), plus acute or chronic effects on the nervous system (Cory-Slechta 2003; Canfield et al., 2004).

Given the high toxicity of this element, this study aims to estimate the lead content in different types of food of plant origin (wheat, wheat flour, onions, corn, corn flour, carrots, beans, potatoes and cabbage) grown in three geographical areas by AAS.

II.4.1. Materials and methods

↳ *Reagents*

Analytical grade chemicals and reagents were used throughout the analysis. Only double distilled water was used for preparation and dilutions of reagents, standards and samples.

↳ *Apparatus*

The study was carried out on an Analytik Jena ContrAA 300 apparatus equipped with an air-acetylene flame and a high-resolution continuum source.

↳ *Digestion of the vegetal material*

20 g of vegetal material was mixed with 10 mL of concentrated HNO_3 and 2.5 mL of 70% $HClO_4$ and allowed to rest for 24h. The resulting mixture was heated on a water bath until complete mineralization. After cooling, double-distilled water was added and then it was heated to remove the nitrous vapor and to reach a low volume (0.5-1.0 mL). When the solution darkened or became yellow during disaggregation, a few drops of HNO_3 or $HClO_4$, respectively, were added. Then 5 mL double-distilled water and 10 mL of 5M HCl solution were added to the solution, and its volume was reduced again to 10 mL. In the end it was filtered and double-distilled water was added up to 50 mL.

II.4.2. Results

The accuracy of the assays, expressed as the consistency between the real value and the analytical result, was verified using the standard addition method. The results obtained and the

calculated recovery are shown in table 25. The analysis of the results emphasized a recovery in between 97.50% and 103.00%.

Table 25. Accuracy of Pb^{+2} determination in foods

Nº	**Sample**	**µg Pb^{+2} added (a)**	**µg Pb^{+2} found (b)**	**b - a subtraction**	**Recovery (%)**
1.	wheat	-	2,12	-	-
2.	wheat	1.00	3.14	1.02	102.00
3.	onions	-	2.02	-	-
4.	onions	1.00	3.05	1.03	103.00
5.	corn	-	2.60	-	-
6.	corn	2.00	4.55	1.95	97.50
7.	carrots	-	2.50	-	-
8.	carrots	2.00	4.54	2.04	102.00
9.	beans	-	2.10	-	-
10.	beans	3.00	5.08	2.97	99.00
11.	potatoes	-	2.25	-	-
12.	potatoes	3.00	5.30	3.05	101.66
13.	cabbage	-	2.00	-	-
14.	cabbage	4.00	5.98	3.98	99.50

For the assay of Pb^{+2} levels in foods, the following types of foods were analyzed: wheat, wheat flour, onions, corn, corn flour, carrots, beans, potatoes, cabbage. The results of the determinations were expressed as µg Pb^{+2}/1000 g of plant product (weighted arithmetic mean).

Food products were sampled from various localities in three areas, grouped by soil types:

- *levigant soil* in Moldavian Plain (soil I): Fântânele (Iaşi), Plopeni (Suceava), Havârna (Botoşani), Dorohoi (Table 26);
- *meadow soil* in Siret and Barlad Valley (soil II): Mărășești, Bacău, Pașcani, Focșani, Tecuci, Roman, Siret (Table 27);
- *forest brown soil* in sub-Carpathian area and Suceava Plateau (soil III): Piatra Neamț, Târgu Neamț, Huși, Vlădeni (Iași) (Table 28).

Table 26. Pb^{+2} levels as µg/1000 g plant products from *levigant soil*

Nº	**Sample**	**Dry product**	**Fresh product**
1.	wheat	90.2	79.0
2.	wheat flour	126.0	114.0
3.	onions	90.0	10.9
4.	corn	135.1	110.0
5.	corn flour	76.2	57.7
6.	carrots	92.5	49.9
7.	beans	102.5	90.0
8.	potatoes	86.6	12.9
9.	cabbage	68.0	57.2

Table 27. Pb^{+2} levels as µg/1000 g plant products from *meadow soil*

N°	Sample	Dry product	Fresh product
1.	wheat	136.6	134.0
2.	wheat flour	142.3	127.1
3.	onions	45.7	10.2
4.	corn	153.0	123.5
5.	corn flour	146.2	133.4
6.	carrots	109.0	33.5
7.	beans	137.2	104.6
8.	potatoes	63.8	13.9
9.	cabbage	97.5	85.8

Table 28. Pb^{+2} levels as µg/1000 g plant products from *forest brown soil*

N°	Sample	Dry product	Fresh product
1.	wheat	185.6	154.7
2.	wheat flour	197.2	161.2
3.	onions	59.0	10.2
4.	corn	199.3	164.0
5.	corn flour	184.6	150.0
6.	carrots	115.0	44.7
7.	beans	158.7	130.0
8.	potatoes	72.2	17.2
9.	cabbage	109.0	83.6

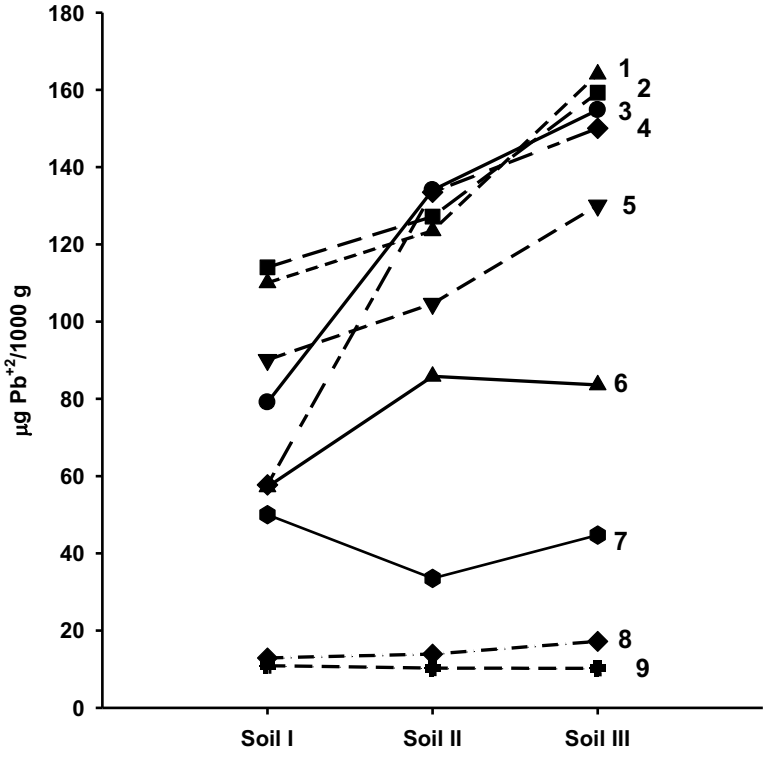


Fig. 17. The average values of Pb^{+2} levels

The average values of Pb^{+2} content as μg/1000 g of fresh vegetable product, by soil and food type, were plotted in figure 17 (1 - corn, 2 - wheat flour, 3 - wheat, 4 - corn flour , 5 - beans, 6 - cabbage, 7 - carrots, 8 - potatoes, 9 - onions).

II.4.3. Discussions

The World Health Organization (WHO) estimates that about a quarter of the diseases facing mankind today occur due to prolonged exposure to environmental pollution (Sanghyuk et al., 2018). Contaminants accumulate in the soil then get transferred to food chain causing serious health hazards to human beings and animals.

In the study, it was established that the assimilation of Pb^{+2} by plant foods was very variable, even for the same type of food. Just as it was stated in literature, it varied based on the geochemical conditions, soil pollution, local level of industrialization, etc (Golia et al., 2008).

The interpretation of the obtained results has been made according to European Commission recommendations (Commission Regulation No 1881/2006) which setting maximum levels for certain contaminants in foodstuffs.

Studies made on samples collected from the area with *levigant soil* show that the highest level of lead can be found in corn (135.1 μg Pb^{+2}/1000 g), while the product with the lowest level is the cabbage (68.0 μg Pb^{+2}/1000 g) in the dry version. Potatoes (86.6 μg Pb^{+2}/1000 g), onion (90.0 μg Pb^{+2}/1000 g) and wheat (90.2 μg Pb^{+2}/1000 g) occupy the middle part of the classification. The analysis of wet products (green) puts wheat flour (114.0 μg Pb^{+2}/1000 g) on the first position, while corn occupies the second position (110.0 μg Pb^{+2}/1000 g). In the last two positions, we can find potatoes (12.9 μg Pb^{+2}/1000 g) and onion (10.9 μg Pb^{+2}/1000 g). The foods with a medium content are cabbage (57.2 μg Pb^{+2}/1000 g), corn flour (57.7 μg Pb^{+2}/1000 g) and wheat (79.0 μg Pb^{+2}/1000 g).

The *meadow soil* is typical of damp areas situated along flowing waters. The highest level of lead in dry product can be found in corn (153.0 μg Pb^{+2}/1000 g), cabbage (97.5 μg Pb^{+2}/1000 g) and carrots (109.0 μg Pb^{+2}/1000 g) have a medium level and onion (45.7 μg Pb^{+2}/1000 g) is the product with the lowest level of lead. In the wet variant, freshly harvested foods, the highest level of lead was found in wheat (134.0 μg Pb^{+2}/1000 g), onion having once again the lowest level (10.2 μg Pb^{+2}/1000 g).

The *forest brown soil* is formed in the areas of beech or oak forests. From the dry products, the corn (199.3 μg Pb^{+2}/1000 g) grown in this area has the highest level of lead from the analysed products, cabbage (109.0 μg Pb^{+2}/1000 g) and carrots (115.0 μg Pb^{+2}/1000 g) have a medium level and onion (59.0 μg Pb^{+2}/1000 g) has the lowest level. Wet products are classified almost identically: corn (164.0 μg Pb^{+2}/1000 g), cabbage (83.6 μg Pb^{+2}/1000 g) and onion (10.2 μg Pb^{+2}/1000 g).

Pb^{2+} levels in fresh product, had the highest values for foods harvested from forest brown soil and meadow soil (except for carrots) while the lowest levels were found in levigant soils.

Out of all the products studied, cereals and beans contained the highest Pb^{+2} levels, unlike carrots, potatoes and onions that assimilated the least amount of cation.

Commission Regulation (EC) No 1881/2006 - Consolidated acts, recommends the maximum permitted level of Pb^{+2} in foods: 200 μg Pb^{+2}/1000 g fresh beans and cereals (wheat,

corn), 100 µg Pb^{+2}/1000 g fresh onions, carrots, potatoes and 300 µg Pb^{+2}/1000 g fresh vegetables of the *Brassicaceae* family (cabbage). None of the foods studied exceeded those recommended limits.

II.4.4. Conclusions

The contamination of foods of vegetable origin with heavy metals can cause some diseases which influence the population's health. The evaluation of the presence and quantity of heavy metals in this type of products represents an important demand which ensures the food safety and its quality control. Studies made through AAS on various foods from different geographical areas with various types of soil have shown that the limit recommended by the European Standards in force regarding the amount of lead did not exceed the acceptable standard for any product.

II.5. Evaluation of *in vitro* reducing effect of several vegetable extracts on the digestive bioavailability of heavy metals

Heavy metals may enter the body via the oral route, inhalation, or dermal absorption and their toxicity manifests in the central and peripheral nervous systems, gastrointestinal, hematopoetic, renal, cardiovascular, and to a lesser extent in the musculoskeletal and reproductive systems (Mao et al., 2019; Ghazala et al., 2016).

Poisoning treatment aims to chelate the metal followed by biochemical transformation of the formed complex in a metabolite easy to remove through the urinary or bilio-faecal routes.

Recent studies have shown that plant products rich in polyphenols, tannins, polysaccharides and anthocyanins may inhibit the absorption of heavy metals in the body and promoting their elimination.

Research conducted on herbs with a high polyphenols content have shown their high capacity to bind and facilitate removal of heavy metals (Ebrahimzadeh et al., 2008). Constant consumption of black tea can decrease the amount of absorbed Hg, derived from oceanic fish (Shim et al., 2009).

Assessing the ability of aqueous extracts of local herbs, to bind and increase the elimination of Cu^{+2}, Cd^{+2}, Ni^{+2}, Pb^{+2}, and Hg^{+2} has required the development and validation of quantitative determination methods through atomic absorption spectroscopy or potentiometry using selective membrane electrodes.

II.5.1. Materials and methods

The evaluation of the complexing capacity of the vegetable extracts has been done by treating an accurately measured volume of standard solution for each studied cation with an accurately measured volume of plant extract. The complexing ability of the extract was given by the difference between the initial concentration and final concentration of the free cation.

↳ *Reagents*

Analytical grade chemicals and reagents were used throughout the analysis. Only double distilled water was used for preparation and dilutions of reagents, standards and samples.

↳ *Apparatus*

The potentiometric measurements were made using a Hanna 301 digital pH/ millivoltmeter and a selective membrane electrode of our own construction. The atomic absorption spectrophotometry studies were made using a Analytik Jena ContrAA 300 spectrophotometer provided with air-acetylene flame atomizer and a high resolution continuous source. The operating parameters had been optimized in accordance with the manufacturer's recommendations.

↳ *The digestion of the vegetable material*

6 g of plant material was treated with 10mL of concentrated HNO_3 and allow to rest for 24 hours. The mixture was heated in a water bath until all the nitrous vapor emission stopped. 4 mL of $HClO_4$ 70% was added and the mixture was heated to yield a smaller volume that was filtered and diluted to 25 mL with double distilled water.

↳ *Preparation of infusions*

6 g of dry plant were added to 100 mL of boiling double distilled water. After 30 minutes of rest, the mixture was filtered and diluted to 100 mL with double distilled water.

↳ *Digestion of infusions*

100 mL of infusion was heated in a water bath to yield a concnetrate. 5 mL of HNO_3 and $HClO_4$ (5:1) mixture are added and it was all digested for two hours at 200°C in a Kjeldahl device. The product was filtered and diluted to 25 mL with double distilled water.

II.5.2. Results

↳ *Estimating the concentration of Cu^{+2}, Cd^{+2}, Ni^{+2}, Pb^{+2}, and Hg^{+2} cations in the studied plant species and their aqueous extracts*

Plant species subjected to this study were analyzed to determine the concentration of existing heavy metals in the plant material used and their ability to release the cations during aqueous extraction. The studies were conducted comparatively through the two previously validated methods: AAS and ISE.

The results obtained for *Crataegus sp.* (**CrS**), *Tilia sp.* (**TS**), *Rosa canina* (**RC**), *Vaccinium myrtillus* (**VM**), *Geranium robertianum* (**GR**), *Mentha piperita* (**MP**), *Cynara cardunculus subsp. Scolymus* (**CC**), *Plantago sp.* (**PS**) and *Coriandrum sativum* (**CoS**) are shown in table 29.

↳ *Assessing the complexing capacity of the prepared extractive solutions for Cu^{+2}, Cd^{+2}, Ni^{+2}, Pb^{+2}, and Hg^{+2} cations*

A standard solution of each cation was prepared in 100 mL infusion (6 g of dried plant/100 mL) adjusted to pH 7.0 with NaOH, and the amount of free metal was determined.

Because no precipitation occurred when the extracts were added, all quantifications of cations were performed using ISE.

The complexing ability of the extract was established by the difference between the initial cation concentration and the concentration of the free cation that was determined using selective membrane electrodes (Table 30).

Table 29. Total concentrations of heavy metal cations in dried plant material (I) and infusion (II) as mg/kg and mg/L (ppm), respectively

Plant species		Total concentrations of heavy metal cations (ppm, averages, n = 3)									
		Cu^{+2}		Cd^{+2}		Ni^{+2}		Pb^{+2}		Hg^{+2}	
		I	II	I	II	I	II	I	II	I	II
CrS	AAS	7.631	0.982	0.150	-	0.712	0.063	0500	-	-	-
	ISE	7.592	0.971	0.144	-	0.683	0.57	0482	-	-	-
TS	AAS	8.014	1.406	0.091	-	0.809	0.115	-	-	-	-
	ISE	7.984	1.390	0.72	-	0.781	0.111	-	-	-	-
RC	AAS	1.943	0.142	0.114	-	0.915	0.053	-	-	-	-
	ISE	1.910	0.140	0.091	-	0.890	0.049	0.161	-	-	-
VM	AAS	4.313	0.472	0.26	-	0.394	0.031	0.140	-	-	-
	ISE	4.303	0.460	0.018	-	0.373	0.029	-	-	-	-
GR	AAS	9.511	0.970	0.128	-	0.681	0.061	-	-	-	-
	ISE	9.482	0.966	0.100	-	0.672	0.057	0.108	-	-	-
MP	AAS	8.154	1.022	0.101	-	1.801	0.190	0.081	-	-	-
	ISE	8.115	1.013	0.090	-	1.771	0.187	0.610	-	-	-
CC	AAS	6.737	3.762	-	-	0.919	0.387	0.592	-	-	-
	ISE	6.608	3.695	-	-	0.890	0.382	-	-	-	-
PS	AAS	6.225	1.801	-	-	0.501	0.162	-	-	-	-
	ISE	6.013	1.742	-	-	0.470	0.145		-	-	-
CoS	AAS	0.862	0.320	0.085	-	0.411	0.123	0.132	-	-	-
	ISE	0.764	0.286	0.079	-	0.381	0.110	0.128	-	-	-

Table 30. Assessment of complexing capacity of the extractive solutions for Cu^{+2}, Cd^{+2}, Ni^{+2}, Pb^{+2} and Hg^{+2} cations

Plant species	Complexed metal concentration (µg/100mL infusion, n = 3)				
	Cu^{+2}	Cd^{+2}	Ni^{+2}	Pb^{+2}	Hg^{+2}
RC	3.14 ± 0.10	5.61 ± 0.17	3.01 ± 0.05	7.51 ± 0.12	6.53 ± 0.13
MP	5.17 ± 0.16	8.34 ± 0.26	4.86 ± 0.09	13.10 ± 0.22	12.87 ± 0.26
GR	7.83 ± 0.25	14.01 ± 0.44	6.93 ± 0.16	18.21 ± 0.30	17.52 ± 0.36
VM	10.11 ± 0.32	19.96 ± 0.63	9.77 ± 0.18	26.15 ± 0.44	24.01 ± 0.49
CrS	-	-	-	-	-
TS	-	-	-	-	-
CC	-	-	-	-	-
PS	-	-	-	-	-
CoS	-	-	-	-	-

↳ *Study of efficiency of extractive solutions under simulated digestive system conditions*

The infusions that proved to have complexing capacity during previous determinations were studied in the next phase under simulated digestive system conditions to quantify the bioavailability of ingested heavy metals in the presence of those extracts (Shim et al., 2009). To that end, a standard solution of the studied cation was prepare in 100 mL infusion (6 g of dried plant/100 mL), it was adjusted to pH 2.0 with HCl. 2 mL porcine pepsin (3 mg/mL) were added and it was incubated for 1 hour at 37ºC. The pH was adjusted to 5.6 with $NaHCO_3$ and 9 mL of pancreatin (0.4 mg/mL), pig bile extract (2.4 mg/mL) and lipase (0.2 mg/mL) was added. The pH was adjusted to 7.0 with NaOH and the mixture was incubated for 2 hours at 37ºC. The free metal content was assessed through ISE and the results shown in table 31 were calculated for 100 mL infusion in light of all the dilutions made.

Table 31. Assessment of complexing capacity of the extractive solutions under simulated digestive system conditions

Plant species	Complexed metal concentration (µg/100 mL infusion, n = 3)				
	Cu^{+2}	Cd^{+2}	Ni^{+2}	Pb^{+2}	Hg^{+2}
VM	8.23 ± 0.11	-	-	1.47 ± 0.04	1.38 ± 0.02
RC	-	-	-		-
MP	-	-	-		-
GR	-	-	-		-

↳ *Chemical composition of extractive aqueous solutions of Vaccinium myrtillus*

The active principles with complexing effect on metal cations under simulated digestive system conditions proved to be the anthocyanins. The anthocyanins are secondary plant metabolites, of flavonoids class, with high solubility and stability in aqueous medium. For the estimation of their concentration and that of their related compounds, the total polyphenols, total flavonoids and total anthocyanins contents were determined (Table 32).

Table 32. Total polyphenols, total flavonoids and total anthocyanins content of queous extract of *Vaccinium Myrtillus* (n = 3)

Total polyphenols mg GAE/100mL extract	Total flavonoids mg QE/100mL extract	Total anthocyanins mg C3GE/100mL extract
15.28 ± 0.74	2.51 ± 0.19	5.60 ± 0.44

- Total polyphenols content was determined (Wrolstad et al., 2005) using Folin-Ciocalteu method, by evaluating the -OH groups of the sample in alkaline medium (Na_2CO_3). The reagent, composed of a phosphotungstic acid and phosphomolybdic acid mixture, got reduced during the oxidation of phenols to a mixture of tungsten and molybdenum blue oxides and their absorbance measured at 765 nm was proportional to the number of phenolic groups of anthocyanins (Fig. 18). Total polyphenols content was expressed as gallic acid equivalents (GAE).

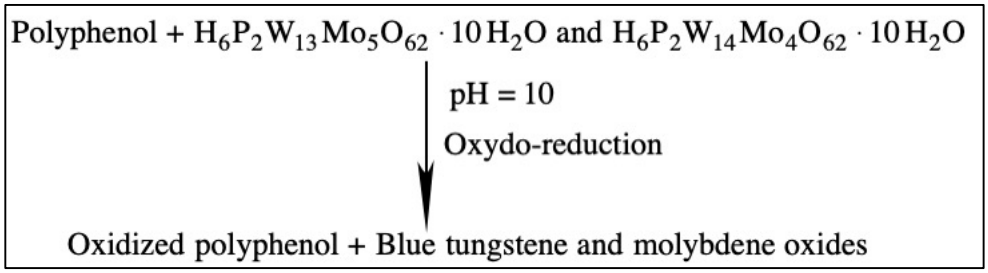


Fig. 18. Reaction of polyphenolic compounds with Folin-Ciocalteu reagent

• Total anthocyanins content was determined based on their property to change color depending on the pH (Giusti et al., 2001) (Fig. 19). Total anthocyanin content was expressed as cyanidin 3-galactoside equivalents (C3GE):

$$\text{mg of anthocyanins} = (A \times M_r) / \varepsilon$$

where $A = (A_{510nm, pH = 1.0} - A_{700nm, pH = 1.0}) - (A_{510nm, pH = 4.5} - A_{700nm, pH = 4.5})$, Mr C3GE = 484.84 and the molar absorptivity of C3GE is ε = 34300.

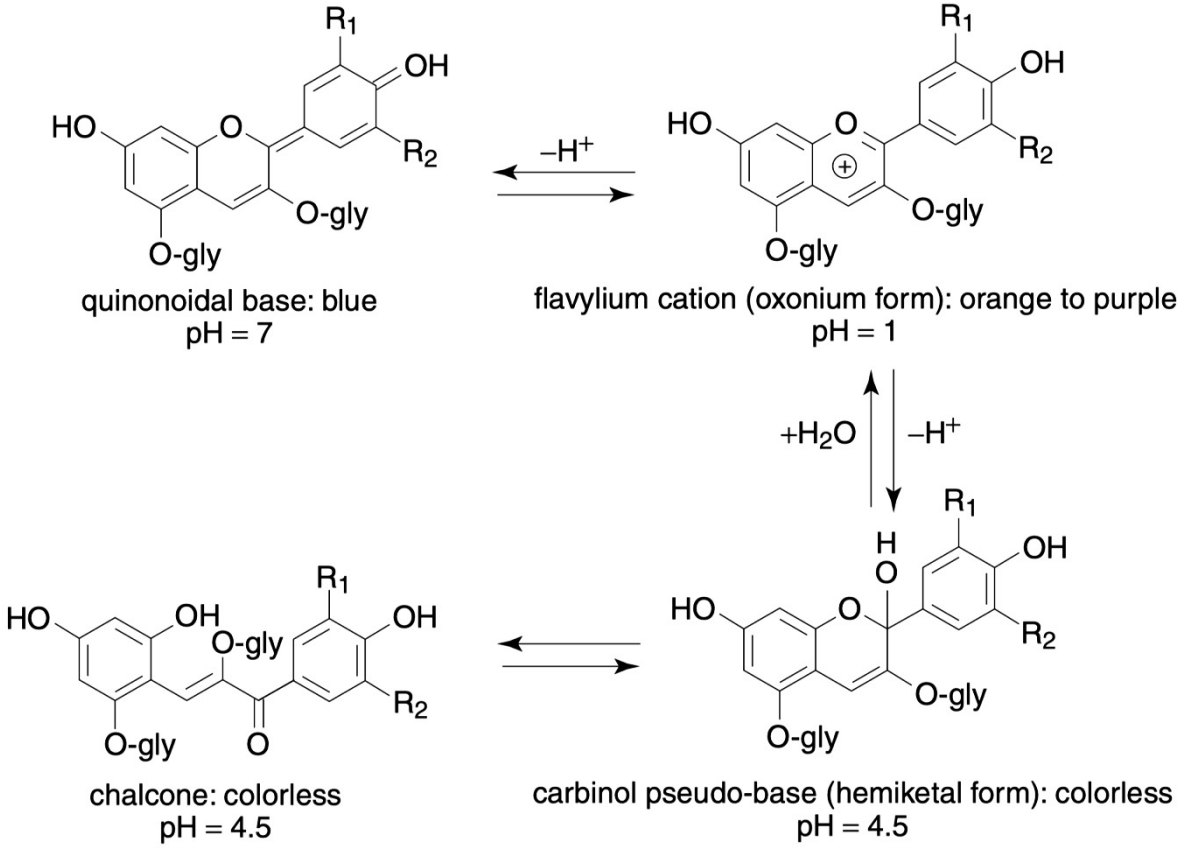


Fig. 19. Predominant structural forms of anthocyanins present at different pH levels (Giusti et al., 2001)

• The total flavonoids content was determined using a colorimetric method (Zhishen et al., 1999) (Fig. 20). 1 mL sample was brought into a 10 mL volumetric flask. 4 mL double

distilled water and 0.3 mL 5% $NaNO_2$ were added and exactly five minutes later 0.3 mL of 10% $AlCl_3$ followed. After one more minute had passed 2 mL of 1M NaOH were added and the mixture was diluted with distilled water up to 10 mL. The absorbance was measure at 510 nm. The total content of flavonoids was expressed as (QE) quercetin equivalents.

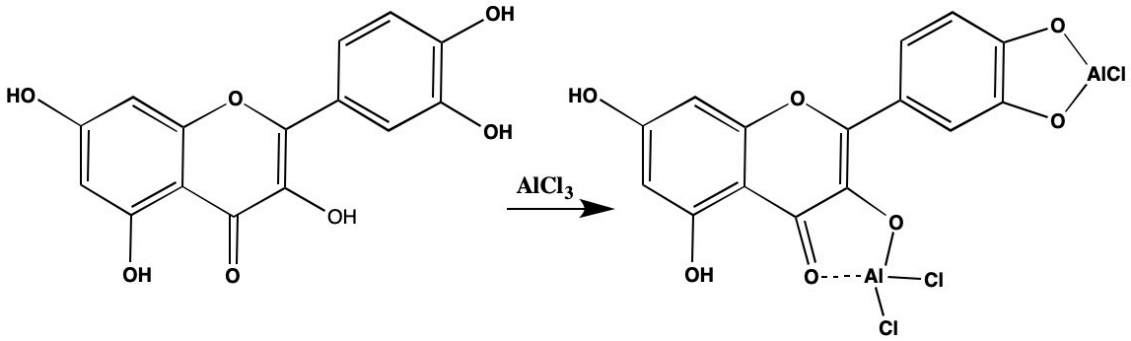


Fig. 20. Formation of flavonoid complexes with $AlCl_3$

II.5.3. Discussions

A mild detoxification that aims to reduce the bioavailability of heavy metals can be done resorting to natural solutions. Table 33 organizes the studied plant species based on the groups of active principles with chelating potential.

