Maria Curie-Sklodowska University Faculty of Chemistry Institute of Chemical Sciences



# **SCIENCE AND INDUSTRY** challenges and opportunities



WYDAWNICTWO UNIWERSYTETU MARII CURIE-SKŁODOWSKIEJ

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## **SCIENCE AND INDUSTRY** challenges and opportunities

**Collective book** 



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SALES DEPARTMENT: tel./faks 81 537-53-02 Internet Bookstore: https://wydawnictwo.umcs.eu e-mail: wydawnictwo@umcs.eu Ladies and Gentelmen, Dear Colleagues,

As a reader of this monograph, you are invited to explore the complex and dynamic relationship between science and industry, a relationship that is shaping the foundations of our modern world.

Whether you are a researcher, an entrepreneur, or a student, you are undoubtedly aware of the growing importance of collaboration between academia and the industrial sector. Therefore, in the pages of the monograph 'Science and industry - challenges and opportunities' that follow, we offer a multifaceted perspective on both the challenges and the opportunities that emerge when knowledge meets practice, from technology transfer and innovation ecosystems to insight, reflection and inspiration.

We believe that it will inspire closer collaboration, foster innovation, and promote practical application of scientific results. May it serve as a bridge between researchers and entrepreneurs, contributing to mutual growth and development.

Wishing you an insightful read!

Organizing Committee

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## THE FATE OF PHTHALATE ACID ESTERS IN THE SOIL-VEGETABLE SYSTEM

**Bożena CZECH, Artur SOKOŁOWSKI**, Maria Curie-Skłodowska University, Faculty of Chemistry, Institute of Chemical Sciences, Department of Radiochemistry and Environmental Chemistry, M. Curie-Skłodowska Sq. 3, 20-031 Lublin, Poland.

**Abstract:** Alkyl aryl and dialkyl phthalate acid esters (PAEs), widely used chemicals in industry and daily life, have been recognized recently as emerging pollutants strongly connected with environmental pollution with plastic. The increased usage of plastic foils in agriculture makes PAEs an essential group of soil and food pollutants. The main route of exposure to PAEs to humans is contaminated food. The presented studies examined the fate of PAEs in the soil-vegetable system. As the tested plants, radish and lettuce were chosen to stress the accumulation of PAEs in different edible parts of vegetables. The results show that some significant differences can be observed among tested conditions. In general, leaves were the sinks for almost all PAEs. The increased bioavailability of five of six priority PAEs in lettuce leaves highlights the potential hazard of lettuce contamination when cultivated in PAEs polluted soil.

**Introduction:** Phthalate acid esters (PAEs), widely used chemicals in industry and daily life have been recognized recently as emerging pollutants [1,2] strongly connected with environmental pollution with plastics [3]. The weak physical bonding to the polymer matrix makes PAEs very mobile in the environment [4]. It was recognized that some PAEs reveal high toxicity for example endocrine disruption. Therefore six PAEs: dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), di-(2-ethyl hexyl) phthalate (DEHP), and di-n-octyl phthalate (DNOP) should be monitored in the environment [5]. The application of plastics in the food industry and foils in agriculture reveals the hazard of food contamination with PAEs [6], and food is one of the main routes of PAEs transport to humans. The bioavailability of PAEs is connected with their interaction with plants: by roots from the soil and leaves from the air [7]. PAEs affect both the morphology and physiology of plants. The ability to generate reactive oxygen species (ROS) and other intra- or extracellular free radicals is responsible for observed oxidative stress [8], which lowered chlorophyll content, and damaged cell membranes [9] inhibiting plant growth and development. In the natural environment, PAEs are degraded by hydrolysis and photodegradation, whereas the most dominant PAEs mechanism is microbial degradation [10]. Considering food safety, the objectives of the presented studies were i) estimation of the fate of six priority PAEs in the soil-vegetable system, ii) estimation of the bioavailability of PAEs to the two different vegetables and their accumulation in the roots and leaves.

**Experimental:** The fate of phthalates in a soil-vegetable system was examined using the agricultural soil (Podborcze, Poland 50°41'47.5" N 22°50'41.3" E) and two vegetables from different groups (root vegetable and leafy vegetable). The soil is defined as acidic brown soil developed from deep loess characterized by high potassium (346.9 mg/kg),

high magnesium (7.3 mg/kg), and medium available phosphorus content (59.3 mg/kg). Carbon (organic 13.567 mg/g, inorganic 5.8  $\mu$ g/g, and total dissolved carbon 33.33 mg/L) and nitrogen (total content 4.9 mg/kg) contents were typical for agricultural soils in this region. The radish (*Raphanus sativus L.*), which was chosen as a vegetable whose roots are consumed, and lettuce (*Lectuca sativa L.*), a leafy vegetable, were cultivated in the pot experiment in the glass containers (no additional PAEs source) using agricultural soil spiked with 1 mL of phthalates mixture containing 1  $\mu$ g/mL of each tested compound. The experiment was conducted in the plant growth chamber (Conviron GEN100 light/dark cycles 12:12 h, temperature 22 °C/18 °C day/night, humidity 65%). After 6 weeks of cultivation, all parts were separated, washed in distilled water, and air-dried. Freeze-dried samples of the soil, roots, and leaves (Alpha 1-2 LDplus Christ) were analyzed by GC-MS/MS as detailed in [11]. Each experiment was performed in triplicate and the mean values were presented.

**Results:** The presence of PAEs has affected the growth of all tested plants (Table 1). In general, in PAEs polluted soil, the growth of the roots was enhanced, which may indicate that at PAEs contamination more available surface for nutrients and water absorption was needed. The lettuce roots were more branched than in the control, whereas the radish roots were larger but empty inside (that was also confirmed by the lower increase in the dry mass). The results stress that at low PAEs contamination, the plants redirected their activity toward root development rather than leaves, confirming the negative effect of PAEs on the tested plants.

Parameter	Control [g]	Soil spiked with PAEs [g]
Fresh mass of radish roots	6.20	15.8
Fresh mass of radish leaves	4.00	6.80
Fresh mass of lettuce roots	5.80	7.50
Fresh mass of lettuce leaves	26.0	25.0
Dry mass of radish roots	0.75	2.80
Dry mass of radish leaves	1.10	2.05
Dry mass of lettuce roots	1.00	1.96
Dry mass of lettuce leaves	4.30	4.98

 Table 1. The mass of the radish and lettuce grown in the control soil and soil spiked with PAEs.

In the next step, the bioavailability of selected PAEs was examined. The amount of tested PAEs in the tested plants was not high (Fig.1) (up to 7.5 ng/g), stressing that tested compounds were accumulated mainly in the soil and their bioavailability to plants was lowered. In general, leaves were the main PAEs sinks independent of plant type. DBP, BBP, and DEHP revealed a tendency to be noted at higher concentrations in the roots of radishes than lettuces, which may be dangerous considering that radish roots are the parts consumed mainly. It is also alarming as, according to the studies [12,13], exposure of rats and mice to DBP led to permanent adverse reproductive effects in males, and DEHP showed synergistic toxicity to microbial composition and function.



Fig.1. Concentration of six priority PAEs in the roots and leaves of radish and lettuce.

What is most alarming, the content of all tested PAEs was the highest in the leaves, with lettuce being the main sink for all compounds independent of PAEs characteristics (no correlations between noted concentrations and logKow or molar mass of PAEs). In general, the content of PAEs in the radish roots was the following: DNOP<DMP<DEHP<BBP<DEP<DBP, and in the lettuce leaves: DNOP<DEP<BBP<DMP<DEHP<DBP.

The bioavailability of tested pollutants was established by the calculation of the Bioavailability Factor (BAF) e.g. ratio of the concentration of pollutant in the plant to its concentration in the soil:

### BAF=C<sub>roots/leaves</sub>/C<sub>soil</sub>,

where:  $C_{roots/leaves}$  are the concentration of PAEs in the roots or leaves of lettuce (ng/g) and  $C_{soil}$  is the PAEs concentration in the soil (ng/g).

The data are presented in Table 2.

Destination	DMP	DEP	DBP	BBP	DEHP	DNOP
Radish roots	0.021	0.068	0.008	0.006	0.048	0.016
Radish leaves	0.089	0.088	0.247	0.125	0.210	0.084
Lettuce roots	0.036	0.112	0.020	0.034	0.025	0.054
Lettuce leaves	0.183	0.107	0.253	0.169	0.239	0.098

Table 2. Bioavailability factor (BAF) values for tested plant subparts cultivated in PAEs polluted soil.

The closer analysis of BAF values revealed that all tested PAEs preferred radish leaves to roots, and the affinity to radish roots was 1.29-30 times lower than to leaves. A similar tendency was noted for lettuce leaves, except DEP, which preferred roots over leaves. The other PAEs were noted at 1.8-12.6 times concentration in the leaves, which was especially seen for DBP and DEHP stressing their toxicity potential for lettuce. DMP, DBP, BBP, DEHP, and DNOP revealed the highest affinity to lettuce leaves, whereas only DEP for lettuce roots. The increased bioavailability of five of six priority PAEs in lettuce leaves highlights the potential hazard of lettuce contamination when cultivated in PAEs polluted soil.

**Conclusions:** The increased usage of plastics in agriculture, such as agrotextiles and greenhouses, results in the contamination of soil with PAEs. Their weak attraction with the polymer matrix highlights their high mobility in the soil. Contaminated food is the

main human exposure to PAEs. The presented studies examined the fate of six priority PAEs in the soil-vegetable system. Under PAEs contamination, an intensive development of the roots was noted, both in radish and lettuce. The roots were shorter but very branched or empty inside. In general, leaves were the main PAEs sinks independent of plant type. DBP, BBP, and DEHP revealed a tendency to be noted at higher concentrations in the roots of radishes than in lettuce, which may be dangerous considering that radish roots are the parts consumed mainly. The bioavailability of tested pollutants was established by the calculation of the Bioavailability Factor (BAF). Considering BAF values, all tested PAEs preferred radish leaves to roots, and the affinity to radish roots was 1.29-30 times lower than to leaves. A similar tendency was noted for lettuce leaves, except DEP, which preferred lettuce roots over leaves. The other PAEs were noted at 1.8-12.6 times increased concentration in the leaves, which was especially seen for DBP and DEHP stressing their toxicity potential for lettuce. The increased bioavailability of five (DMP, DBP, BBP, DEHP, and DNOP) of six priority PAEs in lettuce leaves highlights the potential hazard of lettuce contamination when cultivated in PAEs polluted soil. The results clearly indicate that there is a need to develop new effective methods of reducing PAEs bioavailability to plants and remediation of PAE-polluted soil.

**Acknowledgments:** This study was supported by grant No. 2021/40/Q/NZ8/00006 from the National Science Centre, Poland.

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## THE EFFECT OF BIOCHAR ON THE BIOAVAILABILITY OF PHTHALATES IN THE SOIL-VEGETABLE SYSTEM

**Bożena CZECH**, **Artur Sokolowski**, Maria Curie-Skłodowska University, Faculty of Chemistry, Institute of Chemical Sciences, Department of Radiochemistry and Environmental Chemistry, M. Curie-Skłodowska Sq. 3, 20-031 Lublin, Poland.

**Abstract:** Phthalate acid esters, PAEs, have been recognized recently as emerging pollutants of water, air, and mainly soil. The presence of toxic compounds in the soil may pose a great hazard to the vegetables cultivated in PAEs polluted soil. In the presented studies, the effect of biochar addition on the fate of six priority PAEs was examined. As the tested plants, lettuce – a leafy vegetable, and radish – a root vegetable, were chosen. Sewage sludge-derived biochar, rich in microelements, was applied as a soil additive to mitigate the toxic effect of PAEs. The results indicate that leaves may be the sink for PAEs and the effect of sewage sludge-derived biochar is rather connected with reduced bioaccumulation of PAEs in the plant roots. The observations have an important impact on estimation of the food safety in agriculture under the increased use of plastic foils.

Introduction: Phthalate acid esters, e.g. alkyl aryl and dialkyl phthalates (PAEs) are widely applied plasticizers and solvents that can contaminate all environmental matrix [1]. Their presence in the air, water, or soil was confirmed [2]. The toxicity of PAEs was examined [3] which resulted in the presentation of six PAEs on the priority list of compounds that need to be monitored [4]. As priority PAEs, low molecular weight (or short side chain) PAEs, dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), and butyl benzyl phthalate (BBP) are classified, whereas high molecular weight (or long side chain) are represented by di-(2-ethyl hexyl) phthalate (DEHP) and di-n-octyl phthalate (DNOP) [5]. The increasing number of people worldwide requires increased production of food. The intensive development of agriculture and horticulture uses lots of plastic foils [6] and introduces PAEs into the soil-plant system. Therefore it seems necessary to monitor the fate of PAEs in the soil and vegetables, especially in the context of food safety. The increased need for the production of food is connected with increased usage of fertilizers [7]. In recent years lots of biobased fertilizers have been proposed. Among them, biochar (BC), e.g. the product of the thermochemical conversion of biowastes has gained popularity [8]. As the properties of BC can be tuned up depending on feedstock type, pyrolysis conditions, and any other pre- and post-pyrolysis modifications, a lot of materials were examined as soil additives [9]. The key parameters of BC applied in the soil amendment are high C content, enhanced surface area, porosity, and abundance of surface functional groups. Therefore BC in the soil is a source of nutrients, a habitat for soil microorganisms, and a sorbent of soil contaminants. In the presented studies, we propose to apply wastederived BC as a soil amendment to reduce the bioavailability of PAEs to the vegetables. As a feedstock, sewage sludge (SS) was used. SS is produced in the wastewater treatment plant and contains organic and inorganic compounds, nutrients, pathogens, and heavy metals. Its direct application to the soil is restricted, therefore pyrolysis seems

interesting to overcome the mentioned drawbacks. However, during pyrolysis, some toxic compounds may be formed including polycyclic aromatic hydrocarbons (PAHs) [10]. However, the key parameters is their bioavailability, as only mobile forms may pose a significant hazard to living organisms. Therefore, it seems necessary to examine the effect of waste-derived biochar addition to the soil on the fate of PAEs in the soil-vegetable system.

**Experimental:** BC was produced from sewage sludge from a municipal wastewater treatment plant in Częstochowa via slow pyrolysis at 600 °C (3 h, N<sub>2</sub> atmosphere). The properties of BC were analyzed: pH - digital pH meter HQ430d Benchtop Single Input (HACH, USA), ash content – MagmaTherm (ASTM D3174-12) carbon, hydrogen, and nitrogen content - CHN/CHNS EuroEA3000 Elemental Analyser (EuroVector), total organic carbon (TOC) - Shimadzu SSM-5000A, the surface area and porosity - ASAP 2420 Analyzer (Micromeritics, USA), metal content - Thermo Scientific iCAP<sup>TM</sup> 7000 ICP-OES after microwave mineralization in Start D Microwave Digestion System Milestone. The content of PAHs in BC was determined considering the total fraction (C<sub>tot</sub>) and bioavailable fraction (C<sub>free</sub>) according to the procedure [10]. The results of all analyses are presented in Table 1.

The tests of PAEs fate in BC-amended soil were performed during the pot experiment using agricultural soil and two vegetables from different groups: root vegetable - radish (*Raphanus sativus L.*) and leafy vegetable – lettuce (*Lactuca sativa L.*). The soil samples were spiked with 1 mL of phthalates mixture containing 1  $\mu$ g/mL of each tested compound. Biochar was added at 1 wt%. This dose is commonly used in agriculture [11]. The process was conducted in controlled conditions: light/dark 12:12 h, temperature 22 °C/18 °C day/night, and constant humidity 65% in the Conviron GEN100 plant chamber. The freeze-dried subparts of the vegetables were subjected to GC-MS/MS analysis detailed in [12].

**Results:** The obtained biochar was characterized by basic pH, low surface area, and porosity (Table 1). As can be expected, the content of ash was high (>60%) resulting from the presence of inorganics in SS, and carbon content was rather low (<30%).

Basic	pН	Ash [%]	C [%]	H [%]	N [%]	O [%]
properties	8.06	62.78	23.49	0.57	3.66	9.5
Porosity	S <sub>BET</sub> [m <sup>2</sup> /g]	Vp [cm <sup>3</sup> /g]	D [nm]			
	26.053	0.004706	10.63			
Chemical	H/C	O/C	[N+O]/C	TC	IC	TOC
character				[mg/g]	[mg/g]	[mg/g]
	0.024	0.404	0.560	328.05	1.01	327.03
Hazardous	C <sub>free</sub> [ng/L]	C <sub>tot</sub>	Cr	Cu	Fe	Zn
components	_	[mg/g]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]
	0.461	0.776	768.00	459.61	26,227.13	3396.96

Table 1. The physicochemical properties of BC.

where: C, H, N, O, Cr, Cu, Fe, Zn – content of respective elements, TC – total carbon, IC -inorganic carbon, TOC – total organic carbon,  $C_{free}$  – bioavailable PAHs,  $C_{tot}$  – total PAHs

BC was hydrophilic (high O/C ratio) and abundant in polar surface groups (high (N+O)/C ratio), which may facilitate the interaction of BC with soil constituents and

pollutants [13]. Interestingly, from the agricultural perspective, a high content of Fe and Zn, which are important microelements, was noted in BC. It can be seen that in the PAE-polluted soil, the fresh mass of radish root was increased compared to the control (Fig.1). BC amendment did not affect the fresh mass of both tested plants. However, a decrease in dry mass was noted stressing the role of foliar water in plant cells. No direct negative effects on vegetable yield were observed.



Fig.2. Relative concentration of PAEs after BC amendment.

BC amendment, however, affected the concentration of PAEs noted in individual parts of vegetables (Fig.2). In general, BC lowered the determined concentrations of PAEs, although the effect was dependent on the tested compound and plant. BC did not affect the fate of DMP in any parts of vegetables. When considering the edible parts of vegetables, it should be stressed that the content of DEP, DBP, and DNOP in radish root, and DEP and BBP in lettuce leaves was lowered. Even though the highest reduction of concentration was noted for BBP, which is considered a strong endocrine disrupting compound [14], however, only in lettuce roots. In the case of DBP and DNOP, BC increased their content in the leaves of radish and lettuce, respectively. The results indicate that leaves may be the sink for PAEs and the effect of sewage sludge-derived biochar is rather connected with reduced bioaccumulation of PAEs in the plant roots.

The root uptake of organic hydrophobic compounds such as phthalates strongly depends on the lipid content in the plants' roots [15].

**Conclusions:** The conversion of sewage sludge to biochar may be recommended as the soil additive. PAEs pollution increased the mass of the roots, whereas biochar did not affect the plant growth significantly. The addition of sewage sludge-derived BC increased the fresh mass up to the level observed for the control, which may result from the concentration of microelements. When considering the edible parts of vegetables, it should be stressed that the content of DEP, DBP, and DNOP in radish root, and DEP and BBP in lettuce leaves was lowered. The results indicate that leaves may be the sink for PAEs and the effect of sewage sludge-derived biochar is rather connected with reduced bioaccumulation of PAEs in the plant roots.

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## BIOCHAR AND AQUATIC PLANTS: EVALUATING TOXICITY ACROSS DIVERSE BIOCHAR TYPES

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**Abstract:** Biochar is a carbon rich soil amendment which has gain popularity as a response to the assumptions of closed circulation and sustainable waste treatment. Agricultural waste and sewage sludge are most often used to produce biochar. The use of different raw materials for biochar production may affect not only the properties of the resulting material but also its toxicity. The sewage sludge from wastewater treatment plant may contain heavy metals therefore biochar obtained from it may also contain heavy metals. During the pyrolysis process polycyclic aromatic hydrocarbons (PAHs) may be formed, the amount of which also depends on the type of raw material used. Both heavy metals and PAHs are substances which may be released into the environment and cause some negative effects. It seems necessary to perform ecotoxicity tests to determine the risk of using biochar. Carrying out various tests can provide information about the impact of the material on different types of living organisms like plants, bacteria and animals.

Introduction: Biochar (BC) is a carbon rich material which is obtained from biomass a result of the thermochemical conversion under limited oxygen conditions [1]. In the BC production many different organic wastes, including agricultural wastes for example straw, forestry wastes and municipal solid wastes may be used. BC contain a lot of C and N, but it may also contain P, K and Ca. After BC addition to the soil these elements may be taken up by plants. Other beneficial effects of BC application as soil additive are: improved bioavailability of essential nutrients such as C, K, P and N, increased soil water retention, overall increase in soil fertility which results in increased yields [2]. Due to its properties, BC positively influences the growth of soil microorganisms by supplying nutrients, offering habitat, and altering the surrounding environmental conditions [3]. The raw material used to produce BC has significant impact on the physicochemical properties of the final product [4]. The type of feedstock has also impact on the composition of biochar, especially elemental composition [5] and content of polycyclic aromatic hydrocarbons (PAHs) [6]. These substances may be dangerous for the surrounding environment, and they may affect living organisms and cause biochar toxicity after using biochar as soil amendment [7].

PAHs are aromatic hydrocarbons that have more than two aromatic rings. PAHs are formed during the incomplete combustion of organic matter. So, the main source of them

are burning of biomass, waste and fossil fuels or coke production [8]. PAHs have confirmed toxicity: they are mutagenic, teratogenic, and carcinogenic to all living organisms. Additionally, PAHs cause skin and eye irritation, liver damage and eye cataracts. Therefore, the United States Environmental Protection Agency (USEPA) recognized 16 of PAHs as priority pollutants [9]. PAHs in BC are formed during its production and the main factors which have impact on their content are pyrolysis temperature, heating rate, and residence time in the oven [10]. Heavy metals (HM) are the inorganic components of BC. Heavy metals are the main inorganic substances present in the BC which have impact on its toxicity. They are present in BC only if they were present in the raw material. Additionally, during the pyrolysis their concentration increases due to the decomposition of organic matter [5]. So, the selection of the appropriate raw material has a key impact on the amount of heavy metals in the obtained biochar. There is a high risk of heavy metal contamination associated with the use of sewage sludge as a feedstock for BC production. This material is very popular, but it contains large amounts of heavy metals [11]. When plant residues are used as raw materials in the pyrolysis process, obtained BC contains smaller amounts of heavy metals. Plants residues may be contaminated with heavy metals only when plants grow in contaminated environment [12]. The toxicity of heavy metals to humans is widely known. HM are one of the main factors responsible for the formation of free radicals which disturb the functions of various organs and damage them. They can also cause DNA damage. Additionally, heavy metals act directly on nervous system causing disruption of its operation [13]. The part of these substances called bioavailable fraction is bioavailable for living organisms when BC is used for example as a soil amendment. This fraction of PAHs and heavy metals is not strongly bond with the BC and may be released into the environment [14]. Determination of bioavailable fraction of these substances is the most important to determine the toxicity of BC. To determine whether the use of biochar in the environment will not have undesirable effects, ecotoxicity tests can be carried out. There are many different tests that use plants, animals and bacteria. Some of these tests may be carried out to determine the toxicity of the material in the solid phase and other in the liquid phase. Duckweed Toxkit F test is one of the ecotoxicity test in which the effect of tested material on plants is determined. In this study five different types of BC obtained from agricultural wastes, sewage sludge and sludge from biogas production were tested for toxicity to aquatic plant (Spirodela polyrhiza) using the Duckweed Toxkit F test.

**Experimental:** In the experiment, five types of BC obtained from various raw materials: BC-CR obtained from corn, BC-SF obtained from sunflower, BC-SW obtained from straw, BC-SS obtained from sewage sludge from municipal wastewater treatment plant, and BC-BG obtained from biogas production residues were used. All materials were obtained via slow pyrolysis process at 600 °C, in nitrogen atmosphere. The same conditions of pyrolysis ensured that only the type of raw material has an impact on the properties of the obtained biochars. Next, obtained BC was tested to determine its physicochemical properties like pH, ash content, C, H, N and O content, content of heavy metals and content of two fractions of 16 PAHs: bioavailable fraction (Cfree) and total content (Ctotal).

The Duckweed Toxkit F test was performed according to the procedure ISO Standard 20227. In this test is determined the inhibition of the growth of the first fronds of *Spirodela polyrhiza* germinated from turions, by solution obtained by BC with water.

**Results:** The BC-BG was characterized by the highest (10.7) and BC-SS by the lowest (8.1) pH. However, all tested biochars have alkaline pH (Table 1). Large differences were noted in the elemental composition of individual materials, especially in the carbon content. Biochars derived from plant residues had similar C content ranged from 76.7% (BC-CR) to 86.7% (BC-SF), and materials obtained from sewage sludge and biogas production residues had lower C content 23.5% and 62.9% respectively. Additionally, BC-SS had the greatest O content (9.5%) and ash content (62.8%) of all tested biochars, and BC-SF was characterized by the lowest values for these parameters (4.0% for O content and 6.7% for ash content).

		•	<u> </u>		
Properties	BC-CR	BC-SF	BC-SW	BC-SS	BC-BG
pH	9.6	9.3	10.4	8.1	10.7
C, H, N content [%]	C=76.7 H=2.4 N=3.4	C=86.7 H=1.7 N=1.0	C=77.8 H=1.5 N=1.0	C=23.5 H=0.6 N=3.7	C=62.9 H=1.2 N=2.6
O content [%]	5.2	4.0	4.7	9.5	7.4
Ash content	14.1	6.7	15.0	62.8	25.9

 Table 1. Basic physicochemical properties of obtained biochars.

The plant residues-derived biochars had significantly lower content of bioavailable PAHs with the highest value determined for BC-CR (0.160 ng/L) than BC-SS (0.461 ng/L) and BC-BG (0.857 ng/L). However, BC-SS is the BC which contained the highest concentration of total PAHs (766.49  $\mu$ g/kg) whereas BC-CR contained almost 10 times lower total PAHs content (75.757  $\mu$ g/kg). BC-SS was enriched also in heavy metals, especially Cr, Cu, Ni, Zn, Co and Pb and it was the only biochar that contained Cd (Table 2).

Table 2. Content of heavy metals and two fractions of PAHs in obtained biochars.

	BC-CR	BC-SF	BC-SW	BC-SS	BC-BG
Cr [mg/kg]	0.98474	0.86505	14.268	768.00	6.1607
Cu [mg/kg]	7.6317	16.930	18.902	459.61	20.946
Fe [mg/kg]	476.98	128.77	462.93	26227	2783
Mn [mg/kg]	45.421	16.683	111.95	929.08	632.08
Ni [mg/kg]	0.49237	1.8537	1.9512	226.89	2.4643
Zn [mg/kg]	85.549	343.05	36.585	3396	86.249
Co [mg/kg]	0.12309	0.12358	0.24390	15.815	1.2321
Cd [mg/kg]	<lod< td=""><td><lod< td=""><td><lod< td=""><td>0.97324</td><td><lod< td=""></lod<></td></lod<></td></lod<></td></lod<>	<lod< td=""><td><lod< td=""><td>0.97324</td><td><lod< td=""></lod<></td></lod<></td></lod<>	<lod< td=""><td>0.97324</td><td><lod< td=""></lod<></td></lod<>	0.97324	<lod< td=""></lod<>
Pb [mg/kg]	2.3387	0.37074	1.2195	75.061	1.2321
Cfree [ng/L]	0.160	0.124	0.106	0.461	0.857
Ctotal [µg/kg]	75.757	270.44	462.22	766.49	345.89

The results of Duckweed Toxkit F test (Fig.1) show that all tested biochars had negative impact on the growth of Spirodela *polyrhiza*. Biochars obtained from sewage sludge and biogas production residues inhibited plant growth much more than biochars obtained from agricultural wastes.



Fig.1. Duckweed Toxkit F test results for obtained biochars.

**Conclusions:** Type of raw material used to produce biochar have a major impact on the properties of obtained product. The main differences are in the C content and ash (mineral fraction) content. Obtained biochars also differ in the content of hazardous components like PAHs and heavy metals. These substances may have negative effects on the environment, so it is important to determine its content. Results of one of ecotoxicity tests show that biochars obtained from two types of sludges have significantly greater negative impact on the aquatic plant *Spirodela polyrhiza*. Additionally, the inhibition of growth of this plant is strongly negatively correlated with the content of bioavailable fraction of PAHs in tested biochars.