Table 33. Systematization of plant species according to the active principles with chelating potential

Plant species	Active principles
Crataegus sp. *Coriandrum sativum,* *Cynara cardunculus ssp. Scolymus*	poliphenols
Geranium robertianum *Mentha piperita* *Rosa canina*	tannins
Tilia sp. *Plantago sp.*	polysaccharides
Vaccinium myrtillus	anthocyanins

The possible mechanism of action is the inclusion of the metal into the structure of a complex (based on increased availability to polyvalent metals of hydroxyl, carbonyl, and carboxyl groups and also conjugated double bonds), thus its hydrophilic character increases so that it will not be absorbed, when it reaches the kidney it will be eliminated, and its potential for accumulation in the body will decrease.

Phenolic compounds have been described as electrondonating agents, and therefore they can act as antioxidants (Michalak, 2006), acting as reducing agents, hydrogen donors, and

singlet oxygen quenchers and preventing the evolution of oxidant-free radical and reactive species derived from metal catalysis by Fenton-like reactions (Jung et al., 2003).

Tannins exhibit strong antioxidant properties can result from their ability to chelate transition metal ions (Karamać, 2009).

A polysaccharide-peptide isolated from fruiting bodies of *G. frondosa* which was proved to have mercury eliminating effect both *in vitro* and *in vivo*. This suggests that this could serve as a new resource for developing antidotes for heavy metal intoxication which can be used with safety on a long term basis (Zhang et al., 2018).

Complexes of flavonoids have an impact on the reduction of toxic metals bioavailability. For example, the overload of aluminium leads to neurological disorders. Quercetin complex with aluminium(III) reduces the excess aluminium in diet. Therefore, they seem to be an appropriate antidote in heavy metal poisoning in vivo. Quercetin as a biologically active ligand can be a suitable chelator for molybdenum (VI). Its complex could be used in the case of molybdenum deficiency instead of molybdenum salts which cause toxic effects (Hider et al., 1991; Dobbin et al., 1993).

To eliminate the interfering effect of free heavy metal natural content of the plant material that is transferred to the infusions, determinations were carried out on aqueous extracts. The results showed that the free cations concentration of infusions was not detectable through the used method, as opposed to the extract prepared by digestion when the determinations were done for the total quantity of all forms in which the heavy metal was released from plant material (free and various combination forms).

A comparative analysis of the results (Table 28) revealed a large variation in the heavy metal content of the dried plant material. Cu^{+2} was predominant in all samples at various concentrations, while Hg^{+2} was absent in all samples. Also, the transfer of metal content from plant material to brewing varied from sample to sample. The Cu^{+2} and Ni^{+2} transfer occurred in various proportions while for Cd^{+2} and Pb^{+2} any amount transferred could not be assed through the analytical methods applied. There were significant differences between the results obtained through AAS and ISE.

Of the aqueous extracts tested, those of *Rosa canina*, *Mentha piperita*, *Geranium robertianum* and *Vaccinium myrtillus,* exhibited a measurable complexing capacity for the cations analyzed, while infusions prepared from *Crataegus sp.*, *Tilia sp.*, *Cynara cardunculus subsp. Scolymus*, *Plantago sp.* and *Coriandrum sativum* proved to have no such capacity.

Experimental data showed that among the extracts that previously showed complexing capacity for metal cations, only infusions of *Vaccinium myrtillus* have retained that capacity under simulated digestive system conditions, and it was much reduced and only for Cu^{+2}, Pb^{+2}, and Hg^{+2} cations (Table 29). Extracts of *Rosa canina*, *Mentha piperita* and *Geranium robertianum* that were active previously, had lost their complexing ability or it was reduced to a level undetectable through the method used. One possible explanation is that changing the structure of the active principles involved in the complexation because of the enzyme mix reduces the number of active centers available for attaching metal cations.

The high content of anthocyanins from *Vaccinium myrtillus* (Table 30), compared to the other species studied, involve these active principles into the explanation on complexing metal cations (Erdelyi Pop et al., 2015).

While plants are in need of some transition metals such as copper or zinc, other metals such as lead or cadmium are highly toxic. Therefore, plants need to adjust their metal homeostasis accordingly to limit exposure to the more toxic compounds (Clemens, 2001). It has long been known that metal toxicity can induce anthocyanin biosynthesis in plants in a concentration dependent manner (Nanda-Kumar et al., 1995; Chalker-Scott, 1999).

The metal-chelating ability of anthocyanins has been reported for W (Hale et al., 2002), Al (Schreiber et al., 2010), Cd (Park et al., 2012), Cu (Somaatmadja et al., 2006), Ga (Buchweitz et al., 2012), Fe (Buchweitz et al., 2012), Mo (Hale et al., 2001) and Zn (Park et al., 2012).

The increased complexity of anthocyanins at pH = 7, the pH at which it was performed our study, under a simulated digestive system, was also demonstrated for Fe (Xie et al., 2018).

The study proved that oral administration of infusions of *Vaccinium myrtillus* has a moderate effect on reducing digestive bioavailability of heavy metals.

II.5.4. Conclusions

This study was designed to find the best natural compound that can be safely used for chelating heavy metals.

Heavy metals can be found naturally in foods of vegetable origin, medicinal plants and tobacco or can contaminate these products as a result of human intervention through industrial and agricultural activities. The toxicity of these metals is partly due to the fact that they accumulate in biological tissues, a process known as bioaccumulation.

The complexing ability of aqueous herbal extracts of *Crataegus sp., Tilia spp., Rosa canina, Vaccinium myrtillus, Geranium robertianum, Mentha piperita, Cynara cardunculus subsp. Scolymus, Plantago sp.,* and *Coriandrum sativum* were researched on Cu^{+2}, Cd^{+2}, Ni^{+2}, Pb^{+2} and Hg^{+2} cations.

Out of all the aqueous extracts tested, those derived from *Rosa canina*, *Mentha piperita*, *Geranium robertianum* and *Vaccinium myrtillus* proved to possess a measurable complexing capacity for the studied cations. Under simulated digestive system conditions, the complexing capacity was preserved only for *Vaccinium myrtillus,* in a much lesser extent, and only for Cu^{+2}, Pb^{+2} and Hg^{+2} cations.

Research projects in the field:

- Internal Grant UMF no. 29238/20.12.2013 with title "*In vitro* evaluation of the effect of reducing digestible bioavailability of heavy metals for some plant extracts with possible applications in chelating agent therapy"; Duration 01.02.2014-31.07.2015.

III. Contributions to the development of novel Schiff bases with pharmaceutical applications

III.1. Introduction

The history of the Schiff bases begins in 1864, when the German chemist Hugo Schiff, afterwards a Nobel Prize winner, announced the first synthesis of a compound of this class, as result of the condensation of a primary amine with a carbonyl compound (Schiff, 1864). This discovery represents the debut of the studies concerning an important class of ligands with a special place in the chemistry of coordination combinations and which was named after Schiff.

From a structural point of view a Schiff base is an analogue of an aldehyde and a ketone in which the carbonyl group was replaced with an imine or an azomethine group, in specific conditions. It is a compound which contains as a functional group, a double carbon-nitrogen bond and in which the nitrogen atom is bonded to an aryl or an alkyl radical (Fig. 21).

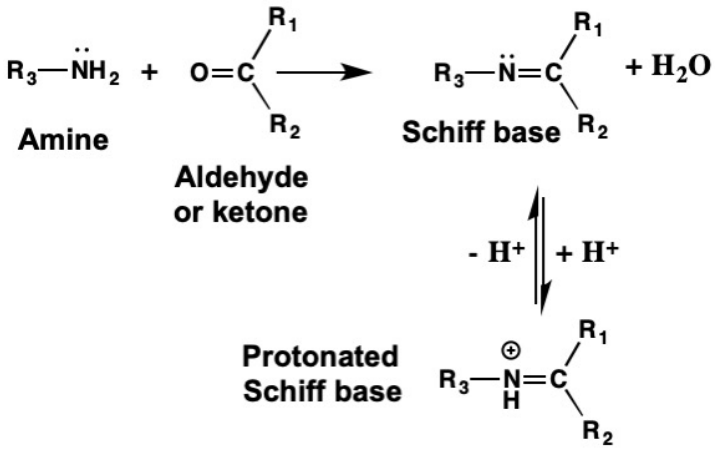


Fig. 21. Synthesis of Schiff base

Schiff bases present themselves in the shape of solid crystalline or liquid greasy substances. They behave as weak bases, forming salts with the acids in anhydride medium, and in acid watery solutions they hydrolyse with the forming of an amine and carbonyl derivate. Most of the Schiff bases are stable in alkaline solutions.

Schiff bases are excellent chelating agents (Jungreis et al., 1969), especially when near the azomethine group there is a functional -OH or -SH group, thus closing a cycle of five or six atoms with the metal ion. Thus the Schiff bases act as bidentate, bis-bidentate, tridentate, tetradentate, respectively polydentate ligands for the metal ions, forming stable complex combinations with a mononuclear, binuclear or polynuclear structure.

The simple synthesis and the structural diversity of the Schiff bases and of the formed metal complexes, as well as their chemical and physical attractive features have been the basis of the research regarding their application in various fields.

Due to their good capacity of coordination, Schiff bases are used as reagents in the spectrophotometric determination of metal ions (Özdemir 2019). The Schiff bases known as azomethine dyes are used for fibre dying (Agathian et al., 2018).

An interesting application of the Schiff bases is their use as corrosion inhibitors, based on their capacity of forming a resistant monolayer on the surface that needs protection (Dasami et al., 2015).

A large number of Schiff bases have been used in potentiometric sensors because they have shown selectivity and sensitivity for metal cations such as Ag (I), Al (III), Cu (II), Ni (II), Pb (II) and Co (II) (Abbaspour et al., 2002; Mahajan et al., 2003; Ganjali et al., 2003; Jain et al., 2005; Jeong et al., 2005; Gupta et al., 2006).

Schiff bases and their complexes are used as catalysers in various chemical processes (Jiao et al., 2016; Wang et al., 2019).

The use of Schiff bases in the medical field is based on their ability to interact with microorganisms. Various studies have shown that the biological activity is determined by the forming of a hydrogen bond with the active centres of the cellular components, through the imino group (Yusof et al., 2015).

Studies made on Schiff bases have shown antibacterial, antiviral, antifungal, antiprotozoal and antihelmintic activities. Also, some of them present significant anticonvulsant or anti-inflammatory activity (Anush et al., 2018).

Certain Schiff bases have shown to be antitumoral and antidiabetic active (Kaczmarek et al., 2018).

The studies were materialized in the following publications:

- Ţântaru G, Marin L, Vieriu M, Panainte AD, Poiata A, Apostu M, Bibire N. The influence of structure on antibacterial activity of some new aniline derived Schiff bases. *Rev Chim (Bucharest)* 2015; 66(12): 1965-1967.
- Ţântaru G, Nechifor M, Apostu M, Vieriu M, Panainte AD, Bibire N. Anti-inflammatory activity of an n, n'-disalicylidenemethylendiamine-derived Schiff bis base and its Copper (II) complex. Rev Med Chir Soc Med *Nat Iaşi* 2015; 119(4): 1195-1198.
- Ţântaru G, Poiata A, Bibire N, Panainte AD, Apostu M, Vieriu M. Synthesis and biological evaluation of a new Schiff base and its Cu(II) Complex. *Rev Chim (Bucharest)* 2017; 68(3): 586-588.
- Ţântaru G, Bibire N, Panainte AD, Vieriu M, Apostu M. Aniline Derived bis-Schiff base - analytical reagent for the assay of Fe (III). *Rev Chim (Bucharest)* 2018; 69(11): 3097-3099.
- Ţântaru G, Apostu M, Bibire N, Vieriu M, Panainte AD. Pyridine-derived Schiff base-analytical reagent for Iron (II) ions. *Med Surg J - Rev Med Chir - Soc Med Nat Iaşi* 2018; 122(3): 640-646.

III.2. Pyridine derived Schiff base - analytical reagents

The Schiff bases can be used as analytical reagents for the quantitative determination of important cations in the body. We have performed an analytical study on a Schiff base: 2-

salicylidene-amino pyridine (SB). It is an organic ligand obtained through the reaction of salicylaldehyde with 2-aminopyridine.

↳ *Synthesis of Schiff base*

The studied Schiff base (Fig. 22) is obtained by condensing the salicilaldehyde with 2-aminopyridine in methanol at reflux.

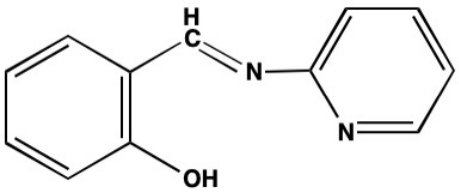


Fig. 22. Chemical structure of 2-(saliciliden) aminopyridine

The synthesized product, the Schiff base type NNO is presented under crystallized form with yellow-orange fine crystals with melting point at 69-70°C, insoluble in water, acetone benzene acetonitrile chloroform, but soluble in methanol and bimethyl sulphoxyde.

Schiff base is characterized by:

- The elemental analysis (Table 34).

Table 34. Elemental analysis

Element %	Calculated	Found
C	78.26	78.49
H	5.43	5.72
N	7.61	7.99

- The spectrum in IR obtained through dispersion in KBr has the following characteristic bands (cm^{-1}): 3400 (OH…N), 3000 (CH aliphatic), 1620 (C=N), 1450 (CH).
- The RMN spectrum ($CDCl_3$, TMS, 25°C) is characterized by the following chemical shifts (ppm): a: δ = 6.89-7.70 (aromatic nucleus, 8H); b: δ = 8.03 (CH=N); c: δ = 13.08 (1H, OH).

III.2.1. Pyridine derived Schiff base - analytical reagent for U(VI)

The greatest health risk from large intakes of uranium is toxic damage to the kidneys, because, in addition to being weakly radioactive, uranium is a toxic metal. Almost all uranium that is ingested is excreted during digestion, but up to 5% is absorbed by the body when the soluble uranyl ion is ingested while only 0.5% is absorbed when insoluble forms of uranium, such as its oxide, are ingested. However, soluble uranium compounds tend to quickly pass through the body whereas insoluble uranium compounds, especially when ingested via dust into the lungs, pose a more serious exposure hazard. After entering the bloodstream, the absorbed uranium tends to bioaccumulate and stay for many years in bone tissue because of uranium's

affinityfor phosphates. The UO_2^{2+} ion represents the uranium (VI) state and is known to form compounds such as the carbonate, chloride and sulfate. UO_2^{2+} also forms complexes with various organic chelating agents, the most commonly encountered of which is uranyl acetate. It is known that when the pH of a uranium (VI) solution is increased that the uranium is converted to a hydrated uranium oxide hydroxide and then at high pH to an anionic hydroxide complex. On addition of carbonate to the system the uranium is converted to a series of carbonate complexes when the pH is increased, one important overall effect of these reactions is to increase the solubility of the uranium in the range pH 6 to 8 (Clarke, 1982; Agrawa, 1985).

Starting from the complexing capacity of the Schiff bases derived by pyridine, the present paper presents the data of a preliminary study regarding the use of 2-(saliciliden)aminopyridine (SB) (Cascaval, 1996)*, as reagent for the spectrophotometric determining of U(VI).*

III.2.1.1. Materials and methods

↳ *Reagents*

All chemicals were of analytical-reagent grade. Stock solution U(VI) 0.1 mg/mL: 0.02109g $UO_2(NO_3)_2 \cdot 6H_2O$ is dissolved in 100 mL distilled water, then from this, standard solutions are prepared through dilution; Reagent solution 10^{-4} M, $5 \cdot 10^{-4}$ M, 10^{-3} M in methanol; Reagent solution 0.1% (m/v); Acetate buffer solution 0.2 M (pH = 5-7);

↳ *Apparatus*

A UV-VIS Hewlett-Packard 8453 spectrophotometer has been used for all determinations

↳ *BS-U(VI): Schiff base - U(VI) complex*

The characterized reagent (SB), through analytically active groups and analytically functional groups, has the ability to complex U(VI) forming a light red complex stable in acetone that has an absorption maximum of 440 nm. 1 mL of solution to be analyzed containing 5-50 µg/mL U(VI), is brought at the pH= 6.0 with acetate buffer, it is treated with 2 mL acetone, 1mL reagent solution 1% (m/v) in methanol. After 10 minutes, the extinction of the light red complex is read at 440 nm against a witness.

III.2.1.2. Results

An analytic study was carried out on the complexing reaction of U(VI), in order to establish the best working conditions.

↳ The complexing reaction of U(VI) is influenced by the value of the pH; in order to follow the variation of the absorbance according to the pH of the medium, buffer solutions were used with pH = 5.0-7.0 and a standard solution of 40 µg/mL U(VI); From figure 23 it results that the best pH for the SB-U(VI) complex is 6.0.

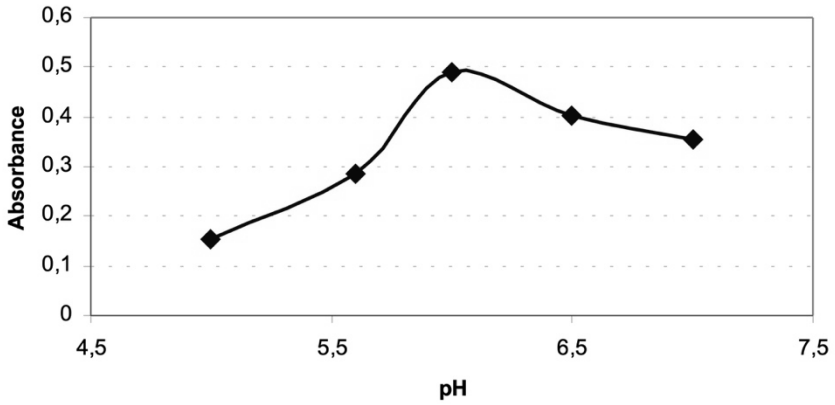


Fig. 23. Influence of the pH

It is studied the stability in time of the SB-U(VI) complex. The results are illustrated in figure 24 From the graphic it results that 10 minutes after the reagent is added, the stability of the complex is maximum and the value of the absorbance is maintained for 15 minutes.

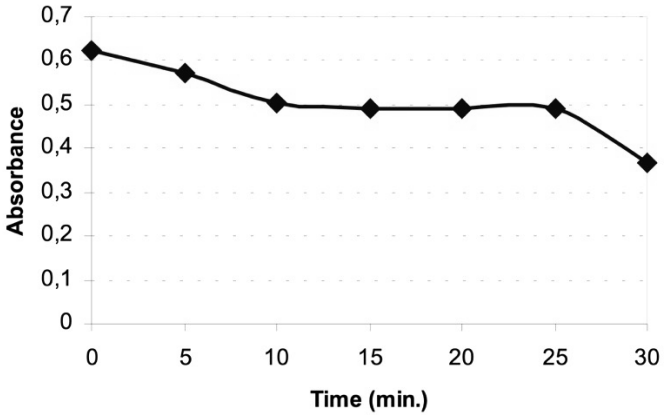


Fig. 24. Stability in time

The combining ratio is established through the method of isomolar series and is 1:3 (M/L) being illustrated in figure 25.

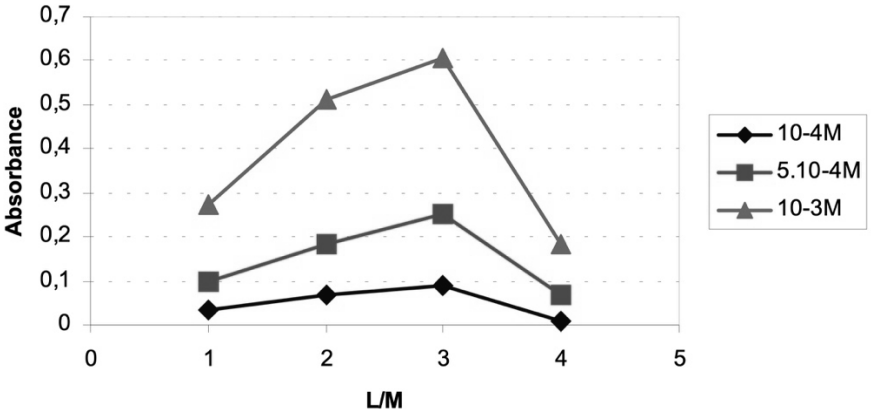


Fig. 25. Combining ratio

The stability constant is determined following the method of Harvey and Manning (Harvey et al., 1950) dissociation degree according to the relations:

$$Ki = \alpha^2 \times C/1-\alpha;\ \alpha = Am-As/Am;\ Ks = 1/Ki$$

were: α = dissociation degree; Am = maximum absorbance (0.6231); As = equilibrium absorbance (0.5015); C = concentration of the solutions of U(VI) and BS (10^{-3} M).

$$\alpha = 0.1951;\ Ki = 4.73\cdot10^{-5};\ Ks = 2.11\cdot10^{-4};\ \varepsilon = 2.5\cdot10^{3}\ mol^{-1}\cdot L\cdot cm^{-1}$$

↳ Calibration curve: the concentration range is set and the Lambert-Beer law is observed. The absorbance is proportional to the concentration in U(VI) for the range 5-50 µg/mL U(VI): correlation coefficient $r^2 = 0.9970$, the slope of the line is 0.0126, the intercept is 0.02038, the detection limit 2,6153 µg/mL, quantification limit 8,7179 µg/mL, SD = 0,0109.

III.2.1.3. Discussions

Following the study carried out on the complexing reaction of U(VI) with 2-(saliciliden) aminopyridine, a new spectrophotometric method of determining it is proposed.

2-(Saliciliden)aminopiridyne is a NNO tridentate Schiff base. Similarly to our study, Schiff base derived from pyridine were used for the spectrophotometric determination of some metals.

Schiff base prepared via condensation of pyridine-2,6-dicarboxaldehyde with 2-aminopyridine and its octahedral complexes with Cr(III), Fe(III), Co(II), Ni(II) and Th(IV) and tetrahedral complexes with Mn(II), Cd(II), Zn(II), and UO_2(II) have been reported (Abd El-halim et al., 2011).

Trivedi (Trivedi et al., 2007) synthesized nickel and copper complexes based on tridentate nitrogen donor ligand 2,6-bis (1-phenyliminoethyl) pyridine. The ligand was prepared by Schiff base condensation of 2,6-diacetyl pyridine with aromatic amines. The reaction of $CuCl_2\cdot2H_2O$ and $NiCl_2\cdot6H_2O$ in 1:1 molar ratio with the ligand in methanol or acetronitrile gave a distorted trigonal bipyramidal copper complex and octahedral nickel complex.

Hexavalent uranium forms coloured complexes with a number of organic chelating agents that can be quantified spectrophotometrically. The range of concentrations of the calibration curve, detection limit and quantification limit is comparable to the values obtained in our study.

An optimized and validated spectrophotometric method has been described for the quantitative analysis of uranyl ion based on the chelation with piroxicam to produce a yellow complex which absorbs maximally at 390 nm. Beer's law is obeyed in the concentration range of $6.75\cdot10^{-2}$-$9.45\cdot10^{-1}$ µg/mL with apparent molar absorptivity $4.11\cdot10^{5}$ $mol^{-1}\cdot L\cdot cm^{-1}$ (Khan et al., 2009).

2,2'-[1,2-phenylenebis(nitrilomethylidene)]bisphenol forms a yellow complex with U(VI). This complex in chloroform shows an intense absorption peak at 413.0 nm. It is observed that Beer's law is obeyed in the range of 2.0-10.0 µg/mL of metal solution with apparent molar absorptivity $3.69\cdot10^{4}$ $mol^{-1}\cdot L\cdot cm^{-1}$ (Satya et al., 2013).

A sensitive method for the determination of uranium using 2-(2-thiazolylazo)-p-cresol was described by Teixeira (Teixeira et al., 1999). The Beer's law is obeyed in the range from 0.30-12.0 μg/mL with a molar absorptivity of $1.31 \cdot 10^4$ $mol^{-1} \cdot L \cdot cm^{-1}$ and features a detection limit of 26 ng/mL at 588 nm.

3,4-dihydroxybenzaldehyde thiosemicarbazone synthesized by Veeranna (Veeranna et al., 2016) was used for the determination of uranium(VI) at pH 7. The developed method can be conveniently applied for the analytical determination of uranium (VI) in the concentration range 0.476-4.760 μg/ml. The molar absorptivity was found to be $2.08 \cdot 10^4$ $mol^{-1} \cdot L \cdot cm^{-1}$.

The developed method for the spectrophotometric determining of U(VI) is selective. The ions Fe(II) are complexed by the studied Schiff base but under different conditions, in the presence of KCl $2.5 \cdot 10^{-2}$ M, pH = 5.6 and the extraction of the chloroform complex, the maximum absorbance being of 525 nm. The ions of Bi(III) under the form of $K[BiI_4]$ are complexed by Schiff base under the form of a complex of pairs of ions that appear as a yellow fine precipitate with a maximum absorbance of 410 nm.

III.2.1.4. Conclusions

The proposed method gives a simple and inexpensive spectrophotometric procedure for determination of uranium.

2-(saliciliden)aminopyridine forms with U(VI) a light red compound, stable in acetone, with maximum absorbtion at 440 nm. The LambertBeer law was followed in the interval of concentrations from 5 to 50 μg/mL. The linear coeficient was 0.9909. The detection limit found was about 2.61 μg/mL and the combination ratio was established using the method of isomolar series at 1:3 (M/L). Molar extinction coefficient was established at $\varepsilon = 2.5 \cdot 10^3$ $mol^{-1} \cdot L \cdot cm^{-1}$.

The obtained results recommend the use of this reagent for the spectrophotometric quantitative determining in VIS of U(VI) with good results.

III.2.2. Pyridine derived Schiff base - analytical reagent for Fe (II)

Iron is an essential element for living organisms, as it is involved in the transport of oxygen and in cellular oxidative processes. It is found in amounts of 5 mg/kg bodyweight for men and 30 mg/kg bodyweight for women. Approximately 2/3 of all iron ions are found in circulating red blood cells in the composition of hemoglobin, while smaller percentage may be found in myoglobin (4%), hemic enzymes (0.2%), transferrin (0.12%) and about 25% is deposited as ferritin and hemosiderin.