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## BIOCHAR WITH IMMOBILIZED BACTERIA – A MODERN MATERIAL IN SOIL REMEDIATION

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**Abstract:** The presented results concern the possibility of using biochar with immobilized bacterial flora to remove phthalic acid esters from the soil-plant system. The immobilized bacterium was *Patulibacter brassicae* and as an immobilization matrix biochar obtained from corn was used. Modified biochar was used as a soil amendment and its impact on the concentration of DMP and DEHP in the soil, and lettuce roots and leaves was determined. Obtained results show that combined processes of PAEs adsorption on biochar and biodegradation by bacteria strain provide high removal efficiency.

**Introduction:** Biochar (BC) is a solid material obtained through the thermochemical conversion of biomass under limited oxygen conditions [1]. Straw and other agricultural wastes and even sewage sludge can be used as raw materials for biochar production [2]. BC has been used as a soil additive for many years. Confirmed beneficial effects of biochar on soil include increasing the pH of acidic soils, enhancing the bioavailability of essential micronutrients such as K, Mg, Ca, P, Cu, Mn, Fe, and N, improving soil moisture retention by reducing water evaporation, increasing soil cation exchange capacity, and stimulating the growth of soil microorganisms [3, 4]. Additionally, BC is used to remove various compounds like dyes, pesticides, and antibiotics from water and soil [5]. BC thanks to its properties has also a positive impact on the development of soil microorganisms by providing nutrients, habitat, and changing properties of the surrounding environment [6]. Due to that BC is used as a matrix for immobilization bacteria that can biodegrade pollutants [7]. Bacteria are immobilized on the BC surface via adsorption on the surface, encapsulation within a porous structure, aggregation by flocculation, and crosslinking [5]. Phthalic acid esters (PAEs) are a group of compounds that are very popular plasticizers used in many household products [8]. The PAEs are categorized into two groups: Low molecular weight (LMW) PAEs which include: dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), and benzyl butyl phthalate (BBP) and high molecular weight (HMW) PAEs such as di-(2ethylhexyl) phthalate (DEHP) and di-n-octyl phthalate (DNOP) [9]. PAEs are only weakly bound to the polymer matrix by hydrogen bonds or van der Waals interactions, so they can be easily washed out from the plastic product and enter the environment [10]. PAEs present in the soil may be taken up by plants and introduced into the food chain. Consumption of fruits and vegetables contaminated with phthalates is the main source of human exposure to these compounds [11]. Health issues associated with phthalates include severe effects such as cancer, disruptions in the nervous and endocrine systems, and developmental disorders in infants and children [12]. DMP, DEP. DBP. BBP. DEHP, and DNOP are classified as compounds requiring special regulatory control by the United States Environmental Protection Agency (USEPA). USEPA additionally listed DEHP as a potential carcinogen [13]. PAEs are removed from the environment via adsorption, coagulation/flocculation, and advanced oxidation processes. However, hydrolysis, photodegradation, and microbial breakdown are the main processes of PAEs degradation in the natural environment [14]. Agromyces, Bacillus, Curvibacter, and Achromobacter are some of the bacteria strains with the ability to decompose PAEs. PAEs are decomposed by bacteria into simpler compounds via different reactions.  $\beta$ -oxidation or hydrolysis of ester bonds leads to the transformation of PAEs into phthalic acid (PA) [15]. Due to transesterification, one ester may be transformed into another, and demethylation such as in the case of conversion of DEP into DMP leads to the shortening of the side chain. Reduction in the size of side chains decreases the steric effect and facilitates the hydrolysis of ester bonds [16]. The use of bacteria immobilized on BC can increase the removal efficiency due to enhanced transfer of contaminants to the microorganisms that decompose them [7]. The ability of bacteria *Patulibacter brassicae* to decompose PAEs was tested in this study. Bacteria were immobilized on BC obtained from corn. Two different PAEs were used in the pot experiment, DMP as the LMW and the smallest of PAEs, and DEHP as the HMW and the most used PAEs. Additionally, these compounds vary strongly in their properties, especially in steric effects due to significant differences in the size of side chains. The use of these compounds was to help determine whether there are differences in the degradation efficiency of different types of PAEs.

## **Experimental:** The experiment had the following steps:

*Biochar production and physicochemical analysis*: BC was obtained from corn stalks via a slow pyrolysis process at 600 °C, in a nitrogen atmosphere. Next BC was tested to determine some basic physicochemical properties like pH, ash content, C, H, N, and O content, specific surface area, and types of functional groups on the surface of BC.

*Bacteria immobilization on BC:* The sterilized BC was used as the matrix for immobilization of bacteria strain *Patulibacter brassicae*. The process of BC sterilization consisted of heating BC for 20 min at 121 °C. The process of bacteria immobilization on BC had the following steps: bacteria was reproduced in LB liquid medium for 24 h, centrifuged for 10 min at 5000 rpm, and washed three times with sterilized sodium chloride saline. Next 5 g of sterilized BC was mixed with 50 mL of sodium chloride saline with bacteria strains and shaken for 24 h on the horizontal shaker. After that time the BC with immobilized bacteria was centrifuged for 10 min at 5000 rpm. The material obtained in this way was used in a pot experiment to determine the ability of the *Patulibacter brassicae* strain to biodegrade PAEs.

*Pot experiment:* the 340 g of soil spiked with 1 mL of solution of DMP and DEHP at a concentration of 1  $\mu$ L mL<sup>-1</sup> each and enriched with 3.4 g of prepared BC was used for lettuce cultivation. Plant cultivation was carried out for 42 days in Conviron GEN100 in constant conditions: 12 h light/12 h darkness, temperature 22 °C/18 °C day/night, and constant humidity 65%. Three times a week plants were watered to keep 65% of the water-holding capacity of the soil. Soil without BC addition was a control sample.

PAEs determination in soil and lettuce: after 6 weeks of cultivation, plants were harvested and washed with distilled water. Lettuce roots and leaves were separated,

frozen at -20 °C, and freeze-dried. Soil samples were air-dried. DMP and DEHP content in lettuce subparts and soil were determined via GC-MS/MS analysis, according to the procedure detailed in [17].

**Results:** The pH of the BC used in this study was alkaline with an exact value of 9.6. The content of basic elements was typical for biochars obtained from plant wastes, C content 76.7%, H content 2.71%, N content 1.3%, and O content 5.18%. The specific surface area was 90 m<sup>2</sup>/g. This material contained various physicochemical groups on the surface. The carbon-containing groups determined by XPS (X-ray photoelectron spectroscopy) analysis were C-H 37% At, C=C sp2 27% At, and C-C sp3 16% At. Among the oxygen-containing groups, C-OH (aliphatic), O=C, and C-OH (aromatic) (their contents were 31% At, 28% At, and 20% At, respectively) were determined. The specific surface area value and oxygen-containing functional groups are very important in the process of immobilization of bacteria on the surface of biochar [18].

Table 1. Relative content of DMP and DEHP in the soil and roots and leaves of the lettuce.

Compound	Soil	Roots	Leaves	
DMP	20.96	22.12	85.71	
DEHP	18.91	71.91	68.19	

The content of two tested compounds in three matrices was reduced in the sample with the addition of modified BC compared to the blank sample (Table 1). The content of DMP in the soil and lettuce roots was reduced by about 80%. It is worth stressing that the DMP content in lettuce leaves was reduced only by 15%. DEHP concentration in the soil with BC was 18.91% of the blank sample concentration. The content in lettuce subparts was 71.91% and 68.19% in roots and leaves, respectively. Simultaneously, the metabolism rates of these two compounds were determined for *Patulibacter brassicae*. The metabolism rate was calculated as the percent of weight loss of individual compounds in the soil, lettuce roots, and leaves in the sample with BC compared to the sum of masses of these compounds from the blank sample. *Patulibacter brassicae* metabolized DMP by 80% and DEHP by 81%. The parts of lettuce plants that grow in the soil enriched with BC weighed 19.32 g (leaves) and 5.69 g (roots). Plants from the blank sample weigh less (18.82 g and 4.47 g of leaves and roots, respectively).

**Conclusions:** The addition of biochar modified with the bacteria *Patulibacter brassicae* to the soil reduced the content of two different PAEs in the soil with similar efficiency. The content of DEHP in the roots was reduced to a much lesser extent than DMP. This may be due to differences in the properties of these two compounds. However, the analysis of the metabolism rates of DMP and DEHP shows that *Patulibacter brassicae* metabolizes both compounds to the same extent. This fact is extremely important from a practical point of view because this bacteria strain does not show selectivity toward PAEs from two different groups LMW, and HMW which had completely different applications and properties. DMP is the compound with the shortest side chain and DEHP is one of the most popular PAEs with the longest side chains. Enriching the soil with biochar has a positive effect also on plant growth. The lettuce plants from the sample with BC addition gained more mass than the plants from the blank sample. The

use of BC modified with bacteria strains that can decompose various contaminants may be a good way to remediate soil. The process of bacteria immobilization on BC is simple and cheap because it does not require many reagents. Additionally, different bacteria strains may be chosen for different contaminants. The other advantage is the fact that BC is used as a soil additive which improves the properties of agricultural soils.

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## CO<sub>2</sub> – ACTIVATED BIOCARBONS OBTAINED FROM COTTON WASTE

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**Abstract:** In this study, biocarbons obtained from cotton waste (lab coats) subjected to the process of physical activation (CO<sub>2</sub>) were characterized. The properties of activated biocarbons were compared with those of the non-activated starting material. All obtained adsorbents obtained are characterized by a developed microporous structure and a small number of mesopores. The tested activated biocarbons are characterized by high specific surface area values in the range from 617 to 715 m<sup>2</sup> g<sup>-1</sup>, while the non-activated biocarbon has a S<sub>BET</sub> of 428 m<sup>2</sup> g<sup>-1</sup>. The carbon content (CHNS analysis) decreases after the activation process. In addition, the obtained materials are characterized by a low degree of crystallinity and have numerous functionalities on the surface, therefore they are potential adsorbents of environmental pollutants (e.g. harmful gases, impurities from aqueous solutions – pharmaceuticals, pesticides, dyes and others).

Introduction: Sustainability and waste management are among the most important challenges of modern society. In response to the growing amount of textile waste and its negative impact on the environment, innovative recycling methods that will not only contribute to reducing the amount of waste but also enable its reuse in industry, environmental protection and various sectors of the economy are increasingly searched [1]. Among textile wastes, cotton waste including worn out laboratory coats, which are a common part of the equipment of numerous research and development facilities, deserves special attention. Instead of treating it as waste, it is possible to turn it into biocarbon - a material with a wide range of application potentials [2]. Biocarbon, produced by pyrolysis processes of organic materials, can act as a carrier of nutrients in the soil, improve soil structure, and act as an effective adsorbent of pollutants. Currently, special attention is paid to methods of activating biocarbons, which allow for the modification of their porous structure thus increasing the specific surface area, which translates into improved adsorption properties. Activation with carbon dioxide  $(CO_2)$  is considered to be one of the most effective methods for obtaining largely porous carbon materials, as it allows to control development of the porous structure of biocarbons and to obtain a material with the desired physicochemical properties. The aim of this study is to investigate the possibility of obtaining biocarbons from waste cotton aprons used in laboratories and to evaluate their physicochemical properties. Particular attention was paid to the  $CO_2$  activation process, which was used to improve the properties of the final biocarbons.

**Experimental:** Used waste lab coats (100% cotton) were cut into small pieces, placed in quartz boats, and then the pyrolysis process was conducted at a temperature of 800 °C

for 1 h in the N<sub>2</sub> atmosphere (flow rate 20 dm<sup>3</sup>/h) using an increase in temperature of 5 °C/min. The resulting biocarbon was called BCW-1. The next stage was activation with CO<sub>2</sub>. Biocarbon was annealed from room temperature to 800 °C (10 °C/min) in a nitrogen atmosphere (flow rate 20 dm<sup>3</sup>/h), then at this temperature the activation process was conducted in the CO<sub>2</sub> atmosphere (flow 10 dm<sup>3</sup>/h) for 3 hours and 5 hours (activated biocarbons were designated BCW-2 and BCW-3, respectively). The furnace was cooled to room temperature in the nitrogen atmosphere. The basic parameters of the determined using low-temperature isotherms of N<sub>2</sub> porous structure were adsorption/desorption (ASAP 2020 volumetric analyzer, Micromeritics Inc., Norcross, GA, USA). The elemental analysis (CHNS) was performed using the Elementar Vario Micro Cube analyzer (Elementar, Langenselbold, Germany) to determine the elementary composition of materials. On the basis of FTIR spectra, the chemical nature of the surface of the obtained biocarbons was determined and functional groups on their surface were identified (Perkin-Elmer Spectrum 400 FT-IR/FT-NIR spectrometer, Perkin-Elmer, Waltham, MA, USA). The degree of order of the biocarbon structure was determined by recording Raman spectra with a spectrometer (Raman Station 400 F, Perkin Elmer, Waltham, MA, USA). All materials were dried before the measurements.

**Results:** Figure 1a presents the isotherms of adsorption/desorption of  $N_2$  of the studied biocarbons. The analyzed isotherms are type I, which indicates the microporous nature of the materials according to the IUPAC classification. The course of the isotherms confirms that the activation of the initial biocarbon with CO<sub>2</sub> affects the development of the porous structure, mostly microporous and to a small extent mesoporous of the activated biocarbons. **a**)



Fig.1. Low-temperature nitrogen adsorption/desorption isotherms (a) and pore size distributions (b).

The structural parameters determined on the basis of adsorption isotherms are presented in Table 1. The analysis of the structural data confirms the conclusions drawn from the course of isotherms. The largest specific surface area (S<sub>BET</sub>) is characterized by BCW-3 biocarbon (715 m<sup>2</sup> g<sup>-1</sup>) compared to BCW-1 and BCW-2. This trend is also observed for the other parameters, i.e. the total pore volume (0.40 cm<sup>3</sup> g<sup>-1</sup>) and the micropore volume (0.29 cm<sup>3</sup> g<sup>-1</sup>) for BCW-3. In the case of non-activated BCW-1 and 3h-activated BCW-2 (shorter activation time), the obtained adsorbents are characterized by less developed porosity and thus lower S<sub>BET</sub> values (BCW-1: 428 m<sup>2</sup> g<sup>-1</sup>, BCW-2: 617 m<sup>2</sup> g<sup>-1</sup>), Vt (BCW-1: 0.22 cm<sup>3</sup> g<sup>-1</sup>, BCW-2: 0.33 cm<sup>3</sup> g<sup>-1</sup>) and V<sub>mi</sub> BCW-1 h: 0.19 cm<sup>3</sup> g<sup>-1</sup>, BCW-2: 0.25 cm<sup>3</sup> g<sup>-1</sup>). To sum up, the parameters of the porous structure increase with the increasing activation time of CO<sub>2</sub>. The micropore dimension obtained from the maximum decomposition function (Fig.1b) for all analyzed biocarbons is the same and amounts to 0.65 nm. The developed microporosity is in the range of 73-86 % (Table 1).

Sample	$S_{BET}$ [m <sup>2</sup> /g]	S <sub>ext</sub> [m <sup>2</sup> /g]	V <sub>ultra</sub> [cm <sup>3</sup> /g]	V <sub>mi</sub> [cm <sup>3</sup> /g]	V <sub>me</sub> [cm <sup>3</sup> /g]	Vt [cm <sup>3</sup> /g]	Pore size [nm]	Microporosity (%)
BCW-1	428	65	0.08	0.19	0.03	0.22	0.65	86
BCW-2	617	160	0.1	0.25	0.08	0.33	0.65	76
BCW-3	715	187	0.11	0.29	0.11	0.40	0.65	73

Table 1. Structural parameters of the tested activated biocarbons.

 $\begin{array}{l} S_{BET} - \text{specific surface area, } S_{ext} - \text{external surface, } V_{ultra} - ultramicropores volume (pores width < 0.7 nm), \\ V_{mi} - \text{micropores volume (pores width < 2 nm), } \\ V_{me} - \text{mesopores volume (pores width 2-50 nm), } \\ V_t - \text{total pores volume, } \\ N_{troporosity} - \text{percentage of micropores in the total pore volume.} \end{array}$ 

The elemental composition of the obtained biocarbons is presented in Table 2. The analysis of the results confirms that activation with  $CO_2$  has a significant effect on the carbon content which decreases from 95.04% to 93.64% with the increasing activation time. A downward trend is also observed in the case of hydrogen content (from 0.638% to 0.566%). A downward trend is not observed in the case of nitrogen and sulfur. In the case of the studied biocarbons, the nitrogen content ranges from 0.129 to 0.427 while the sulphur content ranges from 0.090 to 0.240.

			2 0		
Biocarbon	C (%)	H (%)	N (%)	S (%)	$I_D/I_G$
BCW-1	95.04	0.638	0.140	0.303	1.50
BCW-2	94.21	0.623	0.090	0.427	1.57
BCW-3	93.64	0.566	0.240	0.129	1.50

Table 2. CHNS analysis results and  $I_D/I_G$  of the studied biocarbons.

 $C~(\%)-\text{content of carbon, H}~(\%)-\text{hydrogen content, N}~(\%)-\text{nitrogen content, S}~(\%)-\text{sulfur content, }I_D/I_G-\text{determined from Raman spectra.}$ 

The FTIR spectra (Fig.2a) confirmed the presence of functionalities on the surface of all studied biocarbons. The variable intensity and position of the strands suggest differences in the structure and chemical composition of the non-activated biocarbon (BCW-1) and the activated biocarbons (BCW-2 and BCW-3). The bands in the range of 3800–3300 cm<sup>-1</sup> are characteristic of O-H groups in alcohols or result from the presence of adsorbed moisture. The presence of bands in the range 3000–2800 cm<sup>-1</sup> can be attributed to the asymmetric and symmetric stretches of the C-H bonds (CH<sub>2</sub> and CH<sub>3</sub>). The bands in the range of 1700–1600 cm<sup>-1</sup> can correspond to the presence of C=C (vibrations in aromatic rings) or C=O carbonyl (carboxylic, aldehyde, ketone) groups. The band in the range 1200–1000 cm<sup>-1</sup> can suggest the presence of C-O bonds characteristic of esters. The bands occurring in the area of 1000–400 cm<sup>-1</sup> can correspond to the deformation vibrations of C-H outside the plane of the aromatic rings [3,4].



Fig.2. FT-IR spectra (a) and Raman spectra (b) of the studied biocarbons.

The activation time has a significant impact on the degree of order of the studied biocarbons, which was proved by Raman spectroscopy (Fig.2b). Two broad overlapping bands with maxima at ~1350 cm<sup>-1</sup> and ~1600 cm<sup>-1</sup> (D and G bands), characteristic of carbon materials are observed. The G-band is typical of single crystalline graphite and is derived from the tensile vibrations of sp<sup>2</sup>-hybridized carbon bond pairs in the rings of graphene layers and graphitized carbon material. Defects in the graphene planes and the structure of sp<sup>2</sup> carbon are evidenced by band expansion. The D-band appears with the increase of disorder, which confirms the presence of defects in the structure of graphite with sp<sup>3</sup> hybridization and amorphous nature. The position of the G and D bands and determination of the I<sub>D</sub>/I<sub>G</sub> intensity ratio on their basis allows to describe the structural properties of biocarbons. An increase in the intensity ratio of the I<sub>D</sub>/I<sub>G</sub> peak indicates a decrease in the degree of order of the biocarbon as well as a low degree of crystallinity [5]. The I<sub>D</sub>/I<sub>G</sub> values (1.5-1.57) in Table 2 confirm the presence of a large number of defects and a small degree of crystallinity of the obtained materials.

**Conclusions**: Waste cotton is a good precursor of carbon adsorbents with developed porosity. Due to their large specific surface area, the presence of functionalities and low crystallinity, the studied biocarbons can be successfully used in the future for the adsorption of numerous environmental pollutants.

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## THE EFFECT OF THE METHOD OF CHEMICAL ACTIVATION ON THE PROPERTIES OF BIOCARBONS

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**Abstract:** In this study, the biocarbons, chemically KOH-activated by grinding or wet impregnation were characterized. The grinding method was found to be more effective in developing the surface and porosity of biocarbons. The obtained materials are characterized by high  $S_{BET}$  (up to 1174 m<sup>2</sup>/g) and  $V_p$  (max. 0.56 cm<sup>3</sup>/g), a significant content of fixed carbon (%FC ~ 84 – 86%) and a low ash content (%A ~ 2 – 4%). These are materials with a significant amount of structural defects and have acidic and alkaline functionalities on the surface, which makes them good potential adsorbents of organic pollutants.

**Introduction:** The increase in awareness of anthropogenic pollution of the natural environment causes that the need to search for new, environmentally friendly ways of using natural goods, their processing, recycling and utilization is constantly increasing. The problem is extremely broad, and one of the issues is the search for opportunities to use green waste. It was shown that a wide variety of organic biomass can be successfully converted into value-added materials, namely biocarbons with the potential to adsorb pollutants from the water environment. Each of the types of biomass has different properties and efficiency in the production of biocarbon, the properties of which also depend on the parameters of the pyrolysis process (time, atmosphere, heating rate and temperature). However, the final biocarbon has an undeveloped surface area and porous structure, so it is unusable in adsorption processes. To improve its properties from the point of view of adsorption of water pollution, it is necessary to activate such materials. In the activation process, the structure of biocarbon is "cleaned up" and the pyrolysis residues are thermally degraded, owing to which the existing pores are unblocked and a new porous structure develops. Surface development can be made by applying chemical or physical activation. Choosing the right activation method is not insignificant, because different activation methods give different results. Physical activation ( $CO_2$ ,  $H_2O$ ) is milder and less effective in changing porosity, its course is easier to control and does not cause secondary environmental pollution. On the other hand, chemical activation with the use of concentrated acids, bases or salts is much more effective [1], and allows the introduction of heteroatoms into the structure of the activated material, but requires the use of processes of washing away post-activation residues, which increases significantly the time, costs and water consumption, as well as the generation of toxic wastewater. The aim of the study was to obtain and characterize the structural and surface parameters of biocarbons based on sawdust from mixed trees obtained under different activation conditions using KOH as an activating agent.

**Experimental:** The sawdust of mixed trees was washed with tap and distilled water and then dried for 24h at 105 °C. The dried material was ground and fractionated, a fraction of 1-2 mm was used for the research. The pyrolysis process was conducted in the N<sub>2</sub> atmosphere (flow rate 200 cm<sup>3</sup>/min, heating rate 10 °C/min) up to the temperature of 800 °C, and at this temperature, the heating was continued for 1h. Then the resulting biocarbon (sample INI) was activated with KOH by: (1) grinding (1:4) and activation at 600 and 700 °C (5 °C/min, 2h isothermal heating, samples AB-600<sub>gr</sub> and AB-700<sub>gr</sub>, respectively) and (2) impregnation with the KOH solution (1:4) for 1.5; 4 and 5h (magnetic stirrer, samples AB-1.5<sub>imp</sub>, AB-4<sub>imp</sub> and AB-5<sub>imp</sub>, respectively) and activation (600 °C, 5 °C/min, isotherm - 2h). The activation and cooling process were carried out in the  $N_2$  atmosphere. To determine the structural parameters, data of low-temperature  $N_2$ adsorption/desorption isotherms (Micromeritics ASAP 2405, USA) were used. Thermal analysis was performed using Derivatograph C (Paulik, Paulik, and Erdey, MOM, Hungary). Based on the Raman spectra (Leica Research Grade DMLM microscope, Reflex, Renishaw, UK), the crystal structure of the materials was determined. The surface chemical nature was investigated by Boehm potentiometric titration (716DMS Titrino, Metrohm, Switzerland) and FT-IR spectra (Perkin-Elmer Spectrum 400-FT-IR/FT-NIR, Perkin-Elmer, Waltham, MA, USA).

**Results:** Figure 1 shows the isotherms of  $N_2$  adsorption/desorption of the biocarbons. All isotherms according to the IUPAC classification belong to type I which is characteristic of microporous materials. The analysis of the curves shows that the insertion of KOH by grinding (AB-600<sub>gr</sub>, AB-700<sub>gr</sub>) is much more effective than impregnation with a solution (AB-1.5<sub>imp</sub>, AB-4<sub>imp</sub>, AB-5<sub>imp</sub>). Increasing the impregnation time and activation temperature promotes surface and porosity development.



Fig.1. Low-temperature nitrogen adsorption/desorption isotherms (a) and pore size distributions (b).

Table 1 presents the structural parameters determined for the tested materials. The data analysis confirms the conclusions drawn from the course of the isotherms. The materials ground with KOH are characterized by the largest  $S_{BET}$  (1050 and 1174 m<sup>2</sup>/g),  $V_p$  (~0.5 cm<sup>3</sup>/g) and  $V_{mi}$  (0.42-0.48 cm<sup>3</sup>/g), while the increase in the activation temperature is conducive to the development of the porous structure. Wet-impregnated biocarbons are characterized by slightly lower  $S_{BET}$  (~690 – 805 m<sup>2</sup>/g),  $V_p$  (~0.32 – 0.38 cm<sup>3</sup>/g) and  $V_{mi}$  (0.29 – 0.34 cm<sup>3</sup>/g) values. The impregnation time affects the structure of the obtained activated biocarbon and the obtained results indicate that the optimal impregnation time is 4 hours. However, in all materials, the surface area of the

micropores constitutes ~88%. The average pore radius does not depend on the activation conditions in principle and is 0.95 - 0.97 nm.

	a	a	a	<b>X</b> 7	<b>X</b> 7	D	D '	4 * 1*	
Sampla	SBET	Sext	$S_{mi}$	V <sub>mi</sub>	V <sub>p</sub>	R <sub>av</sub>	Basic	Acidic	рН
Sample	$[m^2/g]$	$[m^2/g]$	$[m^2/g]$	[cm <sup>3</sup> /g]	[cm <sup>3</sup> /g]	[nm]	mgR/g	mgR/g	
INI	342.7	38.6	304.1	0.141	0.166	0.97	n. d.	n. d.	n.d.
$AB-600_{gr}$	1049.5	135.7	913.7	0.424	0.507	0.97	0.318	1.428	6.20
$AB-700_{gr}$	1174.4	129.9	1044.5	0.486	0.560	0.95	0.518	0.934	6.86
AB-1.5 <sub>imp</sub>	690.4	72.8	617.6	0.287	0.328	0.95	0.630	1.000	7.71
AB-4 <sub>imp</sub>	804.7	81.0	723.7	0.337	0.382	0.95	0.525	1.132	7.34
AB-5 <sub>imp</sub>	724.4	74.1	650.3	0.303	0.344	0.95	0.457	1.107	7.04

Table 1. Characteristics of activated biocarbons

 $S_{BET} - \text{specific surface area}, \ S_{ext} - \text{external surface}, \ S_{mi} - \text{micropores surface}, \ V_{mi} - \text{micropores volume}, \ V_p - \text{total pores volume}, \ R_{av} - \text{average pore radius}, \ n.d. - no \ data, \ Basic, \ Acidic - amount \ of \ functionalities.$ 

The materials contained ~1% moisture and a small amount of ashes (~2 - 7%, Table 2). The thermal stability studies showed that the activation of KOH caused, compared with the initial biochar, an increase in the carbon content in the form of volatile structures (%VC), a decrease in the content of carbon fixed as solid structures (%FC) and a decrease in the thermostability index. This indicates a decrease in thermal stability and may be related to the content of surface functional groups increasing during the physical activation process (Table 1).

Sample	%H	%VC	%A	%FC	C <sub>thermo</sub>
INI	0.5	15.4	2.0	82.6	0.843
$AB-600_{gr}$	1.3	25.3	3.7	71.0	0.737
$AB-700_{gr}$	1.4	16.3	3.8	79.9	0.830
AB-1.5 <sub>imp</sub>	0.3	23.7	6.5	69.8	0.746
AB-4 <sub>imp</sub>	0.4	22.2	6.9	70.9	0.762
$AB-5_{imp}$	1.3	20.7	3.8	75.5	0.785

Table 2. Results of thermal analysis of activated biocarbons.