Depending on the way iron they are bound, iron-protein complexes can be classified into three groups: hemoproteins, iron-sulfur proteins and iron-proteins. In hemoproteins, iron is incorporated into a system of four tetrapyrrole rings linked to proteins, such as myoglobin, hemoglobin, cytochrome c and enzymes such as cytochrom-oxidase, catalase and peroxidases. The binding of Fe to proteins occurs at certain sites of amino acids (Grecu et al., 1982; Alcantara et al., 1994; Gower et al., 1993).

This study presents the development and validation of a new spectrophotometric method for the quantitative determination of Fe (II) ions using 2-(saliciliden)aminopyridine (SB) (Cascaval, 1996)*, as reagent, and its application for the analysis of pharmaceutical products.*

III.2.2.1. Materials and methods

↳ *Apparatus*

A UV-VIS Hewlett-Packard 8453 spectrophotometer has been used for all determinations.

↳ *Reagents*

All reagents and solvents were analytical grade. A 0.1 mg/mL Fe (II) stock solution was prepared in distilled water, and then by suitable dilutions a 5-50 μg/mL Fe (II) standard solution was obtained. Reagent solution 0.1% (w/v) was prepared by dissolving 2-salicylidene-amino pyridine (SB) in methanol. A 0.2 M sodium acetate and acetic acid buffer solution with pH = 5.6 has been used.

↳ *Procedure*

The Fe (II) ions react with 2-salicylidene-amino pyridine (SB) at pH = 6.0, and a complex combination is formed. It is extracted in chloroform and its absorbance measured at 525 nm is proportional to the concentration of the cations.

The optimum wavelength for detection was selected and the optimum working conditions were established by studying the influence of pH, formation time, and the stability of the complex. The cation/ligand combination ratio, the stability conditional constant (β_n), and the limit of detection were calculated, and the potential interferers have been evaluated.

III.2.2.2. Results

The optimum working conditions of the complexation reaction have been established by studying the influence of pH, ionic strength, and combining ratio on the complexation reaction.

↳ The complexation reaction was influenced also by the pH of the solution. So 1 mL of 30 μg/mL Fe(II) standard solution was mixed with 1 mL sodium acetate and acetic acid buffer solutions with a pH that varied in the range 3.5-7.0.

0.5 mL KCl solution $2.5 \cdot 10^{-2}$ M and 1 mL 1% (w/v) SB reagent solution. After the extraction of the red complex in 5 mL $CHCl_3$ and separation, anhydrous sodium sulfate was used as desiccant, and the absorbance of the organic layer was measured at λ = 525 nm against a blank sample (Fig. 26).

The optimum pH value for the complexation was established to be 5.6 (Fig. 26). It was achieved using a 0.2 M acetic acidsodium acetate buffer solution.

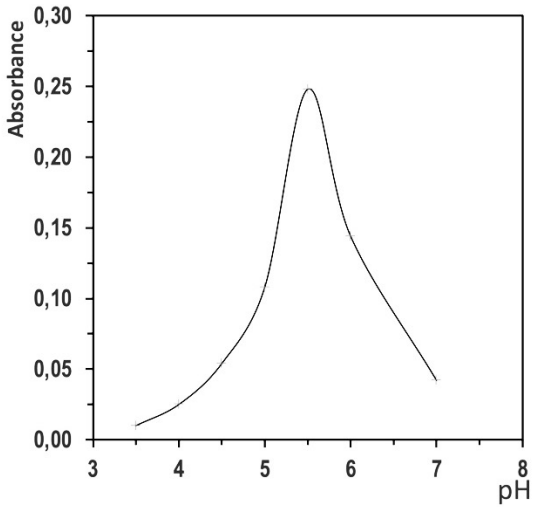


Fig. 26. Influence of pH on the absorbance of the SB-Fe (II) complex

↳ The complexation reaction was influenced by the ionic strength of the solution. Thus, the concentration and volume of the KCl solution that provided the ionic strength that maximized the absorption must be established. 1 mL of 30 μg/mL Fe(II) standard solution was treated at pH = 5.6 (0.2M sodium acetate and acetic acid buffer solution) with 0.5 mL solution with various concentration levels of KCl, 1 mL 1% (w/v) SB reagent solution. After extraction in 5 mL $CHCl_3$ and separation, anhydrous sodium sulfate was used to remove any traces of water, and the absorbance of the organic layer was measured at λ = 525 nm against a blank sample (Table 35).

Table 35. Influence of ionic strenght on absorbance

Fe(II) μg/mL	**KCl**	**1% SB reagent**	**$CHCl_3$**	**$A_{525\ nm}$**
30	$7.5 \cdot 10^{-1}$ M	1 mL	5 mL	0.140
30	$5 \cdot 10^{-1}$ M	1 mL	5 mL	0.147
30	$2.5 \cdot 10^{-1}$ M	1 mL	5 mL	0.155
30	10^{-1} M	1 mL	5 mL	0.167
30	$2.5 \cdot 10^{-2}$ M	1 mL	5 mL	0.248

The concentration and volume of the KCl solution providing the ionic strength for maximum absorbance of the complex was established based on the results shown in table 35. It was established that a volume of 0.5 mL of KCl $2.5 \cdot 10^{-2}$ M solution provided a μ = 0.005526 ionic strength and maximum absorbance.

↳ The influence of the number of extractions in chloroform of the complex combination on its absorbance was evaluated by comparing the results obtained when using 5 mL of organic solvent once versus using 2.5 mL of chloroform twice.

The results of the study on the variation of absorbance according to the number of extractions are shown in table 36, and they prove that one extraction was found to be effective.

Table 36. Absorbance variation depending on the number of extractions

Fe(II) µg/mL	**Buffer solution pH = 5.6**	**$2.5 \cdot 10^{-2}$ M KCl**	**1% SB reagent**	**$CHCl_3$**	**$A_{525\ nm}$**
30	2.5 mL	0.5 mL	1 mL	5 mL	0.250
30	2.5 mL	0.5 mL	1 mL	2.5 mL x 2	0.248

↳ The metal-ligand combination ratio was determined using various volumes of Fe (II) standard solution and mixing then with SB reagent solution with various concentration levels ($7 \cdot 10^{-3}$ M; $5 \cdot 10^{-3}$ M; 10^{-2} M), thus achieving the following combination ratios: 0.2, 0.3, 0.5, 0.7, 1.0, 1.5, 2.0, and 3.0. The mixtures were processed as previously described (Table 37).

Table 37. Absorbance variation depending on combination ratio

Fe(II):SB combination ratio	**$A_{525\ nm}$**		
	$5 \cdot 10^{-3}$ M Fe (II)	**$7.5 \cdot 10^{-3}$ M Fe (II)**	**$5 \cdot 10^{-2}$ M Fe (II)**
1:5	0.169	0.294	0.578
1:3	0.200	0.323	0.702
1:2	0.212	0.391	0.869
1:1.4	0.177	0.315	0.777
1:1	0.092	0.285	0.653
1:0.66	0.069	0.219	0.546

Based on the data obtained (Table 37) the ion: ligand combination ratio was plotted, and its value was 1:2.

↳ The stability constant (K_s) - expressed as $L \cdot cm^{-1} \cdot mol^{-1}$ - of the complex combination was determined using HarveyManning dissociation method based on the instability constant (K_i), according to the following equations (Harvey et al., 1950):

$$Ki = \alpha^2 \times C/1- \alpha;\ \alpha = Am-As/Am;\ Ks = 1/Ki$$

where: α = degree of dissociation, A_m = maximum absorbance, A = equilibrium absorbance, c = molar concentration of Fe (II).

A second method for calculating the stability constant (β_n) was based on the following equation (Mandrescu et al., 2009):

$$\beta_n = (A/A_m)/(1-A/A_m) \cdot n^2 \cdot C^n_L$$

where: A_m = maximum absorbance, A = equilibrium absorbance, n = the coordination number of the ligand, and C_L = molar concentration of the ligand.

Determination of complex stability constant was done using the graph method in order to determine the values for the maximum absorbance (Am = 0.890) and the equilibrium absorbance (A = 0.292), and then to calculate the instability constant of the complex $K_i = 7.3 \cdot 10^{-5}$ and the stability constants $K_s = 1.4 \cdot 10^4$, considering that n = 2, and the

concentration of the Fe (II) was 10^{-2} M. The calculated value of the molar coefficient was $4.16 \cdot 10^4$ $mol^{-1} \cdot L \cdot cm^{-1}$. The second method of calculating the stability constant produced results ($\beta_n = 1.5 \cdot 10^4$ and $1.45 \cdot 10^4$) which were very close to those obtained using the Harvey - Manning dissociation method.

The optimized procedure was: 1 mL Fe(II) standard solutions (5-50 μg/mL) were mixed with 2.5 mL sodium acetate and acetic acid 0.2 M pH = 5.6 buffer solution, 0.5 mL KCl solution $2.5 \cdot 10^{-2}$ M and 1 mL 1% (w/v) SB reagent solution. After one extraction of the red complex in 5 mL $CHCl_3$ and separation, anhydrous sodium sulfate was used as desiccant, and the absorbance of the organic layer was measured after 10 minutes at 525 nm against a blank sample in a 1 cm cuvette.

↳ During the validation of the method, it was established that the absorbance was proportional to the concentration in Fe (II) and the Lambert - Beer Law was obeyed in the 5-50 μg/mL range, with a correlation coefficient of 0.9946. The calibration curve is presented in fig. 3 and the linear equation was: $A = 0.008877 \cdot c - 0.02547$ (correlation coefficient $r^2 = 0.9946$, intercept = 0.008877, slope = 0.02547).

↳ The interference of Fe (III) on the complexation reaction at pH = 5.6 in the presence of KCl ($2.5 \cdot 10^{-2}$ M) has also been ruled out. The method could still be used for the indirect quantitative determination of Fe (III) after the reduction reaction to Fe (II) using reduction agents such as hydroxylamine or ascorbic acid. There were studies conducted that proved that the complexation reaction of Fe (II) using the Schiff base was not interfered by any other cation.

↳ The spectrophotometric determination method for Fe (II) using the Schiff base as reagent has been applied with very good results for the analysis of an antianemic pharmaceutical product formulated as syrup. The following procedure had been used: 2.5 mL syrup sample (7.2 mg/mL Fe (II)) was diluted with distilled water into a 100 mL graduated flask. 1 mL diluted sample solution was diluted even further with distilled water using a 10 mL graduated flask. 1 mL of solution was mixed with 0.5 mL sodium acetate and acetic acid 0.2 M pH = 5.6 buffer solution, 0.5 mL KCl solution $2.5 \cdot 10^{-2}$ M and 1 mL 1% (w/v) SB reagent solution. After one extraction of the red complex in 5 mL $CHCl_3$ and separation, anhydrous sodium sulfate was used as desiccant, and the absorbance of the organic layer was measured after 10 minutes at 525 nm against a blank sample in a 1 cm cuvette.

III.2.2.3. Discussion

Similarly to the proposed new method, Fe (II) forms complex combinations with other Schiff bases pyridine derivate.

Turan (Turan et al., 2017) described a Schiff base derived from the condensation of o-vanillin (3-methoxysalicylaldehyde) and methyl 2-amino-6-methyl-4,5,6,7-tetrahydrothieno [2,3-c]pyridine-3-carboxylate which form an octahedral complex with Fe(II). Isoniazid-p-diethylaminosalicylaldehyde hydrazone in Triton X-100 micellar medium form with Fe(II) a ternary complex. The apparent molar absorptivity at 471 nm was $2.3 \cdot 10^4$ $mol^{-1} \cdot L \cdot cm^{-1}$ (Issopoulos et al., 1992).

The combination ratio 1:2 [M:L] obtained for the 2-salicylidene-aminopyridine complex is also mentioned in the literature for other Schiff base - Fe (II) complexes.

Schiff base ligand derived from 3,3′-diaminobenzidine and salicylaldehyde and its divalent metal complex (Fe(II) were synthesized and characterized. Elemental and spectral data show that complexes are formed with 1:2 [M:L] molar ratio (Guzzi et al., 2013).

Abdel-Rahman (Abdel-Rahman et al., 2017) described synthesis and characterization of a tetradentate ONNO Schiff base ligand namely (1,10-(pyridine-2,3-dimethyliminomethyl) naphthalene-2,20-diol) and selected metal complexes, including Fe(II), as a central metal.

New ligand (Z)-2-(1-methyl-2-(pyridine-2-ylmethylene)hydrazinyl)benzoxazole reacts with Fe(II) ions what results in the formation of mononuclear complexes of 1:2 [M:L] stoichiometry, which crystallizes in the rare space group Ia-3d (Fik et al., 2015).

Several analytical methods have been developed for the quantitative iron analysis with a sensitivity close to our proposed method. These include flux injection analysis, potentiometry, voltammetry and atomic absorption spectrometry.

A simple flow injection method for simultaneous spectrophotometric determination of Fe(II) was proposed by Kozaka (Kozaka et al., 2011). The method is based on a spectrophotometric determination of Fe(II) at 530 nm with using a dedicated reversed-flow injection system. The range of concentrations of the calibration curve was 0.05-4.0 μg/mL.

Abounassif (Abounassif et al., 2011) described a novel poly (vinyl chloride) PVC membrane sensor for Fe^{2+} ions. The sensor is based on the use of newly synthesized chiral 2,6-bis-(carboxamide methyl ester) pyridine derivative as neutral ionophore in plasticized PVC membrane. The sensor displays a fast, stable and near Nernstian response over a relative wide ferrous concentration range ($1 \cdot 10^{-3}$ to $6 \cdot 10^{-6}$ M).

Differential pulse voltammetry was applied to a determination of the Fe(II) and a linear dynamic range from ($9.9 \cdot 10^{-7}$ to $2.9 \cdot 10^{-5}$ M) with a detection limit of $6.13 \cdot 10^{-8}$ M was obtained (Kumar et al., 2017).

A methodology to assay simultaneously iron and nickel present as contaminants in multimineral and multivitamin supplements was investigated by Rovasi (Rovasi et al., 2019). High-resolution continuum source graphite furnace atomic absorption spectrometry and direct solid sample analysis were used. The range of concentrations of the calibration curve was 10-85 μg/mL, $r^2 = 0.9970$.

III.2.2.4. Conclusions

We proposed a new spectrophotometric method for the determination of Fe (II) in antianemia pharmaceutical products, based on our study on the complexation reaction of those cations with 2-salicylidene aminopyridine. The Schiff base that was used as reagent, formed with Fe (II) at pH = 5.6 a red complex extractible in chloroform with a maximum absorption at $\lambda = 525$ nm. The Lambert-Beer Law was obeyed in the concentration range 5-50 μg/mL Fe (II), and the linearity coefficient was $r^2 = 0.9946$. The method has been applied successfully for the analysis of iron (II) ions in antianemia pharmaceutical products, and it could be also used for the indirect analysis of iron (III) ions.

III.3. Aniline derived bis-Schiff base - analytical reagent for Fe (III)

The biological role of metallic ions (Grecu et al., 1982) and that of their ligands in some physiological and pathological processes within the biological systems can be explained through the formation of complexes (Singh et al., 2009). Among the most important in vitro or in vivo biological ligands, there are aminoacids, Schiff bases and bis-Schiff bases, peptides, nucleotides, nucleic acids, proteins porphyrins and other multidentate organic bases (Singh et al., 2009).

Fe(III) can be quantitatively determined directly using a new spectrophotometric method. The optimum conditions for the complexation reaction using a type ONNO bis-Schiff base (Fig. 27) have been determined by studying the following parameters: reaction pH, formation time, stability in time of the complex, cation/ligand combination ratio, conditional stability constant (Cascaval, 1987; Andronescu et al., 2013).

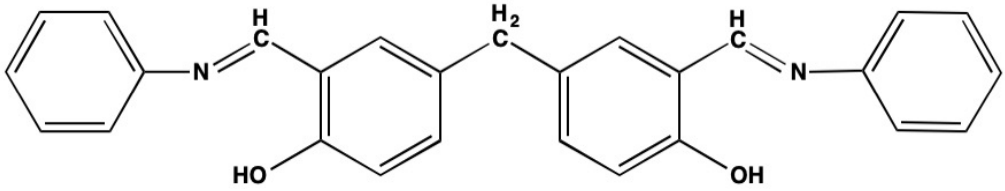


Fig. 27. Chemical structure of 4,4'-methylene bis-salicyliden aniline

III.3.1. Materials and methods

Fe(III) ions formed a complex combination with 4,4'methylen bis-salicylidene aniline (BSB) at pH = 4.5 and its absorbance measured at λ = 520 nm was proportional to the concentration of the ions.

↳ *Reagents*

All chemicals were of analytical-reagent grade. In order to evaluate the performance parameters of the method solutions in the 5-30 µg/mL Fe(III) concentration range have been used.

↳ *Apparatus*

A UV-VIS Hewlett-Packard 8453 spectrophotometer has been used for all determinations.

III.3.2. Results

↳ When establishing the optimum wavelength for the detection, 1 mL of 5-30 µg/mL Fe(III) solution was brought to pH 4.5 using acetate buffer, and 2 mL acetone and 1 mL BSB 1% (w/v) solution in methanol were added. After 10 min, the absorbance of the light-red complex was measured at 520 nm against the blank sample (Fig. 28).

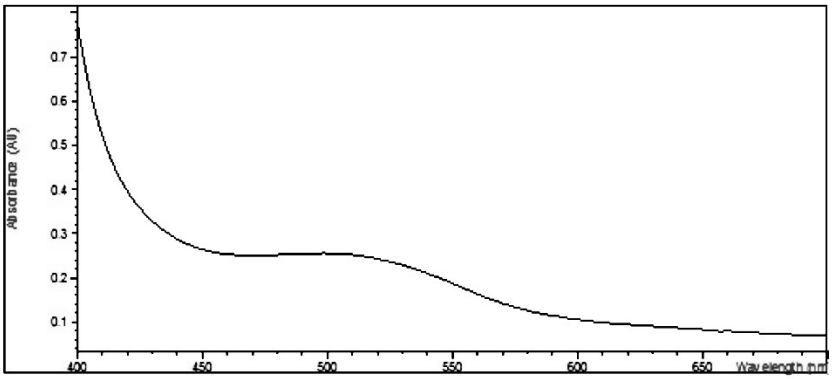


Fig. 28. Absorbtion spectrum of the complex

The absorption spectrum of the complex showed a maximum of absorbance at 520 nm.

➧ The influence of pH on the complexation reaction was studied. Figure 29 shows the absorbance in the 1.0-8.0 pH range. Optimum pH value was established at 4.5.

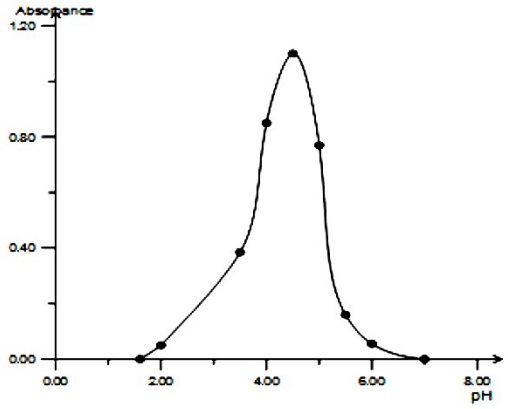


Fig. 29. The influence of pH on the complexation reaction

➧ The value of the stability conditional constant (βn) was established using the formula (Ţântaru et al., 2011):

$$\beta n = (\log C_M \cdot C_L) / (\log A - n \cdot \log V)$$

where: C_M = molar concentration of the metallic ions (Fe(III) ions), C_L = molar concentration of the ligand (BSB), A = absorbance of the metal-ligand complex measured at 520 nm, n = M/L molar ratio; V = the volume of the solution (5 mL); ε = molar extinction coefficient = $5.99 \cdot 10^4$ $mol^{-1} \cdot L \cdot cm^{-1}$.

According to the data collected, the obtained value of the stability conditional constant was $\beta n = 5.74 \cdot 10^{-5}$.

The combination rate was established using the isomolar series method and is 2:1 (M/L)

↳ In order to determine the linearity of the method 5-30 µg/mL Fe (III) solutions have been used. The obtained data was analyzed by linear regression and the calibration curve was obtained (Oprean et al., 2007). Detection and quantification limits were calculated using the following formulas: LOD = 3·Standard error/slope and LOQ = 10·Standard error/slope (Table 38).

Table 38. Linearity of the method

N°	Concentration (µg/mL)	Absorbance (520 nm)				
		I	II	III	IV	Average
1.	5	0.51532	0.51714	0.47974	0.50779	0.50499
2.	10	0.64515	0.63546	0.54848	0.61730	0.61160
3.	15	0.75579	0.76521	0.76276	0.76305	0.76170
4.	20	0.88098	0.88025	0.87866	0.86681	0.87668
5.	25	1.04576	1.03828	0.97101	0.97905	1.00853
6.	30	1.11095	1.15601	1.14776	1.153876	1.14215
Absorbance = 0.0257 x Concentration + 0.3685 *Regression coefficient r^2 = 0.9988, Standard error = 0.003028, Slope 0.0257*						

The obtained data was statistically evaluated (Table 38) and the calibration curve was obtained. The detection limit (LOD) and the quantification limit (LOQ) were calculated: LOD = 0.35 µg/mL and LOQ = 12.56 µg/mL.

↳ In order to determine the precision of the method (Roman et al., 1998; US EPA, 1995), three solutions containing 15, 20 and 25 µg/mL Fe(III) ions were used. Three assays were performed for each concentration level. The assay was performed twice in two different days in order to evaluate the intermediary precision (Table 38).

↳ In order to establish the accuracy of the method three Fe(III) solutions were used for three concentration levels: 15, 20 and 25 µg/mL. For each solution, three determinations were performed (Oprean et al., 2007; ICH, 2005) (Table 39).

Table 39. The method precision and accuracy

Fe(III) (µg/mL)	Method precision		Intermediate precision		Accuracy	
	Absorbance	Recovery %	Absorbance	Recovery %	Absorbance	Recovery %
15	0.75697	100.77	0.75355	99.88	0.75657	100.67
	0.74255	97.03	0.75459	100.15	0.74855	98.59
	0.74696	98.17	0.74566	97.87	0.75096	99.04
20	0.87668	98.87	0.87625	98.78	0.87658	98.85
	0.88193	99.89	0.87855	99.23	0.88096	99.70
	0.88941	101.34	0.88834	101.14	0.87341	98.23
25	0.99752	97.90	0.99584	97.64	0.99852	98.06
	1.00256	98.69	1.01895	101.24	1.01458	100.56
	1.01348	100.39	1.99421	98.24	1.00825	99.57
Statistic. data	*Mean recovery = 99.23%* *RSD = 1.46%*		*Mean recovery = 99.43%* *RSD = 1.30%*		*Mean = 99.25%* *Min = 98.06%* *Max = 100.66%*	

↳ The method was used for the determination of Fe(III) in effervescent tablets from a commercially available pharmaceutical product (Table 40) with multivitamins and multiminerals.

Table 40. Quantitative determination of Fe(III) from effervescent tablets

Pharmaceutical product	Labelled amount (μg/mL)	Assesesd amount (μg/mL)	Recovery (%)	RSD (%)
commercially available effervescent tablets	125	123±1.175	99.27	1.47

III.3.3. Discussions

A new spectrophotometric method for the quantitative determination of Fe (III) was established based on the complexation reaction with a new bis-Schiff base, 4,4'-methylenebis-salicylidene aniline.

The RSD was 1.32%, so the Fe(III) determination method using the VIS spectrophotometric method is precise. The sample concentration was calculated using the calibration curve equation. The relative standard deviation was lower than 2% (RSD = 1.46%) for each set of data.

For the accuracy study, the concentration of the sample was calculated from the experimental value of the absorbance, using the regression curve equation. We observed that the recovery was for the studied concentration range, the mean (minimum was 98.06% and maximum was 100.66% and the relative standard deviation was under 2% (RSD = 1.30%). These values proved that the Fe(III) determination method is accurate.

Several analytical methods have been developed for the quantitative Fe (III) analysis with a sensitivity close to our proposed method. These include spectrophotometric and inductively coupled plasma mass spectrometry

A highly sensitive and selective spectrophotometric method is proposed for direct trace determination of Fe(III) in aqueous solutions. The method is based on the reaction with a new analytical reagent 2-ethanolimino-2-pentylidino-4-one. The Fe(III) complex is detected at 440 nm and Bear-Lambert's law is obeyed in the concentration range 2.0-17.0 μg/mL for Fe(III) (Orabi et al., 2005).

A spectrophotometric method for quantitative determination of Fe^{3+} in a pharmaceutical formulation based on complexation with methylthymol blue was proposed by Totan (Totan et al., 2018). At 628 nm, Lambert-Beer law was obeyed to the concentration area of 2.0-6.0 μg/mL, $r^2 = 0.9997$.

A spectrophotometric method for determination of Fe (III) using green tea extract as reagent is proposed. The method is based on complex formation reaction between Fe and polyphenol compounds from green tea in buffered medium (pH = 4.8). Absorbance of Fe-polyphenol complex formed during the reaction was measured at 570 nm wavelength. The linear dynamic ranges are obtained from $1.0 \cdot 10^{-5}$ to $5.0 \cdot 10^{-4}$ M (Martinović Bevanda et al., 2017).

Whitmire (Whitmire et al., 2011) proposed inductively coupled plasma mass spectrometry method for iron in human plasma. The range of concentrations of the calibration curve was 0.5-20.0 µg/mL.

III.3.4. Conclusions

A new spectrophotometric method for the quantitative determination of Fe (III) was established based on the complexation reaction with a new bis-Schiff base, 4,4'-methylenebis-salicylidene aniline, when a stable complex with an absorption maximum at 520 nm was obtained. The conditions of the complexation reaction were established and the method was validated according to ICH guidelines in terms of linearity, accuracy, precision of the method and the limits of detection and quantification were determined. The method was applied with good results for the quantitative determination of Fe (III) in pharmaceutical products.

III.4. Synthesis and biological evaluation of novel Schiff base

The biological activity of Schiff bases has been attracting the attention of organic chemists and medical researchers for many years. Nowadays, Schiff bases have well known representatives in the groups of anticancer (Venkatesh, 2011), antimicrobial (Hussein et al., 2011; Sathe et al., 2011), anti-inflammatory (Pandey et al., 2011), antiviral, analgesic (Chinnasamy et al. 2010).

III.4.1. Synthesis and biological evaluation of a new Schiff base and its Cu(II) complex

Based on the above-mentioned applications of Schiff bases, this study presents the synthesis, physico-chemical characterization and antimicrobial effects of a new Schiff base and its Cu(II) complex.

III.4.1.1. Materials and methods

↳ *Reagents*

All chemicals and solvents were analytical reagent grade and they were supplied by Merck (Germany) and Chimopar (Romania).

↳ *Apparatus*

The melting points were determined using a Boethius apparatus without correcting the result. The IR spectra (from KBr pellets) were recorded on a FTS-135 BIO-RAD spectrometer. The UV-Vis spectra was obtained on a Hewlett-Packard 8453 UV-VIS spectrophotometer. Elemental analysis of C, H and N was carried out with an Elemental Vario Analyzer. The quantitative determination of Cu(II) was performed using the AAS-IN Carl-Zeiss-Jena spectrometer.

↳ *Synthesis and characterization of N-hydroxy-N'salicylidene-urea - $C_8H_8N_2O_3$*

0.01 mol (0.76g) of hydroxyurea dissolved in 10 mL of methanol was mixed with 0.01 mol (1.06 mL) of salicylaldehyde dissolved in 30 mL of methanol and then it was refluxed for 2-4 h. The reaction mixture was concentrated in vacuo and after the addition of ethyl ether, a brown solid precipitate was collected. It was washed with a 2:1 ether/ethanol mixture and then it was crystallized from a diethyl ether.

The ligand (Fig. 30) was a brown crystalline powder, stable at room temperature, insoluble in water, soluble in ethanol and methanol, very soluble in acetone and dimethylformamide (DMF).

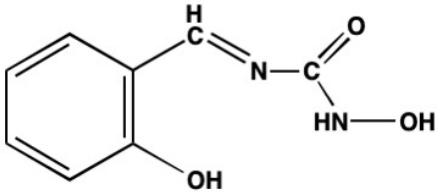


Fig. 30. Chemical structure of N-hydroxy-N'salicylidene-urea

↳ *Synthesis and characterization of Cu(II) complex - $Cu[C_8H_6N_2O_3 \cdot H_2O]$*

The Cu(II) complex was synthesized using the general procedure reported previously (Pignatello et al., 1994). 1.99 g (0.01mol) of $Cu(OAc)_2 \cdot H_2O$ dissolved in 25 mL methanol was added drop wise to 1.8 g (0.01mol) of ligand dissolved in advance in 25 mL methanol. The mixture was stirred at room temperature for 4 hours and then it was evaporated at 90°C, until the solution darkened; sparkling black micro-crystals were filtered, washed with a mixture of ethanol-water (1:1, v/v) and then with ethyl ether.

The Cu(II) complex was a green crystalline powder that was stable at room temperature, insoluble in water, ethanol, benzene or chloroform, soluble in methanol, DMSO and DMF.

↳ *Evaluation of antimicrobial activity*

The antimicrobial activity of the ligand and the copper (II) complex were evaluated against Gram-positive bacteria - *Staphylococcus aureus* ATCC 25923 (*Sa*), *Bacillus cereus* ATCC 14579 (*Bc*), *Bacillus subtilis* ATCC 6633 (*Bs*), and Gram-negative bacteria - *Escherichia coli* ATCC 25922 (*Ec*), *Pseudomonas aeruginosa* ATCC 9027 (*Pa*). Chloramphenicol and Ampicillin were used as reference substances.

The qualitative antimicrobial assay of the compounds was performed using the agar diffusion method according to standard accepted disk sensitivity criteria of The National Committee for Clinical Laboratory Standards (Brown et al., 1979; N.C.C.L.S. 1999) .

The agar dish diffusion procedure is a method approved by the National Committee for Clinical Laboratory Standards and was one of the first methods for evaluating the *in vitro* efficacy of antimicrobial agents. The microbiological assay is one in which the antimicrobial agent placed in a reservoir (paper disc, cylinder), diffuses directly against seeded bacteria.

A standard suspension of each reference strain was prepared from fresh overnight cultures and it was mixed with 15 mL portions of molten nutrient agar in sterile Petri plates, resulting a final concentration of about 10^6 cells/mL. The plates were sliced with solid metal cylinders (6 mm in diameter) and then 0.2 mL samples solutions of ligand and complex and

standard commercial disks of 10 µg Ampicillin and 30 µg Chloramphenicol were transferred into each well. Each microorganism was tested in triplicate and the zones of inhibition around the wells were measured after incubation at 37°C for 24 h. The diameter of the inhibition zones were evaluated as mean ± SD.

III.4.1.2. Results

Schiff base is characterized by:

- The elemental analysis for $C_8H_8N_2O_3$ is presented in table 41

Table 41. Elemental analysis

Element %	Calculated	Found
C	53.34	53.98
H	4.48	4.75
N	15.55	15.69

- Yield 75.7%; m.p. 158-160°C;
- The spectrum in UV-VIS, λ_{max} (DMF - nm) / ε ($mol^{-1} \cdot L\ cm^{-1}$): 282 / 3.10, 325 / 3.27;
- The spectrum in IR obtained through dispersion in KBr has the following characteristic bands (ν, cm^{-1}): 3390 (-OH aril), 1665 (C=O), 1064 (C-N), 1685 (C=N), 1370, 760 (C_6H_5), 1280 (-OH arom);
- The H-NMR spectrum ($CDCl_3$, TMS, 25°C) is characterized by the following chemical shifts (ppm): δ11.31-11.45 (s, 2H, OH), 7.26-7.49 (m, 3H, H-Ar), 8.51 (s, 1H, CH=N), 5.45 (s, 1H, NH).

The Cu(II) complex is characterized by:

- The elemental analysis for $Cu[C_8H_6N_2O_3 \cdot H_2O]$ is presented in table 42.

Table 42. Elemental analysis

Element %	Calculated	Found
C	36.99	37.06
H	3.10	3.25
N	10.78	10.85
Cu	24.47	24.51

- Yield: 65.3%; m.p. 179-180°C;
- The spectrum in UV-VIS, λ_{max} (DMF - nm) / ε ($mol^{-1} \cdot L\ cm^{-1}$): 355 / $8.09 \cdot 10^4$, Ks = $6.91 \cdot 10^5$; solubility (mol/L): $5.08 \cdot 10^{-4}$;
- The spectrum in IR obtained through dispersion in KBr has the following characteristic bands (ν, cm^{-1}): 1610 (C=N), 1668 (C=O), 1055 (C-O), 1360, 1370, 740 (C_6H_4), 528 (Cu(II)-N), 505 (Cu(II)-O);

• The H-NMR spectrum ($CDCl_3$, TMS, 25°C) is characterized by the following chemical shifts (ppm): 7.28-7.50 (m, 5H, H-Ar), 9.78 (s, 1H, CH=N).

The antimicrobial activity was correlated to the ability of the compounds to diffuse through biological membranes to reach its site of action. The cylinder technique was used for testing because it was more sensitive than paper discs technique. The antimicrobial activity was estimated by measuring the diameter of the area inhibited by the Cu (II) complex when compared to that of the ligand. Table 43 summarized the antimicrobial activity of tested compounds against Gram-positive and Gram-negative reference strains, in comparison to Ampicillin and Chloramphenicol.

Table 43. *In vitro* antimicrobial activity of the ligand and Cu(II) complex against Gram-positive and Gram-negative straines through the diameter of the inhibition zone (mm)

Bacteria	**Antimicrobial agent**			
	Ligand	**Cu(II) complex**	**Ampicillin**	**Cloramphenicol**
Pa	22.70±0.29	25.30±0.55	24±0.22	24±0.22
Sa	16.30±0.57	19.66±0.52	30±0.27	29±0.53
Bc	20.20±0.53	25.70±0.53	27±0.52	28±0.42
Bs	18.30±0.42	22.70±0.27	28±0.45	30±0.37
Ec	19.30±0.33	22.50±0.28	23±0.38	26±0.35

III.4.1.3. Discussions

A new Schiff base ligand, N-hydroxy-N'-salicylidene-urea was synthesized through the condensation of salicylaldehyde with hydroxyurea. The antimicrobial activity of the copper (II) complex was evaluated through comparison to the activity of the Schiff base on various bacterial strains.

The structures of the ligand and its complex was confirmed using spectroscopic methods and elemental analysis. The UV-VIS spectrum of the ligand included a large absorption peak at 282 nm that shifted to 355 nm in the UV-VIS spectrum of its Cu(II) complex due to the ligand's coordination with the metallic ion.

The IR spectrum of the ligand included a characteristic band at 1685 cm^{-1} which was due to vibration of C=N group. The shifting of that group to a lower frequency (1610 cm^{-1}) in the spectrum of Cu(II) complex implied the coordination of the metal ion through the nitrogen atom of the azomethine group. It was expected that the coordination of nitrogen to the metal atom would reduce the electron density in the azomethine group and thus lower the C=N group absorption. The band at 1665 cm^{-1} attributed to the vibration of C=O group in the spectrum of the ligand also shifted to a lower frequency (1668 cm^{-1}) in the spectrum of its Cu(II) complex, which implied that the oxygen atom of the C=O group was not linked to the metal ion. The band at 1280 cm^{-1} assigned to the stretching frequency of phenolic C-OH bond and the band at 3390 cm^{-1} both observed in the spectrum of the ligand disappeared from the spectrum of the complex.

Two new bands which were not present in the spectrum of the ligand, appeared in the spectrum of the complex at 505 cm^{-1} and 528 cm^{-1} corresponding to vibration of M-O and M-N groups. The appearance of those bands proved the involvement of N and O atoms in the complexation of Cu(II). The complex exhibited a broad and relatively intense band around 3400 cm^{-1} which indicated the presence of water molecules. That band corresponded to the vibration of O-H stretching. That band was accompanied by two other bands in the 700-800 cm^{-1} range in the spectrum of the complex. That fact suggested that the water molecules were coordinated.

The H-NMR spectrum of the complex possessed significant modifications due to the coordination process in reference to that of the ligand. The proton signal of the -OH (11.45 ppm) and the -NH (5.45 ppm) groups from the structure of the ligand disappeared upon complexation with Cu(II). The aromatic protons did not seem to register significant changes as a result of the coordination process.

The results of the elemental analysis of ligand and its complex were found to be in good agreement with the values that had been theoretically calculated.

The Cu(II) complex showed a higher antimicrobial action than the free ligand and that effect was evident against all reference bacteria tested. The Cu(II) complex was more active than the ligand because of the coordination involving -OH and the nitrogen atom of C=N group.

The association of the ligand with Cu(II) substantially increased the in vitro susceptibility of Gram-positive and Gram-negative tested bacteria. Against *Staphylococcus aureus* ATCC25923, both ligand and its Cu(II) complex exerted the lowest degree of antimicrobial activity when compared to sporulated bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*.

Both tested substances showed at concentrations of 10 μg/mL an antimicrobial profile similar to that of ampicillin and chloramphenicol (30 μg/disk) against *Bacillus cereus*, *Escherichia coli* and *Pseudomonas aeruginosa*. On the contrary, *Staphylococcus cereus* and *Bacillus subtilis* were less sensitive to these compounds than *Bacillus cereus*, *Escherichia coli* and *Pseudomonas aeruginosa*. The data of table 43 shows large inhibition zones of microbial growth. The differences in activity probably reflected the differences in the mode of action of their chemical structures against the bacterial cell.

Several studies proved that Schiff bases and their complexes with various cations which exhibit antibacterial activities have been reported (Faizu et al., 2007; Joginder et al., 2017; Shukla et al., 2017).

III.4.1.4. Conclusions

The research study reported the successful synthesis and antimicrobial activity evaluation of a new Schiff base and its Cu(II) complex. Both substances were physically and chemically characterized through elemental analysis, ^{1}H-NMR, UV-Vis and IR analysis, the ratio of metal/ligand combination, the melting point and the solubility. The tested compounds were active against both gram-positive and gram-negative bacteria. The antimicrobial activity of the complex resembled that of Ampicillin and Chloramphenicol. Also, all tested bacteria revealed a lower sensitivity against the ligand than against the complex.

III.4.2. Anti-inflammatory activity of an N,N'disalicylidenemethylendiamine-derived Schiff bis base and its copper(II) complex

The complex combinations of Schiff bis bases with various cations represent a class of compounds with very important properties from a chemical and biological point of view.

*This paper describes the evaluation of the anti-inflammatory action of a new Schiff bis base (*Cascaval, 1987) *derived from N,N'disalicylidenemethylendiamine and its copper (II) complex (Fig. 31).*

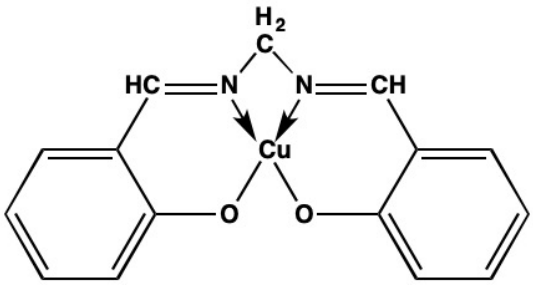


Fig. 31. Chemical structure of copper (II) complex combination

III.4.2.1. Materials and methods

↳ *Reagents*

For the study of the anti-inflammatory activity of the Schiff bis base and its copper (II) complex the following reagents were used: carrageenan kalium salt, indomethacin and carboxymethyl cellulose purchased from Merk - Germany.

↳ *The evaluation of the anti-inflammatory effect*

The evaluation of the anti-inflammatory effect was performed using the carrageenan-induced rat paw oedema assay (Directive 2010/63/Eu). For each substance we used six groups of adult male Wistar rats, 180-200 g, bred in laboratory condition and identically fed. Acute inflammatory paw oedema was induced by injecting 0.2 mL carrageenan 2% solution in the left posterior paw of the rat. Paw volume was determined before and after the test substance was administered and also after 1h, 2h, 4h, 6h, 8h or 24h. A control group of six Wistar received an identical carrageenan injection in order to achieve acute inflammatory paw oedema, but no any other substance. Paw volume of the control group was determined at the same intervals as the test groups.

We used the following groups of rats:

- group I was the control group;
- group II received indomethacin sodium salts 10 mg/Kg i.p.;
- group III received 10 mg/Kg of Schiff bis base;
- group IV received 10 mg/Kg copper (II) complex;
- group V received 5 mg/Kg copper (II) complex.

The anti-inflammatory activity was evaluated as the variation of inflammation volume (tenths of mL). The average sum and standard deviation of that parameter were calculated for every group of rats and then they were compared to those of the control group. The obtained data were statistically analyzed using ANOVA one way test.

In vivo, the anti-inflammatory activity of the copper (II) complex in comparison with that of the Schiff bis base was tested using 2% (w/v) carrageenan potassium salt solutions and 2% (w/v) indomethacin in 0.1% sodium carboxymethyl cellulose solution. The stock solutions of the complex and ligand were prepared in concentrations of 0.1% (w/v) in methanol.

III.4.2.2. Results

The method used to test the inflammation was oedema experimentally induced through carrageenan in rats (adult Wistar rats, male of 180-200 g). The anti-inflammatory activity of the investigated compounds is shown in table 44.

Table 44. Influence of the Schiff bis base and copper (II) complex on carrageenan-induced inflammatory oedema in rats - mean oedema volume variation (tenths of mL) ± SD

Group		Time						
		0	**1h**	**2h**	**4h**	**6h**	**8h**	**24h**
I	*Control*	18.17±1.02	27.83±0.62	30.17±0.32	35.17±1.06	38.83±2.36	38.5±1.80	23.67±1.18
II	*Indomethacin 10 mg/Kg*	22.3±4.05	25.30±2.71	26.90±2.04	25.02±3.21	24.80±2.25	25.32±3.61	24.55±2.77
III	*Schiff bis base 10 mg/Kg*	18.50±0.62	26.33±1.42	28.33±0.80	30.83±0.63 * P<0.05	31.00±3.20 * P<0.001	29.83±3.62 * P<0.001	20.83±0.86 * P<0.01
IV	*Copper (II) complex 10 mg/Kg*	19.67±1.18	28.67±0.86	31.83±1.72	27.17±3.76 * P<0.001 ** P<0.001	22.67±7.14 * P<0.001 ** P<0.001	21.50±7.54 * P<0.001 ** P<0.001	21.33±1.56 * P<0.001 ** P<0.05
V	*Copper (II) complex 5 mg/Kg*	18.83±0.78	25.83±0.47	26.50±080	32.50±0.95 * NS ** NS	31.33±7.78 * P<0.05 ** NS	30.67±9.96 * P<0.001 ** NS	22.50±0.08 * NS ** NS

** P values compared with control group*

*** P values compared with group receiving Schiff bis base*

III.4.2.3. Discussions

In vivo, the anti-inflammatory activity of the metallic complex in comparison with the activity of the Schiff bis base was tested by the method of Winter and co-workers using the Levy technique.

Our study on the anti-inflammatory activity of a new Schiff bis base and of it is copper (II) complex combination showed that the Schiff bis base exhibited significant anti-inflammatory action in acute experimental inflammation when compared to the control group.

The data showed (Table 44) that the anti-inflammatory effect of the Schiff bis base (10 mg/Kg) was statistically significant compared to the control group, after 4h ($P<0.05$). The effect increases at 6h and 8h, respectively ($P<0.001$).

The copper (II) complex presented anti-inflammatory activity compared with the control group, the effect being significantly stronger at a dose of 10 mg/Kg, beginning with 4h ($P<0.001$). The copper (II) complex at a dose of 5 mg/Kg also presented strong anti-inflammatory effect at 4h, 6h and 8h ($P<0.001$), compared with the Schiff bis base (10 mg/Kg).

The copper ions enhanced the anti-inflammatory effect of the Schiff bis base in its complex combination, the effect is stronger at doses of 10 mg/Kg copper(II) complex.

The Schiff bis bases and its copper (II) complex had an anti-inflammatory effect comparable to that of indomethacin.

According to the literature, several studies proved that Schiff bis bases and their complexes with various cations (Cu(II), Mg(II), Ni(II), Co(II) etc.) reduce the synthesis of some chemical mediators of acute inflammation such as leukotrienes which are involved in the formation of free radicals (Tian-Rong et al., 2007; Chen et al., 1987; Colman et al., 2004).

III.4.2.4. Conclusions

The copper(II) complex combination of N, N'-disalicylidenemethylenediamine and the Schiff bis base were investigated for anti-inflammatory activity. Carrageenan-induced paw oedema method was used to determine the anti-inflammatory activity in rats. The results show the Schiff bis bases and its copper (II) complex had an anti-inflammatory effect comparable to that of indomethacin.

III.4.3. The influence of structure on antibacterial activity of some new aniline derived Schiff bases

Among the reported Schiff bases, salicylaldehyde derivatives showed antibacterial and antifungal activity (Felton et al., 1947), but a systematic study regarding their structure-activity relationship wasn't reported so far.

That was the reason why, we have started a complex study by designing Schiff bases that contained the hydroxyl unit in different positions and vicinities, with the aim of clarifying the role of these functionalities in their antimicrobial activity. A model Schiff base without any substituent has been used for comparison, too.

III.4.3.1. Materials and methods

↳ *Reagents*

All chemicals were of analytical-reagent grade. The aniline and aldehyde reagents were purchased from Aldrich and used without further purification. Acetonitrile, methanol and dimethyl formamide were purchased from Carl Roth and used after drying on molecular sieves.

For the study of the antimicrobial activity the following strains were used: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Candida albicans* ATCC

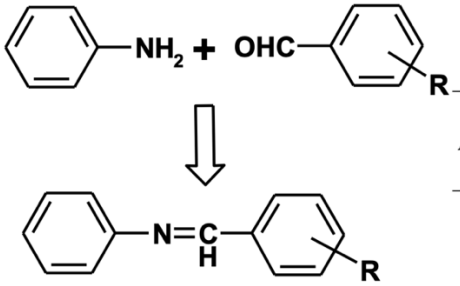
10231. Tetracicline, Ampicillin, Nystatin purchased from Himedia Spaine were used as reference substances.

↳ *Apparatus*

The infrared (IR) spectra were recorded on a FT-IR Bruker Vertex 70 Spectrophotometer in the transmission mode, by using KBr pellets. The proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded by a Bruker Avance DRX 400 MHz spectrometer using $CDCl_3$ as solvent and tetramethylsilane (TMS) as internal reference substance.

↳ *Synthesis of Schiff base*

The azomethine compounds had been synthesized through the condensation reaction of aniline with different aldehydes, in a 1:1 molar ratio (Fig. 32).



Imine code	BsA1	BsA6	BsA5	BsA3
Aldehyde code	A1	A6	A5	A3
R	-H	p-OH	o-OH	p-OH m-OCH_3

Fig. 32. Synthesis of the target azomethines

The reagents were dissolved in acetonitrile to give a 30% solution and the obtained reaction mixture was placed into a round bottom flask fitted with condenser and nitrogen inlet. The reaction mixture has been gently refluxed over night and then concentrated by rotary evaporation. The crude product was recrystallized twice from ethanol to give single crystals by high purity and then dried under vacuum 24 h. The yield was around 90 %.

↳ *Evaluation of antimicrobial activity*

Testing antimicrobial activity While testing the in vitro qualitative antimicrobial activity, the imine compounds had been codified as BsA1, BsA3, BsA5, BsA6, and the aldehyde reagents had been codified as A1, A3, A5, A6.

Testing procedures were validated according to the guidelines of the National Committee for Chinical Laboratory Standards (NCCLS 1990). The reference strains tested were: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231 that were supplied by the Microbiology Department from "Grigore T. Popa" University of Medicine and Pharmacy Iasi, Romania.

Mueller-Hinton agar (Difco) was used for bacteria strains and Sabouraud agar (Difco) was used for Candida. The inoculums were prepared by diluting over sight cultures of the organisms in sterile 0.9% NaCl and adjusting the turbidity to 0.5McFarland (about 10^8 cfu/mL). The media was prepared using 0.5 mL of each tested strain mixed with 15 mL portions of molten agar in a sterile Petri dish. After solidification, 0.1 mL solution of each compound was brought into 8 mm wells drilled into the surface of the medium. The final concentration for all tested compounds was 100 µg/mL. The in vitro activity of the compounds was compared to that

of standard antibiotic discs of ampicillin 10 μg, tetracycline 30 μg and Nystatin 100 μg. The plates were incubated for 24h at 37°C and the diameters of the inhibition zones of the microbial growth around the holes were measured (NCCLS, 1990). Each essay in that experiment had been done twice.

III.4.3.2. Results

- The FTIR spectrum of the BsA1 azomethine is given as an example in figure 33.

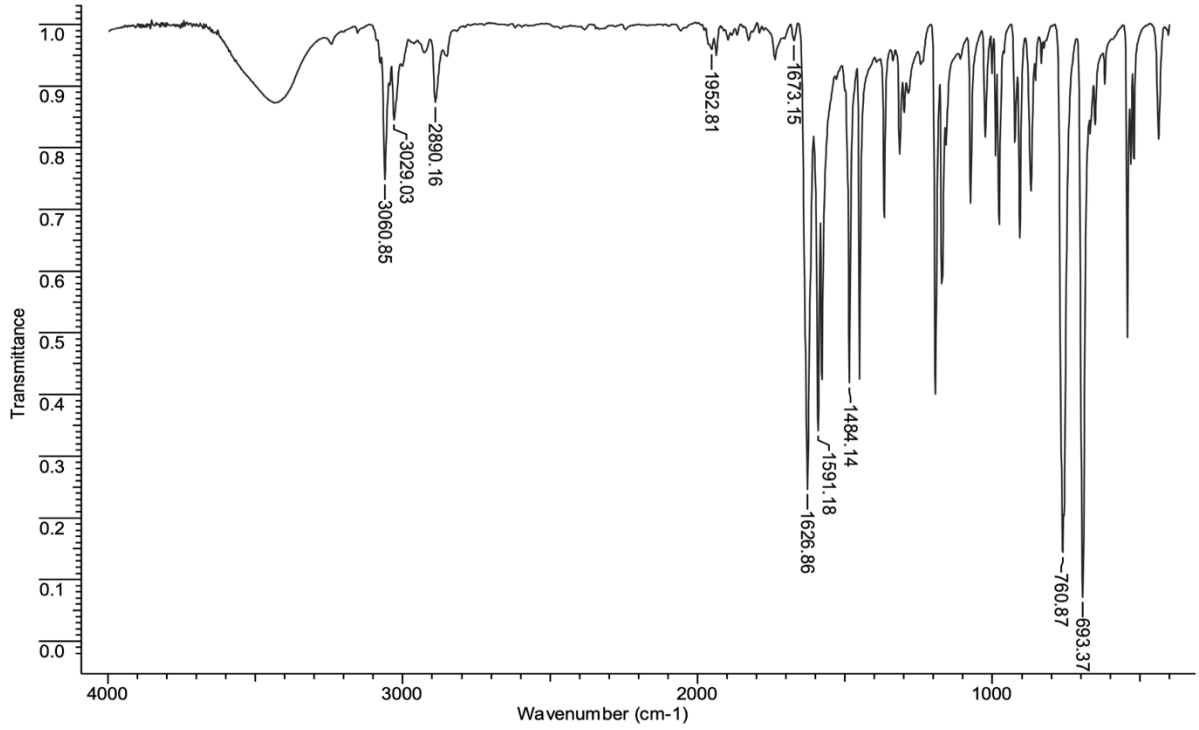


Fig. 33. FTIR spectrum of BsA1

- In figure 34, the ^{1}H-NMR spectrum of the BsA1 is presented as an example.

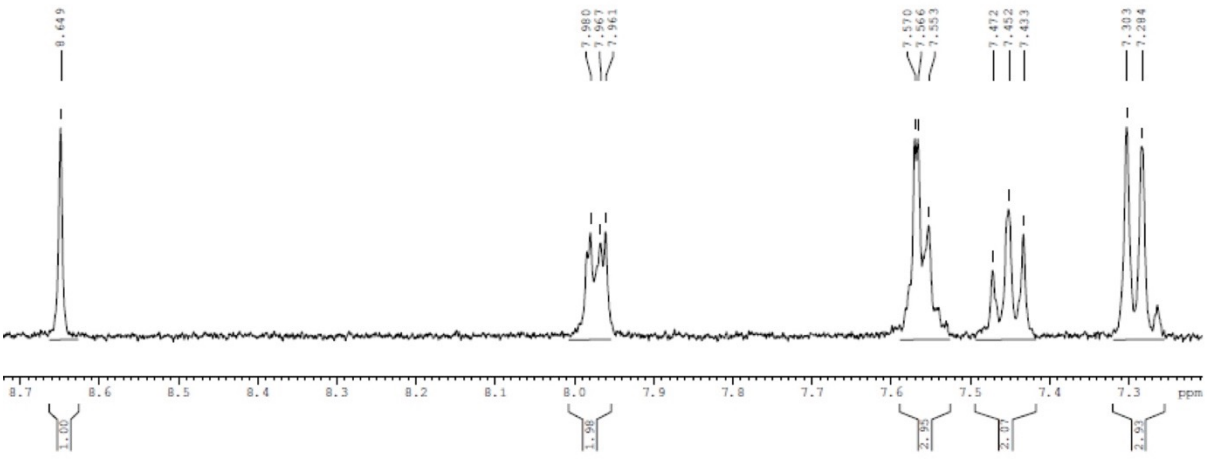


Fig. 34. ^{1}H-NMR spectrum of BsA1

• The antimicrobial activity of the investigated compounds is shown in table 45.

Table 45. *In vitro* antimicrobial activity of the Schiff bases compared to corresponding aldehyde reagents

Nº	**Diameter of inhibition zone (mm)**			
	Sample	***S. aureus* ATTC 25923**	***E. coli* ATTC 25922**	***C. albicans* ATCC 10231**
1.	*A1*	13±0.62	11±0.70	24±0.52
2.	*BsA1*	13±0.47	13±0.62	25±0.51
3.	*A3*	16±0.80	13±0.55	15±0.62
4.	*BsA3*	0	12±0.86	15±0.61
5.	*A5*	0	14±0.72	19±0.47
6.	*BsA5*	15±0.51	7±1.02	21±0.50
7.	*A6*	15±0.52	11±1.18	25±0.57
8.	*BsA6*	16±0.62	20±.060	26±0.61
9.	*Ampicillin*	32±0.52	20±0.57	-
10.	*Tetracycline*	31±0.32	25±0.62	-
11.	*Nystatin*	-	-	22±0.57

III.4.3.3. Discussions

Schiff bases represent an important class of organic compounds which present a wide range of biological activities, including antibacterial, antiviral, antifungal, antimalarial, anti inflammatory, anti-cancer, anti-HIV, antihelmintic and antipyretic.