The content of surface functional groups was determined by the Boehm potentiometric titration. On the surface of the studied biocarbons there are both acidic and basic groups, with a significant predominance of acidic ones (Table 1). This indicates a significant potential of activated materials in the processes of pollutant adsorption from the water environment. The FTIR spectra (Fig.2a) confirmed the presence of functionalities on the biocarbons surface. They differ in intensity and position of the bands, suggesting differences in chemical composition or structural changes in the studied materials. The bands in the range of 3600–3200 cm<sup>-1</sup> are characteristic of O-H groups in alcohols or result from the adsorbed moisture. The bands in the 3000–2800 cm<sup>-1</sup> range can indicate asymmetric stretching and symmetric stretching of C-H bonds (CH<sub>2</sub> and CH<sub>3</sub>). The most intensive band in the  $1700-1600 \text{ cm}^{-1}$  area indicates the presence of C=C (vibrations in aromatic rings) or C=O carbonyl (carboxylic, ketone, aldehyde) groups. The band in the 1200–1000 cm<sup>-1</sup> range can suggest the presence of C-O bonds typical of esters. The bands occurring in the area of 1000–400 cm<sup>-1</sup> (fingerprints, skeletal vibrations) are difficult to assign unambiguously but can indicate deformation vibrations C-H outside the plane of the aromatic rings [2].



Fig.2. FT-IR spectra (a) and Raman spectra (b) of tested materials.

Raman spectroscopy allows to determine the dominant structure and order degree of biocarbons. In the spectra (Fig.2b), two characteristic peaks are observed at ~1300 cm<sup>-1</sup> (D-band) and ~1600 cm<sup>-1</sup> (G-band). The D-band indicates the breathing vibrations in the aromatic rings and is a measure of the disorder of carbon structures. The G-band results from the tensile vibrations of sp<sup>2</sup> carbon pairs in aromatic rings and chain structures [3]. The observed bands are wide, unseparated and the activation has not changed their position. The I<sub>D</sub>/I<sub>G</sub> intensity ratios (Fig.2b) indicate a high level of disorder, the presence of numerous defects and a low degree of crystallinity of the biocarbons.

**Conclusions:** Chemical activation of biocarbons with KOH by the grinding method is more effective than impregnation, leading to a material with a larger specific surface area ( $S_{BET}$  up to 1174 m<sup>2</sup>/g) and greater porosity ( $V_p$  up to 0.56 cm<sup>3</sup>/g). The obtained biocarbons contain a significant share of micropores and numerous functionalities, which promotes the adsorption of organic pollutants. Increasing the activation temperature and impregnation time promotes the development of a porous structure. The decrease in thermal stability and the increase in the content of volatile carbon structures indicate the presence of surface functional groups.

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## THE EFFECT OF THE TEMPERATURE OF THE PYROLYSIS PROCESS ON CHANGES THAT OCCUR IN THE STRUCTURE OF BIOCHAR OBTAINED FROM COFFEE GROUNDS

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**Abstract:** This paper presents the results of tests on biochar obtained from coffee grounds in the pyrolysis process carried out at temperatures of 500 °C, 600 °C and 700 °C. Biochar analyzed for their use as metal sorbents from water solutions and lubricant additives. Analyses were performed using spectroscopic methods, including FTIR and Raman. The FTIR method allowed the identification of functional groups on the surface of biochar that are important from the point of view of sorption processes. The Raman method allowed us to determine the order of the structure and determine the ID/IG quotient. Research conducted using this method is important from the point of view of the use of biochar as additives to lubricants. The conducted research has shown that biochar can be used as metal sorbents from water solutions and as additives to lubricants.

**Introduction:** Coffee, next to water and tea, is one of the most popular beverages in the world, consumed by about 80% of people over 18 years of age in Poland [1,2]. In Poland, coffee consumption per person per year is about 4 kg, which indicates the consumption of at least one cup per day [2]. This indicates the production of approximately 270 tons of coffee grounds per year in Poland. Therefore, coffee grounds constitute waste biomass from which full-value products can be obtained. These products may be biochar obtained through pyrolysis. The biomass pyrolysis process is usually carried out in the temperature range of 500-900 °C, in the presence of an inert gas. Due to their properties, biochar can be used, among others, as metal sorbents from water solutions or as additives to lubricants [3]. The spectroscopic methods can be used to determine changes occurring in biochar as a result of the pyrolysis process and process parameters, including the process temperature [4-6]. An important parameter determining the suitability of biochar for the sorption of metals from water solutions is the presence of functional groups on their surface. The functional groups involved in adsorption may be organooxygen groups, which can be determined by FTIR technique. In addition, this technique can be used to identify the types of bonds present in biochars: C-H, C=C, C=C [4,5]. However, determining the degree of ordering of the biochar structure and determining the  $I_D/I_G$  parameter is possible using the Raman technique [5,6]. The aim of this work was to determine the changes occurring in biochar as a result of the pyrolysis process carried out at temperatures of 500 °C, 600 °C and 700 °C and to evaluate these changes using FTIR and Raman techniques.

**Experimental:** The subject of the study was biochar obtained from coffee grounds in the pyrolysis process carried out at temperatures of 500 °C (BFK\_500), 600 °C (BFK\_600) and 700 °C (BFK\_700), in the presence of the inert gas CO<sub>2</sub>. The tests were carried out
at a laboratory stand equipped with a Czylok furnace, type FCF–V12RM. After the pyrolysis process, the biochas was crushed and sieved to obtain a fraction with a diameter of 200  $\mu$ m. In order to assess the changes occurring in the structure of biochars under the influence of the process temperature, the obtained products were analyzed using spectroscopic methods: (1) Jasco FTIR spectrometer with the following parameters: reflectance mode, diamond crystal pike attachment, (2) Jasco Raman spectroscope with the following parameters: 532 nm laser excitation, 100 second exposure time.

**Results:** The obtained biochar samples were analyzed using Raman and FTIR methods. Figure 1 shows Raman spectra for biochar obtained from coffee grounds at different temperatures. As can be observed, three types of peaks occur in all three analyzed biochar samples.



Fig.1. The Raman spectra of biochar obtained from coffee grounds in the pyrolysis process of a) BFK\_500, b) BFK\_600, c) BFK\_700, in the presence of CO<sub>2</sub>.

The peaks at wavelengths of 1352 cm<sup>-1</sup>, 1355 cm<sup>-1</sup> and 1348 cm<sup>-1</sup> indicate the presence of the D band. This band indicates the presence of defects in the structure attached to the basal plane of graphite and is related to the disorder of the structure [7-9]. These peaks correspond to C–C structures [8]. The peaks at wavelengths of 1582 cm<sup>-1</sup>, 1596 cm<sup>-1</sup> and 1591 cm<sup>-1</sup> indicate the presence of the G band, which is characteristic of samples with a graphite structure and indicates an ordered structure [7-9]. These peaks correspond to C=C structures [8]. The peaks located at 2926 cm<sup>-1</sup>, 2924 cm<sup>-1</sup> and 2919 cm<sup>-1</sup> indicate the presence of 2D graphite phase [7-9]. These peaks correspond to C–H structures. In biochar obtained from coffee at 500 °C, the occurrence of a band with a wavelength of 2083 cm<sup>-1</sup> and 2211 cm<sup>-1</sup> can be observed, which indicates the occurrence of C=O structures [7]. On the other hand, in biochar obtained from coffee grounds at 600 °C, a band with a wavelength of 913 cm<sup>-1</sup>, called the R band, can be observed, which corresponds to the C–C and C–H structures. The peak with a wavelength of  $1061 \text{ cm}^{-1}$  is called the SR band, which corresponds to the C-H structures. Both bands are characteristic for carbon products [8]. Based on the obtained results, the  $I_D/I_G$  coefficient was determined and is presented in Table 1. As can be seen, depending on the temperature, different  $I_D/I_G$  quotient results were obtained, which indicate an ordered structure attributed to the structure of graphite or activated carbons [10]. The change in the  $I_D/I_G$  coefficient in the case of biochar obtained at 600°C, which is lower than the others, may be due to changes in the structure under the influence of the pyrolysis process. This is evidenced by the bands at  $913 \text{ cm}^{-1}$  and  $1061 \text{ cm}^{-1}$ .

No.	Biochar	$I_D/I_G$
1	BFK_500	0.36
2	BFK_600	0.24
3	BFK_700	0.60

Table 1. The  $I_D/I_G$  coefficient of biochar obtained during the pyrolysis process at different temperatures.

The suitability of biochar as heavy metal sorbents was determined using the FTIR method (Fig.2). The obtained FTIR spectra allow for the identification of functional groups present on biochar and changes occurring during the pyrolysis process with increasing temperature.



Fig.2. The FTIR spectra of biochar obtained from coffee grounds in the pyrolysis process a) BFK\_500, b) BFK\_600, c) BFK\_700.

For the analyzed spectra, three band ranges characteristic of biochar can be assigned. For the analyzed spectra, three band ranges characteristic of biochar can be assigned. In the range of  $2500-2000 \text{ cm}^{-1}$  it corresponds to the valence vibrations of the C=C=C system and the triple bonds C=N and C=C. In the range of  $2000-1500 \text{ cm}^{-1}$ , vibrations of C=C, C=O, C=N, N=N double bonds and deformational vibrations of N–H and O–H bonds of water occur. In the range of  $1500-650 \text{ cm}^{-1}$ , valence vibrations of C–C, C–N, C–O bonds and deformation vibrations of C–H occur [4,11]. The studies carried out have shown that as the pyrolysis temperature increases, the organooxygen functional groups are preserved. Additionally, as the process temperature increases above 600 °C in the range of 1996-2114 cm<sup>-1</sup>, C=O groups are formed.

**Conclusions:** The spectral analyses performed allowed us to determine the changes occurring in biochar obtained from coffee grounds under the influence of changes in the temperature of the pyrolysis process. The Raman analysis of biochars allowed the identification of three bands (D, G and 2D) characteristic of materials with a graphite structure and the determination of the  $I_D/I_G$  coefficient. In addition, based on the analyses performed, information was obtained on the degree of structural order in biochar. The D band is associated with a disordered structure, while the G band is

associated with an ordered structure. The conducted research shows that the degree of ordering in biochar changes with the process temperature. The analysis of biochar using the FTIR method allowed the identification of functional groups present on the biochar. Based on the analyses performed, it was observed that with the increase in the process temperature there is no disappearance of organooxygen functional groups. The conducted spectral analyses indicate the possibility of using biochar obtained from coffee grounds as sorbents of metals from water solutions and as additives to lubricants.

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# APPLICATION OF SPECTROSCOPIC METHODS IN THE ANALYSIS OF BIOCHAR FROM RAPESEED OIL PRESS CAKE AND ITS INFLUENCE ON THE TRIBOLOGICAL PROPERTIES OF A POLYMER COMPOSITE FOR RAILWAY TRANSPORT APPLICATIONS

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**Abstract:** This paper presents an evaluation of a polymer composite with lubricating properties that contains biochar, which was obtained by pyrolysing rapeseed oil press cake at temperatures of 500 °C and 700 °C. Spectroscopic analyses confirmed the favourable structure of the biochar, especially after pyrolysis at higher temperatures. Subsequent tribological and mechanical tests demonstrated that the composite with biochar produced at 700 °C exhibited a lower coefficient of friction, higher compressive strength, and higher hardness compared to biochar produced at 500 °C. Furthermore, FTIR analysis revealed negligible chemical alterations both before and after testing, thereby substantiating the material's remarkable chemical stability under frictional conditions.

Introduction: In contemporary rail transport, splash systems are utilised to deliver liquid lubricating oil to the point of contact between the wheel and the rail. Despite their function in facilitating lubrication, these systems have the potential to induce leakage and contaminate the track, as well as soil and groundwater. The non-biodegradable nature of these oils poses a significant environmental threat, and their use is associated with high operating costs and the need for frequent refilling and servicing. Furthermore, the utilisation of grease as a lubricant results in an inconsistent distribution, thereby accelerating the rate of wear and deterioration of both the wheels and the rail infrastructure. An alternative solution is a polymer composite with enhanced lubricating properties, in which the grease is fed in solid form. This alternative eliminates the risk of leaks, reduces material wear and allows for even, permanent lubrication regardless of weather conditions. A further significant consideration is the potential utilisation of rapeseed oil press cake, a by-product of food oil production in Poland, which generates several hundred thousand tonnes annually [1]. Rather than being disposed of, these byproducts can be converted by pyrolysis into biochar with a graphite-like structure. The incorporation of this biochar into the composition of a polymer composite has been demonstrated to enhance its tribological properties, paving the way for the development of innovative, environmentally friendly materials for rail transport in line with the principles of the circular economy [2]. The objective of this study was to evaluate the effect of biochar, obtained by the pyrolysis of rapeseed oil press cake, on the tribological and mechanical properties of the polymer composite. The research was also aimed at verifying the suitability of such a composite as an environmentally friendly solid lubricant for rail transport applications.

**Experimental:** The bio-carbon employed in the present study was obtained by subjecting rapeseed oil press cake to a pyrolysis process. Cascade pyrolysis was conducted in a chamber kiln at two temperatures: 500 °C and 700 °C, in a carbon dioxide atmosphere [3]. The pyrolysis yields were found to be 33.1% at 500 °C and 29.7% at 700 °C. The polymer composite was then prepared by combining epoxy resin, plastic grease, copper powder, titanium dioxide and the biochar obtained by the pyrolysis of rapeseed oil press cake. For the mechanical testing, Z1 hardener was added to the lubricating composite. The material obtained by pyrolysis was ground and then tested using FTIR and Raman techniques. The FTIR spectra were obtained using a Jasco FTIR-6200 spectrometer in reflectance mode, equipped with a Pike attachment and a diamond crystal. Raman spectra were obtained at ambient temperature using an NRS-5100 spectrometer from Jasco, with laser excitation at 532 nm, an objective of 20x, an exposure time of 100 seconds per spectrum, an accumulation count of 20, and a measurement range of 100 cm<sup>-1</sup> to 4000 cm<sup>-1</sup> [2].

**Results:** Fourier-transform infrared spectroscopy (FTIR) was utilised to identify the functional groups present on the surface of the biochar (Fig.1). The spectrum of biochar formed at 500 °C shows the presence of oxygen and aliphatic groups, including the C=C band (1576 cm<sup>-1</sup>), -CH<sub>3</sub>/-CH<sub>2</sub> (1384 cm<sup>-1</sup>), C-O/C-OH (1296-1105 cm<sup>-1</sup>) and ether bridges (1029 cm<sup>-1</sup>), indicating a moderate degree of carbonisation. In the sample pyrolysed at 700 °C, there is a significant weakening of the oxygen group signals and a marked enhancement of the aromatic bands (1570-1475 cm<sup>-1</sup>), indicating a higher degree of aromatisation. Furthermore, the presence of bands associated with condensed carbonyl groups (1860 and 1787 cm<sup>-1</sup>) is observed, and the C-H aromatic bands (901-720 cm<sup>-1</sup>) exhibit greater intensity compared to those observed in the sample from the lower temperature [3].



**Fig. 1.** Comparison of spectra of natural carbon materials obtained pyrolytically from rapeseed oil press cake at a) 500 °C and b) 700 °C.

Raman spectroscopy was utilised to evaluate the carbon structure of materials derived from rapeseed oil press cake at temperatures of 500 °C and 700 °C (Fig.2). The presence of D (~1350 cm<sup>-1</sup>) and G (~1590 cm<sup>-1</sup>) bands, along with the ratio of these bands ( $I_D/I_G$ ), facilitates the estimation of the degree of disorder and graphitisation within the material

[2]. For the biochar produced at 500 °C, an ID/IG ratio of 0.07 was obtained, while for the sample pyrolysed at 700 °C, the ratio was 0.10. This value is comparable to the level of graphite utilised in commercial solid lubricants, for which the typical ratio is approximately 0.15. The analysis of FTIR and Raman spectra indicates that the biochar obtained possesses a favourable chemical structure and degree of aromatisation, thereby confirming its suitability as a functional additive to a lubricating composite for railway applications.



Fig.2. Natural carbon materials obtained pyrolytically from rapeseed oil press cake at a) 500 °C and b) 700 °C.



Fig.3. Comparison of FTIR spectra of the composite with biochar at a) 500 °C before tribological test - black line and after tribological test - gray line b) 700 °C before tribological test - black line and after tribological test - gray line.

A series of tribological and mechanical tests was conducted to provide a comprehensive evaluation of the lubricating composite's performance. Anti-wear tests were performed on a four-ball apparatus. This test was designed to simulate operation in a wheel-rail system, where lubrication film efficiency and durability are crucial. The composite with biochar produced at 500 °C exhibited a coefficient of friction of 0.23 and a friction node temperature of 45.18 °C. However, the composite with biochar produced at 700 °C demonstrated a lower coefficient of friction of 0.21 and a friction node temperature of 44.59 °C, thereby substantiating its superior lubricating properties. Concurrently, hardness measurements were conducted using the Brinell method, which involves

pressing a steel ball into the surface of the material. The composite with biochar produced at 500 °C achieved a hardness of 224 HB, whereas the composite with biochar produced at 700 °C reached 237 HB. Compressive strength was determined using an Instron testing machine. The composite with biochar at 500 °C achieved a compressive stress of 43.08 MPa, while the composite with biochar at 700 °C achieved 47.2 MPa. To assess the chemical changes occurring during operation, FTIR analysis of the composites before and after the friction tests was performed (Fig.3). Following the antiwear test, both variants exhibited an enhancement in the intensity of the bands within the 2800-3100 cm<sup>-1</sup> regions, suggesting an increase in the vibration of the C-H groups. Additionally, the bands at 1460, 1183 and 970 cm<sup>-1</sup> exhibited strengthening, and a novel band emerged at 1420 cm<sup>-1</sup>, suggesting the formation of C-C and C-O bonds resulting from matrix and lubricant degradation. In the biochar composite produced at 700 °C, a decrease in band intensity was observed at 1743 and 2361 cm<sup>-1</sup>, indicating the transformation of oxygen groups.

**Conclusion:** The experimental tests conducted unequivocally demonstrated that the lubricating composite containing biochar, obtained at a temperature of 700 °C, exhibited superior performance properties in comparison to its counterpart, which was formed by pyrolysis at 500 °C. The enhanced hardness, higher compressive strength, and lower coefficient of friction of the former are indicative of its optimised mechanical and tribological structure. Furthermore, analysis of the FTIR spectra indicated that the composite with biochar produced at 700 °C exhibited a reduced number of chemical changes after anti-wear testing, suggesting its enhanced resistance to thermal and oxidative degradation. Conversely, the presence of new bands and an increase in the intensity of carbonyl groups in the composite with biochar produced at 500 °C suggests a higher susceptibility to frictional ageing processes. The outcomes corroborate the elevated chemical and structural stability of both composites, with the composite with biochar produced at 700 °C demonstrating an advantage in the context of long-term utilisation under dynamic loading conditions. These properties make it a promising option for rail transport applications as a durable, lightweight, and efficient solid-state lubricant.

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## SUSTAINABLE TECHNOLOGIES FOR THE SEPARATION OF RARE EARTH ELEMENTS

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**Abstract:** Rare earth elements (REEs) are indispensable components of modern technology, underpinning innovations from smartphones and electric vehicles to renewable energy systems. As the demand for these critical materials continues to grow, the environmental challenges associated with their separation and purification become increasingly pressing. Traditional methods for REE separation are often resource-intensive, involving significant energy consumption, the use of hazardous compounds and generate of large volumes of waste. These processes not only pose environmental risks but also threaten the sustainable supply of REEs. In response to these challenges, the technologies aimed at reducing the environmental footprint of REE separation were evaluated. In this aim the biodegradable complexing agents were used.

Introduction: The rare earth elements (REEs) are a group of 17 elements, consisting of the lanthanoid series (Ln), and scandium (Sc) and yttrium (Y) [1]. Because of its similar ionic radius and chemical characteristics to lanthanoids, yttrium is categorized as a rare earth element (REE) [2]. Despite being a rare earth element and occasionally concentrating into some of the same minerals as the other REEs, scandium has different chemical properties from the other REEs. Based on their electron configurations, REEs are categorized as either heavy REEs or REE oxides or light REEs or REE oxides. While some REEs are not particularly rare in terms of average abundance in the Earth's crust, they are seldom found in concentrations high enough for economic extraction, and global annual production is notably low in comparison to many other metals [3]. The rare earth elements market is dominated by China (60-70% of mining, 85-90% of processing), with smaller contributions from the U.S. (MP Materials), Australia (Lynas), and Myanmar. Global demand is projected to grow at 8-12% annually through 2030, expanding from about \$5-6 billion in 2023 to \$9-12 billion by 2030 [4]. This growth is primarily driven by clean energy technologies (wind turbines, electric vehicles), electronics, and defense applications, with neodymium, praseodymium, dysprosium, and terbium being the most critical elements in demand as well as traditional ones such as magnets, batteries, catalysts, glass industry, ceramics, metal alloys (Fig.1).





The industrial applications for rare earth products include high-tech electronic devices, computers, electronic motors, LCDs, DVDs, magnetic materials, magnetic memory chips, fibre optics, superconductors, mobile phone batteries, precision optics and many energy-saving environmental protection products [4]. Nevertheless, the use of REEs in materials and components have enabled numerous industrial processes and technologies, including those essential to clean-energy initiatives worldwide, and to miniaturised electronics and devices. Refractory products are used extensively in heat-treatment facilities for petrochemical, steel metallurgy, non-ferrous metallurgy, glass, construction materials, chemical fertilizer and ceramics industries. Rare earth products from China are mainly exported to the United States of America, Japan, Europe and Korea.

Experimental: The Sartoflow® Alpha crossflow microfiltration equipment (Sartorius, Göttingen, Germany) was used to separate the yeast biomass (15.5 g/L) and the residual waste frying oil (12.7 g/L) completely from the final culture broth. The microfiltration process was conducted by the Hydrosart<sup>®</sup> type membranes (Sartorius) with 0.2 µm pore sizes and the total filtration area of 0.1 m<sup>2</sup>. After separation of the yeast biomass and the waste frying oil residues, the concentrations of the ionic components were measured in the permeate. The sustainable form of citric acid solution (CA) was used in all experiments on the sorption of REEs. The 907 Titrando titrator equipped with the 800 Dosino type dosing system with the combined pH electrode Metrohm 6.0259.100, 801 type magnetic stirrer and Pt 1000 temperature sensor (Metrohm) was used to perform potentiometric titrations. The static method of adsorption of Ln(III) on lewatit SP112 and Amberlite IRA 458 was performed using the laboratory shakers Elpin type 357 and Elpin type 358A (Elpin Plus, Poland). The pH of the solutions was measured by the pHmeter pHM82 (Radiometer, Copenhagen). The Ln(III) ions concentration was analysed by the inductively coupled plasma optical emission spectrometry using ICP-OES 720-ES (Varian, USA) at 333.749 nm for La(III), 401.224 nm for Nd(III) and 345.600 nm for Ho(III).

**Results:** The circular economy aims to maintain and retain the embedded value of products by creating continuous closed loops of materials and by phasing out waste. The sustainable recovery of rare earth elements (REEs) is a key element in ensuring their availability with minimal environmental impact. As was announced, 47 Strategic Projects will contribute significantly to Europe's green and digital transitions. Among them, Mkango Resources Ltd. (Pulawy) will be involved in the recovery of REEs (Fig.2).

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Fig.2. Strategic processes for EU.

It is well known that alternative sources of methods to recover REEs include: 1) the production of REEs based on their recovery from mineral fertilisers (SecREEts Project) [5] (it has been shown that REEs can be efficiently recovered during fertiliser production, using existing facilities and minimising the environmental impact); 2) recycling of electronic waste and used Ni-MH batteries, using biodegradable agents, which reduces the negative environmental impact [6], 3) 'Urban Mining', i.e. recovery of REEs from urban waste such as used magnets and electronics, is an important direction for the development of a sustainable economy [7]; or 4) coal ash generated from coal combustion in power plants is identified as resource for REEs (it often contains higher concentrations of these elements compared to other sources due to their concentration during the combustion process) [8]. In our research we use CA for recovery Nd(III) and Ho(III) from spent Ni-MH batteries on Lewatit SP112 and Amberlite IRA 458. The results of the influence of the metal:ligand molar ratio on the sorption process of Nd(III) and Ho(III) are shown in Fig.3a.



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Fig.3. The influence of (a) the metal:ligand molar ratio, (b) pH on the sorption process of Nd(III) and Ho(III) on SP112 and IRA 458 as well as (c, d) desorption agent type [6].

The pH effect was presented in Fig.3b. In the examined systems, the kinetic process rate depends on the type of ion exchanger (SP112 or IRA 458). The equilibrium was established after 60 min. for SP112 and 120 min. for IRA 458, after which 85-99% of the Nd(III) and Ho(III) ions were removed, respectively. Kinetic experiments indicated that the pseudo-second order model displayed the best correlation with the adsorption kinetic data from Nd(III) and Ho(III). The sorption occurred most efficiently for pH 2 for SP112 and pH 8 for IRA 458. Taking into account the desorbing agent, the most effective was 2 M HCl and HNO<sub>3</sub>.

**Conclusions:** The efficiency of the adsorption process of rare earth elements (REEs) in the presence of citric acid (CA) is influenced by a number of factors: REE:CA molar ratio, solution pH, phase contact time, and initial concentration. The study made it possible to select optimal process conditions, which varied according to the adsorbent and metal ion studied. The most promising desorbing agent has also been proposed (HCl and HNO<sub>3</sub>).

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## PRECIPITATION OF NICKEL(II) IONS BY TRIMERCAPTOTRIAZINE

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**Abstract:** The precipitation methods was used for nickel removal. 1.5% solution of trimercaptotriazine was used for nickel(II) ions removal from water in the pH range from 7 to 9. The studies have proved an increase in the nickel ions removal with pH increasing from 7 to 9. The maximum nickel(II) ions removal was obtained at pH 9.

**Introduction:** Contact with nickel can cause a variety of side effects on human health, such as allergy, cardiovascular and kidney diseases, lung fibrosis, lung and nasal cancer [1]. This element occurs in the environment due to volcanic eruption, forest fires or rock weathering. However, anthropogenic sources of nickel cannot be ignored. Some sources like dusts are not soluble in water but nickel sulfate emission belongs to water-soluble sources. In the presence of CO, nickel forms carbonyls, that are emitted into the environment [2]. Coal combustion is the leading source, attributing 63.4% of the national total nickel emissions in China; liquid fuels consumption ranks the second, contributing 12.4% of the totals; biofuels burning accounts for 8.4% and the remaining sources together contribute 15.8% of the totals.

The trimercaptoptriazine solution which contain reagent should also precipitate nickel ions in slightly alkaline and neutral solutions. For this reason the trimercaptoptriazine reagent was tested in the pH range 7-9.

**Experimental:** The nickel(II) stock solution was prepared by dissolution of NiCl<sub>2</sub>·6H<sub>2</sub>O purchased from the POCh S.A. company (Gliwice, Poland). 1000 mg/l nickel stock solution was diluted to 100 mg/l. 0.1 M NaOH solution were used for pH adjustment. The 15% solution of trimercaptotriazine was diluted to 1.5%. And then it was used for precipitation of nickel(II) ions. Its chemical structure is presented in Fig.1.



Fig.1. The chemical structure of trimercaptotriazine.

As can be seen in Fig.1 the trimercaptotriazine has three nitrogen and three sulfur atoms. The sulfur atoms can simply react with nickel(II) ions forming a precipitate. The trimercaptotriazine is characterized by low toxicity to fish and living organisms.

The solution of trimercaptotriazine is presented in Fig.2. The Ni(II) ions concentration in the samples was determined using the Atomic Absorption Spectroscopy spectrometer Varian AA240FS. The measurement parameters were: wavelength 232.0 nm, lamp

current 7 mA, air: acetylene flow 13.5:2 l/min. The calibration curve on nickel(II) ions was in the concentration range 0-5 mg/l.



Fig.2. The appearance of the trimercaptotriazine solution.

**Results:** Precipitation of Ni(II) ions (100 mg/l) from the neutral and slightly alkaline solution in pH range 7-9 using 1.5% trimercaptotriazine solution was investigated at the contact time of 1440 min. The volume range of 1.5% trimercaptotriazine solution was 0.1-0.8 ml. The recovery percentage (%R) of nickel(II) ions was calculated using the equation:

### $%R = (C/C_0) \times 100\%$

where:  $C_0$  is the initial concentration of nickel(II) ions [mg/l], C is the concentration of Ni(II) ions calculated from the difference in the solution concentration before and after the precipitation process [mg/l].