In the presented studies a series of aniline derived Schiff bases were synthesised and have been subjected to a screening of their biological *in vitro* activity on bacteria or fungi.

The actual structures of the obtained compounds had been confirmed by Fourier transform infrared (FTIR) and ^{1}H nuclear magnetic resonance (H-NMR) spectroscopy.

All FTIR spectra clearly showed an intense characteristic absorption band around 1625 cm^{-1} and a low intense one around 2890 cm^{-1} due to the -C=N- vibration and axial stretching of the C-H bond in the newly formed azomethine group, while the bands characteristic for the functional groups of the reagents (the aldehyde group around 1670 cm^{-1} and the amine group around 3350 cm^{-1} , respectively), had disappeared. The weak absorption peaks of the 1952-1673 cm^{-1} range were assigned to the C-H outof-plane (γ_{C-H}) deformations of the phenylene rings, while the strong absorption peaks within the 1591-1484 cm^{-1} and 760-693 cm^{-1} domains were attributed to the $\nu_{C=C}$ and γ_{C-H} of the 1,4-phenylene rings.

In the ^{1}H-NMR spectra, the new azomethine obtained was confirmed by the presence of the chemical shift specific to the azomethine proton around 8.6ppm and the absence of the signals characteristic to carbonyl (around 9.8ppm) and amine (3.55ppm) protons. In the aromatic region (7-8ppm), there were present all the signals belonging to the aromatic protons in the right integral ratio.

Among the new combinations, the activity of BsA6 was greater against *E. coli* and *C. albicans*. While the model compound BsA1 presented the same activity as its aldehyde reagent, in the case of imines possessing hydroxyl unit a slight increase of the biological activity against

S.aureus and *C.albicans*, was observed when compared to its aldehyde reagent. Interesting enough the ortho position of the hydroxyl unit to the carbonyl group led to a decreased activity against *E. coli*, while the same group in para positions led to a drastically increased activity against the same strain. The introduction of a methoxy unit in meta position (BsA3) inhibited the activity against *S. aureus* and maintained the activity against *E. coli* and *C. albicans* of the corresponding aldehyde reagent.

All these data suggested a strong influence of the functional groups and their positions and encouraged us to continue the study by using other substituents.

According to the literature, several studies proved that Schiff bases and their complexes with various cations showed moderate-to-good activity against *Staphylococcus aureus*, *Escherichia coli* or *Candida albicans* (Goszczyńska et al., 2015; Durmuş et al., 2017; Shaygan et al., 2018; Kuddushi et al., 2018).

III.4.3.4. Conclusions

In this research, we successfully reported the synthesis of some of the aniline derived Schiff bases. The structures of the synthesized compounds were proposed by FTIR and 1H-NMR studies. Antibacterial activities of the compounds were examined against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*.

IV. Contributions to the analytical profile of the bisoprolol fumarate

IV.1. Introduction

Arterial hypertension affects many people and is a major risk factor for stroke, myocardial infarction, vascular disease, and chronic kidney disease. Numerous classes of drugs are available to help lower blood pressure: diuretics, beta-blockers, calcium-channel blockers, central agonists, peripheral adrenergic inhibitors, vasodilators, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers. Beta-blockers, also known as beta antagonists, beta-adrenergic blocking agents, or beta- adrenergic antagonists, are drugs that are prescribed to treat several different types of conditions, including hypertension, angina, some abnormal heart rhythms, heart attack, anxiety, migraine, glaucoma, and overactive thyroid symptoms (DiNicolantonio et al., 2015).

Bisoprolol fumarate (BF) is one of the most widely used beta-blockers in the treatment of cardiovascular diseases, chemically known as (±)1-[4-[[2-(1-methylethoxy) ethoxy] methyl] phenoxy]-3-[(1methylethyl)amino]-2-propanol(E)-2-butenedioate (2:1). It possesses an asymmetric carbon atom in its structure and is provided as a racemic mixture. The S(−) enantiomer is responsible for most of the beta-blocking activity (Fig. 35).

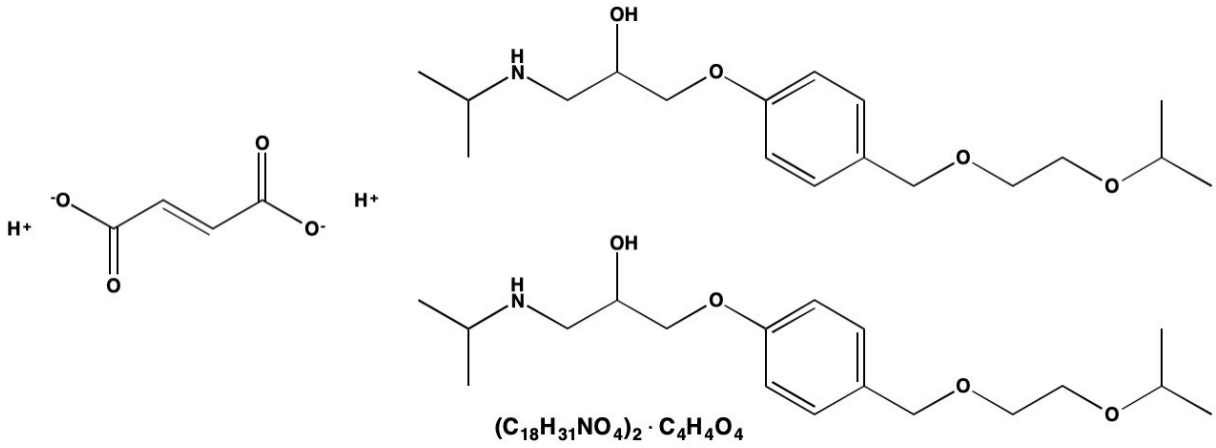


Fig. 35. Chemical structure of bisoprolol fumarate

The two substituents present in the para position of the benzene ring might be the reason for its β1-adrenergic receptor selectivity. BF is well absorbed following oral administration. The absolute bioavailability for a 10 mg dose is greater than 80%. Absorption is not influenced by meals. The first pass metabolism of bisoprolol fumarate is less than 20% and it is eliminated through renal and non-renal pathways, as about 50% of the dose is found unchanged in the urine and the remainder is eliminated as inactive metabolites (Fares et al., 2012; Trobec et al., 2016).

Bisoprolol fumarate - $(C_{18}H_{31}NO_4)_2 \cdot C_4H_4O_4$ - has a molecular weight of 766.97. It is a white crystalline powder which is approximately equally hydrophilic and lipophilic, and is readily soluble in water, methanol, ethanol and chloroform.

Several analytical methods for the determination of BF in bulk, dosage forms, and human plasma have been proposed, such as high performance liquid chromatography **(**Tilea et al., 2016; Kondratova et al., 2017; Turner et al., 2019), UV-VIS spectrophotometry (Kumbhar et al., 2012; Ulu et al., 2012), electrophoresis (Hercegová et al., 1998; Wang et al., 2009) and voltammetry (Bozal et al., 2013; Zil'berga et al., 2016)

The main objective of this work was developing and validating simple, rapid, accurate and economical methods for the determination of bisoprolol using high performance liquid chromatography (HPLC) and UV-VIS spectrophotometry.

The studies were materialized in the following publications:

- Pește G, Bibire N, Apostu M, Vlase A, Oniscu C. A new liquid chromatography - tandem mass spectrometry method for determination of bisoprolol in human plasma samples. *Journal of Biomedicine and Biotechnology*, Volume 2009; Article ID 736327: 8 pag.
- Panainte (Gudruman) AD, Bibire N, Ţântaru G, Apostu M, Vieriu M, Dorneanu V. Validation of a new spectrophotometric method for the assay of bisoprolol fumarate using tropaeolin 00. *Rev Chim (Bucharest)* 2013; 64(4): 393-396.
- Panainte AD, Bibire N, Ţântaru G, Apostu M, Vieriu M, Dorneanu V. A new method for the assay of bisoprolol using bromocresol green. *Rev Chim (Bucharest)* 2014; 65(8): 916-920.
- Panainte AD, Agoroaei L, Bibire N, Ţântaru G, Apostu M, Vieriu M, Spac AF. A HPLC method for the determination of bisoprolol in tablets and its application to a bioequivalence study. *Rev Chim (Bucharest)* 2015; 66(11): 1791-1795.
- Panainte AD, Ţântaru G, Bibire N, Apostu M, Vieriu M. Experimental reaserch for the determination of bisoprolol fumarate in bulk and dosage forms using liquid chromatography. *Med Surg J - Rev Med Chir - Soc Med Nat Iaşi* 2017; 121(2): 433-437.
- Panainte AD, Popa G, Vieriu M, Bibire N, Ţântaru G, Creteanu A, Apostu M. Evaluation of qualitative and quantitative stability parameters of a new tablet formulation containing bisoprolol fumarate. *Farmacia* 2018; 66(3): 487-493.
- Panainte AD, Vieriu M, Ţântaru G, Apostu M, Bibire N, Avasilcăi L, Morariu ID. RP-HPLC analysis method of bisoprolol fumarate in a new tablet formulation. *Med Surg J - Rev Med Chir - Soc Med Nat Iaşi* 2018; 122(3): 634-639.

IV.2. Development of high-performance liquid chromatography methods

High-performance liquid chromatography (HPLC) is an analytical technique to separate, identify and quantify components in a mixture. HPLC coupled with mass spectrometry (MS) detection is one of the most powerful analytical tools for organic compound analysis. The advantages of using HPLC/MS methods over HPLC methods include: selectivity, chromatographic integrity, peak assignment, structural information, and rapid method development.

IV.2.1. Development and validation of a HPLC method for for determination of bisoprolol in human plasma samples

The objectives of this work were to develop and to validate a simple, accurate, rapid and economic liquid chromatography tandem mass spectrometry method for the determination of bisoprolol in human plasma samples, using liquid-liquid extraction (metoprolol as internal standard) and to present some of this method applications.

IV.2.1.1. Materials and methods

↳ *Apparatus*

All analyses were performed using the Agilent 1100 LC/MSD Trap XCT system. The system compenents included the Agilent 1100 Degasser, Agilent 1100 Binary Pump, Agilent 1100 Autosampler, Agilent 1100 Mass Selective Detector. The Bruker Daltonik software was used for system control and data acquisition. The separation was performed using a reverse phase column (Zorbax SB-C18 Solvent Saver Plus, 3 × 100 mm, 3.5 μm, supplied by Agilent,).

↳ *Reagents*

All solvents and other chemicals (acetonitrile, methanol, sodium hydroxide, tert-butyl methyl ether, water, formic acid) were HPLC grade provided by Merck's Chemical Co., Darmstadt, Germany. The reference substances of bisoprolol and metoprolol (internal standard) were supplied from the USP Pharmacopoeia. The human plasma was obtained from Center for Blood Drawing and Preservation, Iasi, Romania.

↳ *The development of the LC-tandem Mass Spectrometry method*

The LC-MS/MS method for determination of bisoprolol in human plasma samples described in this paper was performed using a mobile phase consist in mixture 0.1% formic acid solution (pH=3) - acetonitrile (50-50, v/v). The LC system was operated at 0.3 mL/min, using the binary pump. The column temperature was 40°C. The injection volume was 5 μL and represented no more 5% of the total sample available for injection. Short run times of about 3 minutes were achieved for both bisoprolol and internal standard, metoprolol. Bisoprolol was eluted at 1.7 minutes and metoprolol at 1.9 minutes.

To minimize undesirable fragmentation voltages were tested from 80 to 200 V. At 100 V, the MS response of bisoprolol and metoprolol showed both minimal undesirable fragmentation and highest response. The mass transition ion-pair was followed as m/z 326.2 → 116.1 for bisoprolol and m/z 268.2 → 191.0, for metoprolol (Bhatt et al., 2007).

The protonated molecular ion of bisoprolol [M+H] + (m/z 326.3) was tested to give the highest sensitivity. Based on the optimization results, m/z 116.2 was selected as the quantifier ion. Also, the protonated molecular ion of metoprolol [M+H] + (m/z 268) was tested and m/z 116.2 was selected as the quantifier ion. We have chosen products ions with the same m/z value for bisoprolol and metoprolol, respectively, because a possible suppression effect would influence the quantification of both the analyte and internal standard in the same way.

Other mass spectrometric parameters (gas temperature, gas pressure and gas flow) were adjusted to get a maximum signal for bisoprolol. The nebulizing gas flow rate was set at 10

l/min, drying gaz temperature at 350°C, and the capillary voltage at 4000 V. The response of bisoprolol and metoprolol were measured by MRM in the positive ionization mode with a collision energy of 20 V.

IV.2.1.2. Results

↳ *Validation of the LC-tandem Mass Spectrometry method*

The method was validated according Guidance for Industry: Bioanalytical Method Validation (CDER 2001). The parameters usually examined in the validation process are selectivity/specificity, linearity, limit of quantification, accuracy and precision, stability.

• *Selectivity*. The reversed-phase HPLC method described in this paper has been tested for possible interferences from other plasma factors. Plasma aliquots from six different sources were assessed for analysis in order to investigate the plasma components behavior (Fig. 36).

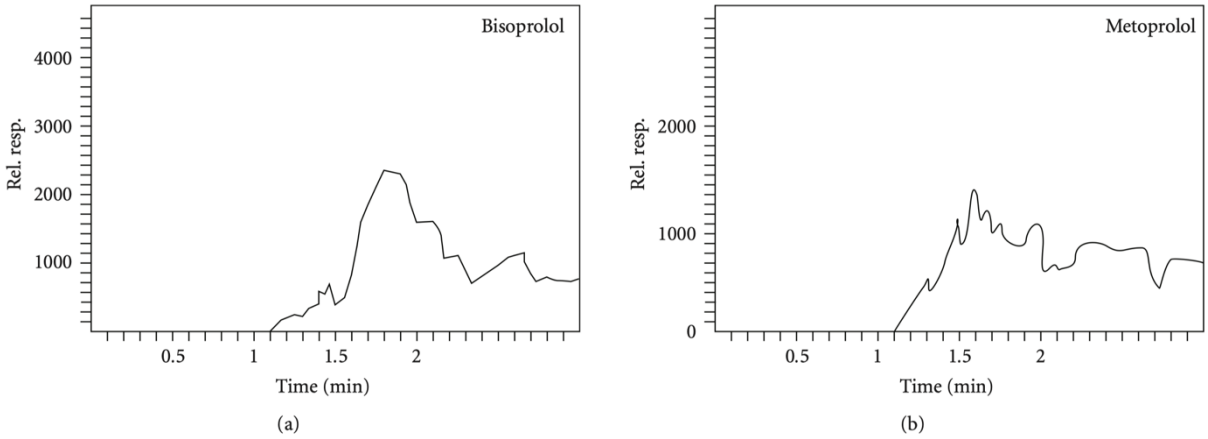


Fig. 36. Chromatogram recorded for blank plasma sample

• *Linearity and lower limit of quantification*. The linearity was investigated for a bisoprolol theoreticals concentrations range between 1 ng/mL and 100 ng/mL and the calibration curve was derived by plotting the peak-height ratios of the analyte and the internal standard against the concentration of bisoprolol, using linear regression analysis (Fig. 37).

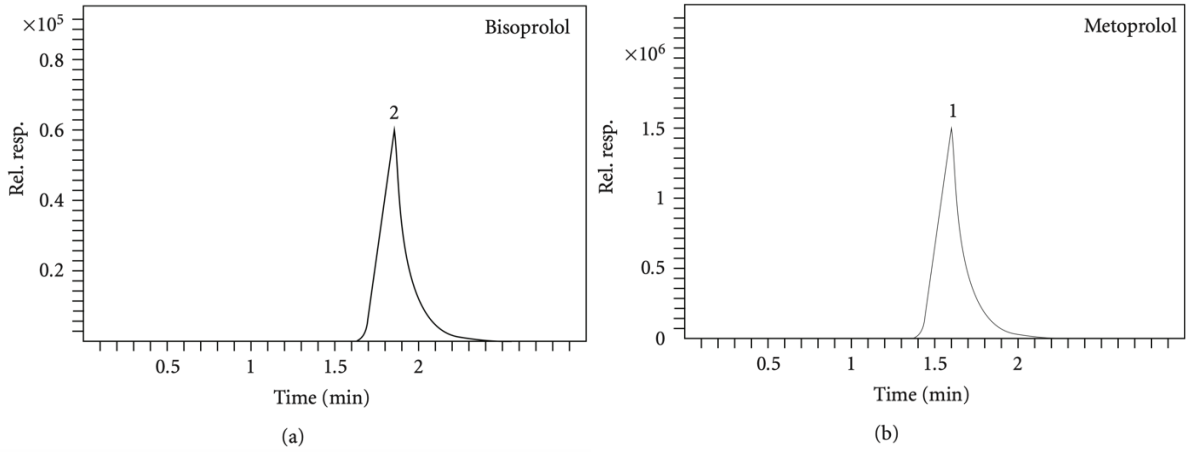


Fig. 38. Chromatogram recorded for plasma containing (a) bisoprolol (0.990 ng/mL, lower limit of quatification) and internal standard (b)

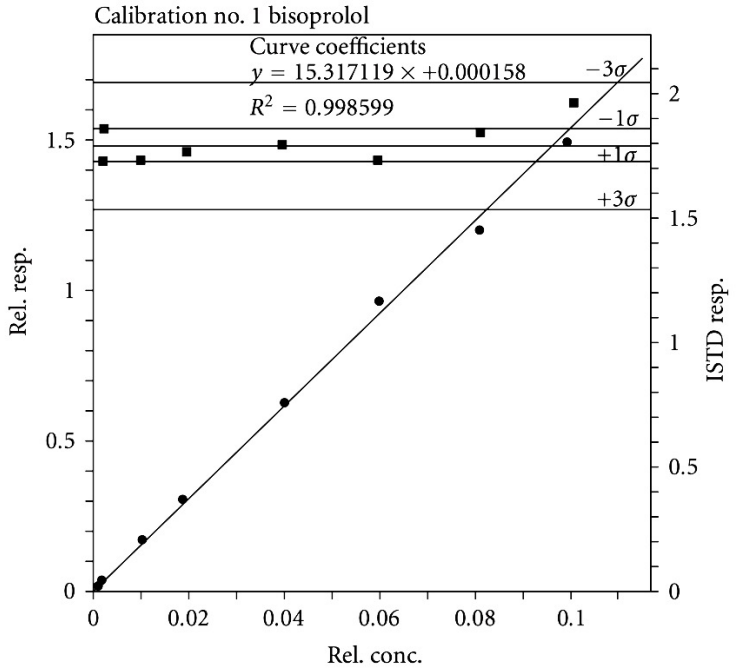


Fig. 37. The bisoprolol calibration curve obtained for plasma samplee

The lower limit of quantification, that is, the lowest standard level with a coefficient of variation less than 20%, is for bisoprolol 0.990 ng/mL with 41.433 signal to noise ratio. The bioanalytical method proved to be sensitive, allowing a precise quantification of concentrations as low as 1 ng/mL (Fig. 38). Results are presented in table 46.

Table 46. Lower limit of quatification

	Analyte concentration (0.990 ng/mL)		
	Conc. (ng/mL)	**% nominal**	**Signal/noise ratio**
1	0.989	99.945	44.500
2	1.407	142.094	36.000
3	0.958	96.802	46.100
4	1.167	117.863	31.700
5	1.175	118.723	41.500
6	1.241	125.400	48.800
N	6	6	6
Mean	1.156	116.804	41.433
SD (±)	0.166		
CV (%)	14.339		
Acceptance criteria			
4 out of 6 LLQC must be 100 ± 20% nominal value Mean % nominal 100 ± 20% CV (%) ≤ 20% Signal/noise ratio ≥ 5			

SD = standard deviation; CV = coefficient of variation

• *Accuracy and Precision*. Accuracy of the analitycal method represents the degree of closeness of the determined values of an analyte to the nominal/or known true value declared from an individual sample. The accuracy of a bioanalytical method is expressed as a percentage of the nominal value (% nominal).

Precision of the analitycal method represents the degree of dispersal of the values determined of an analyte, from a series of samples processed and analyzed individually from a homogeneous volume of biological matrix. Precision of a bioanalytical method is expressed as the coefficient of variation of the concerned series of determinations, CV (%).

The accuracy and precision of this method were calculated for three concentrations of bisoprolol in human plasma. Six replicate samples having bisoprolol theoreticals concentrations of 3 ng/mL (QC1), 25 ng/mL (QC2) and 75 ng/mL (QC3) were injected into the system. Table 47 summarizes the results obtained for the intraday parameters.

Table 47. Evaluation of intraday precision and accuracy for bisoprolol spiked quality control sample

	C_{th} = 3 ng/mL		C_{th} = 25 ng/mL		C_{th} = 75 ng/mL	
	C_{exp} (ng/mL)	% nominal	C_{exp} (ng/mL)	% nominal	C_{exp} (ng/mL)	% nominal
1	2.912	93.301	22.524	91.263	69.203	93.468
2	3.003	101.439	24.862	100.737	68.525	92.552
3	3.262	110.192	23.762	96.281	66.781	90.196
4	2.829	95.568	25.189	102.062	76.691	103.580
5	3.018	101.960	21.739	88.085	65.844	88.931
6	2.854	96.431	21.371	86.593	74.009	99.958
Mean	2.980	100.664	23.241	94.170	70.176	94.781
SD (±)	0.158		1.610		4.268	
CV (%)	5.296		6.927		6.082	
Acceptance criteria						
67% total QCs must be 100 ± 15% nominal value 50% QCs per level must be 100 ± 15% nominal value Mean % nominal 100 ± 15% CV (%) ≤ 15%						

C_{th} = theoretical concentration; C_{exp} = experimental concentration; SD = standard deviation; CV = coefficient of variation

Table 48. Evaluation of interday precision and accuracy for bisoprolol spiked quality control sample

	C_{th} = 3 ng/mL		C_{th} = 25 ng/mL		C_{th} = 75 ng/mL	
	C_{exp} (ng/mL)	% nominal	C_{exp} (ng/mL)	% nominal	C_{exp} (ng/mL)	% nominal
1	3.015	101.860	24.331	98.586	70.921	95.788
2	2.879	97.276	23.332	94.539	77.073	104.097
3	3.257	110.048	23.109	93.636	77.932	105.256
4	2.964	100.136	24.013	97.297	70.590	95.340
5	2.943	99.439	28.411	115.117	75.582	102.083
6	3.152	106.480	24.910	100.933	74.865	101.115
Mean	3.035	102.540	24.684	100.018	74.494	100.613
SD (±)	0.142		1.940		3.092	
CV (%)	4.686		7.860		4.151	
Acceptance criteria						
67% total QCs must be 100 ± 15% nominal value 50% QCs per level must be 100 ± 15% nominal value Mean % nominal 100 ± 15% CV (%) ≤ 15%						

C_{th} = theoretical concentration; C_{exp} = experimental concentration; SD = standard deviation; CV = coefficient of variation

The interday precision and accuracy was evaluated also using six aliquots for each quality control sample concentration, prepared and analysed in six different days. The results are presented in table 48.

• *Stability Tests*. To test stability, a series of standards samples was prepared from freshly made stock solutions in the same solvent used for the assay. The lowest and highest concentration of the quality control (3 ng/mL and 75 ng/mL), including the analyte and internal standard (when appropiate) were used. Human plasma samples of each concentration were prepared in enough volume to have multiple aliquots.The aliquots of each concentration were processed and quantified immediately in order to provide the reference (fresh) values and other six aliquots of each concentration were processed for the desired tests.

The following subsections present the procedure carried out and the corresponding results.

a. *Stability of the analyte after sample processing at room temperature*.

Samples prepared at low (QC1) and high (QC3) quality control levels were submitted to the extraction procedure and kept at room temperature under ambient laboratory conditions (stability samples). A calibration curve and 6 replicates of low and high quality control samples (comparison samples) were freshly processed and analyzed with 6 replicates of stability samples in a single run. Concentrations were calculated to determine % change over time (Table 49).

Table 49. Stability of analyte following sample processing at room temperature

Analyte: Bisoprolol ***Biological matrix: Human plasma*** ***Storage condition: 31 hours at room temperature***						
	QC1 (3 ng/mL)			**QC3 (75 ng/mL)**		
	Comparison samples		**Stability samples**	**Comparison samples**		**Stability samples**
	Measured	**% nominal**	**Measured**	**Measured**	**% nominal**	**Measured**
	Conc.		Conc.	Conc.		Conc.
1	2.682	90.611	2.838	80.224	108.352	82.798
2	2.330	78.719	2.639	82.755	111.771	63.262
3	3.082	104.134	2.434	78.821	106.457	75.379
4	2.380	80.392	2.079	79.826	107.815	80.616
5	2.117	71.504	2.047	91.323	123.343	58.511
6	2.619	88.475	2.745	84.105	113.595	79.410
N	6	6	6	6	6	6
Mean	2.535	85.639	2.464	82.842	111.889	73.329
SD (±)	0.338		0.338	4.597		10.049
CV (%)	13.314		13.734	5.549		13.704
% Change		- 2.808			- 11.483	
Acceptance criteria						
67% comparison samples must be 100 ± 15% nominal value Mean % nominal of comparison samples 100 ± 15% CV (%) ≤ 15% % Change ± 15%						

SD = standard deviation; CV = coefficient of variation

b. *Stability of analyte in biological matrix at room temperature*.

Samples were prepared at low (QC1) and high (QC3) quality control levels. Six replicates of low and high quality control samples were left at room temperature for approximately 4 hours (stability samples). A calibration curve and 6 replicates of low and high quality control samples (comparison samples) were freshly processed with 6 replicates of stability samples and analyzed in a single run.

Concentrations were calculated to determine % change over time (Table 50).

Table 50. Stability of analyte in biological matrix at room temperature

Analyte: Bisoprolol ***Biological matrix: Human plasma*** ***Storage condition: 4 hours at room temperature***						
	QC1 (3 ng/mL)			**QC3 (75 ng/mL)**		
	Comparison samples		**Stability samples**	**Comparison samples**		**Stability samples**
	Measured	**% nominal**	**Measured**	**Measured**	**% nominal**	**Measured**
	Conc.		Conc.	Conc.		Conc.
1	3.509	118.561	3.540	61.764	83.419	72.020
2	2.839	95.898	3.502	74.312	100.368	67.462
3	3.453	116.639	3.281	73.697	99.537	90.117
4	3.187	107.684	3.514	72.186	97.495	73.715
5	4.310	145.604	3.616	68.557	92.595	69.225
6	3.123	105.510	3.688	75.832	102.421	69.012
N	6	6	6	6	6	6
Mean	3.403	114.493	3.523	71.058	95.973	73.592
SD (±)	0.506		0.138	5.182		8.405
CV (%)	14.864		3.925	7.292		11.421
% Change		3.522			3.566	
Acceptance criteria						
67% comparison samples must be 100 ± 15% nominal value Mean % nominal of comparison samples 100 ± 15% CV (%) ≤ 15% % Change ± 15%						

SD = standard deviation; CV = coefficient of variation

c. *Stability of analyte in biological matrix after 3 freezethaw cycles*.