Fig.3. Influence of 1.5% trimercaptotriazine solution on %R nickel(II) ions in pH range 7-9.

The influence of pH values on nickel(II) ions removal by using 1.5% trimercaptotriazine solution is presented in Fig.3. The removal percentage of nickel(II) ions depends on pH values. The %R values increase sharply in pH 7 and 8. In the pH range of 7-8, increasing the solution volume from 0.1 to 0.8 increases the percentage of nickel ions removal to 60%. At pH 9, an increase in the percentage of removal can be observed with the amount of solution added. The maximum value of %R is estimated at pH 9 for 0.4 ml of trimercaptotriazine solution. Increasing the volume of 1.5% trimercaptotriazine solution in the range of 0.5-0.8 ml causes decreasing the removal percentage to 48%. As can be seen in Fig.4 the precipitate is yellow.



Fig.4. The appearance of precipitate trimercaptotriazine with nickel(II) ions.



Fig.5. Influence of volume of 1.5% trimercaptotriazine solution on equilibrium pH values.

Adding 1.5% trimercaptotriazine solution causes changes in the equilibrium pH value of nickel(II) ions solution. The influence of volume of 1.5% trimercaptotriazine solution on equilibrium pH values is showed in Fig.5. The greatest increase in the equilibrium pH value can be observed for an initial pH of 7. However, over the entire pH range of 7-9, adding 1.5% trimercaptotriazine solution causes an increase in the equilibrium pH value. The amount of nickel(II) ions precipitated by volume trimercaptotriazine solution q [mg/ml] is calculated from below equation:

### $\mathbf{q} = [(\mathbf{C}_0 \cdot \mathbf{C}_e) \cdot \mathbf{V}_S] / \mathbf{V}_T$

where:  $C_0$  is the initial concentration of nickel(II) ions [mg/l],  $C_e$  is the concentration of Ni(II) ions in the solution after the precipitation process [mg/l],  $V_S$  is volume of nickel(II) ions solution [1],  $V_T$  is volume of 1,5% trimercaptotriazine solution [ml].

The influence of volume of 1,5% trimercaptotriazine solution on q [mg/ml] parameter is shown in Fig.6. As can be seen from Fig.6, the best results of nickel(II) ions precipitation were obtained at pH 9. In acidic media the trimercaptotriazine exist in hydrogen form according with reaction in Fig.7. The hydrogen form of trimercaptotriazine is slightly soluble in water and precipitates as a sludge. This reaction is reversible in an alkaline solution.



Fig.6. Influence of pH values on the nickel(II) ions precipitation.



**Conclusions:** The investigations of nickel(II) ions removal proved that nickel(II) ions can be precipitated with the 1.5% solution of trimercaptotriazine. The best results of nickel(II) ions precipitation were obtained at pH 9. This pH indicates the possibility for removal of nickel(II) ions from alkaline water and wastewater solutions.

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## SORPTION OF ZINC(II) IONS ON LEWATIT S5428 ION EXCHANGE RESIN BY THE COLUMN METHOD

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**Abstract:** The paper investigated the adsorption of the packed-bed column with the strongly basic anion exchange (SBA) resin Lewatit S5428 for Zn(II) ions removal. The glass columns were filled with 10 ml of swollen adsorbent beads of spherical shape. The impact of inlet Zn(II) (10, 25, 50 mg/L) and HCl (0.1, 1, 3, 6 mol/L) concentrations on the breakthrough curves was examined, with a focus on the adsorption performance of Zn(II) on the Lewatit S5428 ion exchanger. The column parameters demonstrate that the sorption efficiency of Zn(II) is optimal at 3 mol/L HCl, and that the working ion exchange capacities ( $C_w$ ) decrease with the decreasing initial Zn(II) concentration ( $C_0$ ). The comparison of the obtained results with those from other sources in the literature was made, and it was observed that the Lewatit S5428 adsorbent could be utilised for the continuous recovery of Zn(II) ions from aqueous solutions. However, it was observed that the sorption efficiency of Lewatit S5428 was smaller to other SBA resins.

Introduction: From a technological point of view, column studies are of particular importance. The column method, also known as the packed bed or fixed bed method, is a conventional adsorption technique [1]. In the context of column studies, the most applicable technique is the fixed-bed column due to the sorption-desorption cycles [2]. In the column process, the solution of the adsorbate is passed through the column at a constant flow rate, filled with an appropriate amount of the swollen adsorbent. The eluate (solution introduced at the top of the column) is continuously passed through the column with the adsorbent [3]. The development of industrial wastewater treatment systems for metal removal and/or separation is predicated based on laboratory column tests. In order to verify the applicability of a column sorption system on an industrial scale, it is necessary to obtain the breakthrough curve and determine the breakthrough point of the column in a laboratory setting. The column breakthrough point is defined as the time taken for the adsorbate appeared as the first appear at the column outlet. This is the time when the mass transfer zone reaches the final part of the sorbent bed [1,3]. In the field of environmental contaminant adsorption, the batch experiment, also known as the static method, is a more frequently employed approach in comparison to the column experiment [4]. Despite its technological limitations, it remains the prevailing approach. This is primarily due to the fact that it is less space-consuming, does not necessitate the use of sophisticated apparatus, does not consume a very large amount of time, requires a small amount of adsorbent and can be operated in single, series and in parallel [2,4]. Consequently, the experimental analysis can provide readily all the parameters required to assess adsorbent efficiency towards a specific contaminant. By contrast, the column approach is a more intricate process, necessitating the establishment of a laboratory environment, substantial resources such as laboratory space and time, and the execution of multiple trials [1]. On the other hand, the column method provides

a more accurate simulation of the actual environment during the removal of contaminants. It is straightforward to operate and design, ensures a large yield, facilitates a faster adsorption process, and is effective in treating large quantities of wastewater. Consequently, it is more suitable for determining the transport properties of contaminants in the laboratory-scale settings [4]. However, the drawback of time and space demanding, reduces the informative benefit of the column method greatly. In addition, some derivative issues including the bulk density of packed adsorbent and the length and diameter of columns, etc. arise concerning in the column experiments which can cause that assessment of the contaminant transport might be inappropriate [3]. The column method is less frequently used than the batch method for the removal of heavy metal ions. Zinc has been effectively removed from chloride and chloride sulphate solutions by the batch method using the ion exchangers such as Purolite C-100 MH [5], Amberlite IRA410, Purolite A103S, Purolite NRW700, Purolite A400MBOH, Purolite A600MB [6], Purolite S985, Purolite A500, AV-17-8 and AM-2B [7] and from the cyanide effluents on  $201 \times 7$  and 301 resins [8]. The other examples of Zn(II) removal from end-of-life PCBs, aqueous solution and industrial effluents using Lewatit TP207, Lewatit MonoPlus M500, Lewatit VPOC1071, Lewatit MonoPlus MP64, Amberlite IRA743, Lewatit TP208 and Lewatit TP260, Amberlite IR120, Lewatit MonoPlus TP220, Purolite S984, Lewatit MonoPlus SR7, Purolite A400TL, Dowex PSR2, Dowex PSR3, Purolite A830 ion exchangers and adsorbent Lewatit AF5 have been reviewed by Wołowicz et al. [9]. Among the column methods for metal removal (Fig.1), the fixed bed column is generally employed. As reported in the literature, the impact of the experimental parameters such as bed height, initial concentration, column length and diameter, pH, temperature and flow rate have been studied to examine the adsorption efficiency of metals.



**Fig.1.** Schematic diagram of the fixed bed adsorption column and typical breakthrough curve (U is the total leakage volume until the breakthrough point (ml),  $C_0$  and C are the initial and equilibrium M(II) ions concentrations (mg/L), U is the leakage volume corresponding to  $C/C_0 = 0.5$  in (ml),  $V_j$  is the adsorbents bed volume placed in the columns (ml)).

For example, zinc was extracted from the *Azolla filiculoids* based aqueous solution by utilising a fixed-bed column as the adsorbent, with the column measuring 285 cm in height, possessing a diameter of 2.5 cm, and containing an adsorbent mass ranging from 2.5 to 7.5 g. The employed flow rate was 480 ml/h. The zinc sorption capacity was determined to be 31.3 mg/g at a flow rate of 480 ml/h and pH 6.2. The decrease in the pH of the influent to 4, and the decrease in the influent Zn(II) concentration to 50 mg/L, with an increase in the flow rate to 800 ml/h (residence time: 2.6 min), resulted in the decreased sorption capacities by 15.3%, 7.7% and 9.6%, respectively [10].

Mohan et al. [11] also employed the fixed-bed column for the removal of Zn(II), Cu(II), Pb(II) and Mn(II) by the rice husk adsorbents (raw and phosphate-treated rice husk). The researchers reported that the removal of M(II) was influenced by two factors: the pH of the influent solution and the water flow rate. The column height was set at 30 cm, the diameter at 2.5 cm, and the adsorbent dose at 36 g. The sorption process was initiated using a 10 mg/L solution containing Zn(II), and the solution was passed through the column at a flow rate of 20 ml/min. With the increasing bed height (10-30 cm), the breakthrough time also increased from 1.3 to 3.5 h for Pb(II), from 4 to 9 h for Cu(II), from 12.5 to 25.4 h for Zn(II) and from 3 to 11 h for Mn(II), respectively [11]. Furthermore, studies on the effect of column height and adsorbent mass on Zn(II) removal on zeolite NaY demonstrate that adsorption gradually increases with the increasing column height and adsorbent mass [12]. In light of rare data concerning the removal of Zn(II) ions from water and wastewater by the dynamic method, particularly from an application and knowledge perspective, the objective of this study was to examine the impact of the inlet Zn(II) and HCl concentrations on the Zn(II) removal efficiency of the Lewatit S5428 ion exchanger.

**Experimental:** Sorption of Zn(II) ions by the dynamic method was performed using the ion exchange columns. Columns with a diameter of 1 cm and a height of 25 cm were connected to glass balloons with rubber tubes. The columns were filled with 10 mL of swollen Lewatit S5428 sorbent and the glass balloons were filled with the solutions of 10, 25, 50 mg Zn(II)/L concentrations at pH 4. Moreover, the effect of HCl concentration such as 0.1, 1, 3, 6 mol/L on the sorption efficiency was analyzed. The solutions were introduced into the columns and the column effluent was collected in fractions until the initial Zn(II) ion concentration was reached. The flow rate was gravitational and equal to 0.4 cm<sup>3</sup>/min. Then the Zn(II) ion concentrations were determined by the flame atomic absorption spectroscopy (FAAS) and the breakthrough curves, the column parameters such as weight (D<sub>w</sub>) and bed volume (D<sub>b</sub>) coefficients and working ion exchange capacity (C<sub>w</sub>) were determined using the formulae given below:

- the weight distribution coefficient  $(D_w)$  (mL/g):

$$D_{w} = \frac{U'' - U_{o} - V}{m_{j}}$$
(1)

- the bed distribution coefficient (D<sub>b</sub>):

$$D_b = D_w \times d_z \tag{2}$$

- the working ion exchange capacity (C<sub>w</sub>) (g/mL):

$$C_w = \frac{U_p \times C_o}{v_j} \tag{3}$$

where: U'' – the eluate volume for C/C<sub>0</sub>=0.5 mL, U<sub>0</sub> – the dead volume of the column (the volume of liquid in the column between the lower edge of the ion exchanger bed and the column outlet, under the process conditions of U<sub>0</sub>=2 mL), V – the free (intergranular) volume of the ion exchanger bed (approx. 0.4 of the bed volume) (mL),  $m_j$  – the dry mass of the ion exchanger in the column (g),  $d_z$  – the sorbent density (g/mL); U<sub>p</sub> – the volume of the eluate up to the breakthrough of the column (L); C<sub>0</sub> – the initial concentration of Zn(II) in the solution (g/L); V<sub>j</sub> – the volume of the sorbent in the column (mL).

Lewatit S5428 is a strongly basic anion exchanger with a macroporous structure and the quaternary amine (type 1) functional groups. It has a cross-linked polyacrylate matrix. The total capacity was 0.85 val/L and the average bead size was in the range of 0.4-1.6 mm. The operating temperature and pH ranges were 80°C and 0-12, respectively.

**Results:** The dynamic method was used to study the sorption of Zn(II) ions on Lewatit S5428. The effects of Zn(II) initial (10, 25 and 50 mg Zn(II)/L, pH 4.00) and HCl (0.1; 1.0; 3.0; 6.0 mol/L HCl - 25 mg/L) concentrations on the sorption properties of Lewatit S5428 were studied. The results of the tests performed were presented in the form of breakthrough curve graphs as shown in Fig.1.



Fig.1. Breakthrough curves obtained during the Zn(II) sorption of on Lewatit S5428.

The breakthrough curves have the typical "S" shape. The effect of HCl on the sorption parameters was observed. The volume of eluate collected at the breakthrough point of the column increased with the increasing HCl concentration. The most optimal HCl concentration for Zn(II) removal by Lewatit S5428 was 3 mol/L, which was in agreement with the results obtained by the batch method. In this case, the working ion exchange capacity was 3.6×10<sup>-4</sup> g/mL. The C<sub>w</sub> values for the other HCl concentrations were  $5 \times 10^{-5}$  g/mL (0.1 mol/L HCl) <  $7.5 \times 10^{-5}$  g/mL (1 mol/L HCl) <  $1.3 \times 10^{-4}$  g/mL  $(6 \text{ mol/L HCl}) < 3.6 \times 10^{-4} \text{ g/mL}$  (3 mol/L HCl). The weight and bed distribution coefficients were in the range of 8.6 to 99.3 mL/g and 1.93 to 22.3 mL/g, respectively. The changes in the tendency of the values of  $D_w$  and  $D_b$  were the same as those of  $C_w$ . In the systems of different initial Zn(II) concentrations the obtained sorption parameters were lower compared to those 0.1-6.0 mol/L HCl-25 mg Zn(II)/L. The working ion exchange capacity and the weight and bed distribution coefficients decreased with the initial Zn(II) concentration increase (Fig.2). High concentrations quickly saturated the resin, resulting in the breakthrough point of the column being observed after a shorter time. The increase in the Zn(II) concentration modified the adsorption rate through the bed and decreased the working adsorption capacity. In the system of the highest Zn(II) concentration the  $C_w$ ,  $D_w$ ,  $D_b$  parameters were the lowest. These results indicate that the Zn(II) sorption is low under such experimental conditions.



Fig.2. Weight and bed distribution coefficients obtained for the Zn(II) sorption on Lewatit S5428 as a function of initial Zn(II) concentration (pH 4).

In the aqueous solution, Zn(II) occurs in the form of various complexes, the nature of which is dependent on the pH and the Cl<sup>-</sup> concentration [13]. The adsorption efficiency of Zn(II) is found to be contingent upon the form and electrical charge of these complexes. In the chloride solutions, Zn(II) exists in the forms of Zn<sup>2+</sup>, ZnCl<sup>+</sup>, ZnCl<sub>2</sub>, ZnCl<sub>3</sub><sup>-</sup>, ZnCl<sub>4</sub><sup>2-</sup>. The greatest sorption efficiency of Zn(II) in the 3 mol/L HCl solution is related to an increase in the fractions of anionic forms of Zn(II) in the solution, which increases the sorption ability on the Lewatit S5428 resin. In contrast, with the concentrated 6 mol/L HCl solution, the competition effect was responsible for reduction in the Zn(II) sorption. The sorption process of Zn(II) on the strongly basic anion exchange resin can be delineated as an ion-exchange mechanism.

**Conclusions:** The adsorption of Zn(II) ions on Lewatit S5428 was influenced by the initial concentration of Zn(II) and HCl. The results of the column experiment showed that Lewatit S5428 was not an effective adsorbent for the uptake of Zn(II) from the aqueous acidic medium at pH 4, with the maximum uptake capacity equal to  $2 \times 10^{-5}$  g/mL at an initial Zn(I) concentration of 10 mg/L. The Zn(II) uptake was more efficient in the HCl-25 mg Zn(II)/L system, and the largest working ion exchange capacities were  $3.6 \times 10^{-4}$  g/mL for 3 mol/L HCl.

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## ALGINATE-BASED BIOCOMPOSITE BEADS AS A POTENTIAL FOR CRITICAL RAW MATERIAL RECOVERY

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**Abstract:** The main objectives of this study were the characterization and evaluation of alginate@chitosan biocomposites cross-linked with calcium ions, with an emphasis on their adsorption capabilities as biosorbent materials for critical raw material recovery with specific targeting of  $La^{3+}$  and  $Ho^{3+}$  ions. To comprehend their effects on the adsorption behavior, we performed and analyzed the adsorption kinetics, isotherms, thermodynamics, and characterization approaches.

**Introduction:** Innovation in the application of biopolymers for functional materials is driven by the transition to sustainable industrial practices. Due to their availability, affordability, and versatility for use in food, medicine, and environmental remediation, polysaccharides stand out among biopolymers. Alginate, an anionic polysaccharide discovered by E.C.C. Stanford in 1881 [1], is very adaptable because it interacts with different metal cations and is also biocompatible and biodegradable. Alginate can selectively coordinate metal cations across multiple oxidation states thanks to the special structure of (1,4)-linked  $\beta$ -D-Mannuronate (M) and  $\alpha$ -L-Guluronate (G) blocks [2]. The hydroxyl and carboxylate groups of its backbone are essential for the formation of crosslinked hydrogel structures with unique porosities and mechanical properties. The ability to absorb metallic or cationic ions through ion exchange between cross-linking cations and target contaminants such as heavy metals, dyes, or critical raw materials is one of the factors that makes it suitable for use in environmental settings. However, alginate gel has many drawbacks, including poor mechanical qualities, excessive stiffness, and fragility. To improve the mechanical and thermal stability and swelling properties of traditional alginate gels, alginate-based organic and inorganic compounds are being developed [3]. To improve properties, alginate is subjected to cross-linking with multivalent metal cations, such as Ca<sup>2+</sup>, or polyamines, such as chitosan, with which it interacts ionically, leading to the formation of tough gels or insoluble polymer structures. Chitosan is a linear copolymer of  $\beta$ -(1–4)-2-acetamido-2-deoxy- $\beta$ -D-glucose and  $\beta$ -(1– 4)-2-amine-2-deoxyb- $\beta$ -D-glucose, which is produced primarily by deacetylating chitin, a naturally occurring polysaccharide found in crustacean shells. The polyelectrolyte complex, which is produced by ionic interactions between the protonated amino groups of chitosan and the carboxylate groups of alginate, has been shown to have improved mechanical properties and a decreased propensity to swelling when compared to their constituent polymers [4].

In this study, alginate@chitosan biocomposites were synthesized and characterized toward the uptake of  $La^{3+}$  and  $Ho^{3+}$  ions from aqueous solutions.

**Experimental:** The alginate-based biocomposite, i.e., alginate with chitosan (ALG@CS), was obtained by cross-linking with calcium ions using a peristaltic pump

(BT100S-1, Lead Fluid Ltd., China) to produce beads. The structure of the dry samples was studied with a scanning electron microscope (SEM). The particle size distributions of the beads were estimated using sieve analysis. Furthermore, pH<sub>pzc</sub> measurements were done. The kinetic and equilibrium adsorption process of La<sup>3+</sup> and Ho<sup>3+</sup> ions was carried out using the following conditions: kinetic studies (m= 0.05 g, V= 20 cm<sup>3</sup>, C<sub>0</sub>=100 mg/dm<sup>3</sup>, T=293 K, t=1-1440 min) and equilibrium studies (m=0.05 g, V= 20 cm<sup>3</sup>, C<sub>0</sub>=25-1000 mg/dm<sup>3</sup>, T=293 K, t=24 h). The adsorption studies were performed in the batch mode using an orbital laboratory shaker (SK-O33-Pro, DLAB Scientific, China) with a speed of 180 rpm and an amplitude 8.

**Results:** The surface morphology and macro shape of synthesized alginate@chitosan (ALG@CS) were analyzed using scanning electron microscopy analysis (Fig.1).



Fig.1. SEM images of ALG@CS in three magnifications: 25 000 x, 5 000 x, and 100 x.

The obtained SEM images showed a smooth, slightly porous structure of the adsorbent with chitosan irregular particles. The synthesized adsorbent assumes a spherical shape, the diameter of which decreased four-fold after air drying from 2.4 to 0.6 mm. To confirm the bead size, the sieve analysis was carried out using sieves with a diameter of  $200 \times 50$  mm and mesh sizes of 0.5, 0.6, 0.71, and 0.8 mm. The fraction of 0.6 mm had the largest contribution, equal to 85.67%. The other fractions, i.e., 0.5 mm, 0.71 mm, and 0.8 mm, made up only a small percentage of 5.07%, 9.22%, and 0.04%, respectively. The pH<sub>pzc</sub> measurement reveals that synthesized alginate@chitosan beads have this value equal to 7.18. This value close to neutral pH suggests that both acidic (-COOH from alginate) and basic (-NH<sub>2</sub> from chitosan, -OH from alginate and chitosan) functional groups are present on the surface of ALG@CS biocomposite. The kinetic studies revealed that the adsorption process is very time-dependent (Fig.2a-b). Increasing the contact time resulted in the adsorption efficiency increase regardless of the initial solution concentration. For 50, 100, and 200 mg/L, the equilibrium points were achieved after 120, 180, and 480 minutes for La<sup>3+</sup> and after 60, 120, and 360 minutes for Ho<sup>3+</sup>. The kinetic experimental data were fitted to the non-linear pseudo-first order (PFO) and pseudo-second order (PSO) (Fig.2c-d). It can be seen that  $La^{3+}$  and Ho<sup>3+</sup> uptake by the ALG@CS follows the reaction mechanism typical of pseudo-second order reactions. Additionally, the estimated experimental values of the parameter  $q_e$  confirm the best fit and should match the data derived from the models that are presented. Accordingly, the  $q_e$  values and the  $q_2$  values assessed for the non-linear PSO kinetic model were quite similar. The values of the  $k_2$  parameter for the non-linear PSO equation were

 $4.60 \times 10^{-4}$  g/mg·min for La<sup>3+</sup> and  $3.60 \times 10^{-4}$  g/mg·min for Ho<sup>3+</sup>.The experimental results were then compared to non-linear isotherm models, including Temkin, Freundlich, and Langmuir (Fig.2e-f). According to the Langmuir model, the maximum surface coverage parameter, q<sub>m</sub>, yielded values that show an efficient metal ion adsorption process onto the ALG@CS. The maximum q<sub>m</sub> values were obtained for Ho<sup>3+</sup> equal 151.16 mg/g; for La<sup>3+</sup> equal 133.10 mg/g. Considering the determination coefficient R<sup>2</sup>, the best fitting was obtained for the Temkin model (R<sup>2</sup>= 0.954-0.972).



**Fig.2.** Contact time effect (a, b), non-linear fitting of kinetic models (c, d), and non-linear fitting of isotherm models to experimental adsorption data (e, f) of La<sup>3+</sup> and Ho<sup>3+</sup> ions by ALG@CS.

In the next step, the thermodynamic parameters such as free enthalpy of adsorption ( $\Delta G^{\circ}$ ), entropy of adsorption ( $\Delta S^{\circ}$ ), or enthalpy of adsorption ( $\Delta H^{\circ}$ ) were calculated based on the temperature effect studies. It was noted that increasing the temperature from 293 to 333 K caused an increase in the equilibrium capacity (q<sub>e</sub>) and the sorption performance (%S). It was demonstrated that temperature growth resulted in an equilibrium capacity increase by 12.5% during La<sup>3+</sup> adsorption and by 14% during Ho<sup>3+</sup> adsorption.

Matal	a [ma/a]	$\Delta H^{\circ}$	$\Delta S^{\circ}$	$\mathbb{R}^2$	$\Delta G^{\circ} [kJ/mol]$		
Wietai q <sub>e</sub>	q <sub>e</sub> [mg/g]	[kJ/mol]	[J/mol·K]		293 K	313 K	333 K
La <sup>3+</sup>	145.90	6.17	88.43	0.999	-19.75	-21.50	-23.28
Ho <sup>3+</sup>	168.44	23.63	147.17	0.999	-19.47	-22.49	-25.35

Table 1. Thermodynamic data of La<sup>3+</sup> and Ho<sup>3+</sup> adsorption onto ALG@CS biocomposite.

The results of the thermodynamic calculation are shown in Table 1. The endothermic nature of the La<sup>3+</sup> and Ho<sup>3+</sup> adsorption process with the biocomposite was explained by positive values of the  $\Delta$ H° parameter, which were in the range 6.17-23.63 kJ/mol. Furthermore, negative values of the  $\Delta$ G° parameter were achieved at all temperatures, indicating that the La<sup>3+</sup> and Ho<sup>3+</sup> adsorption is spontaneous and beneficial. The  $\Delta$ G° parameter confirmed that the adsorption process is more effective at higher temperatures.

**Conclusions:** In summary, the alginate@chitosan biocomposite was successfully prepared and studied as a potential adsorbent for critical raw materials such as  $La^{3+}$  and  $Ho^{3+}$  ions. The non-linear kinetics and isotherm confirmed in pseudo-second order ( $R^2 \ge 0.972$ ) and Temkin models ( $R^2 \ge 0.954$ ), indicating that the metal adsorption took place onto a heterogeneous surface on alginate@chitosan adsorbent. The maximum adsorption capacity of ALG@CS at 333 K was 145.90 mg/g for  $La^{3+}$  and 168.44 mg/g for Ho<sup>3+</sup>. Thermodynamic studies revealed that the adsorption of metal ions onto alginate@chitosan beads was endothermic and favorable at higher temperatures. The prepared adsorbent had good adsorption properties toward the studied metal ions and was expected to be widely applied in the critical raw materials recovery from waste solutions.

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# SYNTHESIS AND CHARACTERIZATION OF POLYUREA-CROSSLINKED BIOPOLYMER AEROGEL BEADS FOR ENVIRONMENTAL DECONTAMINATION

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Abstract: In this work, polyurea-cross-linked alginate aerogel beads loaded with cellulose and chitosan were produced for possible application in environmental remediation. Two triisocyanates were used as cross-linkers, that is, aliphatic (Desmodur Z4470) and aromatic (Desmodur RE). The chemical structure and the nanoporous structure of the aerogels were analyzed using ATR/FT-IR spectroscopy and N<sub>2</sub>-sorption measurements. Bulk and skeletal densities were also measured. The potential adsorption abilities of these aerogels for La(III) ions were demonstrated using batch adsorption experiments.

Introduction: Aerogels can be defined as open, non-fluid colloidal or polymer networks expanded with a gas; they can be formed from wet gels by removing all swelling agents without volume reduction or network compaction [1]. They are known for their very low densities, high specific surface areas, low thermal conductivity, and a wide range of applications in several sectors, including, but not limited to, thermal insulation, catalysis, pharmaceutics, biomedicine, environmental remediation, and the food industry [2]. Aerogels can be manufactured from inorganic compounds, synthetic polymers, biopolymers (polysaccharides, and proteins) or any combination of the above. Kistler synthesized the first aerogels in 1931 [3]. The process of obtaining aerogels involves two stages: the sol-gel process, during which a gel is formed, and the drying step, during which the solvent is removed from the pores of the gel. The drying method, e.g., conventional drying, freeze drying, or supercritical drying, affects the nanostructure of the material. Conventional drying usually leads to shrinkage and collapse of the network, while freeze-drying minimizes such phenomena, although it carries the risk of damage from the formation of ice crystals. Supercritical drying, using supercritical carbon dioxide, best preserves the original gel morphology [2]. Alginate aerogels are one of the biopolymer-based aerogels that deserve much attention. To improve the limitations of alginate aerogels and thus increase application potential, the potential for application, X-aerogel technology (i.e., cross-linking with polyurea) has been applied [4-6]. X-alginate aerogels have improved mechanical strength and stability in natural waters [7] compared to alginate aerogels.

**Experimental:** Alginate (Alg) wet-gel beads modified with cellulose (Cel) or chitosan (Chit) were produced by dripping a suspension of Cel and Chit in aqueous Alg solutions into 200 mM of  $CaCl_2$  solution. After aging for 24 h, the beads were separated from the

CaCl<sub>2</sub> solution. Next, the solvent exchange was performed at room temperature by immersing the beads in acetonitrile/water mixtures and dry acetonitrile. Subsequently, a solution of triisocyanate Desmodur Z4470 (IPDI) and Desmodur (RE) in MeCN was added to the Alg-Cel and Alg-Chit wet-gel beads. After the cross-linking reaction was completed (72 h; 343 K), the beads were dried in an autoclave (E3100, Quorum Technologies, East Sussex, UK) at 318 K using supercritical CO<sub>2</sub>. The resulting aerogel beads were named X-Alg-Cel-IPDI, X-Alg-Cel-RE, X-Alg-Chit-IPDI, and X-Alg-Chit-RE. The potential application of aerogel beads in La(III) sorption (C<sub>0</sub>=200 mg/dm<sup>3</sup>, m=0.025 g, V=20 cm<sup>3</sup>, pH=2-6, t=24 h) was evaluated.

**Results:** X-alginate aerogel beads, X-Alg-Cel-IPDI, X-Alg-Cel-RE, X-Alg-Chit-IPDI, and X-Alg-Chit-RE, with average diameters of 2.46±0.01, 2.496±0.007, 2.626±0.004, and 2.631±0.001 mm, respectively, were characterized by ATR/FT-IR spectroscopy (Fig.1). Selected material properties are presented in Table 1.



Fig.1. Physical appearance and ATR/FT-IR spectra of (a) X-Alg-Cel-IPDI, X-Alg-Cel-RE, (b) X-Alg-Chit-IPDI, and X-Alg-Chit-RE, as indicated.

The ATR/FT-IR spectra indicate the successful cross-linking of the alginate-based gels with aliphatic (from IPDI) and aromatic (from RE) polyurea. The spectra show the

prominent peaks in the range of 3500-1000 cm<sup>-1</sup> corresponding to characteristic peaks of alginate, cellulose and chitosan (stretching vibrations of O-H groups around 3300 cm<sup>-1</sup>, symmetric and asymmetric stretching vibrations of O-C=O<sup>-</sup> groups coordinated with Ca<sup>2+</sup> ions around 1600 cm<sup>-1</sup> and 1420 cm<sup>-1</sup>, respectively, and stretching vibrations of C-O-C groups on the sugar ring around 1080 cm<sup>-1</sup> and 1030 cm<sup>-1</sup>). The bands at 3373 cm<sup>-1</sup> and 1563 cm<sup>-1</sup> were assigned to the polyurea N-H stretching and bending vibrations, respectively. The presence of bands around 2260 cm<sup>-1</sup>, assigned to N-C=O groups, indicates that not all isocyanate groups have reacted during cross-linking. For IPDI-derived aerogels, the isocyanurate carbonyl stretches around 1695 cm<sup>-1,</sup> and the stretching vibrations of -CH<sub>2</sub> groups around 2950 cm<sup>-1</sup> were observed. For RE-derived aerogels, the stretching vibrations of the aromatic double bonds C=C at 1506 and 1410 cm<sup>-1</sup> and the C–H in-plane bending in the region of 1250-1000 cm<sup>-1</sup> were observed.

Sample	Bulk density [g/cm <sup>3</sup> ]	Skeletal density [g/cm <sup>3</sup> ]	Porosity [%]	BET surface area [m <sup>2</sup> /g]	V <sub>total</sub> [cm <sup>3</sup> /g]	Average pore diameter [nm]
X-Alg-Cel-IPDI	$0.11 \pm 0.01$	$1.42 \pm 0.02$	92	348	8.3	18
X-Alg-Cel-RE	$0.22 \pm 0.02$	$1.37 \pm 0.02$	84	260	3.7	11
X-Alg-Chit-IPDI	0.12±0.01	$1.41 \pm 0.01$	92	359	7.9	19
X-Alg-Chit-RE	$0.24 \pm 0.05$	$1.360 \pm 0.009$	83	257	3.5	9.2

 Table 1. Selected material properties of X-alginate aerogel beads.

X-alginate aerogels are highly porous, with porosities up to 92% v/v for X-Alg-Cel-IPDI and X-Alg-Chit-IPDI, 84% v/v for X-Alg-Cel-RE, and 83% v/v for X-Alg-Chit-RE (Table 2). The materials are also characterized by high surface area, the highest for X-Alg-Chit-IPDI (359  $m^2/g$ ) and the lowest for X-Alg-Chit-RE (257  $m^2/g$ ). The average pore diameter, between 9.2 and 19 nm, reveals the presence of mesopores in the X-alginate aerogel beads. Skeletal densities were similar for all aerogels, while bulk densities were lower for IPDI-derived materials (0.11-0.12 vs 0.22-0.24 for RE-derived materials). To assess the potential application of X-Alg-Cel-IPDI, X-Alg-Cel-RE, X-Alg-Chit-IPDI, and X-Alg-Chit-RE aerogels in environmental issues, the preliminary sorption of lanthanum(III) from aqueous solutions was carried out. In the sorption process, the following conditions were maintained: initial La(III) concentration 200 mg/dm<sup>3</sup>, solution pH in the range 2-6, aerogel mass 0.025 g, solution volume 10 cm<sup>3</sup>, temperature 20°C, and shaking time 24 h. Depending on the type of triisocyanate used for cross-linking Alg-Cel and ALG-Chit wet-gel beads, different values of the parameter  $q_e$  were obtained in the range of pH=2-6 (Fig.2). The maximum values of the equilibrium capacity  $q_e$  and the distribution coefficient  $K_d$  were obtained for pH equal to 3 for the X-Alg-Cel-IPDI and X-Alg-Chit-IPDI and pH 5 for X-Alg-Cel-RE and X-Alg-Chit-RE. The higher  $K_d$  values were obtained for X-alginate aerogels modified with chitosan and were equal to 75.20 cm<sup>3</sup>/g for X-Alg-Chit-IPDI and 39.58 cm<sup>3</sup>/g for X-Alg-Chit-RE. On the other hand, the  $K_d$  values for X-Alg-Cel-IPDI and X-Alg-Cel-RE were 29.18 and 10.69 cm<sup>3</sup>/g, respectively.





Fig.2. The pH adsorption tests of La(III) ions by X-alginate aerogels, as indicated.

**Conclusions:** In this study, polyurea-cross-linked alginate/cellulose and alginate/chitosan aerogel beads were successfully prepared via the reaction of preformed alginate gels with an aliphatic (Desmodur Z4470) or aromatic (Desmodur RE) triisocyanate, which was confirmed by ATR/FT-IR spectroscopy. The aerogels had low densities, high surface areas, and high porosity. The preliminary sorption studies for the removal of La(III) from water were promising for the application of these aerogels in environmental issues.

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# ADSORBENTS CONTAINING LIGNIN AS A BIODEGRADABLE ADDITIVE ENHANCING DYES REMOVAL - A REVIEW OF RECENT DEVELOPMENTS

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**Abstract:** The aim of this paper is to present recent developments in the synthesis, characterisation and application of lignin-containing adsorbents for the removal of dyes from model solutions and wastewaters. A review of the world literature of the past few years was made.

**Introduction:** Dyes enter wastewaters as a consequence of the colouring of various products from the textile, paper and chemical industries, plastic production, inks, paints, varnishes, cosmetics or foodstuffs (Fig.1). The amount of dyes in industrial effluents can vary widely, from 7-8% during dye and paint production branches, through 10% in the paper industry up to 54% in effluents from the textile industry [1].



Fig.1. The use of dyes in various industrial fields.

The direct disposal of untreated dye-containing effluent into natural water bodies has an adverse effect on the photosynthetic activity in aquatic ecosystems. It creates mutagenic or teratogenic effects on aquatic organisms and fish species due to the existence of metals and aromatics in water. Further, the presence of dyes in the environment has mild to severe toxic effects on human health, including carcinogenic, mutagenic, allergic, and dermatitis effects. Azo type dyes are particularly hazardous as they can degrade to toxic aromatic amines under certain conditions [1,2]. According to The Ecological and Toxicological Association of Dyes and Organic Pigments Manufacturers (ETAD), 98% of 3000 tested dyes exhibit  $LD_{50}$  (the dose at which a substance is lethal for 50% of animals tested) value >1 mg/L [3]. Therefore, the removal of these pollutants from

wastewater to protect the environment and human health is urgently required. Various treatment processes are available for contaminant removal: chemical precipitation, ion exchange, adsorption, solvent extraction, membrane filtration, advanced oxidation, reverse osmosis, etc. However, the use of each of these methods in separation has merits and disadvantages. Among them, adsorption is one of the most popular and recognized techniques [4,5]. The adsorption technique as the physical method provided 86.8-99% yield of dye effluents purification [1]. It has been estimated that the average cost of wastewater treatment by adsorption is US\$ 5.0-200/m<sup>3</sup>, while other technologies are in the range of US\$ 10.0-450/m<sup>3</sup> [6]. At present, various adsorbents such as biosorbents, carbon-based adsorbents, transition metal based oxides, and polymer-based adsorbents are used to treat dye-containing wastewater [7]. Of particular interest to scientific teams from around the world is research into the synthesis of new adsorptive materials containing biodegradable additives such as lignin, starch or cellulose. The aforementioned biocomponents can be derived from waste, as can lignin. Biomass from the pulp and paper industry can be a source of lignin. The lignin content in lignocellulosic biomass varies depending on the plant source. In wood, lignin typically makes up 20–30% of the dry weight, with softwoods generally containing more lignin than hardwoods [8]. Composed of phenylpropane units linked by various bond types, lignin's complex structure presents both opportunities and challenges for its utilization. Its rich aromatic backbone and abundant hydroxyl, carbonyl, methoxy, and carboxyl groups, makes it a good substrate for the synthesis of adsorbents that are selective towards dyes, metal ions or other toxic substances present in the environment. This literature review demonstrated that it is possible to recover all the major biopolymers, such as cellulose, hemicellulose, and lignin, from lignocellulosic biomass and convert them into valuable products like biosorbents [8-11]. The following are some examples from the literature of efficient dye removal from aqueous solutions using adsorbents with a lignin biocomponent. Du et al. [12] modified lignin by grafting the glycidyltrimethylammonium chloride GTMAC into quaternized lignin (QL) and applied for Rhodamine B (RB), Methylene Blue (MB), Acid Blue 92 (AB-92) and Congo Red (CR) dyes. The maximum adsorption capacities of OL for RB, MB, AB-92 and CR were 41.85, 49.47, 110.53 and 134.61 mg/g, respectively. In a study reported by Zhang et al. [13], organosolv lignin was used for MB adsorption. It was found that the MB adsorption on organosolv lignin is a pH-dependent process. A wide pH range (from 5.0 to 9.0) was utilized for adsorption. At 20 °C, it was found that 40.02 mg of MB was In another study reported by Dai et al. [14], retained by organosolv lignin [13]. mesoporous lignin-calcium (LC) microspheres were fabricated by a simple flocculationsedimentation approach to remove MB from aqueous solution. The LC microspheres exhibited favorable and uniform adsorption efficacy across the pH spectrum of 3 to 11. The adsorption of MB by LC was consistent with the pseudo second-order and Langmuir models, with a maximum adsorption capacity of 803.9 mg/g [14]. The amino-silanemodified lignins (ASLs) with primary (P), secondary (S), and tertiary (T) amine groups were prepared via biphasic organic synthesis and the adsorption behavior towards MB and CR was investigated. The maximum adsorption capacities of ASLs for MB was 187.3 mg/g for P-ASL, 164.75 mg/g for S-ASL, and 166.39 mg/g for T-ASL. The maximum adsorption capacities of ASLs for CR were 293.26 mg/g for P-ASL, 257.07 mg/g for S-ASL, and 165.56 mg/g for T-ASL. The adsorption capacities of PASL for the two dyes were much higher than those of S-ASL and T-ASL, which may

be due to the dense amine groups in P-ASL providing abundant active sites. The dve adsorption followed second-order kinetics. Langmuir and Temkin isotherm models, and spontaneous processes. The hypothesized adsorption mechanisms of MB and CR mainly involve electrostatic, hydrogen bonding, NH $-\pi$ , and  $\pi-\pi$  interaction [15]. The novel chitosan-lignin hydrogel composite beads (CL), in a 1:1 ratio, were applied for Direct Blue 218 dye removal [16]. Under optimized conditions, including a pH of 4, the CL beads achieved a remarkable 96% dve removal efficiency compared to 94.8% for pure chitosan (C), within a 120-minute contact time. According to the equilibrium data, the Langmuir isothermal model provided the best fit with a maximum adsorption capacity of 75.75 mg/g (C) and 93.5 mg/g (CL) and the adsorption process followed the pseudosecond-order kinetic model [16]. The studies performed by Saini et al. [17] focused on fabricating and utilizing a lignin-steel sludge magnetic carbon composite (SLNa) to efficient adsorption of two dyes from an aqueous medium. The SLNa composite was fabricated through a simple two-step strategy involving the activation and thermal treatment of lignin and steel sludge. The synergistic combination of lignin and steel sludge resulted in composite's high adsorption potential for Methylene Blue (MB; 153.92 mg/g) and Acid Orange 7 (AO7; 64.84 mg/g) in batch mode. Additionally, the rapid adsorption kinetics and effective dye removal for MB (>99%) and AO7 (72%) from simulated effluent revealed the potential of fabricated adsorbent for water remediation. Furthermore, the composite's applicability for continuous adsorption was also evaluated through a series of dynamic flow experiments using fixed-bed column systems. The demonstrated efficacy of SLNa, viz., 48.18 and 15.00 mg/g for MB and AO7, respectively, at high adsorbent loading and lower dye concentrations, highlights its potential for scalable water treatment applications. The fixed bed column adsorption studies were conducted and study uncovered improved MB adsorption, reaching 48.18 mg/g under conditions of low pollutant concentration (5 mg/L) and high adsorbent loading (50 mg) [17]. The bio-based sodium alginate/lignin (SA/Lig) composite hydrogel beads were fabricated by facile cross-linking with calcium ions and were used for the efficient removal of MB. The maximum adsorption capacity of 254.3 mg/g (removal efficiency of 84.8%) was obtained for SA/Lig containing 20% of lignin, under the optimal conditions of pH 12, and temperature 45 °C. The calculated thermodynamic and kinetic parameters revealed that adsorption is an endothermic process, and chemical adsorption is the rate-limiting step [18]. A novel lignin-based adsorbent, P(ClAPTA-AL), was successfully synthesized through radical polymerization and evaluation of its potential for dye adsorption was investigated. The hydrogel was based on aminated lignin (AL) and poly(3-acryloamidopropyl)-trimethylammonium chloride P(ClAPTA). The optimal conditions of Alizarin Red S removal were: pH 12.0, 20 °C, 120 min contact time, and adsorbent-to-ARS mass ratio of 10, resulting in a high adsorption capacity of 3889 mg/g. Kinetic studies showed an adsorption process followed a pseudosecond-order model, confirming chemisorption as the predominant mechanism, while thermodynamic analysis revealed a spontaneous and endothermic adsorption process. Furthermore, promissory results demonstrated high reusability, with adsorption efficiency remaining at ~99% until the fourth cycle and maintaining 81.1% after seven cycles [19]. The crystal violet (CV) dye elimination was performed using a lignin copper ferrite (LCF) adsorbent. The highest adsorption potential (97%) was executed at mild operating conditions, with a 5 min contact time at room temperature and pH 8. The adsorption capacity towards CV decreases from 79 to 70% when the temperature

increase from 25 to 70 °C, indicating an exothermic adsorption process. It was found that the adsorption efficiency decreased from 97 to 71% and 82% in the presence of 1 M NaCl and LiCl. The maximum adsorption capacity was found to be 34.129 mg/g. LCF can be reuse the five cycles with removal efficiency 86%, and this reaches 60% after 10 cycles [20].

**Conclusions:** In summary, bio-based polymers containing such additives as lignin used in environmental decontamination show significant potential combined with inherent limitations. Although modifications of lignin are often necessary to improve adsorption capacity, and performance varies depending on specific conditions, their costeffectiveness, environmental friendliness and versatility offer an important alternative to conventional approaches. The price of their production is also an important aspect. Further research needs to focus on the further development of these types of materials, the use of waste sources as raw materials for their production and improved performance in order to fully realise their potential in the face of global pollution challenges.

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# MICROPLASTIC INDENTIFICATION IN NILE WATERS IN THE LUXOR AREA - RAMAN SPECTROSCOPY STUDY

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**Abstract:** Microplastics pose a significant threat to aquatic ecosystems, including rivers of key hydrological and ecological importance, such as the Nile. The aim of this study was to identify and characterise microplastics fished from the main stream from the surface of the Nile waters in Upper Egypt, using Raman spectroscopy. Samples were obtained from areas of Luxor, including urban and more wild areas, to account for variability in contamination, and then analysed to determine their chemical composition. The results showed the presence of polymers, but also organic fibres, with the highest concentrations of microplastics recorded in regions with intense anthropogenic activity.

**Introduction:** The Nile River, which is the world's longest river and the main source of water for millions of people in Africa, plays a key role in the ecosystem and economy of Egypt. However, in recent years, the growing problem of plastic pollution, including microplastics, has posed a serious threat to both the environment and human health. Microplastics, i.e. pieces/fibres or plastic particles with a diameter/length of less than 5 mm, enter the Nile from municipal, industrial and agricultural waste, as well as through sewage and erosion processes (Fig.1).



Fig.1. Sources of pollution in the Nile.

These microscopic pollutants do not biodegrade, but accumulate in water, river sediments and aquatic organisms, with far-reaching consequences for the entire ecosystem. Fishing for microplastic-contaminated fish, consumption of contaminated water and the penetration of toxic substances into living organisms lead to the accumulation of these particles in the food chain [1,2]. In addition, microplastics can absorb dangerous chemicals, such as heavy metals and endocrine active compounds, which can cause serious health effects, including endocrine disruption and cancer. Despite the growing threat, environmental awareness in Egypt is still at a very low level. The lack of an effective recycling policy, inadequate waste management and low environmental education of the public means that the microplastic problem remains largely ignored. In many cities, including Cairo and Luxor, rubbish is often dumped directly into the river, leading to its systematic pollution. Lack of proper regulation and little public involvement in tackling the plastic crisis mean that the Nile, the heart of Egypt, is gradually turning into a toxic stream full of microplastic pollution. To prevent further degradation of the Nile ecosystem, extensive scientific research on microplastics and effective measures to reduce their emissions are needed [2]. Educating the public, improving the waste management system and implementing eco-friendly alternatives to plastic are key steps that can help protect one of Africa's most important natural resources.

**Experimental:** The microplastic research process began by catching a concentrated sample (50 ml) using a plankton mesh with 20 micrometer pores, which effectively isolated the fine plastic particles from the test environment [3,4]. After collection, the samples were chemically cleaned with perhydrol (hydrogen peroxide,  $H_2O_2$ ), which removed organic contaminants, leaving only fibres. The cleaned microplastics were then dried and prepared accordingly for further analysis [3,4]. The next step was to spread the fibres under the Keyence microscope between two pieces of tape, which ensured their stabilisation and allowed accurate observation without the risk of displacement. The samples prepared in this way were then subjected to spectroscopic measurements, during which a ×50 magnification objective and a 785 nm wavelength laser beam were used. This made it possible to obtain Raman spectra, which were then compared with databases to identify the type of polymers present in the samples [3,4].

**Results**: The Raman spectroscopy analyses confirmed, that polypropylene (PP) is the most commonly identified material (Fig.1). Raman spectra showed distinct bands characteristic for this polymer, the proposed assignment can be as follows: 841 cm<sup>-1</sup> band is assigned to CH vibrations associated with the tactical structure of PP, the band at 973 cm<sup>-1</sup> is attributed to C-C stretching vibrations in the main chain, the main contributions to the band at 1152 cm<sup>-1</sup> are related to stretching vibration CC of the skeleton, stretching vibration of C–CH<sub>3</sub> groups, bending CH and rocking CH<sub>3</sub>, and bands in the range 2850-3000 cm<sup>-1</sup> are assigned to C-H stretching vibrations. For polyethylene terephthalate (PET), the most characteristic bands are observed in the spectral range 600 – 1750 cm<sup>-1</sup> (Fig.2). The band at around 860 cm<sup>-1</sup> is attributed to C–H out of-plane bending and C–C stretching and C(O)–O ester stretching vibrations, respectively, and band at 1618 cm<sup>-1</sup> corresponds to C=C stretching vibrations in the aromatic ring. The Raman band at 1726 cm<sup>-1</sup> is attributed to stretching vibration of C=O carbonyl group in ester. This band is the most characteristic feature of PET polymer [4].



Fig.2. Raman spectra of polypropylene.



Fig.3. Raman spectra of PET.

**Conclusions:** Studies carried out on different sections of the Nile River showed significant differences in the abundance and characteristics of microplastic fibers depending on location. The highest density of fibers was found in the section of the river flowing through the city of Luxor, where the predominant types were polypropylene and polyethylene terephthalate fibers, commonly used in textiles and packaging. In contrast, samples taken near agricultural regions had lower fiber abundance, but were characterized by significantly higher fluorescence levels. This may indicate their organic origin or the presence of organic substances associated with agricultural practices. The results suggest that urban human activities, particularly those related to waste and wastewater management and tourism, have a greater impact on microplastic pollution of the river than agricultural activities.

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# REMOVAL OF MICROPOLLUTANTS BY LEMNACEAE PLANTS

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**Abstract:** In recent decades, the pollution of the aquatic environment with personal care products and industrial chemicals has increased. These pollutants disrupt the natural ecosystems and harm living organisms in the aquatic environment. Besides other methods such as sorption, membrane methods, or advanced oxidation, phytoremediation can efficiently remove micropollutants. Lemnaceae plants, known as duckweeds, water lentils, or water lenses play an important role in the ecology of water reservoirs by preventing water eutrophication and reducing pollutants through the phytoremediation process. The results of conducted studies indicate the potential of duckweeds to remove benzotriazoles (BTRs), benzotriazole UV filters (BUVs), contaminants of emerging concerns (CECs), and phthalates. The efficiency of the micropollutants removal by Lemnaceae plants: *Lemna minor, Spirodela polyrhiza*, and *Wolffia arrhiza* varied from 65% to 100%.

**Introduction:** Lemnaceae plants, genus Lemna, and related genera are the most miniature flowering plants [1]. Despite their small size, they have unique properties like rapid vegetative propagation and relative growth rate, reflecting adaptation to local conditions. Duckweed species grow at all latitudes, except Antarctica [2], and are relatively not demanding about soil, water, sun exposure, or temperature conditions. They are highly effective in a relatively short period, inexpensive, and do not require a large area for their cultivation. Lemnaceae plants are among the fastest-growing higher plants in natural and in vitro conditions [3]. Due to the high yield of Lemnaceae plants' biomass and relatively easy harvesting, they can be used as feedstock for biofuels and animal feed, efficient absorption of nitrogen and phosphate-containing pollutants, heavy metals, and various organic chemicals [2,4].

This paper reviews the literature on the removal of micropollutants by plants from species *L. minor* (size 3mm, 1 root), *S. polyrhiza* (size 1cm, 7-11 roots), and *W. arrhiza* (0.5mm, no roots) [2,5]. Micropollutants are natural or anthropogenic compounds, whose concentrations in the environment range from nanograms to micrograms per liter or kilogram [6]. One of the leading environmental protection challenges is elaborating and improving micropollutant removal efficiency in wastewater purifying processes. Besides chemical and physicochemical methods of purifying polluted waters, phytoremediation and other biological methods are efficient, ecologically friendly, and inexpensive ways of purifying water and wastewater [7]. Contaminants of Emerging concern (CECs) are chemicals and toxins found in aquatic systems that may impact ecological and human health. Benzotriazoles are organic pollutants, frequently used in metal corrosion inhibitors and UV stabilizers.

*Wastewater treatment using floating plants.* Lemnaceae plants found in most freshwater systems can efficiently absorb nitrogen- and phosphate-containing pollutants, heavy metals, and various organic chemicals [8,12,13]. The floating plants' roots and raft material is a surface that acts as a natural water filter and removes pollutants, and thus, the excess of nutrient uptake and degradation occurs. The floating plants reduce turbulence and facilitate pollutants to settle. From the Lemnaceae plants, *L. minor, S. polyrhiza,* and *W. arrhiza* have been extensively tested in terms of efficiency, kinetics, and mechanism of micropollutant removal [9, 14-20]. Table 1 summarizes literature data on the removal of selected groups of micropollutants by the mentioned plants.

Micropollutants/	The name	No. of days	Initial	Efficiency of	Half-life,
literature	of the used	of the	concentration	micropollutant	t <sub>1/2</sub>
	Lemnaceae	conducted	(µg/L)	removal	(days)
	plant	experiment			-
Benzotriazoles/	L. minor	36	150	Batch: 100%	1.6±0.3
[14]				except 4MBTR	(5ClBTR)
				(48.2 ±4.1%)	to
					25±3.6
			20, 200, 2000	Continuous	(4MBTR)
				flow: from 26%	
				(4MBTR) to	
				72% (5ClBTR)	
Benzotriazole	L. minor	14	100	97-99%	1.84-2.73
UV filters/ [15]		_			
Phthalates/ [18]	L. minor	7	100	$\sim 80\%$ to more	0.85-3.12
				than 99%	
Contaminants of	L. minor	14	100	89-98%	0.12-1.20
emerging			500		
concern/[9]	a		100	<b>20 2 0 4 20</b>	
Benzotriazoles/	S. polyrhiza	14	100	70.5-94.5%	3.33-7.92
[16]	a		100	1000/	1 05 1 55
Benzotriazole	S. polyrhiza	14	100	~100%	1.37-1.55
UV filters/ [15]	117 1:	1.4	100	02 1000/	0.00
Benzotriazoles/	w. arrhiza	14	100	92-100%	0.98-
[20]				(except	36.19
Deventrianala	W	1.4	100	4MB1K)	2 90 0 19
LIV filters/[15]	w. arrniza	14	100	03-92%	5.60-9.18
UV IIIters/ [13]	W. amhi-a	7	0.1.100	78.0.00.70/	0.95 2.12
r nulaiates/ [18]	w. arrniza	/	0.1-100	10.9-99.1%	0.63- 5.12
Contaminants of	W arrhiza	14	100	93-99.6%	0.30-0.57
emerging	ττ. αττιίζα	1-7	500	<i>yJyJ</i> .070	0.50-0.57
concern/[9]			500		
				1	1

Table1. Experimental conditions and characteristics of micropollutant removal by the L. minor, S. polyrhiza,
and W. arrhiza plants.