Samples were prepared at low (QC1) and high (QC3) quality control levels, aliquoted and frozen at −25 ± 10 ◦ C. Some of the aliquots of quality control samples were subjected to three freeze-thaw cycles (stability samples). The remaining aliquots were not thawed (comparison samples). A calibration curve and 6 replicates of low and high quality control samples (comparison samples) were freshly processed with 6 replicates of stability samples and analyzed in a single run. Concentrations were calculated to determine% change over freeze-thaw cycles. Results are presented in table 51.

Table 51. Stability of analyte in biological matrix after 3 freeze-thaw cycles at -25± 10°C

Analyte: Bisoprolol ***Biological matrix: Human plasma*** ***Storage condition: -25 ± 10°C***						
	QC1 (3 ng/mL)			**QC3 (75 ng/mL)**		
	Comparison samples		**Stability samples**	**Comparison samples**		**Stability samples**
	Measured	**% nominal**	**Measured**	**Measured**	**% nominal**	**Measured**
	Conc.		Conc.	Conc.		Conc.
1	3.015	101.860	2.934	70.921	95.788	74.754
2	2.879	97.276	3.239	77.073	104.097	73.574
3	3.257	110.048	3.096	77.932	105.256	77.879
4	2.964	100.136	3.180	70.590	95.340	83.159
5	2.943	99.439	3.049	75.582	102.083	78.939
6	3.152	106.480	3.063	74.865	101.115	86.426
N	6	6	6	6	6	6
Mean	3.035	102.540	3.093	74.494	100.613	79.121
SD (±)	0.142		0.107	3.092		4.921
CV (%)	4.686		3.454	4.151		6.220
% Change		1.920			6.212	
Acceptance criteria						
67% comparison samples must be 100 ± 15% nominal value Mean % nominal of comparison samples 100 ± 15% CV (%) ≤ 15% % Change ± 15%						

SD = standard deviation; CV = coefficient of variation

➧ *The applications of LC-tandem Mass Spectrometry method*

The assay has proven to be suitable to determine the bisoprolol concentration in the bioequivalence study of Bisoprolol 10 mg coated tablets produced by Antibiotice S.A. (referred to as test drug) versus Concor® 10 mg coated tablets produced by Merck (referred to as reference drug). In figure 39, average bisoprolol concentrations recorded for 22 volunteers are plotted against time for both test and reference drugs.

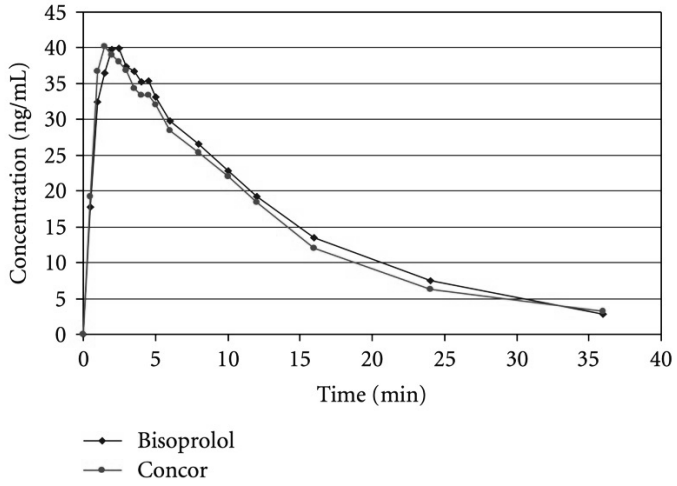


Fig. 39. Average bisoprolol concentration recorded for the test and reference drugs in the bioechivalence study performed on 22 healty volunters

The concentration profiles are similarly, fitting the results obtained for the in vitro dissolution test (Fig. 40).

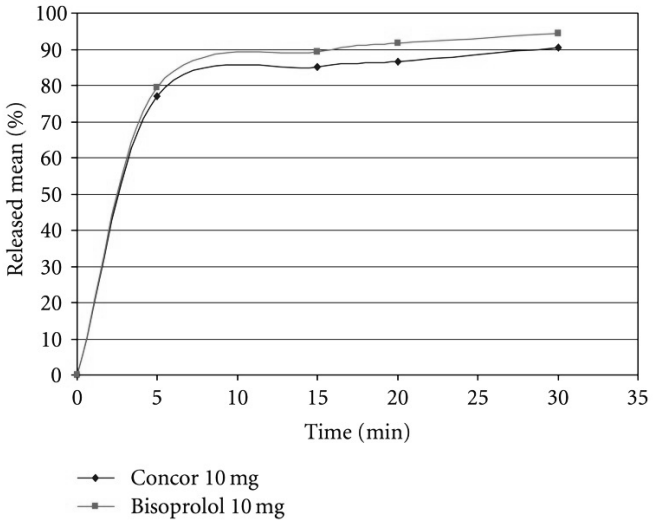


Fig. 40. Average dissolved bisoprolol recorded for test and reference drugs recorded for the in vitro dissolution test.

IV.2.1.3. Discussions

LC separation of bisoprolol and internal standard, metoprolol, has been carried out using the mobiles phases consisting of differents aqueous solutions and methanol or acetonitrile as organic phase. Hernardo et al. (Hernardo et al., 2004) used a acetonitrile as organic mobile phase to lead to shorter retention times and better resolution of the bisoprolol and internal standard. Formic acid solution as additive in water was used by Li et al. (Li et al., 2007) to improve the sensitivity of MS detection.

The method was validated according Guidance for Industry: Bioanalytical Method Validation.

The bioanalytical method proved to be selective. As it can be seen in figure 36, no overlapping peaks were detected at bisoprolol and internal standard retention time, 1.7 minutes and 1.9 minutes, respectively.

The least-square linear regression revealed that the relationship was linear in the investigated domain, with a correlation coefficient of 0.998599, meeting the acceptance criteria ($r^2 \geq 0, 990$), as it can be seen in figure 37.

The lower limit of quantification, that is, the lowest standard level with a coefficient of variation less than 20%, is for bisoprolol 0.990 ng/mL with 41.433 signal to noise ratio. The bioanalytical method proved to be sensitive, allowing a precise quantification of concentrations as low as 1 ng/mL (Fig. 38).

Intra-and interday precision of analysis was <8% and accuracy range was from 94.170% to 102.540% (Table 46; Table 47).

The values for the investigated parameters proved to be lower than the one reported by Oniscu (Oniscu et al., 2007), employing a HPLC method with fluorescence detection. Also, Liu

(Liu et al., 2007) reported an accuracy ranged from 89.4%-113%, employing a precipitation with acetonitrile procedure for plasma sample preparations.

Recovery of bisoprolol was evaluated by comparing analyte response of six extracted samples of low, medium, and high quality control samples to those of six appropriately diluted standard solutions. Mean recovery values for bisoprolol are 76.529, 78.479, and 79.863% at low, medium and high quality control levels, respectively.

For internal standard, mean internal standard response of eighteen extracted samples was compared to the mean internal standard responses of eighteen appropriately diluted internal standard solutions. Mean recovery value for the internal standard is 90.568%.

Bisoprolol is found to be stable for 31 hours at room temperature under ambient laboratory conditions after sample processing with % changes (ratio between mean concentration of stability samples and mean concentration of comparison samples) of -11.483 and -2.808% (Table 48).

In human plasma bisoprolol is found to be stable for 4 hours at room temperature with % changes of 3.522 and 3.566% (Table 49).

After three freeze-thaw cycles, bisoprolol is found to be stable in human plasma with % changes of 1.920 and 6.212% (Table 50).

Several high-performance liquid chromatographic methods are described for the determination of bisoprolol in human plasma. The inferior limit of analytical ranges of the methods was close to our proposed method: 10-2000 ng/mL (Ulu et al., 2012), 1-250 ng/mL (Rodina et al., 2018), 0.5-50 ng/mL (Logoyda et al., 2018)

The assay has proven to be suitable to determine the bisoprolol concentration in the bioequivalence study of Bisoprolol 10 mg coated tablets produced by Antibiotice S.A. (referred to as test drug) versus Concor® 10 mg coated tablets produced by Merck (referred to as reference drug).

Based on the determined bisoprolol concentrations, the calculated pharmacokinetic parameters demonstrated that the drug produced by Antibiotice S.A. is bioequivalent with the one produced by Merck

IV.2.1.4. Conclusions

LC-tandem mass spectrometry method described and validated above is sensitive, accurate, precise, rapid, and efficient. The developed method can be applied for the determination of bisoprolol from human plasma samples (e.g., for pharmacokinetic parameters).

IV.2.2. Development and validation of novel HPLC methods for determination of bisoprolol in bulk and pharmaceutical dosage forms

In table 52 we can see the summarized presentation of the HPLC methods developed and validated for the quantitative determination of bisoprolol in bulk and pharmaceutical formulations.

Table 52. Development and validation of novel HPLC methods for determination of bisoprolol in bulk and pharmaceutical dosage forms

Columns	Mobile phase/ Detection	Results/Discussion
Zorbax SB-C18 (100×3mm, 3.5µm)	Methanol, acetonitrile and 45mM potassium dihydrogen phosphate buffer, pH = 3.0 (30:25:45, v/v) - 0.3mL/min UV, 225 nm	A sensitive HPLC method with UV detection was developed and validated for bisoprolol fumarate determination from tablets. The retention time for bisoprolol was 2.32 min. The calibration graph was linear in the concentration range 0.3-10µg/mL (r^2 = 0.9995). LOD and LOQ of bisoprolol fumarate were 0.10 and 0.28µg/mL. The intra and interday precision of measurements were lower than the accepted criteria (CV ≤15%) and concerning the accuracy of the method, the recovery variation was in between 99.33 and 102.00%.
Zorbax SB-C18 (100×3mm, 3.5µm)	Phosphate buffer pH = 3.5 and acetonitrile (70:30, v/v) - 1mL/min UV, 225 nm	The study described the development and validation of a novel fast and accurate HPLC method for the quantitative determination of bisoprolol in bulk and pharmaceutical formulations. The retention time for bisoprolol was 1.158 min. The calibration graph was linear in the concentration range 5-90 µg/mL (r^2 = 0.9996). The LOD and LOQ were 1.63 µg/mL and 4.94 µg/mL, respectively. The intra and interday precision of measurements were lower than the accepted criteria (RSD ≤ 2%). The recovery values of HPLC determination of bisoprolol from tablets proved that none of the excipients influenced the results of the analysis.
Zorbax SB-C18 (100×3mm, 3.5µm)	Methanol and 0.02M potassium acetate (40:60, v/v) - 1mL/min UV, 225 nm	The RP-HPLC method we have developed for quantitative analysis of bisoprolol fumarate in pharmaceutical products. The retention time for bisoprolol was 2.38 min. A linear response was observed over the concentration range 5-80 µg/mL of bisoprolol fumarate (r^2 = 0.9993). The LOD and LOQ were 1.15 and 3.81µg/mL. The relative standard deviations did not exceed 1%. The accuracy of the method was proved and the mean recovery of drug was in the range 99.74-100.02%.
Agilent Eclipse XDB-C18 (150×4.6mm, 5 µm)	Acetonitrile 10mM and phosphate buffer solution pH = 4.5 (10:90, v/v) - 1mL/min UV, 227 nm	The method is suitable for routine analysis of bisoprolol fumarate in bulk or in tablet dosage forms. The retention time for bisoprolol was 3.52 min. The linearity of the HPLC method used in the study was tested over the 0.5 - 20 µg/mL range (r^2 = 0.9983). The LOD and LOQ were 2.79 and 3.07 µg/mL, respectively. The relative standard deviation evaluated for the precision of the method was lower than 1%. Values for accuracy and precision of the method are in the range of the acceptability for accuracy (97.0-103.0%). The % RSD values found in precision study showed that the proposed method provides acceptable intra- and inter-day variations for bisoprolol fumarate determination.

IV. 3. Development of spectrophotometric methods

Spectrophotometric methods UV-VIS absorption are fast, robust, precise and universally accepted in pharmaceutical analysis. Most spectrophotometric methods for bisoprolol determination are in UV range. In literature there are few methods for the analysis of bisoprolol in visible domain that include reactions with either 7,7,8,8-tetracyano qumodimethane (Ulu et al., 2012), tropaeolin 000 or chromazurol S (Ayad et al., 2013).

IV.3.1. Development and validation of a spectrophotometric method for the assay of bisoprolol using tropaeolin 00

The aim of the present investigation was to develop a new rapid and reproducible method for the quantitative determination of bisoprolol fumarate in pharmaceutical products.

The method was based on the formation of yellow coloured ion pair complex between bisoprolol and tropaeolin 00 (Fig. 41) which was extracted in dichloromethane and then spectrophotometrically analysed at 412 nm.

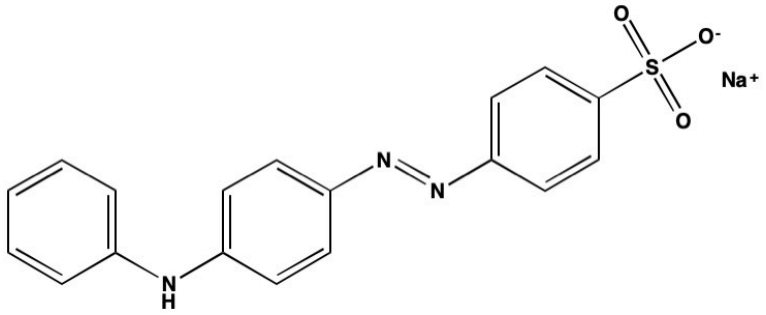


Fig. 41. Chemical structure of tropaeolin 00

IV.3.1.2. Materials and methods

↳ *Apparatus*

Absorbance was measured in quartz cuvettes using a Hewlett Packard 8453 UV-VIS spectrophotometer while maintaining the temperature at 25°C.

↳ *Reagents*

All chemicals were of analytical-reagent grade. Bisoprolol fumarate pure substance provided by Unichem Laboratories LTD, India.

↳ *Assay procedure*

1 mL 0.05 M hydrochloric acid and 1 mL 0.01% (w/v) tropaeolin 00 aqueous solution were added to each 1.0 mL of bisoprolol fumarate solution with a concentration in between 5-30 μg/mL. The complex was then extracted using dichloromethane. Fifteen minutes later the absorbance was measured at 412 nm, using as reference a blank sample prepared in the same conditions.

IV.3.1.2. Results

The basic spectrophotometric conditions were designed to be simple and easy to use and reproduce and were selected after testing the different conditions that affect spectrophotometric analysis like chemical and instrumental factors.

↳ *The influence of the concentration of reagents* upon maximum absorption was investigated. The optimal concentration of the tropaeolin 00 aqueous solution was found to be 0.01% (w/v) and 0.05 M hydrochloric acid was used with best results according to the data shown in table 53.

Table 53. Effect of the reagents concentration

Hydrochloric acid (mol/L)	Bisoprolol concentration		Tropaeolin 00 (%)	Bisoprolol concentration	
	5 μg/mL	30 μg/mL		5 μg/mL	30 μg/mL
	Absorbance			Absorbance	
0.001	0.0568	0.4025	0.005	0.0698	0.4103
0.005	0.0572	0.4105	0.01	0.0702	0.4121
0.01	0.0627	0.4112	0.05	0.0712	0.4131
0.05	0.0712	0.4129	0.1	0.0710	0.4125
0.1	0.0702	0.4105	0.5	0.0698	0.4110
0.5	0.0697	0.4102	1	0.0692	0.4023

↳ *Stability of the complex* was evaluated at ambient temperature without protection of light. The absorbance was measured after 15 min have passed since dichloromethane extraction according to the experimental data from table 54.

Table 54. Study of the stability of the complex

Time (min)	Absorbance	
	Bisoprolol concentration	
	5 μg/mL	30 μg/mL
10	0.0705	0.4102
15	0.0716	0.4125
20	0.0712	0.4123
25	0.0706	0.4112
30	0.0685	0.4053
40	0.0682	0.4026
60	0.0682	0.3925

↳*Validation of the method*

Validation of the method was carried out following the linearity, detection limit, quantification limit, precision and accuracy.

• *Linearity* was assessed by analyzing the obtained data shown in table 55 by linear regression and the calibration curve (Fig. 42) was obtained.

Table 55. Linearity determination

Bisoprolol concentration	Absorbance						Mean
	I	II	III	IV	V	VI	
5 μg/mL	0.0703	0.0698	0.0705	0.0697	0.0680	0.0620	0.0684
10 μg/mL	0.1316	0.1354	0.1405	0.1357	0.1372	0.1380	0.1371
15 μg/mL	0.1998	0.2096	0.2013	0.2087	0.2008	0.1979	0.2014
20 μg/mL	0.2698	0.2711	0.2654	0.2685	0.2701	0.2685	0.2689
25 μg/mL	0.3430	0.3461	0.3397	0.3413	0.3453	0.3431	0.3431
30 μg/mL	0.4119	0.4099	0.4120	0.4099	0.4198	0.4198	0.4128

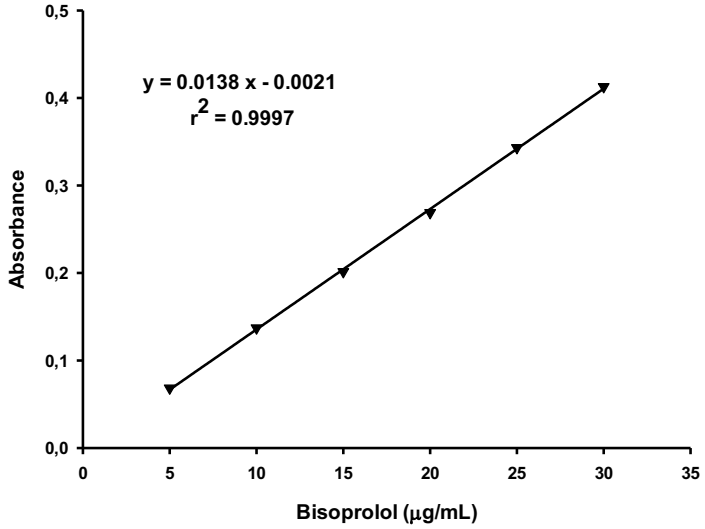


Fig. 42. Calibration curve

The method presented a good linearity in the concentration range 5-30 μg/mL.

Regresion equation: Abs. = 0.0138 · Conc. - 0.0021; $r^2 = 0.9997$

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the following equations:

LOD = 3·SD/Slope = 0.67 μg/mL; LOQ = 10·SD/Slope = 2.23 μg/mL

where: SD = standard deviation (0.002866117); slope = the slope of the calibration curve equation (0.0021).

• *Method precision* was evaluated through repeatability and reproducibility. Using the experimental data the sample concentration was calculated using the calibration curve equation. Three solutions of 10 μg/mL, 15 μg/mL and 20 μg/mL bisoprolol were used. Three assays were performed for each concentration on the same day in order to assess repeatability. Three sets of assays were performed on different days in order to evaluate reproducibility (Table 56).

Table 56. Precision of the method

Bisoprolol concentration	Repetability		Reproductibility	
	Absorbance	Recovery (%)	Absorbance	Recovery (%)
10 μg/mL	0.1381	100.39	0.1373	101.01
	0.1375	101.47	0.1380	101.52
	0.1376	101.54	0.1369	100.72
15 μg/mL	0.2078	101.71	0.2081	101.54
	0.2068	101.23	0.2105	102.27
	0.2081	101.86	0.2068	100.91
20 μg/mL	0.2749	101.67	0.2753	100.51
	0.2721	99.66	0.2723	99.42
	0.2758	10.00	0.2746	100.25
Statistical data	*Mean = 101.05%* *SD = 0.71* *RSD = 0.70%*		*Mean = 100.95%* *SD = 0.87* *RSD = 0.86%*	

While studying the precision of the method, for all sets of data shown in table 56, the relative standard deviation was lower than 2% (RSD = 0.78), which proved that the proposed method was precise.

• *In order to establish the accuracy* of the method, bisoprolol solutions of 10 μg/mL, 11.25 μg/mL, 15.00 μg/mL and 18.75 μg/mL were analyzed. For each concentration, three determinations were performed (Table 57).

Table 57. Accuracy of the method

Theoretical conc. (μg/mL)	Absorbance	Calculated conc. (μg/mL)	Recovery (%)
10.00	0.1374	10.14	101.41
	0.1367	10.06	100.60
	0.1353	9.98	9.87
11.25	0.1538	11.30	100.44
	0.1525	11.21	99.64
	0.1519	11.16	99,20
15.00	0.2081	15.28	101.88
	0.2047	15.03	100.23
	0.2016	14.88	99.23
18.75	0.2588	18.91	100.85
	0.2548	18.62	99.31
	0.2574	18.81	100.32
Statistical data			*Mean = 100.30* *Min = 99.20* *Max = 101.88*

It was established that the recovery for the studied concentration range was in between 99.20% and 101.88% and the mean was 100.3%. These values proved that the proposed method was accurate.

IV.3.1.3. Discussions

Bisoprolol fumarate is one of the most used beta-blockers in the treatment of cardiovascular diseases.

Chromatographic techniques such as high performance liquid chromatography (Suryanarayana et al., 2016), reversed-phase high performance liquid chromatography (Patel et al., 2019), liquid chromatography-electrospray ionization-mass spectrometry (Tilea et al., 2014; Hemavathi et al., 2017) have been applied for the quantification of bisoprolol fumarate in bulk, biological fluids and commercial formulations. Though the chromatographic methods are sensitive the instrumentations are burdensome and necessitate critical experimental conditions. Hence these techniques are not applied for routine analysis of bisoprolol fumarate.

In view of the fact that spectrophotometric procedures are simple, fairly accurate and precise and cost-effective, they are still popular for the routine analysis in quality control laboratories. Rudwan have described first order derivative spectrophotometric method for the estimation of bisoprolol fumarate in pharmaceutical formulation (Rudwan et al., 2017). El-Didamony determined bisoprolol fumarate spectrophotometrically based on the ion pair complexation reaction with bromothymol blue and bromocresol green (El-Didamony et al., 2015).

The validation of analytical methods is required to obtain high-quality data. For the pharmaceutical industry, method validation is crucial to ensure the product quality as regards both therapeutic efficacy and patient safety.

The aim of this work was the development and optimization of a simple spectrophotometric method for the determination of bisoprolol fumarate. The results of validation parameters reveal the good linearity, sensitivity, accuracy, precision and robustness of the proposed methods.

IV.3.1.4. Conclusions

This study describes the successful development of a simple spectrophotometric method for the determination of bisoprolol fumarate. The proposed method is based on the formation of a colored complex combination with tropaeolin 00.

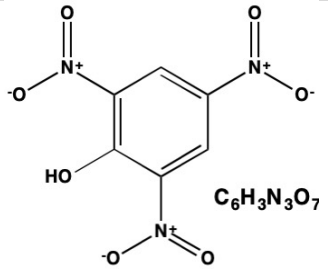
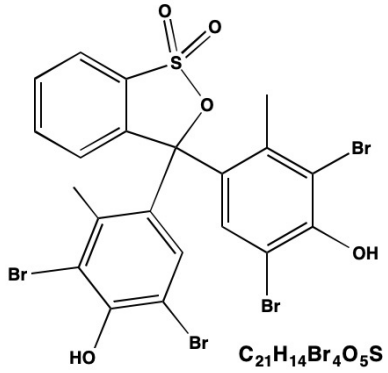
The values of specific absorbance for bisoprolol and reaction product in dichlormethane solutions are $A_{1cm}^{1\%}$, λ_{223nm} = 395 and $A_{1cm}^{1\%}$, λ_{223nm} = 1095 respectively. So, the sensibility of this method is three times greater than of the direct UV determination of bisoprolol.

All analytical reagents used are inexpensive, quite stable and widely available in analytical laboratory. Complex procedures are not required. The earlier reported methods involved costlier techniques. The methods are suitable for routine analysis in quality control laboratories.

IV.3.2. Development and validation of novel spectrophotometric methods for determination of bisoprolol in pharmaceutical dosage forms

In table 58 we can see the summarized presentation of the spectrophotometric methods developed and validated for the quantitative determination of bisoprolol in bulk and pharmaceutical formulations.

Table 58. Development and validation of novel spectrophotometric methods for determination of bisoprolol in pharmaceutical dosage forms

Reactive / λ_{max}	Results/Discussion
 $C_6H_3N_3O_7$ ***Picric acid*** *VIS, 420 nm*	A spectrophotometric method for quantitative determination of bisoprolol was developed based on the formation of a complex combination between bisoprolol and picric acid. The complex combination of bisoprolol and picric acid has a maximum absorbance peak at 420 nm. The method presented a good linearity in the concentration range 5-120 µg/mL (r^2 = 0.9992). LOD and LOQ of bisoprolol fumarate were 0.89 and 2.97 µg/mL. The RSD for the precision of the method was 1.74 and for the intermediate precision 1.43, and recovery values ranged between 98.25-101.48%.
 $C_{21}H_{14}Br_4O_5S$ ***Bromocresol green*** *VIS, 402 nm*	A new VIS spectrophotometric method was developed for the assay of bisoprolol in pharmaceutical preparations using bromocresol green in hydrochloric acidic medium. The reaction product showed a maximum absorbance at 402 nm proportional with the concentration of bisoprolol. The optimum conditions for the reaction were established. The developed method was validated. The method showed a good linearity in the concentration range of 7-80 µg/mL (r^2 = 0.9998). The detection limit (LOD) was 1.78 µg/mL and the quantification limit (LOQ) was 5.41 µg/mL. The precision and the accuracy were determined; mean recovery was 100.11% in the 98.35-101.57% concentration range.

V. Contributions to the analytical profile of the lisinopril

V.1. Introduction

Lisinopril is a drug of the angiotensin converting enzyme (ACE) inhibitor class that is primarily used in treatment of hypertension, congestive heart failure, heart attacks and also in preventing renal and retinal complications of diabetes. Historically, lisinopril was the third ACE inhibitor, after captopril and enalapril, and was introduced into therapy in the early 1990s (discovered and developed by the Merck Sharp & Dohme Research Laboratories).

Lisinopril has a number of properties that distinguish it from other ACE inhibitors: it is hydrophilic, has long half-life and tissue penetration and is not metabolized by the liver (Cavalieri et al., 2007; Circelli et al., 2012).

Lisinopril is chemically (2S)-1-[(2S)-6-amino-2-[[(1S)-1-carboxy-3-phenylpropyl] amino]hexanoyl] pyrrolidine-2-carboxylic acid (Fig. 43).