The efficiency of phytoremediation of micropollutants depends on the type of target compound, their concentration, and the type of wetland. In the analysed studies, the efficiency of Lemnaceae plants in removing micropollutants is high and ranges from 65% to 100%, except for 4MBTR, which was removed in 48% in the batch experiments and in 26% in continuous flow experiments. The benzotriazoles 1H-benzotriazole (BTR), 4-methyl-1H-benzotriazole (4MBTR), 5-methyl-1H-benzotriazole (5MBTR), and 5-chlorobenzotriazole (5ClBTR) were studied in batch and continuous-flow

L. minor systems. There was no observed inhibition on the specific growth rate of L. minor for concentrations up to 200  $\mu$ gL<sup>-1</sup> [14]. These compounds were also tested in S. polyrhiza and W. arrhiza systems [16, 20]. The tested BTRs were significantly removed in batch experiments with L. minor, W. arrhiza, and S. polyrhiza plants, apart from 4MBTR, which had a removal efficiency between 26% and 48%. Five benzotriazole UV filters: 2-(2-hydroxy-5-methylphenyl)benzotriazole (UV-P), 2-tertbutyl-6-(5-chloro-2H-benzotriazol-2-yl)-4-methylphenol (UV-326), 2.4-di-tert-butyl-6-(5-chloro-2H-benzotriazol-2-yl)phenol (UV-327), 2-(2H-benzotriazol-2-yl)-4,6-di-tertpentylphenol (UV-328), and 2-(2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)phenol (UV-329) were removed in L. minor, S. polyrhiza, and systems W. arrhiza [15]. Their removal efficiency was between 65% and 100%. The most effective plant in removing BUVs was S. polyrhiza, while W. arrhiza had the lowest efficiency. L.minor and W. arrhiza were analysed as agents removing six compounds from the group contaminants of emerging concern: bisphenol A, triclosan, N,N-diethyl-mtoluamide (DEET), diethylstilbestrol, estrogen, and estradiol [9]. In this case, W. arrhiza proved to be more effective in removing CECs. Its efficiency ranged from 93 % to almost 100 %, while for L. minor it ranged from 89 % to 98%. The eight phthalates (phthalic acid esters) commonly used in industry: dimethyl phthalate (DMP), diethyl phthalate (DEP), dipropyl phthalate (DnPP), dibutyl phthalate (DnBP), diisobutyl phthalate (DIBP), bis(2-ethylhexyl) phthalate (DEHP), diisononyl phthalate (DINP), and diizoheptyl phthalate (DIHP) were tested in L. minor and W. arrhizal systems [18]. Both plants showed similar removal efficiencies ranging from approximately 80 to over 99%. The highest removal efficiency was recorded for phthalic acid esters with the smallest molecules and the highest hydrophilicity (DMP, DEP), while compounds with the largest molecular weights and hydrophobic properties (DEHP, DINP) were removed the least. The effectiveness of phytoremediation depends on the Lemnaceae plant's welfare, like the pH of its living environment. The optimal pH for the duckweed planting is usually given as 6.5 - 7.5 [17-19]. The optimization of cultivation conditions described in the analysed works showed that in the case of L. minor and W. arrhiza, a pH close to neutral is the most beneficial for plants, however, in the case of S. polyrhiza, a pH of 9 turned out to be better [15]. In most cases, the leading mechanism of micropollutant removal was plant uptake. The partial mechanisms were bioconcentration, biodegradation, sorption hydrolysis, and photolysis. In most cases, the rate of target micropollutants and half-lives were calculated in a pseudo-first-order kinetic model. Lemnaceae plants try to survive and adapt when they interact with micropollutants. Depending on the plant and the concentration of the micropollutant, plants may display normal growth or may manifest a reduction of their amount, deformation, and changes in the color of their leaves, and lower concentration of chlorophylls. Because plants try to fight the toxicity from micropollutants, an increase in the activity of antioxidant enzymes can be exhibited. The enzymatic and non-enzymatic responses of plants to abiotic stress allow them to survive in toxic conditions through the phytoremediation of micropollutants [17].

**Conclusions:** In most cases, the efficiency of Lemnaceae plants (*L. minor, S. polyrhiza,* and *W. arrhiza*) in removing tested micropollutants from the groups of BTRs, BUVs, CECs, and phthalates was higher than 65% and reach almost 100 % in many cases. Floating plants from the duckweed family can be an alternative to traditional methods of

effective wastewater treatment by absorbing nutrients and pollutants. The influence of micropollutants on the growth dynamics of Lemnaceae plants depends on the type of micropollutant tested. The biological activity of the micropollutants depends on their chemical structure, physical properties, and bioavailability in aquatic systems. Inhibitory effects on Lemnaceae plant growth characterized all of the micropollutants used in the experiment to a different extent. The results from research on the removal of micropollutants by Lemnaceae plants can contribute to the approach to the treatment and phytoremediation of wastewater in an eco-friendly and cost-effective manner, and further research on floating treatment wetlands, which use artificial floating platforms where plant organisms can utilize nutrients and micropollutants present in the aquatic environment.

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## INCORPORATION OF MAGNETIC PARTICLES WITH AMINO FUNCTIONALITIES INTO ALGINATE MATRICES TO IMPROVE MECHANICAL PROPERTIES OF THE RESULTING MAGNETIC HYDROGELS

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**Abstract:** Amine-functionalized iron microparticles were obtained by anchoring the aminopropyltriethoxysilane (APTES) and trimethoxysilylpropyldiethylenetriamine (TMPET) monomers to the particles' surface. The microparticles obtained in this way were dispersed in an alginate matrix during the chemical cross-linking process, ultimately creating magnetic composite hydrogels. The impact of the amine-modification on the magnetorheological properties of the obtained hydrogels was assessed.

**Introduction:** The integration of magnetic nanoparticles into hydrogel networks has emerged as a highly promising strategy for engineering multifunctional, stimuliresponsive biomaterials. Particularly, magnetic hydrogels, also referred to as ferrogels, can exhibit broadly tunable physicochemical properties under the action of external magnetic field, enabling the controlled and fast actuation, as well as the dynamic modulation of structural and functional characteristics [1]. Such features make them particularly attractive for a wide range of biomedical applications, including controlled drug delivery, magnetically-induced hyperthermia, or tissue engineering scaffolds [1,2].Owing to their inherent biocompatibility, biodegradability, and ability to form hydrogels under physiologically mild conditions, Alginate-based matrices are among the most widely studied platforms designed for incorporation of magnetic phases [2]. The incorporation of superparamagnetic nanoparticles (e.g., Fe, Fe<sub>2</sub>O<sub>3</sub>, Fe<sub>3</sub>O<sub>4</sub>) within alginate matrices allows for the development of smart systems capable of responding to external magnetic stimuli, by remarkable changes in mechanical strength, porosity, or other properties which are important in the design of advanced biomedical systems [3,4]. Beyond their biomedical utility, magnetic hydrogels are increasingly investigated for applications in environmental remediation, soft robotics, and microfluidic systems, where remote controllability and adaptability are essential [5,6]. The ongoing development of such magneto-responsive systems represents a significant step forward in design of modern stimuli-responsive materials with broad functional potential. One of the key aspects of such composite systems is the appropriate design of interactions between the continuous hydrogel phase and the discontinuous magnetic phase (usually composed of nanoparticles or microparticles). In this work, we undertake this attempt by investigation of the role of appropriate functionalization of magnetic microparticles' surface so that they can provide stronger interactions with the neighboring alginate chains, and thus influence the properties of the entire hydrogel.

**Experimental:** Iron microparticles (IMPs) were functionalized in the following way: 5 g of IMPs were placed in a vial and mixed with 100 mL of EtOH, acidified with 1.43 mL

of 1.75 M HCl. The suspension was sonicated for 5 min. After that time 5 mmol of aminopropyltriethoxysilane (APTES) or trimethoxysilylpropyl-diethylenetriamine (TMPET) was added and sonicated for 5 min and left for 25 min. This sequence (5 min of sonication + 25 min of relaxation) was repeated 5 times more. The functionalized IMPs were separated from the solution by magnet, washed two times with absolute ethanol and dried overnight at 40 °C. The magnetic hydrogels were prepared following our previously published procedure [7]. Magnetorheological tests of the obtained composite hydrogels were run at 25 °C using the MCR 301 magneto rheometer (Anton Paar) with plate-plate geometry [7]. The composite hydrogels were also tested in the presence of magnetic field (35 kA m<sup>-1</sup> and 282 kA m<sup>-1</sup>). The formulas of both monomers used to functionalize the magnetic particles are shown in Fig.1.



**Fig.1.** Structural formula of the organosilica monomers used to functionalize iron magnetic particles: aminopropyltriethoxysilane - APTES (left) and trimethoxysilylpropyldiethylenetriamine - TMPET (right).

**Results:** The SEM images of initial, APTES- and TMPET-functionalized iron particles are presented in Fig.2. As it can be seen, the morphology and aggregation degree of the particles have not changed after the functionalization. From our previous studies it is clear that such a functionalization procedure introduces amino groups onto the surface [7].



Fig.2. SEM microphotographs of the iron microparticles (initial – left, APTES-functionalized – middle, TMPET functionalized – right).

Magnetorheological characterization of the obtained hydrogels evidencing changes in values of storage modulus (G') and loss modulus (G") upon action of magnetic field is presented in Fig.3. The values of G' and G" are constant in the range of shear strain values up to ~5% and ~1%, respectively (so called linear viscoelastic region, LVR), indicating the elastic structure of the hydrogel. The presence of the weaker magnetic field (35 kA m<sup>-1</sup>) does not significantly changes G' and G" values, whereas the stronger magnetic field (282 kA m<sup>-1</sup>) induces remarkable changes, particularly for the higher concentration of incorporated microparticles (i.e. 0.069 g/mL, see samples A2 and A4).

Another characteristic feature in the magnetological properties of the tested composite hydrogels is the shift of the so-called yielding point (i.e. the point of intersection of the G' and G" curves, at which dissipation of energy is maximal) several times towards higher shear strain values, which is again the most pronounced for the higher concentration of incorporated microparticles (i.e. 0.069 g/mL, see samples A2 and A4). The yielding point reflects to the critical stress related to transition from elastic deformation to plastic flow or irreversible deformation, indicating the limit beyond which a hydrogel no longer behaves in a purely elastic manner. Since the value of yielding point may be linked with crosslinking degree it can be concluded that hydrogels with a higher content of magnetic can be more intensively cross-linked. Magnetic particles moving upon magnetic field pull the cross-linked alginate network and the intensity of this forced movement of the alginate network (reflected by shift of yielding point) depends on the number of magnetic particles in the vicinity of which a local increase in cross-linking density occurs.



Fig.3. Values G' and G" moduli as a function of shear strain for 1% alginate hydrogels with various types and amounts of functionalized IMPs in the absence (0 kA m<sup>-1</sup> - blue curves) and presence of magnetic field (35 kA m<sup>-1</sup> - orange curves and 282 kA m<sup>-1</sup> - green curves).

The variation of the viscoelastic moduli as a function of frequency is presented in Fig.4. Both, G' and G" exhibit only slight dependence on the frequency across all rang studied (0.1–10 Hz). Again, G' is substantially higher than G", indicating a predominantly elastic behavior, characteristic of cross-linked polymeric networks [8] and soft biological tissues [9]. Finally, it should be noted that the rheological properties of alginate hydrogels obtained here are different from those of obtained by us previously [7], which suggests the important role of ensuring exactly the same reagents, synthesis and functionalization protocols and rheological testing regime.



**Fig.4.** Values G' and G" moduli as a function of frequency for 1% alginate hydrogels with amount of functionalized IMPs in the absence (0 kA m<sup>-1</sup> - blue curves) and presence of magnetic field (35 kA m<sup>-1</sup> - orange curves and 282 kA m<sup>-1</sup> - green curves).

**Conclusions:** The incorporation of amine-functionalized iron particles into alginate hydrogels imparted magnetic responsiveness to the resulting composite materials. The presence of a strong magnetic field significantly increased both viscoelastic moduli (G' and G") and shifted the yielding point toward higher shear strain values, particularly in hydrogels with a higher content of magnetic particles. These observed effects, resulting from the incorporation of the functionalized magnetic phase, may offer useful means to control the mechanical properties of the composite hydrogels.

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## OBTAINING DOUBLE LAYERED HYDROXIDES AS POTENTIAL CARRIERS OF ACTIVE SUBSTANCES

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**Abstract:** The aim was to obtain new materials based on layered double hydroxides (LDH), which are characterized by high stability and good sorption properties. LDH, like clay minerals, can act as carriers of active substances with controlled release. A method for the synthesis of layered double hydroxides containing metal cations: Mg, Fe, Cu, Zn, Al, in various stoichiometric ratios and  $CO_3^{2-}$  or Cl<sup>-</sup> anions is developed.

Introduction: Double layered hydroxides are inorganic materials with numerous applications. Their layered structure resembles clay minerals. The similarity in structure results from the presence of cationic layers, composed of the cations of divalent metals -M(II), for example: Mg<sup>2+</sup>, Zn<sup>2+</sup>, Cu<sup>2+</sup> and trivalent - M(III), such as: Al<sup>3+</sup>, Fe<sup>3+</sup>. These layers are octahedrally surrounded by the OH<sup>-</sup> groups. In the interlayer space, there are exchangeable ions, which are the anions:  $NO_3^{-}$ ,  $CO_3^{2-}$ ,  $Cl^{-}$  or  $PO_4^{3-}$  and water molecules coordinated with them. The M II/M III cation ratio in LDHs is most often from 2 to 3, although it can range from 1 to about 51. Different charge density of the layer, determined by the cation ratio and the natural flexibility of the crystal structure, enables the formation of LDHs intercalated with a large variety of inorganic anions [1-10]. The general chemical formula of LDH can be written as  $[M^{2+}_{1-x}M^{3+}_{x}(OH)_2]^{x+}(A^{y-})_{x/y}$  zH<sub>2</sub>O, where  $M^{2+} = Mg$ , Zn, Co, Ni, Cu, Mn and  $M^{3+} = Al$ , Ga, Cr, Co, Fe, V, Y, Mn, and Ayis the intercalated anion. LDHs have an excellent ability to accept organic and inorganic anions. Due to their structure, they have a large surface area, high anion exchange capacity comparable to ion exchange resins and good thermal stability. These features make LDHs useful in: electrochemistry, electrocatalysis, catalysis, ion exchange, sorption, as corrosion inhibitors or as nanocontainers for herbicides for agricultural purposes [6-10]. Biological and medical studies have shown that LDH containing the cations: Zn Al, Ca Al, Mg Al have very small toxicity and are even "health-friendly" [11]. The other very important features of LDHs are the ability to intercalate different types of anions (inorganic, organic, biomolecules, and even genes), high biocompatibility, and easy biodegradability [5]. Owing to this, LDHs can be materials with biomedical applications, such as drug delivery, gene delivery, biosensors, advanced cosmetics, or special food additives [6]. LDHs can contribute to improving the stability of some cosmetic ingredients or hydrodispersibility [11,12]. The example is lycopene described in the literature, for which the presence in the LDH structure increases its resistance to oxidation [7] or overall durability. Double layered hydroxides can be used as additives in cosmetic products, as they are effective as sorbents for substances that

prevent skin photoaging. The ZnTi-CO<sub>3</sub>-LDH composite was used as a carrier for 1-(4-(1.1-dimethylethyl)phenyl)-3-(4-methoxyphenyl)-1.3-propanedione (known as Avobenzone/AVB) to improve stability and synergistically enhance UV absorption in antiaging cosmetics [2]. The photocatalytic properties of LDH were also analyzed and compared with TiO<sub>2</sub> and ZnO. They were found to be very effective for the use in cosmetic formulations, as they offer smaller photoreactivity compared to cosmetic ZnO and  $TiO_2$  [9]. LDHs can be carriers of bacteriostatic agents, the example of which is the incorporation of kojic acid and cinnamic acid into Zn-Ti LDH [13,14]. LDHs are used as carriers for organic dye anions (Ponceau SX and acid green (AG)) [3], forming a new type of hydrophobic organic-inorganic composite pigment with the sandwich structure. In this case, LDH played a protective role, providing photostability, good hydrophobicity and biocompatibility of pigments as well as the possibility of colour control and more effective performance in terms of hiding power and colour saturation [15,16]. Macroporous LDHs synthesized by the co-precipitation method were used to immobilize iron(III) porphyrin [17,18]. Recently, monodisperse hierarchical LDHs on the silica beads with core-shell structures were developed for immobilizing metalloporphyrin. Successful intercalation of metallophthalocyanines into the ZnAl LDH interlayer was also obtained. However, these materials were used only as catalysts for oxidation of various organic substrates. Due to such great interest in double layered hydroxides in various fields, including the cosmetic industry, the attempt was made to obtain LDHs containing such cations as: Mg, Cu, Fe, Al, Zn with various combinations in terms of composition and stoichiometric ratio. The selected systems are not only safe for the human body. However, they can also perform important functions, for example copper has bactericidal properties and zinc is a known antioxidant. Therefore, they can potentially be materials for further research on application in cosmetics or medicines.

**Experimental:** Double layered hydroxides were obtained by the co-precipitation method. For this purpose, the solutions of Na<sub>2</sub>CO<sub>3</sub>, NaOH and nitrates of magnesium, aluminum(III), copper(II), zinc, iron(II) and iron(III), were prepared. They were slowly mixed in portions at the appropriate stoichiometric ratios. The pH of the mixture was controlled by adding the NaOH solution, so that its value was close to pH=10. After adding the entire portion of nitrate solutions, the reaction mixture was stirred using a magnetic stirrer for 4 hours, and heated to about 160 °C degrees all the time. After this time, the mixture was cooled and then filtered under the pressure by means of a Schott crucible. The material was left to dry in an oven at 75 °C.

**Results:** The obtained materials - double-layered hydroxides:  $Mg_2Al_1$ -CO<sub>3</sub>,  $Mg_2Al_1$ -Cl,  $Mg_1Cu_{0.5}Zn_{0.5}Al_1$ -CO<sub>3</sub>,  $Mg_1Cu_{0.5}Zn_{0.5}Al_1$ -Cl,  $Mg_2Al_{0.5}Fe_{0.5}$ -CO<sub>3</sub>,  $Mg_2Al_{0.5}Fe_{0.5}$ -Cl were examined by X-ray diffraction to confirm their structure. The XRD spectrum (Fig.1) shows the distinct peaks-reflections: 003, 006, 009, 015, 018, 110, 113.



Fig.1. Typical XRD patterns of different LDH materials obtained by the co-precipitation method resulting in formation of LDH-CO<sub>3</sub> form (a) and subsequent carbonate-to-chloride anion exchange (LDH-Cl form) conducted at room temperature for 24 h in the NaCl solution (b). d – the basal spacing values of the respective LDHs.

Based on the analyses of the position of characteristic bands for LDH in the XRD diffraction pattern (Fig.1), the structure can be identified and the properties of the material can be assessed. The most important is the (003) reflection, which indicates an ordered layer structure [19]. Based on its position, the interlayer distance (d-spacing) in the LDH structure can be determined, which depends on the type of interlayer anions  $(CO_3^{2-}, Cl^-, NO_3^-, OH^-, etc.)$  and the degree of hydration. The shifts in the position of this band confirm the efficiency of ion exchange in the interlayer space. The higher-order reflections, located at higher 2theta values, indicate the ordering of the crystalline layer structure and allow the determination of the distance between cations in these layers.

**Conclusions:** There were obtained the double layered hydroxides:  $Mg_2Al_1$ -CO<sub>3</sub>,  $Mg_2Al_1$ -Cl,  $Mg_1Cu_{0.5}Zn_{0.5}Al_1$ -CO<sub>3</sub>,  $Mg_1Cu_{0.5}Zn_{0.5}Al_1$ -Cl,  $Mg_2Al_{0.5}Fe_{0.5}$ -CO<sub>3</sub>,  $Mg_2Al_{0.5}Fe_{0.5}$ -Cl, which were confirmed by the XRD studies. In the next stage, these materials will be tested for their use as sorbents and carriers with controlled release of active substances, such as anthocyanins, chlorophyll, vitamin C and others.

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# LYOPHILIZED AGAR AND ITS HYBRID SILICA COMPOSITES AS A DRUG CARRIER

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**Abstract:** The scientists work methodically to provide biologically inert materials (i.e. the carriers) that are capable of delivering biologically active compounds directly into inflamed tissue in a controlled manner without adverse effects on the cured organism. The possibility of preparing a lyophilized agar-hybrid silica-drug delivery system for the controlled release of diclofenac sodium in aqueous solution is investigated. The morphology of the system is characterized by scanning electron microscopy and X-ray powder diffraction. The composite is formed without recrystallization of the drug. The agar-diclofenac system releases 10 mg of the drug in the first 10 minutes to reach ca. 25 mg after 2 hours. The hybrid silica significantly slows down the release kinetics. The composite releases from 10 mg to 17.5 mg of diclofenac sodium in 2 h. No 'burst release' is observed.

**Introduction:** Agar is a widely known, versatile, biocompatible and safe-for-oral-use mixture of polysaccharides of natural origin [1]. When added to water and heated above 80 °C, it dissolves. When the solution is cooled below 40 °C, it forms a gel. Even in small amounts of only 0.5%, gelling occurs and is completely reversible. The use of agar, its derivatives and composites has been reported in medical application for drug delivery in systems consisting of tetracycline [2], metformin [3], doxorubicin [4,5], ascorbic acid [6] and diltiazem hydrochloride [7]. Diclofenac is an anti-inflammatory drug that has some adverse effects with prolonged oral administration, including gastric ulcers [8]. Little is known about the use of freeze-dried agar as a drug carrier. However, it is possible to use it as a template for the production of silica [9]. Previous studies have shown that polymer-silica composites have favorable release kinetics [10]. The hypothesis is that the diclofenac is uniformly dispersed in the agar matrix. Upon contact with water, the lyophilized agar is slowly rehydrated, and the drug is transferred into the release medium. The role of the hybrid silica is to slow down this process for favorable control of the drug release.

**Experimental:** Agar (Sigma-Aldrich) aqueous (deionized water) solution (2.36 % w/w) was heated to 80 °C. With constant stirring, 500 mg of diclofenac sodium (Sigma) was added. The solution was stirred vigorously for about 20 min. The hot solution was divided into 10 portions and poured into plastic containers with airtight lids. After cooling to room temperature, the solution gelled and was frozen at approximately 18 °C. The samples were freeze-dried (5 Pa, -45 °C) to obtain a constant mass. An agar-diclofenac system was prepared in the form of white, opaque rolls (sample name D1). The agar-diclofenac-hybrid silica composites were prepared by soaking the rolls with solutions of silica (TEOS, tetraethyl orthosilicate, Acros Organics) and hybrid silica (APTES, (3-aminopropylo)trietoxysilane, Sigma-Aldrich) precursor's. TEOS and

APTES were mixed in a molar ratio of 2:1 and 4:1 (samples D2 and D3, respectively). The soaked rollers for condensation of the silica precursors were exposed to ammonia vapors for 72 hours and then dried at room temperature in a vacuum (Vacucell dryer) for at least 1.5 hours to evacuate water vapor and ammonia. The morphology of the investigated samples was characterized by scanning electron microscopy (SEM, FEI Quanta 3D FEG, 5kV, 0.7 mPa – 4 mPa) and X-ray powder diffraction (XRD, PANanalytical Empyrean,  $\lambda_{CuK\alpha} = 1.54056$  Å, ICDD PDF4+). The release of diclofenac sodium from all samples under study was carried out for 2 h into the 250 mL of buffered solution (pH= 6.8, [11]) at (37±0.5) °C with constant stirring. The concentration of diclofenac sodium was estimated via UV-Vis spectra measurements (Varian Cary 100 Bio,  $\lambda_{max} = 276$  nm).

**Results:** SEM images of the freeze-dried agar gel with sodium diclofenac are shown in Fig.1a-b. They show that the D1 sample consists of flake-like objects of the order of a few micrometres in size, which are interconnected and form larger structures with a non-uniform surface. Fig.1a shows areas that appear to be 'smoother' and 'rougher', unevenly distributed. It should be noted that the SEM is not used to determine surface roughness.



Fig.1. SEM microimages of D1 (a, b), D2 (c, d) and D3 (e, f) samples.

The morphology of the D2 sample is different from that of the D1 sample. The D2 sample no longer consists of 'smooth flakes' (Fig.1c-d). At 5000x magnification (Fig.1d), numerous objects appear on the 'flakes'. Their size does not exceed 1  $\mu$ m. The structure of this material resembles a cauliflower flower. At 250x magnification (Fig.1c), areas with varied structures can be recognized, which are covered with numerous smaller objects (probably the silica species). In the D2 material (Fig.1c-d), the proportion of the hybrid-silica precursor, i.e. APTES, is higher. The structure of this material visible at 5000x magnification (Fig.1d) has fewer objects < 1  $\mu$ m in comparison with D3. The subtle structure of the 'flakes' is lost. Instead, larger fragments of the analysed material look as if they are 'melted together' or covered with a substance that ensures that the sharper edges in the D2 micrographs are not as numerous as in the D3 sample.

None of the micrographs show that microcrystallites were present in the samples analysed. It is also difficult to recognize in the morphology of the samples examined that any systematic structural element has been repeated in the spatial structure of the material.

The diffraction pattern for diclofenac (Fig.2) contains numerous reflexes of small halfwidth with high relative intensity. The noise/signal ratio is very favorable. The most intense reflections corresponds very well with diffraction patterns already published [12-14]. In contrast to the diffraction pattern of pure diclofenac, the signal recorded for the D1 sample has the characteristics of diffraction patterns recorded for amorphous substances (Fig.2inset).



Fig.2. XRD diffractograms of diclofenac sodium and samples under study (inset).

The signal-to-noise ratio is clearly unfavorable. There are no intense reflections in the diffraction pattern, and those that can be read have a relatively large half-width. Reflections are visible at 2 $\Theta$  angles of 13°, 19°, and 28.5°. Contrary to the diffractograms presented for a physical mixture of diclofenac with an amorphous substance [14], there are no intense reflections in the diffraction pattern. It may indicate that diclofenac sodium dissolved during the process and no recrystallization took place during the relatively long gelation and freeze-drying time. a similar diffraction pattern to that for D1 has been previously presented for agarose [15] and for agar film [16]. The first two reflections in the diffraction pattern of the D1 sample can be attributed to agar, while the reflection at 28.5° probably originates from diclofenac. a broad reflection at a value of  $2\Theta = 23^{\circ}$  for D2 and D3 is similar for XRD patterns for amorphous silica [9]. It can, therefore, be assumed that neither the process of impregnation with silica precursor solutions, nor their condensation, nor degassing in a vacuum dryer influenced the recrystallization of the drug. Since the agar dissolved neither in TEOS nor in the TEOS-APTES mixture, it can be assumed that the drug is molecularly dispersed in the agar matrix. The release profiles presented in Fig.3 indicate that there is no 'burst release' [17] for composites D2 and D3 (modified with hybrid silica) in contrast to the D1 system.



Fig.3. Release kinetics of diclofenac sodium to buffered release medium for samples D1, D2 and D3.

**Conclusions:** The system consists of a continuous agar-hybrid silica phase and the drug as a dispersed phase. No recrystallization of diclofenac occurred during the synthesis. The freeze-dried agar is well wetted with the hybrid silica precursors. The presence of the hybrid silica in the system significantly slows down the release of the drug into the aqueous medium. Within two hours, a small but therapeutic amount of diclofenac sodium is released linearly from the system (approx. 20 mg). The study shows the great potential of agar-hybrid silica-drug materials for medical applications. In order to meet the very strict requirements of the pharmaceutical industry, the synthesis process presented must be further optimized (increasing the amount of drug) and its biocompatibility researched.

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# VOLTAMMETRIC TRACE ANALYSIS OF Hg(II) VIA ELECTRODE MATERIALS BASED ON NANOPARTICLES

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**Abstract:** Mercury is one of the most harmful heavy metals, and its presence can cause many very serious health problems. For this reason, the content of Hg(II) in food products is carefully controlled, which requires very sensitive instrumental techniques. Stripping voltammetry, combined with a properly designed sensor, is an excellent analytical tool for this purpose. Metal oxides nanoparticles are gaining increasing popularity due to their excellent catalytic properties. The procedures using different nanoparticles presented in the literature allow for the determination of Hg(II) at trace level and are characterized by excellent analytical performance. In addition, they were used to determine this element in fish, blood serum, certified reference materials, and water samples. Moreover, our studies allowed us to obtain promising results for the sensor based on Fe<sub>3</sub>O<sub>4</sub> magnetic nanoparticles, and carbonaceous material in a trace analysis of Hg(II) ions.

**Introduction:** The pervasive environmental threat posed by heavy metal ions (HMIs) stems from their inherent toxicity, resistance to degradation, and propensity for bioaccumulation. Mercury, among HMIs, holds a unique position as one of the longestrecognized and extensively investigated toxicants. Its presence and harmful properties lead to significant contamination of environmental matrices such as water, sediment, and soil, disrupting the ecosystem function and posing severe risks to human and wildlife health. Anthropogenic activities, driven by urbanization and industrialization, have dramatically increased Hg<sup>2+</sup> release. Notably, Hg<sup>2+</sup> accumulation within the food web results in severe adverse health outcomes, including nephrotoxicity, neurotoxicity, gastrointestinal disruption, neuromuscular dysfunction, and potential carcinogenic effects [1-4]. Due to the high toxicity of mercury ions, it is crucial to monitor their presence in food products. It should be emphasized that the permissible content of this element in drinking water is very low, and in the case of WHO standards, it is only  $6 \mu g$  $L^{-1}$  [5]. For this reason, it is necessary to develop sensitive analytical procedures that will allow for analysis of this element at the trace concentration level. There are many different instrumental methods that are distinguished by good analytical parameters for mercury analysis. These include techniques such as cold vapour atomic absorption spectrometry (CV-AAS) [6], inductively coupled plasma mass spectrometry (ICP-MS) [7], atomic fluorescence spectrometry (AFS) [8], and cold vapor atomic fluorescence spectrometry (CV-AFS) [9,10]. However, these procedures are often very expensive and require a time-consuming sample preparation step before measurement, which significantly extends the analysis duration. Stripping voltammetry (SV) is becoming increasingly popular due to its low analysis cost, high sensitivity, and simple sample preparation. In the case of SV, very low limits of detection (LODs) and quantification

(LOOs) and wide linear ranges of the calibration curves are achieved due to the accumulation of the analyte on the surface of the working electrode before the analytical signal recording step. This enables the monitoring of HMIs, such as Hg(II), to be conducted in a wide range of concentrations at the trace level. Nanoparticles (NPs) of different materials such as hydroxyapatite [4], different metal oxides such as Fe<sub>3</sub>O<sub>4</sub>@Au [11], Fe<sub>3</sub>O<sub>4</sub> and MnO<sub>2</sub> [3], SnO<sub>2</sub> [1], PdO [12] as well as Au-nanoparticles [9] are increasingly utilized as electrode materials, especially in various types of composites. Metal oxides are often used to design voltammetric sensors for HMIs detection due to their remarkable properties such as high adsorption capability, thermal and mechanical stability, and biological compatibility. Notably, iron(II, III) oxide magnetic nanoparticles  $(Fe_3O_4 NP_5)$  are characterized in particular by the above-mentioned advantages of nanoparticles and exhibit supermagnetic properties. However, they tend to aggregate and easily oxides [13-16]. The use of carbon material such as multi-walled carbon nanotubes (MWCNTs) is one of the ways to counteract the disadvantages of  $Fe_3O_4$  magnetic nanoparticles by providing good conductivity, an additional number of active sites, and improving the homogeneity of Nps distribution on their surface [15,16]. In the case of other metal oxides such as  $SnO_2$ , the presence of reduced graphene oxide (rGOS) rich in functional groups feasibly endows high electron mobility, enhancing the potential window and catalytic activity towards Hg(II) ions [1]. The modulation of surface properties can also be provided for nanoparticles by their surface coating with an organic, stabilizing agent. For example, polymer films stabilize  $Fe_3O_4$  magnetic nanoparticles against oxidation, promote their homogenous distribution, and also allow for the introduction of various functional groups [13,14].

**Experimental:** Stripping voltammetry measurements were performed in a standard 10 mL electrochemical cell using a three-electrode setup and a  $\mu$ Autolab analyzer controlled by GPES 4.9 software. Prior to the ink application, a glassy carbon electrode (GCE) was meticulously polished using 2000-grit silicon carbide paper, 0.3  $\mu$ m alumina suspension, and a polishing pad, with 2-minute ultrasonic cleaning in demineralized water between each step. The 1  $\mu$ L of ink containing Fe<sub>3</sub>O<sub>4</sub> nanoparticle and carbon material was applied onto the dried GCE surface. After that, the resulting sensor was dried in a laboratory drier before electrochemical measurements.

**Results:** In Table 1, the analytical performance in the trace analysis of Hg(II) ions of selected voltammetric procedures using sensors based on nanoparticles were shown. The developed procedures were used in the measurement of mercury content in fish, blood serum, certified reference materials, and water samples. The lowest LOD=1.0 nmol L<sup>-1</sup> and wide enough linear range of calibration curve were achieved using magnetic carbon paste electrode modified with halloysite nanotubes-iron oxide–manganese oxide nanocomposite (MCPE/HNTs-Fe<sub>3</sub>O<sub>4</sub>–MnO<sub>2</sub>) [3]. Moreover, this sensor utilizes an unusual measurement procedure.

	Table 1. Comparison of selecte	d voltammetric	procedures	parameters for dete	rmination of Hg(II)	ions.
r						

CII (ID)

(Technique)	Analytical nonomotors	Determined ion	Amplication	Ref.
Sensor	Anarytical parameters	Hg(II)	Application	
(SWV)	LOD [nmol L <sup>-1</sup> ]	141.0	tap water and	[4]

GCE/AV-HA	Linear range [nmol L <sup>-1</sup> ]	200.0-210000.0	industry waste samples	
(DPASV) CPE-NP/PdO	LOD [nmol L <sup>-1</sup> ] Linear range [nmol L <sup>-1</sup> ]	19.30 250.0-150000.0	water samples	[12]
(DPASV) GCE/ErGO/AuNPs	LOD [nmol L <sup>-1</sup> ]* Linear range [nmol L <sup>-1</sup> ]*	3.0 10.0-75.0	tap, lake, and river water samples	[9]
(DPASV) SPCE/T- COOH/CA/Fe <sub>3</sub> O <sub>4</sub> @Au	LOD [nmol L <sup>-1</sup> ]* Linear range [nmol L <sup>-1</sup> ]*	2.49 4.99-997.06 997.06-10967.65	water and fish samples, certified reference material	[11]
(DPV) GCE/rGOS@SnO <sub>2</sub>	LOD [nmol L <sup>-1</sup> ] Linear range [nmol L <sup>-1</sup> ]	1.27 250.0-705300.0	blood serum, fish extract, and drinking water samples	[1]
(DPASV) MCPE/HNTs-Fe <sub>3</sub> O <sub>4</sub> MnO <sub>2</sub>	LOD [nmol L <sup>-1</sup> ]* Linear range [nmol L <sup>-1</sup> ]*	1.00 2.49-747.79	water samples	[3]

SWV - square wave voltammetry; DPASV – differential-pulse anodic stripping voltammetry; DPV – differential-pulse voltammetry; GCE/AV-HA – glassy carbon electrode modified with hydroxyapatite (HA) nanoparticles, biosynthesized using Aloe vera plant (Av) extract; CPE-NP/PdO - carbon paste electrode modified with a palladium oxide supported onto natural phosphate; GCE/ErGO/AuNPs – glassy carbon electrode modified with electrochemically reduced graphene oxide and Au nanoparticles; SPCE/T-COOH/CA/Fe<sub>3</sub>O<sub>4</sub>@Au - screen-printed carbon electrode modified with thymine acetic acid anchored with cysteamine-conjugated core shell Fe<sub>3</sub>O<sub>4</sub>@Au anoparticles; GCE/rGOS@SnO<sub>2</sub> – glassy carbon electrode modified with reduced graphene oxide and nanoparticles of tin (IV) oxide; MCPE/HNTs-Fe<sub>3</sub>O<sub>4</sub>-MnO<sub>2</sub> – magnetic carbon paste electrode modified with composite used for extraction of Hg(II) - halloysite nanotubes-ironoxide-manganese oxide nanocomposite. \*The units of analytical parameters presented in the publication have been converted to nmol L<sup>-1</sup>.

In this procedure, the obtained composite (HNTs-Fe<sub>3</sub>O<sub>4</sub>-MnO<sub>2</sub>) was used for extraction of Hg(II) from the tested solution acidified to pH=3.5 and then magnetically separated through an external magnet from the supernatant. The separated material was used to prepare paste electrodes. Then, the prepared sensor was rinsed with demineralized water and used for voltammetric measurements. It should be emphasized that before the actual measurement, a negative potential was applied to the working electrode to reduce the adsorbed mercury to a metallic form, which was subjected to oxidation at the signal recording stage [3]. Despite the very low detection limit of the discussed procedure, sensor preparation is a time-consuming task. Sensors that do not require additional extraction steps (other sensors are shown in Table 1) seem to be more user-friendly for routine analyses. For example, glassy carbon electrode modified with reduced graphene oxide and nanoparticles of tin (IV) oxide (GCE/rGOS@SnO<sub>2</sub>) [1] as well as screenprinted carbon electrode modified with thymine acetic acid anchored with cysteamineconjugated core shell Fe<sub>3</sub>O<sub>4</sub>@Au nanoparticles (SPCE/T-COOH/CA/Fe<sub>3</sub>O<sub>4</sub>@Au) [11] could be an excellent alternatives, which allow to obtain only slightly higher LODs then  $MCPE/HNT_{s}-Fe_{3}O_{4}-MnO_{2}$ . However, this comparison indicates that the examples described in the literature (Table 1) demonstrate good catalytic properties of  $Fe_3O_4$  in the context of Hg(II) determination [3,11]. As a result of our preliminary studies, we have obtained promising data indicating excellent catalytic properties of the sensor based on  $Fe_3O_4$  magnetic nanoparticles, and carbonaceous material in a trace analysis of Hg(II) ions. This procedure is currently at the optimization stage.

**Conclusions:** The selected voltammetric procedures for the determination of mercury ions presented in the literature (Table 1) are characterized by wide linear ranges of calibration curves and low LODs. Moreover, they can be successfully used for the analysis of Hg(II) content in fish, blood serum, certified reference materials, and water samples. The most significant seem to be the procedures using  $Fe_3O_4$  magnetic nanoparticles [3,11], which allow achieving wide linear ranges of calibration curves and low LODs. The preliminary studies has shown a catalytic activity  $Fe_3O_4$  magnetic nanoparticles and carbon material in the enhancement of the Hg(II) signal.

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# VOLTAMMETRIC PROCEDURES FOR SIMULTANEOUS DETERMINATION OF Cd(II) AND Pb(II) AT THE TRACE CONCENTRATION LEVEL VIA COMPOSITE ELECTRODE MATERIALS BASED ON MAGNETIC NANOPARTICLES

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Abstract: The progressive industrialization increases the risk of exposure to Cd(II) and Pb(II) in drinking water and can contribute to the development of many chronic diseases, including cancer. Therefore, the constant monitoring of cadmium and lead in food and water samples, using sensitive tools such as stripping voltammetry, is necessary. This technique, combined with a thoughtful way of designing a sensor based on magnetic nanoparticles, allows for the achievement of very low detection limits and high sensitivities in the trace analysis of Cd(II) and Pb(II) in the environmental and food samples. Despite many advantages, magnetic nanoparticles tend to aggregate and oxidize, and as a result, their activity decreases over time. To counteract those problems, it is possible to cover the nanoparticles with a special polymer coating or carbonaceous supporting material. The sensors presented in the literature using both concepts are characterized by very good analytical performance for both Cd(II) and Pb(II) ions and have been successfully used to determine their content in environmental samples. In addition, our studies have shown the possibility to improve the analytical performance of sensors based on Fe<sub>3</sub>O<sub>4</sub> magnetic nanoparticles even further and shorten the analysis duration.

Introduction: Cadmium and lead, being toxic heavy metals ions (HMIs), pose significant health risks for both humans and animals. The exposure to their presence occurs through environmental contamination of air, water, food, and consumer goods. Those ions also have the ability to penetrate soils and groundwater, contributing to their contamination [1-3]. Due to the high toxicity of Cd(II) and Pb(II) and potential for bioaccumulation, regulations have been developed specifying its maximum permissible concentrations in food and drinking water. The WHO has established maximum limits for cadmium and lead content in drinking water, specifically for 3 mg  $L^{-1}$  and 10 mg  $L^{-1}$ , respectively [4]. Consequently, there is a clear need for the development of highly sensitive methods for fast and simple trace analysis of those elements in consumable products. Stripping voltammetry (SV) is a highly sensitive analytical technique breaking records in achieving very low detection limits of various compounds and heavy metal ions [2,5,6]. Its exceptional sensitivity stems from the ability to preconcentrate the analyte on the working electrode surface before the actual measurement of the signal (Fig.1). This preconcentration step allows for the achievement of very low limits of detection (LODs) and limits of quantification (LOOs), as well as high sensitivities within a relatively short, single measurement time [7]. The vital element in stripping voltammetry is the working electrode, which plays a crucial role in determining a specific compound or ion. Therefore, intensive research is being conducted to find new electrode materials that would establish a new boundaries for simultaneous and highly sensitive analysis of heavy metal ions, such as Cd(II) and Pb(II).



Fig.1. SV measurement simplified scheme.

Magnetic nanoparticles (MNPs) are experiencing a surge in utilization, owing to their multifaceted, advantageous properties. Notably, iron(II, III) oxide magnetic nanoparticles (Fe<sub>3</sub>O<sub>4</sub> NPs) have garnered significant attention due to their inherent properties. High biocompatibility, low toxicity, simple preparation methods, and remarkable adsorptive and catalytic functionalities render them suitable for quantitative analysis of heavy metal ions. Consequently, they are a promising electrode material for the electrochemical determination of Cd(II) and Pb(II). However, the inherent limitations of MNPs include their vulnerability to oxidative degradation and propensity for agglomeration, which results in a reduction of their catalytic efficiency [1,2,8,9]. The challenges associated with magnetic nanoparticles can be overcome through two distinct approaches. One involves the application of a highly conductive carbon material, which serves to increase the density of active sites available to conduct electrode reaction and promote even more dispersion of MNPs. Alternatively, coating the nanoparticles with an organic polymer film effectively prevents oxidative degradation, enhances homogeneity, and allows for the introduction of functional groups that facilitate the analyte deposition [1,8,9].

**Experimental:** Stripping voltammetry measurements were conducted using a threeelectrode system with a conventional 10 mL electrochemical cell. The experiments were performed utilizing a  $\mu$ Autolab electrochemical analyser, controlled by GPES 4.9 software. Before applying the ink onto the glassy carbon electrode (GCE), its surface was polished with silicon carbide paper (SiC-paper, #2000), alumina particle suspension (0.3  $\mu$ m), and a polishing pad. Before each step, GCE was sonicated in demineralized water using an ultrasonic bath for 2 minutes. Then, after the electrode was completely dry, ink prepared with commercially available chemical reagents (Fe<sub>3</sub>O<sub>4</sub> nanoparticles and carbon material) was applied onto its surface. The sensor prepared in this way was dried in a laboratory dryer and was ready to conduct experiments.

**Results:** Table 1 summarizes selected magnetic nanoparticles based SV procedures for simultaneous determination of Cd(II) and Pb(II). Presented examples are characterized

with very low LODs ( $10^{-9}$ - $10^{-10}$  mol L<sup>-1</sup>) and high sensitivities for both Cd(II) and Pb(II) ions [1,2,8,10-13].

(Technique)		Determi	ned ions	A 1' 4'	D.C
Sensor	Analytical parameters	Cd(II)	Pb(II)	Application	Ref.
(SWASV) GCE/BiNPs@CoFe <sub>2</sub> O <sub>4</sub>	LOD [nmol L <sup>-1</sup> ]* Sensitivity [µA/µmol L <sup>-1</sup> ]*	8.2 42.7	7.3 50.0	tap water samples	[10]
(SWASV) GCE/C <sub>3</sub> N <sub>4</sub> /Bi <sub>2</sub> O <sub>3</sub> /Fe <sub>3</sub> O <sub>4</sub>	LOD [nmol $L^{-1}$ ]* Sensitivity [ $\mu$ A/ $\mu$ mol $L^{-1}$ ]*	3.0 51.1	1.0 82.5	lake water samples	[11]
(SWASV) mGCE/GSH@Fe <sub>3</sub> O <sub>4</sub>	LOD [nmol L <sup>-1</sup> ]* Sensitivity [µA/µmol L <sup>-1</sup> ]*	1.5 8.7	0.9 28.2	environmental water samples	[1]
(SWASV) mCPE/Fe <sub>3</sub> O <sub>4</sub> @G <sub>2</sub> -PAD	LOD [nmol L <sup>-1</sup> ]* Sensitivity [µA/µmol L <sup>-1</sup> ]*	1.9 31.2	0.8 73.6	river and lake water samples, sewage samples	[12]
(SWASV) GCE/GO-Fe <sub>3</sub> O <sub>4</sub> - PAMAM	LOD [nmol L <sup>-1</sup> ]* Sensitivity [µA/µmol L <sup>-1</sup> ]*	0.6 21.2	0.6 32.1	river and lake water samples	[13]
(DPAdSV) GCE/CoFe <sub>2</sub> O <sub>4</sub> @CTS	LOD [nmol L <sup>-1</sup> ]* Sensitivity [µA/µmol L <sup>-1</sup> ]*	2.8 3.3	0.2 43.3	environmental water samples	[2]
(SWASV) GCE/CD/LSG/MWCNTs /Fe2O4/BiF	LOD [nmol L <sup>-1</sup> ]* Sensitivity [µA/µmol L <sup>-1</sup> ]*	0.9 10.9	0.3 43.5	tap water samples	[8]

 Table 1. Comparison of selected voltammetric procedures analytical parameters for simultaneous determination of Cd(II) and Pb(II).

SWASV - square wave anodic stripping voltammetry; DPAdSV - differential pulse adsorptive stripping voltammetry; GCE/BiNPs@CoFe2O4 - glassy carbon electrode modified with a composite based on cobalt ferrite and bismuth nanoparticles; GCE/C<sub>3</sub>N<sub>4</sub>/Bi<sub>2</sub>O<sub>3</sub>/Fe<sub>3</sub>O<sub>4</sub> - glassy carbon electrode modified with a composite based on carbon nitride, bismuth oxide and Fe<sub>3</sub>O<sub>4</sub> magnetic nanoparticles; mGCE/GSH@Fe<sub>3</sub>O<sub>4</sub> – magnetic glassy carbon electrode modified with glutathione and Fe<sub>3</sub>O<sub>4</sub> magnetic nanoparticles; mCPE/Fe<sub>3</sub>O<sub>4</sub>@G<sub>2</sub>-PAD - magnetic carbon paste electrode modified with a polyaminoamide dendrimer functionalized with Fe<sub>3</sub>O<sub>4</sub> magnetic nanoparticles; GCE/GO-Fe<sub>3</sub>O<sub>4</sub>-PAMAM – glassy carbon electrode modified with graphene oxide, Fe<sub>3</sub>O<sub>4</sub> nanoparticles and polyamidoamine dendrimer; GCE/CoFe<sub>2</sub>O<sub>4</sub>@CTS - glassy carbon electrode modified with small-sized nanoparticles core-schell chitosan (CTS) and cobalt ferrite (CoFe<sub>2</sub>O<sub>4</sub>@CTS): GCE/CD/LSG/MWCNTs/Fe<sub>3</sub>O<sub>4</sub>/BiF – glassy carbon electrode modified with laser-scribed graphene, multi-walled carbon nanotubes (MWCNTs), coated with chitosan, doped with  $Fe_3O_4$  nanoparticles and covered with a bismuth film. \*The units of analytical parameters presented in the publication have been converted to nmol  $L^{-1}$  and

\*The units of analytical parameters presented in the publication have been converted to nmol  $L^{-1}$  and uA/umol  $L^{-1}$ .

The lowest detection limit for Cd(II) ions was achieved for the sensor using a glassy with graphene oxide, Fe<sub>3</sub>O<sub>4</sub> nanoparticles and carbon electrode modified polyamidoamine dendrimer (GCE/GO-Fe<sub>3</sub>O<sub>4</sub>-PAMAM), and for Pb(II) - using a glassy carbon electrode modified with small-sized core-shell type nanoparticles of chitosan and cobalt ferrite (GCE/CoFe<sub>2</sub>O<sub>4</sub>@CTS). In the case of both ions, the highest sensitivities were achieved for the glassy carbon electrode modified with a composite based on bismuth carbon nitride. oxide. and Fe<sub>3</sub>O<sub>4</sub> magnetic nanoparticles  $(GCE/C_3N_4/Bi_2O_3/Fe_3O_4)$ . It should be noted that most of the procedures presented in Table 1 utilize long analyte accumulation time up to 300 s, which significantly extends the analysis duration. Our preliminary studies revealed that it's possible to shorten this time over by half and achieve a drastic incensement of sensitivity for both, Cd(II) and

Pb(II) ions by using a composite based on carbon material, functionalized  $Fe_3O_4$  magnetic nanoparticles. Current research is focused on optimizing this procedure.

**Conclusions:** The selected SV procedures presented in the literature for the simultaneous determination of Cd(II) and Pb(II), using sensors based on magnetic nanoparticles, are characterized by high sensitivities and low LODs [1,2,8,10-13]. Furthermore, they have been validated for the determination of these ions in environmental water samples. By the conjunction of magnetic nanoparticles with organic coating or a carbon support, it's possible to create sensors that offer significantly enhanced analytical performance for the trace analysis of cadmium and lead in water samples. The procedures that currently allow for achieving the best analytical parameters utilized the above-mentioned methods of nanoparticle modification. The preliminary studies show that a composite sensor based on carbon material and Fe<sub>3</sub>O<sub>4</sub> magnetic nanoparticles probably obtain even better analytical performance and shorten the analysis time by up to half after optimization.

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## THE EFFECT OF pH ON THE KINETICS OF THE ELECTRODE PROCESS IN THE PRESENCE OF L-DOPA

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**Abstract:** The electroreduction of  $Zn^{2+}$  ions on a mercury electrode from a NaClO<sub>4</sub> solution at both pH=2.0 and pH=6.0 is a quasi-reversible process. The introduction of L-DOPA to a solution at pH=2.0 causes a slight inhibition of this process, and to a solution at pH=6.0 causes its significant acceleration. The stepwise nature of the analyzed electrode process was also demonstrated in both solutions, both in the absence and presence of L-DOPA.

Introduction: The kinetics of electrode reactions involving a mercury electrode depend on many factors. These include pH, temperature, and the presence of organic substances that, when adsorbed on the electrode surface, can accelerate or inhibit the electroreduction process. The acceleration effect ("cap-pair") occurs when an unstable active complex is formed on the mercury surface between the organic compound adsorbed on it and the depolarizer ions, facilitating the exchange of charge during the electrode reaction. For such a complex to form, the organic compound must meet three conditions: 1) contain atoms with free electron pairs that are capable of forming a coordination bond with the depolarizer ions, 2) be electrochemically inactive at the reduction potential of the depolarizer ions, and 3) cause an increase in the differential capacitance of the electrode/solution interface in this potential range, compared to the capacitance value corresponding to the base electrolyte [1]. In turn, the inhibition can be explained by the blocking of the electrode surface by the adsorbing molecules of organic compounds on it. This makes it difficult for depolarizing ions to access the electrode and, consequently, inhibits the electrode process. It is also possible that the adsorbed molecules do not affect the electrode process. The study were conducted to determine the effect of pH on the kinetic parameters of the electroreduction process of Zn<sup>2+</sup> ions in the presence of L-DOPA on a mercury electrode, using sodium perchlorate as the supporting electrolyte. The adsorption measurements and the determined kinetic parameters of the process allowed us to assess whether the drug adsorbs on the surface of the electrode and how its adsorption, affects the electroreduction rate. The pH values at which the measurements were conducted are pH=2.0, corresponding to the pH of gastric juice in the human body, and pH=6.0, which is close to the natural pH of the skin. L-DOPA is considered the gold standard in the treatment of motor symptoms of Parkinson's disease [2]. Studies involving L-DOPA indicate interactions between it and zinc ions [3]. The use of metal chelates, such as L-DOPA -  $Zn^{2+}$ , instead of large doses of L-DOPA alone, allows for optimal dosing and avoids the side effects of the drug. The obtained results can be successfully used as model systems for biological systems in explaining the pharmacological action of the drug.

**Experimental:** An electrochemical analyzer  $\mu$ Autolab by Eco Chemie was used for the study, equipped with a three-electrode system: a mercury electrode as the working electrode, a Ag/AgCl electrode as the reference electrode, and a platinum wire as the auxiliary electrode. SWV and CV voltammetry, DC polarography, and electrochemical impedance spectroscopy (EIS) were used.  $5.0 \cdot 10^{-3}$  mol·dm<sup>-3</sup> Zn<sup>2+</sup> was used as the depolarizer, 1 mol·dm<sup>-3</sup> NaClO<sub>4</sub> as the supporting electrolyte, and L-DOPA ( $5.0 \cdot 10^{-5} - 5.0 \cdot 10^{-3}$  mol·dm<sup>-3</sup>) as the substance affecting the change in the kinetics of the electrode reaction. All solutions were prepared immediately before the measurement using deionized water and analytical-grade reagents, and deoxygenated by purging them with nitrogen during the measurements. The experiments were conducted at the constant temperature of 298 K.

**Results:** Using a frequency of 800 Hz, differential capacitance curves of the double layer at the mercury/sodium perchlorate interface were recorded in solutions with pH=2.0 and pH=6.0 and with the addition of L-DOPA. These curves are presented in Fig.1. As can be observed, in the potential range of  $Zn^{2+}$  reduction (around -0.950 V), the presence of L-DOPA leads to an increase in the differential capacitance of the double layer compared to the capacitance of the supporting electrolyte. Thus, one of the conditions of the cap-pair rule is fulfilled. However, this increase is more pronounced in the solution with pH = 6.0.



**Fig.1.** Differential capacitance curves of the double layer at the mercury/NaClO<sub>4</sub> interface in a solution at pH=2.0 and pH=6.0 in the absence and the presence of increasing amounts of L-DOPA.

The results of the measurements conducted using CV and SWV voltammetry provide a qualitative assessment of the influence of L-DOPA on the kinetics of  $Zn^{2+}$  ion electroreduction in the analyzed systems (Table 1). Based on the CV voltammetry results, it can be observed that in the absence of the drug, the analyzed electrode process is quasi-reversible in both pH=2.0 and pH=6.0 solutions ( $\Delta E_{pH=2.0}=75$ mV,  $\Delta E_{pH=6.0}=76$ mV). The addition of increasing amounts of L-DOPA slightly decreases the reversibility of the  $Zn^{2+}+2e \rightarrow Zn_{(Hg)}$  reaction in the pH=2.0 solution, while in the pH=6.0 solution, it noticeably increases. The slight inhibitory effect of L-DOPA on the kinetics of  $Zn^{2+}$  electroreduction in the pH=2.0 solution is also indicated by a slight decrease in

SWV peak currents. In contrast, the catalytic effect of the drug in the pH=6.0 solution is evident from a significant increase in SWV peak currents.

<b>Table 1.</b> The changes in the difference between the anodic peak potential and the cathodic peak potentia	al
$\Delta E_{CV} = E_a - E_k$ and the peak currents in SWV for the electroreduction of $Zn^{2+}$ ions in 1 mol·dm <sup>-3</sup> NaClO <sub>4</sub> at p	эH =
2.0 and $pH = 6.0$ , as well as in the presence of L-DOPA.	

c [mol/dm <sup>-3</sup> ]	$\Delta E_{CV}$	[mV]	-I <sub>SWV</sub> [µA]		
CL-DOPA [IIIOI dill ]	pH=2.0	pH=6.0	pH=2.0	pH=6.0	
0	75	76	43.68	45.34	
5.0.10-5	76	75	42.39	47.03	
1.0.10-4	77	73	41.93	49.80	
5.0.10-4	80	65	39.50	60.36	
1.0.10-3	83	60	39.34	73.73	
2.5.10-3	85	53	39.62	96.10	
5.0 10-3	85	49	39.40	117.1	

Based on the CV curves, the values of reversible half-wave potentials were determined, which change only slightly with variations in drug concentration in both the pH=2.0 and pH=6.0 solutions. This indicates the formation of unstable depolarizer ion-L-DOPA complexes in the solution or their complete absence [4]. The increase in the activation resistance value associated with the electrode reaction, determined from EIS spectra, with the increasing concentration of L-DOPA in the solution at pH=2.0 confirms its inhibitory effect on the Zn<sup>2+</sup> electroreduction rate. In turn, a significant decrease in the value of this parameter in the presence of the drug in the solution at pH=6.0 indicates the catalytic effect of L-DOPA (Fig.2).



**Fig.2.** Impedance diagrams measured at the formal potential for the electroreduction of Zn<sup>2+</sup> in 1 mol dm<sup>-3</sup> NaClO<sub>4</sub> and in the presence L-DOPA at pH=2.0 and pH=6.0.

Based on the obtained measurement results, the values of the  $Zn^{2+}$  electroreduction rate constants in the presence of L-DOPA were determined as a function of the electrode potential [4]. The nonlinear nature of these changes indicates the two-step nature of the analyzed electrode reaction. Subsequently, using these relationships and the known values of formal potentials, the standard rate constants for the first and the second stages of  $Zn^{2+}$  electroreduction were calculated (Fig.3).