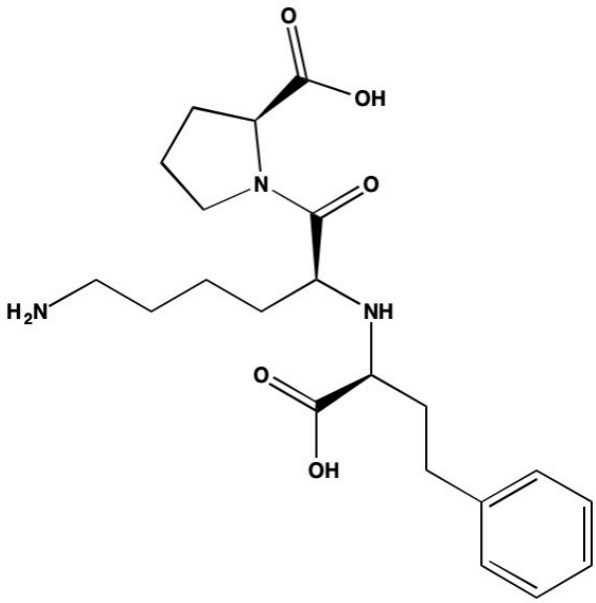


Fig. 43. Chemical structure of lisinopril

Lisinopril is a white to off-white crystalline powder. It is soluble in water (1/10) and methyl alcohol (1/70), but it is practically insoluble in ethyl alcohol, acetone, chloroform and ether. Lisinopril can be expected to be "highly soluble" at 37°C over the entire pH-range 1-7.4. The low bioavailability of lisinopril is in line with its low permeability (Cristea, 2000).

Several analytical methods for the determination of lisinopril in bulk, dosage forms, and human plasma have been proposed, such as high performance liquid chromatography (Stoimenova et al., 2017), UV-VIS spectrophotometry (Nasir et al., 2017), spectrofluorimetry (Derayea et al., 2017; Derayea et al., 2018), potentiometry (Hasanzadeh et al., 2014; Olalowo et al., 2015) and voltammetry (Valezi et al., 2017)

The main objective of this work was developing and validating simple, rapid and economical methods for the determination of lisinopril in bulk powders and pharmaceutical formulations, with high degree of accuracy and precision, using high performance liquid chromatography (HPLC) and UV-VIS spectrophotometry.

The studies were materialized in the following publications:

- Vieriu M, Bibire N, Apostu M, Tociu C, Dorneanu V, Vlase C. Evaluation of some pharmaceutical formulation of lisinopril through dissolution testing. *Rev Med Chir Soc Med Nat Iaşi* 2010; 114(3): 900-903.
- Vieriu M, Bibire N, Ţântaru G, Apostu M, Mandrescu M, Gudruman AD, Dorneanu V. A new method for the assay of lisinopril using molybdophosphoric acid. *Ovidius University Annals of Chemistry* 2011; 22(1): 32-36
- Vieriu M, Bibire N, Apostu M, Panainte AD, Znagovan A, Anghel L, Ţântaru G. Spectrophotometric determination method for lisinopril using permanganate in alkaline medium. *Rev Chim (Bucharest)* 2014; 65(10): 1146-1148.
- Vieriu M, Ţântaru G, Apostu M, Panainte AD, Agoroaei L, Uncu L, Bibire N. A new spectrometric method for quantitative determination through molecular absorption of lisinopril. *Rev Chim (Bucharest)* 2015; 66(10): 1563-1566.
- Vieriu M, Ţântaru G, Apostu M, Panainte AD, Bibire N. A new spectrophotometric method for the assay of lisinopril. *Rev Med Chir Soc Med Nat Iaşi* 2015; 119(4): 1199-1204.

V.2. Evaluation of some pharmaceutical formulations of lisinopril through dissolution testing

The study of dissolution in vitro is considered a fundamental requirement in the pharmaceutical industry in order to assure the quality of solid pharmaceutical forms for oral use, guarantee the quality from lot to lot, orientate the development of new formulations and secure the uniformity, quality and performance of the drug even after modifications.

On a parallel basis, this allows formulation, optimization in the development phase and, in the same way, it allows stability studies, manufacturing process monitoring, and the establishment of *in vivo* / *in vitro* correlations (Koytchev et al., 2004; Shin et al., 2007). The USP 29 dissolution specification for lisinopril tablets is not less than 80% (Q) dissolved in 30 minutes in 900 mL HC1 0.1M. We compared the dissolution profiles of Lisinopril 20 mg tablets (Antibiotice S.A. Iaşi) and Zestril 20 mg tablets (Astra Zeneca).

V.2.1. Materials and methods

↳ *Apparatus*

Dissolution tests were performed according to USP 41 (USP 41, 2018) using dissolution tester Erweka DT 808 LH and HPLC 1200 Agilent system with DAD/ MWD detector.

↳ *Reagents*

All solvents and other chemicals were HPLC grade provided by Merck's Chemical Co., Darmstadt, Germany.

↳ *The development of the HPLC method*

Determination of dissolved active substances was performed using a HPLC method. Lisinopril was separated on a C18 column using a mobile phase consisting of a mixture of phosphate buffer pH = 2 containing 0.125% sodium hexanesulphonate and acetonitrile (72 : 28, v/v), at a flow rate of 1 mL/min.

Detection was performed at 215 nm on an Agilent 1200 HPLC system. The injection volume was 20 µL. In these chromatographic conditions, the retention time of lisinopril was 1.9 min (Sagirli et al., 2004; Qin et al., 2007).

↳ *The dissolution profile* of the pharmaceutical formulations was studied using various dissolution medias. First, the comparative dissolution profiles of the drugs were performed using USP dissolution media (HC1 0.1N) at stirring speed of 50 rpm, then the dissolution profiles were obtained using three different dissolution media : pH 1.2 buffer, pH 4.5 buffer and then pH 6.8 buffer at a stirring speed of 75 rpm.

The dissolution media volume was maintained at 900 mL in all the cases (USP 41, 2018).

V.2.2. Results

Comparative dissolution profile of Lisinopril 20 mg tablets produced by Antibiotice S.A. Iaşi (A) and Zestril 20 mg tablets produced by Astra Zeneca (B) gave the results shown in table 59 when performed accordingly to USP 41.

The results show that both products have similar dissolution profile as it can also be seen from the graphical representation in figure 44.

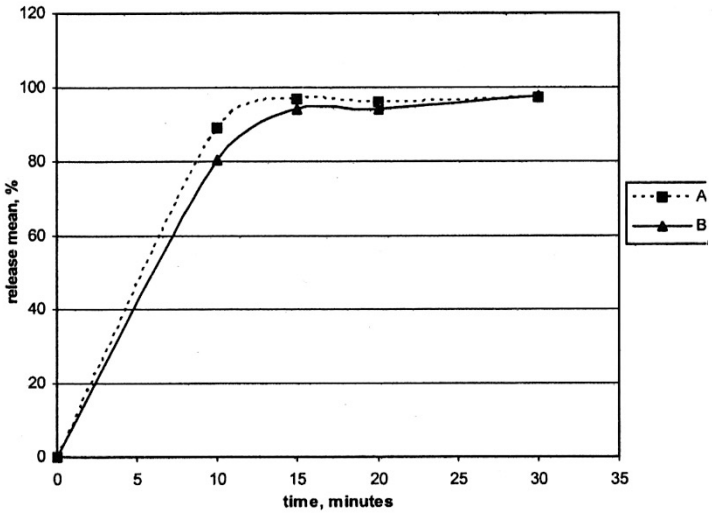


Fig. 44. Comparative dissolution profiles in USP dissolution media

Table 59. Dissolution test in USP dissolution media

Time (min)	% released lisinopril			
	10	**15**	**20**	**30**
A	89.1054	96.7536	95.7648	97.1445
B	80.5794	93.8223	94.0334	97.5769

Because lisinopril is a third class active substance, we performed dissolution tests in standard media at three pH values using the paddle apparatus at 75 rpm.:

• pH = 1.2 - the results are shown in table 60 and they are graphically represented in figure 45;

Table 60. Dissolution test in pH 1.2 dissolution media

Time (min)	% released lisinopril			
	10	**15**	**20**	**30**
A	76.5515	93.5989	94.0598	95.8547
B	63.3411	91.8680	92.7665	97.3693

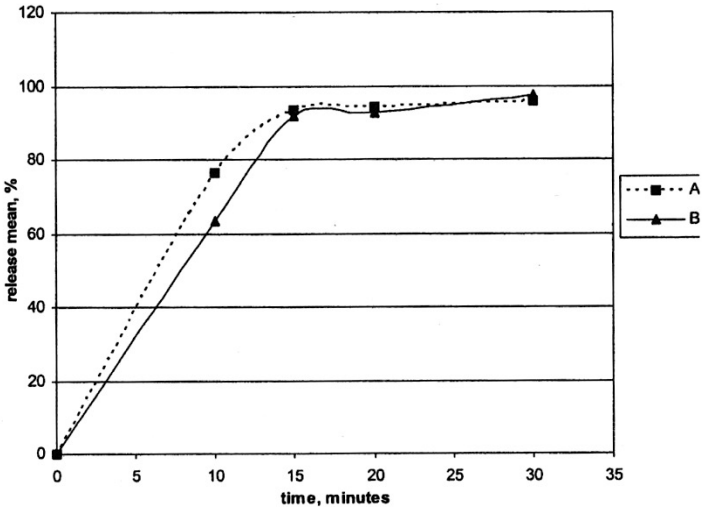


Fig. 45. Comparative dissolution profiles in pH 1.2 dissolution media

• pH = 4.5 - the results are shown in table 61 and they are graphically represented in figure 46;

Table 61. Dissolution test in pH 4.5 dissolution media

Time (min)	% released lisinopril			
	10	**15**	**20**	**30**
A	51.1896	96.7726	97.7318	98.5634
B	75.5164	93.0365	95.4984	98.3925

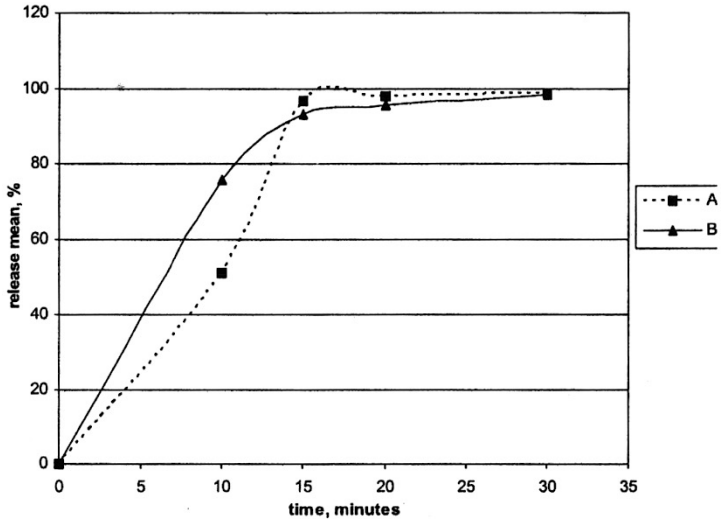


Fig. 46. Comparative dissolution profiles in pH 4.5 dissolution media

• pH = 6.8 - the results are shown in table 62 and they are graphically represented in figure 47.

Table 62. Dissolution test in pH 6.8 dissolution media

Time (min)	% released lisinopril			
	10	**15**	**20**	**30**
A	87.1772	93.5230	94.1995	98.3643
B	93.1250	95.2389	95.9041	100.1474

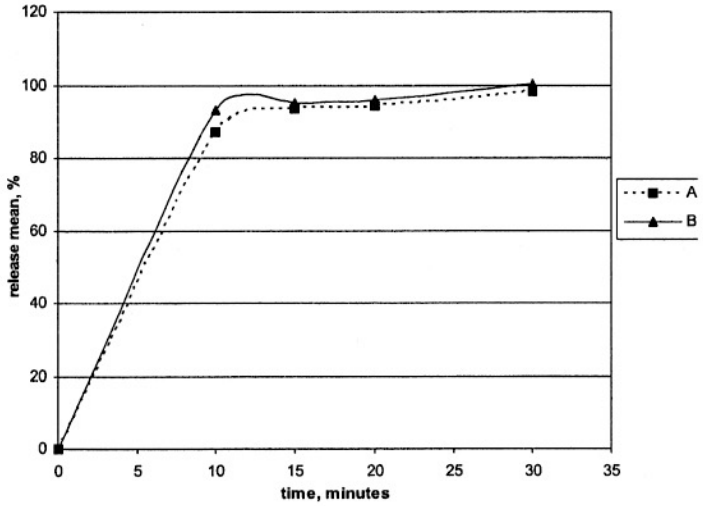


Fig. 47. Comparative dissolution profiles in pH 6.8 dissolution media

V.2.3. Discussions

Dissolution testing has emerged as a very important tool in the pharmaceutical industry. The in vitro dissolution test is the most useful quality control test for pharmaceutical dosage forms such as tablets and capsules from the standpoint of bioavailability and physical consistency. Because bioavailability is largely dependent upon having the drug in dissolved state, for poorly soluble drugs the dissolution test can give an indication of poor bioavailability and reduced efficacy (Anand et al., 2011; Kovács et al., 2014; Speer et al., 2019).

The dissolution method should be sufficiently rugged and reproducible for daily operations, capable of being transferred between laboratories, and adequately discriminating to distinguish any changes that could affect the product's *in vivo* performance (Siew, 2016).

Dissolution profiles should be generated using at least three dissolution media, for example, pH 1.2, 4.5, and 6.8 buffers (Anand et al., 2011).

While comparing dissolution profile of the pharmaceutical formulations with lisinopril using 900 mL pH 1.2 buffer as dissolution media and stirring speed of 75 rpm, the results are shown in table 60 prove that both products released more than 85 % within 15 min. of testing and that they have similar dissolution profiles. This can also be observed in figure 45.

Each lisinopril product released a percent greater than 90% when tested in a pH 4.5 buffer as dissolution media and at stirring speed of 75 rpm. The percent increases when the released lisinopril is quantified after 30 min. - over 98% (Table 61, Fig. 46).

The last dissolution test was performed at an almost neutral pH value (pH 6.8 buffer was used as dissolution media). Both products released more than 85% lisinopril in the first 15 min. as can be seen from table 62 and figure 47.

The dissolution requirements according to The United States Pharmacopeia (USP 41, 2018) and Food and Drug Administration (FDA, 2018) is that the amount of drug released (dissolution) should not be less than 80% of the labelled amount at 30 min.

In almost every situation involving dissolution test results (whether new lab results or data from documents), the need will emerge to compare dissolution profiles, either with other test results or with pre-determined specifications. Such comparisons can be performed in multiple ways, to various degrees of statistical rigour (Wang et al., 2015).

Our studies led to the following results: both products present a dissolution percentage of more than 85% (Q) of the labelled amount after 30 min. for lisinopril. The dissolution profiles of the studied products are almost identical.

V.2.4. Conclusion

The results of dissolution test of the two brands of lisinopril complies within the official specification and passed the test which states that the amount of drug release (active ingredient) in solution should not be less than 80% of the labelled amount at 30 min. The products develop similar dissolution profiles, thus the bioequivalence of Lisinopril 20 mg tablets (Antibiotice S.A. Iaşi) and Zestril 20 mg tablets (Astra Zeneca) was demonstrate *in vitro*.

V.3. Development and validation of novel spectrophotometric methods for determination of lisinopril in pharmaceutical dosage forms

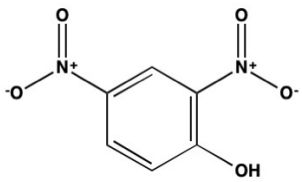
VIS Spectrophotometry presents some advantages which have found their usefulness in the analysis of lisinopril as well: the determinations are fast, small quantities of complex samples can be used, and they present a high sensitivity and offer a high degree of accuracy of the measurement results.

The development of specific VIS methods in the quantitative estimation of lisinopril in bioequivalence studies or quality control of some pharmaceutical forms using phosphomolybdic acid, phosphotungstic acid, potassium permanganate or 2,4-dinitrophenol as

reactants. The spectrophotometric methods validation has been made respecting recommendations regarding the validation of analytical procedures.

In table 63 we can see the summarized presentation of the spectrophotometric methods developed and validated for the quantitative determination of lisinopril in bulk and pharmaceutical formulations.

Table 63. Development and validation of novel spectrophotometric methods for determination of lisinopril in bulk and pharmaceutical dosage forms

Reactive / λ_{max}	**Results/Discussions**
$H_3Mo_{12}O_{40}P$ ***Molybdophosphoric acid*** *369 nm*	A new UV-VIS molecular absorbance spectrometric method was developed for the assay of lisinopril using molybdophosphoric acid in hydrochloric acid medium. The reaction product showed a maximum absorbance at 369 nm. The method showed a good linearity in the range of 8.0 - 32 µg/mL (r^2 = 0.9995). The detection limit (LOD) was 2.33 µg/mL and the quantification limit (LOQ) was 7.79 µg/mL. The precision and the accuracy were determined, (RSD = 1.64%); mean recovery was 100.59% in the 98.51-102.39% concentration range.
$KMnO_4$ ***Potassium permanganate*** *608 nm*	The method was based on the oxidation reaction with potassium permanganate in alkaline medium when potassium manganate was obtained. The maximum absorbance was measured at 608 nm. The method presented a good linearity in the concentration range 2-14 µg/mL (r^2 = 0.9995). The detection limit (LOD) was 0.54 µg/mL and the quantification limit (LOQ) was 1.83 µg/mL. The RSD for the precision of the method was 1.50 and the recovery values was ranged between 99.18-100.74%.
$H_5O_{41}PW_{12}$ ***Phosphotungstic acid*** *335 nm*	The method was based on the precipitation reaction with phosphotungstic acid in acidic medium. The maximum absorbance of the precipitate was measured at 335nm. The method presented a good linearity in the concentration range 9.0-33 µg/mL (r^2 = 0.9995). The detection limit (LOD) was 2.24 µg/mL and the quantification limit (LOQ) was 7.47 µg/mL.The RSD for the precision of the method was 0.42, the RSD for the intermediate precision was 1.11, and the recovery values were ranged between 99.15-100.97%.
 ***2,4-dinitrophenol*** *400 nm*	A UV-VIS spectrophotometric method was developed for the assay of lisinopril using 2,4-dinitrophenol. The reaction product showed a maximum absorbance at 400 nm. The analytical method was validated by establishing the linearity domain in the range of 2.0-14 µg/mL (r^2 = 0.9995), the detection limit was 0.42 µg/mL, the quantification limit was 1.42 µg/mL lower than the lowest concentration from the linearity domain, method precision RSD% = 1.61% and the accuracy evaluated through recovery was 100.39%.

VI. Modern analytical methods used for quantification of some pharmaceutical substances

VI.1. Introduction

Research and development of pharmaceutical compounds has led to a revolution in the field of human health. Methods of analysis for concentration estimation in active substances, excipients and impurities were developed so that pharmaceutical products could serve the purpose they were inserted into therapy. Degradation and alteration can appear in the stages of synthesis, conditioning, wrapping, transport or storage.

Bioavailability and pharmacokinetics studies provide information regarding the fate of the drug compound, including in biological mediums, confirming the aimed action mechanism, the dosage and the administration plan, the efficacy rate, the safety and the association between the benefit and the total risk of the drug.

The instrumental techniques of quantification and detection have a decisive role in the quality control of drugs and in their biological profile study.

In this chapter one can see the summarised description of a few HPLC determination methods of some drug compounds in biological mediums (alprazolam, clopidogrel) or pharmaceutical dosage forms (piroxicam). We have also studied the separation through ion exchange chromatography of haemoglobin hydrolysed.

The studied spectrophotometric methods were applied in the quantitative determination of Fe (II) from pharmaceutical products or of hydroxyurea from biological liquids. The disposal of miconazole nitrate from products (films) with dermal application was also quantified through a spectrophotometric method.

The studies were materialized in the following publications:

- Bibire N, Ţântaru G, Apostu M, Agoroaei L, Vieriu M, Panainte AD, Vlase A. A New bioanalytical method for the determinationof alprazolam in human plasma. *Rev Chim (Bucharest)* 2013; 64(6): 587-592.
- Bibire N, Vieriu M, Ţântaru G, Apostu M, Agoroaei L, Panainte AD, Znagovan A, Vlase A. A new and sensitive LC-MS/MS method for the determination of clopidogrel in human plasma. *Rev Chim (Bucharest)* 2014; 65(7): 807-810.
- Panainte AD, Vieriu M, Ţântaru G, Apostu M, Bibire N. Fast HPLC Method for the determination of piroxicam and its application to stability study. *Rev Chim (Bucharest)* 2017; 68(4): 701-706.
- Panainte AD, Morariu ID, Bibire N, Vieriu M, Ţântaru G, Luca AC, Apostu M. Studies on chromatographic fractioning by cations exchangers of a bovine hemoglobin hydrolysate. *Rev Chim (Bucharest)* 2018; 69(10): 2794-2798.
- Ţântaru G, Apostu M. Analytical and biological implications of complex combinations of hydroxyurea with Iron (II). *Rev Chim (Bucharest)* 2010; 61 (7): 632-635.
- Bîrsan M, Apostu M, Todoran N, Antonoaea P, Rusu A, Ciurba A. Development of dermal films containing miconazole nitrate. *Molecules* 2018; 23, 1640; doi:10.3390/molecules23071640.

VI.2. High-performance liquid chromatography metods

High-performance liquid chromatography (HPLC) was introduced for the first time in 1980 as an official method of analysis of pharmaceutical substances in bulk (United States Pharmacopoeia, 1980). It is an advanced type of liquid chromatography used in the separation of some complex mixes encountered in chemical and biological systems or in the individual recognition of molecules. Its high precision and specificity have determined it to become one of the most used, or even, the main analysis method in various pharmacopoeias (USP, Ph. Eur.).

Through the adequate choice of the mobile phase, the type of column and detector, as well as through the establishment of the best analysis conditions, the work time was considerably shortened, the obtained results justifying the choice of this type of analysis.

VI.2.1. A new bioanalytical method for the determination of alprazolam in human plasma

Alprazolam (8-chloro-1-methyl-6-phenyl-4H-[1,2,4] triazole [4,3,-α]-[1,4] benzodiazepine) (Fig. 48) is a new generation 1.4-benzodiazepine belonging to the nitrogen heterocycle compounds class. It is a benzodiazepine mainly used to treat anxiety disorders. On a short time basis it is used to palliate symptoms of anxiety or anxiety associated to symptoms of depression (Pieróga et al., 2018). Besides this, alprazolam is also used to treat panic disturbances with or without agoraphobia (Dawson et al., 1984; Ait-Daoud et al., 2018; Strawn et al., 2019).

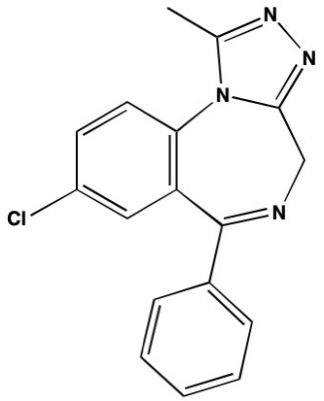


Fig. 48. Chemical structure of alprazolam ($C_{17}H_{13}ClN_4$)

Several methods for the quantitative determination of alprazolam in human plasma have been reported. Mainly, liquid chromatography methods with UV detection were described (Pérez-Lozano et al., 2004; Bazmi et al., 2016). LC-MS methods have been widely accepted as the most used method for the quantitative determination of drugs due to their sensitivity and specificity (Marin et al., 2010; Furugena et al., 2019).

This study reports a sensitive, specific and reproducible liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the quantitative determination of alprazolam in human plasma (EDTA-K 3 , anticoagulant), using lorazepam as internal standard.

All analyses were performed using the Agilent 1200 Triple Quad 6410A System. The system components included the Agilent 1200 Degasser, Agilent 1200 Binary Pump, Agilent 1200 Autosampler, Agilent 1200 Mass Selective Detector. The Agilent MassHunter software was used for system control and data acquisition.

The LC-MS/MS method was performed using a reverse phase column Zorbax SB-CN (4.6 x 100mm, 3.5 μm). The mobile phase consisted of a mixture of 0.5% formic acid solution (pH = 3) and acetonitrile (30:70, v/v). The LC system was operated at 0.8 mL/min, using the binary pump. The column temperature was 40°C. The injection volume was 10 μL and represented no more 5% of the total sample available for injection.

Alprazolam and lorazepam were monitored at m/z transitions of 309→ 281 and 321→ 275 for lorazepam, respectively, with dwell times of 100 ms and collision energy of 14, for each.

LC-MS/MS peaks were observed at approximately 2 min for both the alprazolam and lorazepam, respectively. Integration was performed using the Agilent MassHunter software associated with the mass spectrometer.

The proposed method has been validated for the concentration range in between 0.1 and 50 ng/mL and a correlation coefficient of 0.9991. The precision and accuracy were within 12% for intra-day and inter-days assays. The overall recoveries for alprazolam and lorazepam were 76% and 88%, respectively. The overall time of one analysis was 4 minutes. The assay has proven to be sensitive, specific and reproducible, suitable for the quantitative determination of alprazolam in human plasma.

VI.2.2. A new and sensitive LC-MS/MS method for the determination of clopidogrel in human plasma

Clopidogrel is a thienopyridine derivative: [methyl(+)(S)-α-(2-chloro-phenyl)-6,7-dihydrothienol[3,2-c]pyridin5(4H)-acetate hydrogen sulfate] (Fig. 49). It is a potent antiplatelet drug prescribed for the prevention of vascular thrombotic events. Following oral administration, clopidogrel is rapidly absorbed and undergoes extensive hepatic metabolism.

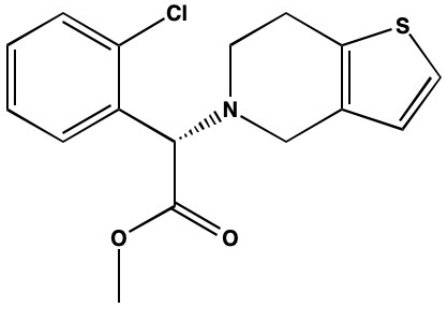


Fig. 49. Chemical structure of clopidogrel ($C_{16}H_{16}ClNO_2S$)

Several assay methods are available for determination of clopidogrel, including HPLC-UV (Mitakos et al., 2002; Takahashi et al., 2008, Anuta et al., 2015, Deshmukh et al., 2019).

The HPLC assays are not sensitive enough to measure clopidogrel levels in biological fluids after oral administration of therapeutic doses. Recently, LC-MS/MS has been increasingly utilized as it can analyze test samples regardless of their purity in biological substances and measure the blood drug concentrations with high sensitivity and selectivity (Ramakrishna et al., 2006; Peer et al., 2012; Liu et al., 2018; Turner et al., 2018).

This study reports a simple and sensitive LC-MS/ MS method for the determination of clopidogrel in human plasma. This assay can be used for the therapeutic drug monitoring of clopidogrel. The parameters usually examined in the validation process are selectivity/specificity, linearity, limit of quantification, accuracy and precision.

All analyses were performed using the Agilent 1200 Triple Quad 6410 System. The system components included the Agilent 1200 Degasser, Agilent 1200 Binary Pump, Agilent 1200 Autosampler. The separation was performed using a reverse phase column Zorbax SB-C8, supplied by Agilent, USA.

The mobile phase consisted of a mixture of 50:50 (v/v) mixture of acetonitrile and 0.1% formic acid aqueous solution, with a flow rate of 1 mL/min.

Detection was performed by positive ion electrospray tandem mass spectrometry on a HPLC Agilent 1200 Triple Ouad 6410 System. The mass transition of the ion-pair was followed for m/z 322.1→212.1, for clopidogrel and m/z 327→217, for clopidogrel-d3 (internal standard), in multiple reaction monitoring mode (MRM).

The proposed method has been validated for a linear range of 10-10000 mg/mL and the value of the regression coefficient was ≥ 0.99. The precision (CV%) and accuracy (%) were lower than 6.1 % for intra- and inter-days assays. The overall recoveries for clopidogrel and clopidogrel-d3 were 81 and 84%, respectively. The overall time of the analysis was 7 min. The method is sensitive, specific and reproducible and can be used for the therapeutic drug monitoring of clopidogrel.

VI.2.3. Fast HPLC method for the determination of piroxicam and its application to stability study

Piroxicam is a nonsteroidal anti-inflammatory drug from the oxicam class, which shows chemopreventive and chemosuppressive effects against various cancer cell lines in animal models. It is used to treat various painful conditions such as injuries and dental inflammation Piroxicam works by reducing hormones that cause inflammation and pain in the body. The anti-inflammatory effect of piroxicam result from the reversible inhibition of cyclooxygenase, causing the peripheral inhibition of prostaglandin synthesis.

The chemical name of piroxicam shown in figure 50 is 4-hydroxy-2-methyl-1,1-dioxo-N-pyridin-2-yl-1λ6,2-benzothiazine-3-carboxamide. Piroxicam is official in European Pharmacopoeia, British Pharmacopoeia, United States Pharmacopeia, and Indian Pharmacopoeia.

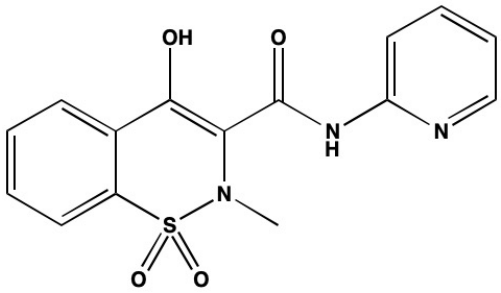


Fig. 50. Chemical structure of piroxicam ($C_{15}H_{13}N_3O_4S$)

Piroxicam may be quantitatively determined from tablets and biological fluids using various analytical methods such as high performance liquid chromatography (Vijay et al., 2010; Calvo et al., 2016), supercritical fluid chromatography (Li et al., 2018), UV-VIS spectrophotometry (Bonazzi et al., 1995; El-Ries et al, 2003), electrophoresis (Gül Dal et al., 2014; Otarola et al., 2015).

This study described the development and validation of a novel fast and accurate HPLC method with UV detection for the quantitative determination of piroxicam in bulk and pharmaceutical formulations.

All analyses were performed using the Agilent Technologies 1100 High Performance Liquid Chromatograph equipped with Diode Array Detector.

The chromatographic separation of piroxicam was achieved using isocratic elution on a SB-C 18 Eclipse (150 x 4.6 mm, 5µm) reverse phase stainless steel column. The temperature of the column and autosampler was maintained constant at 25°C. A 50 µL sample loop injector was used. The detection was done spectrophotometrically 360nm. The mobile phase was a mixture of acetonitrile and water (50:50) used at a flow rate of 0.50mL/min. The retention time for piroxicam was 2.55 min.

The method was validated in accordance with the current ICH guidelines in terms of linearity, limit of detection, limit of quantification, precision, accuracy, recovery and system suitability.

The calibration graph was linear in the concentration range 5-90 µg/mL (r^2 = 0.9993, LOD = 1.04 µg/ mL, LOQ = 1.15 µg/mL).

The system suitability was evaluated using piroxicam standard solution. Because RSD area ≤ 2% and RSD retention time ≤ 1.5%, we concluded that the chromatographic system was robust. The intra- and inter-day precision of measurements for three different concentration levels (36, 45, and 54 µg/ mL) were lower than the accepted criteria (RSD ≤ 2%). The proposed method was accurate because the maximum value of RSD(%) was lower than 2% and mean recovery yield was 100.83 with values in between 99.08% and 101.98% (table 4).

The proposed method was used to for the analysis of piroxicam in pharmaceutical formulations used for the treatment of arthritis.

VI.2.4. Studies on chromatographic fractioning by cations exchangers of a bovine hemoglobin hydrolysate

Enzymatic protein hydrolysis is used in the food industry to improve organoleptic and functional properties such as solubility, emulsification, gelling or for large-scale preparation of protein hydrolysates used in hypoallergenic diet of children and in nutritional therapy (Temussi, 2012). Blood proteins are also used in the pharmaceutical industry. For example, hemoglobin is the main constituent of Proferrin® (Colorado Biolabs, Inc., CO, USA), a heme iron supplement used to address iron deficiency. Many beneficial health effects have been described for theose molecules, (e.g., antimicrobial, antioxidative, antithrombotic, antihypertensive and immunomodulatory) (Adje et al., 2011).

One of the most common ways to obtain biopeptides from protein sources is enzymatic hydrolysis. Active peptides obtained during enzymatic hydrolysis of food proteins are often intermediates and are generally found in a complex peptide population in which their concentration is very poor. Their preparation requires rather complicated fractioning and purification techniques.

In order to simplify those problems, numerous researches have been carried out using biphasic organic solvent extraction, systems that can isolate these active peptides from protein hydrolysates. In 1985, Brantl (Brantl et al., 1986) have isolated for the first time biologically active peptides based on in vitro enzymatic hydrolysis of bovine hemoglobin, a method that has been used extensively. Currently, 150 fragments of hemoglobin have been isolated, and the biological activity of 18 of them has already been determined. Bovine hemoglobin was selected for use in bioactive hydrolysates with potential use as functional food ingredients for the prevention of various disorders such as hypertension, obesity and diabetes (Li-Chan, 2015; Bah et al., 2013; Danquah et al., 2012).

The objective of the study was to fractionate using ion exchange chromatography a bovine peptic hydrolysate hemoglobin in order to obtain fractions with a small number of peptides.

The HPLC system used included a Waters TM 600 pump controller and a Waters 996 diode bar detector. A Vydak C4 column (250 × 4.6 mm, 3.5 μm) was used for separation, because it was suitable for the separation of large peptides, with 20 to 30 amino acids.

The mobile phase consisted in an A eluent containing water and trifluoroacetic acid (TFA) in a 1000:1 (v/v) ratio, and a B eluent composed of acetonitrile, water, and TFA in a 600:400:1 (v/v) ratio. The solutions were degassed continuously, thanks to a membrane degasser. Prior to injection, the samples were filtered through a 0.2 μm cellulose acetate filters (AIT Chromato). Separation was carried out in an elution gradient at a flow rate of 1 mL/min. The gradient consisted of a linear increase of the eluent B ratio in the mobile phase from 0 to 60% for the first 30 min, and then from 67 to 87% during the next 35 min.

The detection was done using mass spectrometry, and the retention time, size and distribution of the peptides were determined. All spectrometric measurements were performed in positive ionization mode on a Quatro II triplequadripol equipped with an electrospray ion source.

The bovine hemoglobin used in this study is a model substrate because it combines exceptional features such as its structure and its perfectly known sequences and the possibility

of being obtained in pure state. The 2.5 minute hydrolysate showed a peptide population between 35 and 38 which was composed of large peptides. The MS analysis of the isolated protein revealed a series of multiple charged species of bovine α- and β-globin chains. The majority of the sequences were identified.

VI.3. Spectrophotometric metods

An important group of methods with a significant share in pharmacopoeia is represented by the spectrophotometric methods based on the natural absorption of UV-VIS radiations or on the achievement of coloured derivates through colour reactions with adequate reagents. The advantages of these methods are a reduced time and reagents requirement, accuracy and sensitivity of the determinations.

VI.3.1. Analytical and biological implications of complex combinations of hydroxyurea with iron (II)

Hydroxyurea (Hy) inhibits the enzyme ribonucleotide reductase, responsible for the conversion of the ribonucleotides in deoxyribonucleotides, blocking the AND synthesis and determining the topping of the cellular division (Nyholm et al., 1993; Staake et al., 2016). For this reason, it is used in the treatment of numerous types of cancer (Fang et al., 2019). Hy reduces the plasmatic clearance of ferum, as well as its use by the erythrocytes being the cause of some severe effects such as anemia (Fields et al., 2019). The pharmacological effects are determined by the structural modifications from inside the molecules due to the solvent (Glover et al., 1999, Singh et al., 2016). In contact with water, Hy has several tautomeric forms in equilibrium (Fig. 51) in which the solvent acts as a catalyser on the >N-H bond resulting in the formation of the >N–OH bond.

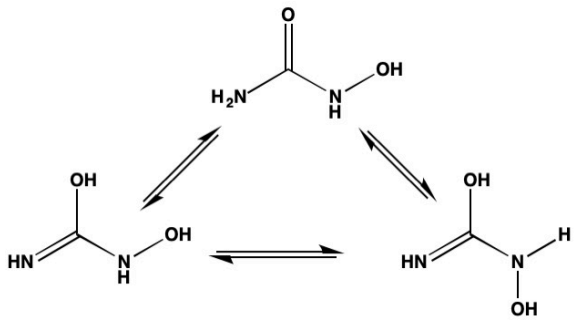


Fig. 51. Conformational and tautometric system of hydroxyureas (Hy)

The IR spectra have a strong absorbance at 1650 cm^{-1} due to the presence of the >C=N bond (the same as for the Schiff bases and bis bases used as reagents for determining some cations). Studies on these tautomeric forms have been carried out through NMR, HPLC and quantitative determinations (James et al., 2006).

Different structural forms can prevail "in vivo" depending on the polarity of the medium and the prevailing structure influences the transport, the passing through membranes, the partition and the interaction with the ions (Parker et al., 1977).

Based on these tautomeric forms in aqueous solutions, an in vitro study has been carried out regarding the complexing capacity of Hy with Fe(II).

The complex formed by Hy with the Fe(II) ions is stable in water for a pH = 7.4-7.6 with a maximum of absorption at λ = 267nm ($\varepsilon = 3.4559 \cdot 10^4$ $mol^{-1} \cdot L \cdot cm^{-1}$). The combination rate has been established using the isomolar series method at 1:1 (M/L). The calculated value of the stability constant is $1.26 \cdot 10^6$. The Lambert - Beer law is observed in the concentration interval 10-60 μg Fe(II), r^2 = 0.9999. The detection limit (LOD) is 2.71μg/mL and the quantification limit (LOQ) is 9.04 μg/mL.

Under the same reaction conditions, Hy forms complex combinations with other ions: Fe(III) with maximum absorption at λ_{max} = 386 nm and λ_{max} = 578 nm, but also Co(II) with λ_{max} = 297 nm and λ_{max} = 430 nm (Nigovic et al., 2005). Under different reaction conditions, the hydroxyurea complexes the Al(III) and Mg(II) ions, these reactions being used for the quantitative determination (El-Kosasy, 2003). N-hydroxyurea can form a bidentate complex combination with Zn(II) that strongly inhibits the metalloenzyme (Temperini et al., 2006). The volatile residual solvents from the pharmaceutical products determine the degradation of the hydroxyurea in urea and lactic acid which interferes with its determination (Restituto et al., 2006).

The proposed method has been successfully applied to determine Fe(II) from soft gelatin capsules. The statistical analysis of the results shows a significant accuracy and precision for the proposed method. The proposed method was applied to determine the Hy which is 80 % excreted, through the biological fluid, according to literature data. From the excreted Hy, the Hy-Fe (II) complex presence is determined for the first time. The presence of the Hy-Fe(II) complex in the bilogical fluid of the patients in a percentage of 20% explains the appearance of anemia as a side effect of the treatment with hydroxyurea for a long period of time.

VI.3.2. Development of dermal films containing miconazole nitrate

The development of pharmaceutical forms begins with the preformulation process, which establishes the physical and chemical, pharmaceutical and technological, pharmacological and toxicological features of substances (Zulkifli et al., 2017). As an active pharmaceutical ingredient, miconazole nitrate (MN) is a broad spectrum antifungal agent which has already been studied in the form of mucoadhesive tablets, polymeric matrices (chitosan, gelatine, gum arabic, alginate, carbopol or acrylic resins), microgels, nano-lipid gels or nano-vesicles formulated to treat oral or vaginal candidiasis (Gupta et al., 2016; Kenechukwuet al., 2017). In addition to its antifungal activity, MN also has an antimicrobial action - the imidazole class (Fig. 52), which makes it frequently applied on skin mucosa to heal fungal infections (Ozeki et al., 2016).

This study aims to develop new antifungal dermal films based on their mechanical properties (elongation, adhesion, behaviour towards vapour moisture) and the in vitro availability of miconazole nitrate, used as a pharmaceutical active ingredient in various concentrations.

The three polymeric films prepared were translucent or shiny, with the surface of 63.585 cm^2, 0.20 - 0.30 mm thickness, and content of miconazole nitrate of 3.931 or 15.726 $mg \cdot cm^2$. The ability of MN to be released from the polymeric matrix of the films was evaluated using the Franz diffusion cell method (Park et al., 2016; Shah et al., 2010), through a synthetic membrane and acceptor media with pH 7.4.

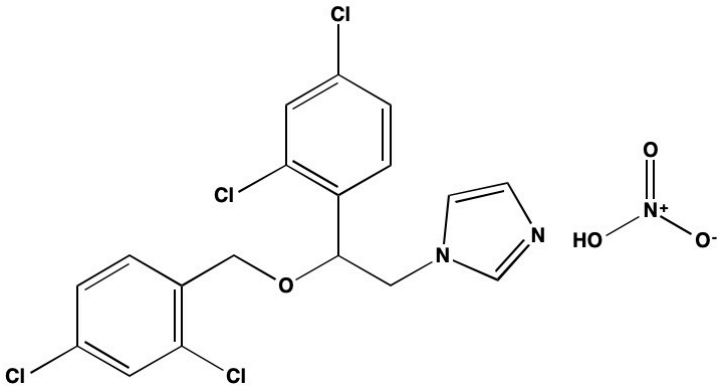


Fig. 52. Chemical structure of miconazole nitrate ($C_{18}H_{15}Cl_4N_3O_4$)

Franz cells of 14 mL capacity and 1.8 cm diameter were used. The diffusion membrane (Teknokroma Ø 25 mm × 0.45 μm) was prepared before each determination by maintaining it in a phosphate buffering solution pH 7.4.

The samples analysed consisted of disk shaped films with diameter of 1.8 cm (surface of 2.54 cm^2). The media consisted in a buffer solution pH 7.4 to which was added 0.045% sodium lauryl sulphate.

During the determination: the diffusion cells were maintained at 32 ± 0.5 °C, by heating in recirculated water, with oscillating (electromagnetic) stirring. The samples taken (5 mL) were replaced each time with fresh media, and the MN content was assessed by ultraviolet (UV)-spectrophotometric method, at λ = 273 nm (Clarke's, 1989).

The calculations considered equation:

$$MN = m(n-1) + 14C_{pn} - 9C_p(n-1)$$

where: MN (mg)-mass of miconazole nitrate released at moment *n* (collection *n*), C_{pn}-the concentration of miconazole nitrate expressed in mg/mL for sample collected at moment n, 14-the volume of Franz cell expressed in mL, 9-the volume expressed in mL of the acceptor media that remained in Franz cell after the collection of the 5 mL sample.

VI.4. Conclusions

The studies have aimed to develop new methods of quantitative determination through high-performance liquid chromatography (HPLC) or VIS spectrophotometry for some of the pharmaceutical and biomedical compounds of interest. The methods were validated and applied at their quantitative estimation in pharmaceutical dosage forms or biological mediums.

VII. The evolution and development of the professional, scientific and academic career

My entire activity as member of the teaching staff and researcher took place in the Analytical Chemistry Discipline at the Faculty of Pharmacy, "Grigore T. Popa" University of Medicine and Pharmacy, Iaşi. Along with the members of this discipline, a team in which I find many values of my own, I have participated in didactic and scientific activities, research and collaboration in the field of analytical chemistry activities.

Profesional achievements

In my vision, a successful member of the teaching staff must have technical and cultural abilities for the future, to notice the evolutions of the academic environment and to be willing to continuously learn and develop in order to adapt in case of the appearance of possible opportunities. His or her professional value is defined by the recipient of his or her actions, the student, thus being considered a good teacher when he or she generates actual value in the quality of graduates, fact which shall reflect in his or her reputation.

I have begun my didactic activity in 1996, when I was employed, through examination, on the function of University assistant in the Analytical Chemistry Discipline at the Faculty of Pharmacy, "Grigore T. Popa" University of Medicine and Pharmacy, Iași. In the same year I have also begun the preparation through residency in General Pharmacy specialisation.

From the position of teaching staff member I have actually participated in the preparation and development of practical activities, as well as in the processing through seminars of analytical chemistry knowledge accumulated by 1st, 2nd and 3rd year Pharmacy students, 4th year Medical Bioengineering students and Clinical Pharmacy residents. I have contributed to the establishment of some new practical activities, especially in the field of instrumental analysis, in order to familiarise students with the latest methods which underlie the physical-chemical control of the drug (optic methods, electrochemical methods, chromatographic methods, etc.).

In 2006 I have been promoted, through examination, to lecturer and have become the holder of the course in Analytical Chemistry - module II, 3rd year Faculty of Pharmacy, but I have also taught/ I teach Analytical Chemistry courses to 1st and 2nd year students, as well as to the ones from Pharmacy Assistant specialisation.

From 2014 until now I have been an Associate Professor of the same discipline, position which I have taken through examination.

Throughout this entire time I have tried to improve my didactic abilities which could be emphasised through a teaching technique based on learning through discovery, through cooperation and questioning, the notions learned in classes ensuring the assimilation of knowledge mandatory for the pharmacist profession.

I have published 7 textbooks as main author or co-author, which have had as purpose the improvement of the didactic and professional activity and represent a transposition of the knowledge acquired throughout research and documenting activities into teaching materials.

Scientific achievements

Scientific research is a fundamental component of a teacher's activity and represents one of the main evaluation criteria of the qualification and appreciation of the academic performance. Scientific research is the base of the higher education process, which ensures the training of highly qualified human resources.

The main themes that I have approached in my scientific research activity until now refer to:

- The elaboration and validation of new analysis methods for medicinal substances, pharmaceutical forms, active principles from medicinal plants, mycotoxins;
- The insertion of new analytical reagents and electrochemical sensors in order to determine certain ions and pharmaceutical substances.

↳ In 1996 I have begun my research activity with my enrolment in the doctoral program, under the guidance of Prof. V. Dorneanu, with the thesis *"Analytical research regarding new methods of quantitative determination of inhibitory medical substances of gastric secretion (Antihistamines H_2)"*.

Researches made during the doctoral studies had as purpose finding new methods of analysis for a group of medicines frequently used in the treatment of gastric and duodenal ulcer: ranitidine, nizatidine and famotidine.

For each of the medical compounds present in the study I have made an analytic profile based on the dates synthesised from the consulted specialty literature.

The study of physical-chemical proprieties has led to achieving new methods of quantitative determination through UV-VIS spectrophotometry and potentiometry, thus extending the possibilities of quantitative estimation of the three antihistamines H_2 in pharmaceutical dosage forms or biological liquids.

A part of the results presented in the doctoral thesis have been obtained during the research project called:

- *Analytical research in order to insert new methods of physical-chemical control of certain inhibitory medical substances of the H_2 receptors of histamine;* Contract no. 1290/1996 and Amendment no. 1627/29.10.1997 signed with the Ministry of Research and Technology for the period 1997-1999.

↳ In the postdoctoral period, along with the procurement of certain reagents which were not previously available and with the modern equipment, I went deeply into research in the field of *selective membrane electrodes based on inclusion polymeric matrices.*

I have built, after a personal idea, and tested a series of electrochemical sensors with PVC matrix for ranitidine, nizatidine, famotidine, scopolamine and lisinopril. All electrodes have found applications in pharmaceutical dosage forms.

↳ Research regarding *heavy metals and their biotransfer* from various types of soil in tobacco or vegetable origin foods has represented another direction of study. In order to quantify some of the most contaminated heavy metals I have elaborated and validated methods of analysis through atomic absorption spectroscopy (AAS) or direct potentiometry using selective membrane electrodes.

In the same field I have led, as project director, a study regarding the evaluation of the real capacity of some vegetable watery extracts to bind and favour the removal of heavy metals from the body, as an alternative to the classic chelating agents therapy:

- Contract no. 29238/20.12.2013 - *"In vitro" evaluation of the effect of reduction of the digestive bioavailability of heavy metals for some vegetable extracts with possible applications in the therapy with chelating agents*; Financer: "Grigore T. Popa" University of Medicine and Pharmacy, Iași through the internal research grant program.

A "*light detoxification*" which aims to reduce the bioavailability of heavy metals introduced on a daily basis into the body through food, inevitably affected by the current level of industrialisation and pollution, can be made through natural solutions. Recent research have proven that vegetable products rich in fibres, pectin, polyphenols and sulphur-containing amino acids can inhibit the absorption of heavy metals into the body, favouring their elimination.

Studies have followed the effects of watery vegetal extracts coming from hawthorn (*Crataegus sp.*), linden (*Tilia sp.*), rosehip (*Rosa canina*), blueberries (*Vaccinium myrtillus*), herb-Robert (*Geranium robertianum*), mint (*Mentha piperita*), artichoke (*Cynara cardunculus subsp. scolymus*), plantain (*Plantago sp.)* and coriander (*Coriandrum sativum*) on cations Cd^{+2}, Cu^{+2}, Pb^{+2}, Hg^{+2} and Ni^{+2}.

↳ Studies regarding the synthesis and analytical and biological applications of Schiff bases and their complexes with some metal cations have been a preoccupation of the research collective in the Analytical Chemistry discipline.

I have synthesized and characterized Schiff bases derived from pyridine, aniline, hydroxyurea and methylenediamine which have been submitted, along with a part of their metal complexes, to a screening of the biological activity and proprieties with analytical applications.

↳ In the following research projects, along with the discipline or faculty researchers, I have contributed to the *elaboration and validation* of new analysis methods for pharmaceutical substances, active principles in medicinal plants, mycotoxins:

- CNCSIS no. 1222/2005 - *Ochratoxins in foods - risk factor for human health;* Financer*:* CNCSIS, Period: 2005-2006;
- CEEX no. 10246/2006 - *Hepato - nephrotic mycotoxins (aflatoxins, ochratoxins, sterigmatocystin, citrinin) risk factors for human and animal health; studii epidemiological, morphological and prevention strategy studies.* Financer: National Project Management Centre, Period: 2006-2008;
- PNCDI, Program 4 (PC) nr. 62-065/2008 - *Complex characterisation of some cytostatic active extracts in Claviceps purpurea strains obtained through parasexual hybridization technology, in order to use it in veterinary therapeutics;* Financer: National Project Management Centre, Period: 2008-2010.

↳ I have been part of the *guidance team* in the research activity of some PhD students, thus contributing to choosing and adapting the best study methods of their research themes.

↳ Within the Analytical Chemistry Science Circle I have tried to offer from my experience to young enthusiastic researchers who wished to be part of this team.

In summary, I have published 22 papers in ISI indexed journals (10 as main author), 25 in BDI journals and I have presented 58 papers at different national and international congresses.

Academic achievements

I have been the Scientific Secretary (2008-2012), and the Pro Dean (2012-2016) of the Faculty of Pharmacy, moments when I have actively participated, among other attributions, in the elaboration and application of the Faculty of Pharmacy Strategic Development Plan and in the carrying out the ARACIS periodical evaluations of the Faculty study programs.

I have been an internal auditor for the Quality Management System from 2008.

Over the years, I have been involved in different activities in the university community, of which I mention here:

- member of the Scientific Committee of University (2008-2016);
- member of the Faculty Council (2008-2016);
- member of the Faculty Committee CEAC (2012-2016);
- member of the Senat of University (2016-present);
- member of the Organizing Committee and Scientific Committee of national scientific congresses.

Future directions in scientific activity

The doctoral training is part of the educational strategy and represents the progress of knowledge through original research. The PhD student is considered a researcher in the beginning of his or her career, his or her training being a professional experience which motivates him or her and trains him or her to be capable to:

- Independently conduct research activities;
- Openly and thoroughly analyse scientific events;
- Determine the required steps in order to gain a new synthesis in the field;
- Bring an original contribution to science and research - this feature being the one which clearly distinguishes the PhD from the other superior educational levels.

As a PhD supervisor I intend to continue to develop with the PhD students my previous research themes and continuously improve them. The research shall focus on multidisciplinary and interdisciplinary collaboration, in order to generate renown value which would maximise the opportunities of partnerships with the aim of revaluating all financing forms.

To the previous themes, in order to develop the scientific research activities, I shall add some new ones:

- The modelling and correlation of physical-chemical parameters of some newly synthesised chemical entities with a structure with the purpose of identifying their utility in pharmaceutical analysis or in the development of pharmaceutical formulations;
- The exploration of particular aspects of modern methods of analysis such as gas chromatography, liquid chromatography, capillary electrophoresis, atomic absorption spectroscopy, in order to emphasize some new possibilities of using them in the analysis of pharmaceutical substances of interest or of biological compounds.

Due to the fact that the subject of electrochemical sensors is one of a continuous interest, aspect shown by the abundance of studies published in prestigious specialty publications in analytical chemistry, I shall continue to develop selective membrane electrodes for new therapeutic molecules or biological compounds.

In the pharmaceutical area, high performance liquid chromatography is essential in the research and development of new medicine, in pharmacokinetics studies and in the quality control of raw materials and finished products. Thus, the HPLC technique is crucial in the pharmaceutical industry, as well as in the control laboratories of the field regulatory authorities.

The HPLC methods developed until the present moment are "mono analyte" methods. Due to the fact that the samples, especially in the pharmacokinetics studies or in the medicine interactions, are complex ones most of the time, I consider that the development of "multi analyte" separation and identification methods represent a promising research direction.

The perspective of administrating some vegetal extracts which would complex and favour the elimination of heavy metals from the body makes the continuation of this research theme on another batch of medicinal plants very appealing, in order to identify the *phytocomplex* which would present a maximum efficiency of the effect.

Professional activity development

The development of the didactic activity aims to continuously improve the teaching methodology, by supporting and involving the students in the learning and research process and by ensuring an information exchange at a national and international level, the role of a professor being that of generating and offering knowledge.

The student is the fundament of the activities in the academic area, the relationship with him or her having to be based on the mentor - future specialist concept, due to the fact that the graduate could become our future colleague.

In the activity with students I intend to modernise the teaching process through:

- The identification of new methods and technologies of training, transmitting, assimilation and evaluation of knowledge understanding;
- The continuous adaptation and revision of teaching materials so that they correspond to international tendencies and particularities;
- The diversification of interactive teaching methods, based on collaborative creativity and educational partnership;
- The active involvement of students in classes and applications, using teaching methods focused on learning through discovery and team study.

I hope that these intentions, once materialised, will stimulate the interest of students in the field of analytical chemistry and will offer them the specific abilities for the profession of pharmacist who can carry out his activity in a medicine research centre, in an analysis laboratory, in the pharmaceutical industry or in the cosmetics industry.

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