**Fig.3.** The dependences of individual standard rate constants  $k_{s1}$  and  $k_{s2}$  for the electroreduction of  $Zn^{2+}$  in 1 mol·dm<sup>-3</sup> NaClO<sub>4</sub> vs. L-DOPA concentrations at pH=2.0 (O) and pH=6.0 ( $\bullet$ ). Dashed lines indicate the values of  $k_{s1}$  and  $k_{s2}$  relating to the solution without L-DOPA.

An increase in L-DOPA concentration in the solution at pH=2.0 causes a slight decrease in the value of  $k_{s1}$  and a somewhat more pronounced decrease in  $k_{s2}$ . In the solution at pH=6.0, a significant acceleration of both the first and second stages of charge transfer between the electrode and the depolarizer ions is evident. It is worth noting that the values corresponding to the first electron transfer step are always lower than those for the second electron transfer. Therefore, the first step determines the overall process rate, both in the presence and absence of the drug.

**Conclusions:** The analysis of the influence of L-DOPA on the kinetics of zinc ion electrodeposition on mercury in NaClO<sub>4</sub> solutions at different pH levels indicated an accelerating effect of the drug at pH=6.0 and an inhibitory effect at pH=2.0. The observed changes increase with rising L-DOPA concentration and are significantly more pronounced in the case of the catalytic effect. The differences in the effect of L-DOPA on the  $Zn^{2+}$  electroreduction kinetics at pH=2.0 and pH=6.0 are likely due to the different forms of the compound depending on the pH. This results in changes in the adsorption properties of L-DOPA on mercury and alterations in its ability to form active complexes between the adsorbed forms of the drug and  $Zn^{2+}$  ions on the electrode surface. Consequently, it can be assumed that in the solution at pH=6.0, active complexes are formed on mercury, facilitating charge transfer during the analyzed electrode process. In the solution at pH=2.0, the cationic form of L-DOPA adsorbed on the surface, lacking a free electron pair at the nitrogen atom, does not form an active complex and instead blocks the access of depolarizer ions to the electrode, thus hindering the electrode reaction.

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## THE ROLE OF SELECTED NEUROTRANSMITTERS IN THE KINETICS OF ELECTRODE REACTION

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**Abstract:** Based on measurements conducted using SWV and CV voltammetry as well as EIS impedance spectroscopy, the catalytic effect of  $\gamma$ -aminobutyric acid on the electroreduction rate of Zn<sup>2+</sup> ions and the inhibitory effect of L-aspartic acid were demonstrated. These effects increased with the concentration of the neurotransmitter. A small inhibitory effect of glycine was also proven, but it was not dependent on changes in its concentration.

Introduction: Zinc, as a trace element, is essential for life. Maintaining zinc homeostasis is crucial for proper brain function, and its deficiency or excess can brain damage and exacerbate neurological contribute to disorders [1]. Amino acid neurotransmitters are amino acids present in the body that serve, among other functions, as carriers of information in the nervous system. The interaction between amino acid neurotransmitters and zinc plays a significant role in the functioning of the nervous system, particularly in synaptic processes and neuropal neuroplasticity [2]. The study presented below focuses on qualitative determination how three selected compounds (Fig.1):  $\gamma$ -aminobutyric acid (GABA), glycine (GLY), and L-aspartic acid (LAA) which act as amino acid neurotransmitters, affect the rate of  $Zn^{2+}$  ion electroreduction on mercury electrode. Due to the similar working potential of mercury electrode to potential at the cell membrane interface, the obtained results can be considered as a helpful in explaining the role of zinc in modulating amino acid neurotransmitters.



**Experimental:** In the study the electrochemical analyzer  $\mu$ Autolab with FRA module (EcoChemie, Netherlands) was used, cooperating with an electrode stand (MTM Kraków) adapted for use with a working electrode with controlled growth of a drop (CGMDE). Measurements were conducted in thermostated vessels using a threeelectrode system: a mercury electrode (working electrode), a silver/silver chloride electrode with saturated NaCl (reference electrode), and a platinum spiral (auxiliary electrode). The CGMDE mercury electrode was used either as a dropping mercury electrode (DME) or a hanging mercury drop electrode (HMDE). The study employed square wave voltammetry (SWV), cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). For the preparation of the solutions used in the study, analytical-grade reagents were used:  $\gamma$ -aminobutyric acid, glycine and L-aspartic acid from Sigma, Zn(NO<sub>3</sub>)<sub>2</sub>, acetic acid, and sodium acetate from Fluka. In the measurements, Zn<sup>2+</sup> ions at a concentration of 5.0·10<sup>-3</sup> mol·dm<sup>-3</sup> served as the depolarizer, while an acetate buffer with pH=6.0 was used as the supporting electrolyte. The analyzed amino acid neurotransmitters were used in the concentration range of 1.0·10<sup>-4</sup> mol·dm<sup>-3</sup> to 1·10<sup>-1</sup> mol·dm<sup>-3</sup>. All solutions were freshly prepared before each measurement using purified water from a Merck Millipore system and deoxygenated with nitrogen for 20 minutes.

**Results:** To qualitatively determine the influence of three selected amino neurotransmitters: GABA, GLY, and LAA on the rate of  $Zn^{2+}$  ion electroreduction on a mercury electrode, SWV and CV voltammograms were recorded, and EIS spectra were obtained at formal potentials. As an example, for the baseline solution  $(5.0 \cdot 10^{-3} \text{ mol}\cdot\text{dm}^{-3} \text{ Zn}^{2+})$  in acetate buffer) and for the highest applied neurotransmitter concentration of  $1 \cdot 10^{-1} \text{ mol}\cdot\text{dm}^{-3}$ , they are shown in Figs.2-4.



**Fig.2.** SWV voltammograms of the electroreduction of 5.0<sup>-</sup>10<sup>-3</sup> mol·dm<sup>-3</sup>Zn<sup>2+</sup> in the acetate buffer and in the presence of GABA, GLY, and LAA at a concentration of 1<sup>-</sup>10<sup>-1</sup> mol·dm<sup>-3</sup>.



Fig.3. CV voltammograms of the electroreduction of 5.0 10<sup>-3</sup> mol·dm<sup>-3</sup> Zn<sup>2+</sup> in the acetate buffer and in the presence of GABA, GLY, and LAA at concentration of 1 10<sup>-1</sup> mol·dm<sup>-3</sup>.



**Fig.4.** Impedance diagrams measured at the formal potential for the electroreduction of  $Zn^{2+}$  in the acetate buffer and in the presence  $1.0 \cdot 10^{-1}$  mol.dm<sup>-3</sup> of  $\Box$ -aminobutyric acid (GABA), glycine (GLY) and L-aspartic acid (LAA).

Table 1 shows the changes in SWV peak heights, the difference between anodic and cathodic peak potentials ( $\Delta E$ ) in CV voltammograms, and variations in activation resistance related to the electrode reaction ( $R_a$ ) obtained from EIS spectra as a function of neurotransmitter concentration. The increase in GABA concentration causes a gradual rise in the height of SWV peaks and a decrease in the values of  $\Delta E$  and  $R_a$ , which

indicates the catalytic effect of this compound on the kinetics of zinc electrodeposition on mercury [3].

The presence of glycine only slightly affects the mentioned parameters: it slightly decreases the height of the SWV peaks and increases the values of  $\Delta E$  and  $R_a$ . It can therefore be inferred that it slightly slows down the electrode reaction. It is worth noting that the increase in GLY concentration does not affect the magnitude of the inhibitory effect. LAA has more pronounced delaying effects on the electroreduction process, and the observed inhibition increases gradually with the drug concentration.

CI	$c_{\rm NI}$									
		GABA			GLY			LAA		
[mol:dm <sup>-3</sup> ]	$\Delta E_{CV}$	-I <sub>SWV</sub>	Ra <sub>EIS</sub>	$\Delta E_{CV}$	-I <sub>SWV</sub>	Ra <sub>EIS</sub>	$\Delta E_{CV}$	-I <sub>SWV</sub>	R <sub>a EIS</sub>	
[mor um ]	[mV]	[µA]	$[\Omega cm^2]$	[mV]	[µA]	$[\Omega cm^2]$	[mV]	[µA]	$[\Omega cm^2]$	
0	70	22.63	5.85	70	22.63	5.85	70	22.63	5.85	
1.0.10-4	69	22.89	5.85	69	22.36	6.25	70	22.34	5.95	
5.0.10-4	69	22.49	5.83	70	22.49	6.22	-	-	-	
1.0 10-3	69	22.66	5.8	71	22.23	6.01	70	22.07	5.95	
2.5.10-3	68	23.47	5.72	-	-	-	-	-	-	
5.0.10-3	67	23.81	5.38	69	22.48	6.22	71	22.04	6.12	
1.0.10-2	66	24.61	4.92	70	21.75	6.22	72	21.49	6.15	
2.5.10-2	60	27.58	3.79	-	-	-	-	-	-	
5.0 10-2	56	29.46	2.94	-	-	-	73	21.19	6.48	
7.5.10-2	53	30.97	2.51	-	-	-	74	19.91	6.50	
1.0.10-1	52	32.19	2.24	71	22.15	6.23	74	13.38	6.54	

**Table 1.** The changes in the difference between the anodic peak potential and the cathodic peak potential  $\Delta E_{CV}=E_a-E_k$ , the peak currents in SWV and the activation resistance (R<sub>a</sub>) obtained from EIS spectra of the electroreduction of Zn<sup>2+</sup> as a function of neurotransmitters concentration  $c_{NT}$ .

**Conclusions:** The increase in concentration of  $\gamma$ -aminobutyric acid accelerates the electroreduction of zinc ions on a mercury electrode in the acetate buffer solution with pH=6.0. This is evidenced by the increased peak heights in SWV and a decrease in the distance between the anodic and cathodic peaks on the CV voltammograms, as well as a reduction in the activation resistance value of the electrode reaction determined from the EIS spectra. a different effect is observed with the increase in the concentration of L-aspartic acid. Here, a slowing down of the analyzed electrode process is observed. A slightly different, very small, and concentration-independent effect can be observed in the presence of glycine. The electroreduction of Zn<sup>2+</sup> ions is slightly inhibited. The reasons for such different influences should be sought in the adsorption properties of the studied compounds. Adsorption analysis of  $\gamma$ -aminobutyric acid, glycine, and L-aspartic acid will be the subject of further studies.

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## A NEW TYPE OF NITRATE ION-SELECTIVE ELECTRODE – THE APPLICATION OF PERINONE POLYMER-BASED HYBRID MATERIAL AS AN INTERMEDIATE LAYER

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**Abstract:** The present work shows the application of a new type of hybrid material based on polymer and carbon material, which was used as an intermediate layer. The hybrid material consisted of perinone polymer (applied by potentiodynamic polymerization) and multi-walled carbon nanotubes (applied by drop-casting). The comparison electrodes were an unmodified electrode and an electrode coated only with perinone polymer. Glassy carbon electrodes coated with such a solid contact were covered with a nitrate ion-sensitive membrane. The use of carbon material allowed to obtain much better stability – the drift for the electrode coated with GCE/PPer/ISMM perinone polymer was 0.24 mV/min, while for the electrode coated with GCE/PPer:MWCNTs/ISM hybrid material was equal to 0.11 mV/min. Similar results were obtained for potential reversibility.

**Introduction:** Compounds containing nitrate ions (NO<sub>3</sub><sup>-</sup>) can easily be found in soil and water, which in itself does not constitute a threat as long as their concentration remains at a low, stable level. The problem arises when their amount exceeds acceptable standards. Excess nitrates should be especially monitored in industrial and agricultural areas, as they end up in the soil and groundwater along with fertilizers and wastewater, harming the fauna, flora, and humans. Long-term exposure to high concentrations of nitrates leads to soil degradation, causing acidification and leaching of nutrients, which reduces crop yields. In aquatic ecosystems, excess nitrates contribute to excessive algal growth, reducing the amount of oxygen in the water. As a result, there is a massive dieoff of fish and other aquatic organisms [1]. According to WHO recommendations, the maximum concentration of nitrate above which it becomes dangerous for humans is  $50 \text{ mg/L NO}_3$ . Long-term consumption of drinking water with nitrate levels above the standard can increase the risk of cancer and lead to methemoglobinemia, known as "blue baby disease" [2]. Therefore, it is extremely important to determine nitrate ions. One of the methods used for this purpose is potentiometry, based on the use of ion-selective electrodes (ISEs). Of the various types of these electrodes, the most popular are liquid contact electrodes (LCISEs) and solid contact electrodes (SCISEs). There is an increasing trend to move away from the design of LCISEs in favor of SCISEs - the lack of an internal solution in these electrodes greatly facilitates measurements and their operation but also presents new challenges [3]. The lack of internal electrolyte leads to reduced efficiency of ion transfer and transduction into charges, which results in poor stability and potential reversibility of such electrodes. To improve their parameters, solid contact materials such as carbon materials, conducting polymers, as well as composite and hybrid materials are used in the construction of electrodes [4,5]. This improves ionto-electron conductivity, resulting in better potential stability and reversibility [6]. This paper describes the use of hybrid materials in the role of a solid contact. They consist of two components – perinone polymer (PPer) and multi-walled carbon nanotubes (MWCNTs), which were implemented in two ways, differing in the order of application of each component.

**Experimental:** Before the application of the mediation layers, the glassy carbon electrodes were carefully polished on moistened Al<sub>2</sub>O<sub>3</sub> placed on a special felt. The electrodes were then cleaned of alumina residue in an ultrasonic bath, followed by rinsing with distilled water, and degreasing by immersion in tetrahydrofuran (THF). A suspension of multi-walled carbon nanotubes was prepared by dispersing 15 mg of nanomaterial in 3 ml of THF. The mixture was homogenized for 2 hours in an ultrasonic bath. Meanwhile, a membrane mixture was prepared, consisting of 62% NPOE (2-nitrophenyl octyl ether), 32% PVC (poly(vinyl chloride)), and 6% TDMANO<sub>3</sub> (tridodecylmethylammonium nitrate) dissolved in THF (0.3 g of the components in 3 ml of THF). Four types of electrodes were obtained: (1) GCE/ISM - an unmodified GCE electrode with an ion-sensitive membrane: (2) GCE/PPer/ISM – an electrode to which a layer of perinone polymer was applied by potentiodynamic polymerization in 10 cycles using cyclic voltammetry (the detailed procedure is described in publication [7]); (3) GCE/MWCNTs:PPer/ISM – a GCE electrode first coated with a layer of MWCNTs (10  $\mu$ L of a 5 mg/mL suspension was drop-casted) and then subjected to PPer application for 10 cycles; (4) GCE/PPer:MWCNTs/ISM – an electrode first coated with a layer of perinone polymer for 10 cycles and then coated with a 10  $\mu$ L suspension of carbon nanotubes. The membrane on each of the prepared electrodes (directly on the GCE – control electrode, or on the intermediate layer) was drop-casted in three steps every 30 minutes, 50 µL for each layer.

**Results:** After conditioning the electrodes in  $1 \cdot 10^{-3}$  M potassium nitrate solution for several days, calibration was performed - it was aimed at evaluating the correctness of individual electrode performance, including determining the sensitivity (slope), detection limit, and working range of the electrodes. Calibration was performed in the concentration range from  $1 \cdot 10^{-7} - 1 \cdot 10^{-1}$  M by appropriate additions of KNO<sub>3</sub> standards of  $1 \cdot 10^{-3}$ ,  $1 \cdot 10^{-2}$ ,  $1 \cdot 10^{-1}$  and 1 M to 50 mL of distilled water. The determined parameters calculated from the characteristics of the ISEs are listed in Table 1. The introduction of intermediate layers improved the response of the electrodes by increasing their sensitivity, but the change was not significant relative to GCE/ISM.

Electrode	Slope [mV/decade]	Linearity range [M]	Detection limit [M]
GCE/ISM	-55.78	$1 \cdot 10^{-5} - 1 \cdot 10^{-1}$	5.14.10-6
GCE/PPer/ISM	-57.54	$5 \cdot 10^{-6} - 1 \cdot 10^{-1}$	3.12.10-6
GCE/MWCNTs:PPer/ISM	-57.96	$5 \cdot 10^{-6} - 1 \cdot 10^{-1}$	2.12.10-6
GCE/PPer:MWCNTs/ISM	-58.14	$5 \cdot 10^{-6} - 1 \cdot 10^{-1}$	2.43.10-6

Table 1. Parameters determined on the basis of electrodes' response.

The most important parameters determining whether the introduced intermediate layer fulfills the specified requirements are the reversibility and stability of the potential. We

determine the reversibility of the potential by alternately measuring the potential in KNO<sub>3</sub> solutions of  $1 \cdot 10^{-3}$  and  $1 \cdot 10^{-4}$  M concentrations. Based on the results, standard deviations from the average value for each concentration are calculated (the values of these parameters are placed in Table 2). A graph showing the potential reversibility of all tested electrodes is presented in Fig.1.

 Table 2. Potential reversibility of tested electrodes expressed as standard deviation from mean potential value measured in successive measurements (n=3).

Standard deviation from mean potential value for certain NO <sub>3</sub> <sup>-</sup> concentration	GCE/ ISM	GCE/PPer/ISM	GCE/MWCNTs:PPer/ISM	GCE/PPer:MWCN Ts/ISM
1·10 <sup>-3</sup> M	7.07	1.18	1.31	1.86
1·10 <sup>-4</sup> M	9.16	1.83	2.61	2.06



Fig.1. Reversibility of the electrodes' potential.

The stability of the potential was determined in a solution of  $1x10^{-3}$  M KNO<sub>3</sub> by measuring the potential for a period of one hour, and then the drift value was calculated as the difference in potential ( $\Delta E$ ) per unit time ( $\Delta t$ ). The values of this parameter are included in Table 3.

 Table 3. Short-term potential stability obtained for tested electrodes.

Electrode	GCE/ISM	GCE/PPer/ISM	GCE/MWCNTs:PPer/ ISM	GCE/PPer:MWCN Ts/ISM
Potential drift [mV/min]	0.48	0.24	0.11	0.11

Based on the analysis of the results obtained for both potential reversibility and stability measurements, improvements in the transfer process and ion-to-charge conversion were obtained for all of the modified electrodes in comparison to GCE/ISM. The best stability result was obtained for electrodes modified with hybrid materials, as evidenced by more
than 4 times lower potential drift for GCE/MWCNTs:PPer/ISM and GCE/PPer:MWCNTs/ISM (for both it is 0.11 mV/min) compared to GCE/ISM, where the drift equals 0.48 mV/min. We can draw similar conclusions from the deviation values for potential reversibility – the introduction of an intermediate layer improved potential reversibility – in this case, the electrode modified only with perinone polymer performed best.

Measurements were also carried out to determine the effect of the external conditions on the potential values (gas and light) in a  $1 \cdot 10^{-3}$  KNO<sub>3</sub> solution. For this purpose, alternating measurements were carried out in darkness and light, as well as in the presence of gases (O<sub>2</sub>, CO<sub>2</sub>) and without them. The study showed that the electrodes modified with the hybrid material showed better resistance to changes in conditions than the unmodified electrode and the PPer-modified electrode.

**Conclusions:** The purpose of this study was to determine the possibility of using a hybrid material as a solid contact in SCISEs. It was investigated whether the introduction of an additional carbon material into the intermediate layer in the form of PPer would have a beneficial effect and further improve the performance. It was shown that there were no significant changes in the response of the electrodes (each of the modified electrodes showed very good slope, linearity, and detection limit). The greatest changes were observed in potential stability (these parameters improved significantly over GCE/ISM). The hybrid material provided more than 2 times better stability than a solid contact consisting of PPer alone. In the case of potential reversibility, for SCISE modified with the perinone polymer, better reversibility values were obtained, but the hybrid material was more stable under different measurement conditions, giving a stable potential regardless of illumination and the presence of gases.

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## APPLICATION OF A NOVEL SOLID BISMUTH MICROELECTRODE ARRAY FOR THE DETERMINATION OF INORGANIC IONS BY ANODIC AND ADSORPTIVE STRIPPING VOLTAMMETRY

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**Abstract:** This article presents the results of a study on the determination of low concentrations of inorganic ions by anodic and adsorptive stripping voltammetry using a new type of working electrode - a solid bismuth microelectrode array. The presented solid metal microelectrode array is an interesting alternative to the commonly used metal film electrodes and solid metal electrodes of conventional sizes.

Introduction: Microelectrodes are a specific group of working electrodes used in stripping analysis and their use provides several advantages. These advantages include: the possibility of carrying out measurements in unmixed solutions (due to the occurrence of spherical diffusion on the surface of microelectrodes), which simplifies the measurement procedure and makes it possible to carry out the measurements in field conditions. The small sizes of the working microelectrodes enable to provide the analysis of samples of very small volume and to carry out the measurements from solutions with a low concentration of supporting electrolyte and from solutions of organic solvents [1,2]. In addition, as compared to working electrodes of conventional sizes, the use of microelectrodes results in a more favourable signal-to-noise ratio. A particular example of a working microelectrodes are solid metal microelectrodes. In addition to the advantages mentioned above, this type of microelectrodes offers the following benefits: simplification of the measurement procedure and elimination of toxic wastes, since the metal ions used to form a film of this metal on the surface of the working microelectrode need not to be added to the supporting electrolyte solution. In addition, the amount of electrode material (e.g. metallic bismuth) required for their construction is limited by a very small size of the proposed working microelectrodes. The main disadvantage of working with microelectrodes is the low, noise-sensitive intensity of the recorded current. To overcome this drawback, arrays of tens or even hundreds of microelectrodes are constructed. The recorded currents are amplified because the current of such arrays are the sum of currents of the individual microelectrodes that make up the arrays, and therefore are more resistant to interferences. The reason for designing solid metal microelectrodes was to find an alternative to metal film electrodes. During measurements using metal film electrodes, toxic ions of a given metal are introduced into the supporting electrolyte to reduce them and form a thin metal film on a given, usually carbon, substrate. In addition, the working microelectrodes have found the application in the measurements carried out using double preconcentration and double stripping steps mode, where it is necessary to use the microelectrode as a second working electrode. Furthermore, the solid metal microelectrodes offer an interesting alternative to conventionally sized solid metal electrodes, due to the small amount of metal required for their construction. The solid metal working microelectrodes discussed here have found the application in the determination of inorganic ions by anodic and adsorptive stripping voltammetry.

**Experimental:** The measurements were conducted using  $\mu$ Autolab analyser made by Eco Chemie (the Netherlands). A traditional three-electrode cell with a volume of 10 mL was used. A solid bismuth microelectrode array was polished daily using an abrasive paper of 2500 grit and then cleaned in the ultrasonic cleaner Sonic-3 (Polsonic, Poland). Pt wire and Ag/AgCl/NaCl<sub>sat</sub>, were used as an auxiliary and reference electrode, respectively.

**Results**: Preliminary studies were carried out to investigate whether the presented smallsized working electrode exhibited microelectrode properties. The microelectrode properties were assessed by examining the rate of analyte transport to the working electrode surface under conditions where the solution was mixed and unmixed during the preconcentration stage. It was observed that the analytical signals obtained from solutions unmixed during the deposition step were about three to four times lower as compared to the mixed one, confirming the microelectrode properties of the solid bismuth microelectrode array. It was furthermore observed that the so-called activation step proposed previously [3] was crucial for the application of a solid bismuth microelectrode array in terms of obtaining well-shaped, higher and reproducible analytical signals of ions of interest. The activation step is based on the application of a short, high-negative-potential pulse to the metal-working microelectrode, during which the reduction of bismuth oxides is carried out. Bismuth oxides can be formed as a result of oxidation of electrode material with oxygen dissolved in the analyzed solution. In the course of the presented studies it was found that the application of the activation step significantly increased the sensitivity of determinations.

Determination of Cd(II) and Pb(II) by anodic stripping voltammetry: the solid bismuth microelectrode array was used for the simultaneous determination of Cd(II) and Pb(II) by anodic stripping voltammetry. A solution containing 0.05 mol/L acetate buffer (pH 4.6) was used as a supporting electrolyte. Measurements were carried out at the following change of microelectrode potential: each measurement was started with an activation step conducted at a potential of -2.5 V for 1 s. Then, the deposition step was carried out at a potential of -0.9 V within 60 s. The square wave voltammogram was recorded with a potential change in the range from -1.0 V to -0.3 V. Under the optimized conditions, the calibration graph for Cd(II) determination for deposition time of 60 s was linear in the range from  $5 \times 10^{-9}$  to  $2 \times 10^{-7}$  mol/L, while the calibration graph for Pb(II) determination was linear in the range from  $2 \times 10^{-9}$  to  $2 \times 10^{-7}$  mol/L. The interference studies indicated that a 100-fold excess of V(V), Ni(II), Co(II), Fe(III), Mn(II), Zn(II), a 10-fold excess of Mo(VI) and Cu(II) did not significantly influence the analytical signals of Cd(II) and Pb(II).

Determination of Co(II) by adsorptive stripping voltammetry: furthermore, a new type of solid bismuth microelectrode array was used for Co(II) determination by adsorptive stripping voltammetry. The measurements were conducted from a supporting electrolyte containing 0.1 mol/L ammonium buffer (pH 9.4). The complexing agent for Co(II) was nioxime at a concentration of  $5 \times 10^{-4}$  mol/L. The measurements were carried out with the following change in the potential applied to the microelectrode: first, a short potential

pulse of -3.0 V was applied to the microelectrode for 3 s. This stage is known as the microelectrode activation. This was followed by an accumulation step of Co(II)-nioxime complexes on the surface of a working microelectrode carried out at a potential of -0.7 V for 120 s. The analytical signal was obtained while a potential was changed in the range from -0.7 V to -1.3 V using the square wave technique. The measurements were performed without solution deoxygenation. After optimization of the analytical procedure, the calibration curve was found to be a straight line in the concentration range from  $5 \times 10^{-10}$  to  $1 \times 10^{-7}$  mol/L. It was found that a 1000-fold excess of V(V), Cu(II). W(VI), Mo(VI), Fe(III), Mn(II), Zn(II) ions, a 10-fold excess of Pb(II) and Ni(II) ions in 1:1 ratio to Co(II) concentration did not interfere with the analytical signal of Co(II). The correctness of the discussed procedures was checked by determining Co(II), Cd(II) and Pb(II) ions in certified reference materials. The agreement of the obtained results with the certified values led to the conclusion that the developed analytical procedures using a new type of solid bismuth microelectrode array can be used to determine these ions in environmental water samples. The validation results of the discussed analytical procedures are summarized in Table 1.

Determined ion	Certified material		
Cd(II)	6.77 (4.8%)	$7.11\pm0.45$	TM 26.5
Pb(II)	9.88 (4.3%)	$10.1\pm0.8$	TM 26.5
Co(II)	28.7 (4.2%)	$27.7\pm1.6$	TM 25.5

 

 Table 1. Summary of the results of Cd(II), Pb(II) and Co(II) ions determinations in certified reference materials performed using a solid bismuth microelectrode array.

(The relative standard deviation value is given in brackets.)

**Conclusions:** The presented solid bismuth microelectrode array is a particular voltammetric sensor. The main advantages resulting from its application are the extended operating life and the environmental friendliness due to the elimination of the bismuth film formation step and the simplification of a composition of the supporting electrolyte. The application of a solid bismuth microelectrode array offers the possibility of determining (i) metal ions by anodic stripping voltammetry that is limited by the oxidation potential of the electrode material and (ii) metal ions in the form of complexes with a complexing agent adsorbed on the surface of working microelectrode in the range of negative values of the accumulation potential using their reduction process. The short activation step introduced to the standard measurement procedure and carried out at high negative potential values, results in well-shaped, higher and reproducible analytical signals.

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### CHALLENGES IN MYCOTOXINS DETERMINATION IN HIGHLY PIGMENTED FRUITS

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**Abstract:** Mycotoxins are food and feed contaminants. They exhibit high toxicity to living organisms. Therefore, their concentration in products intended for consumption should be strictly monitored. Due to the increasingly stringent standards related to mycotoxin content in food, the requirements for mycotoxin determination methods are constantly increasing. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is a gold standard considering the determination of mycotoxins and many other groups of compounds. However, this technique is burdened with some limitations connected to e.g. matrix effect (ME). Thus, we proposed polymeric materials that can be applied during the sample preparation step toward mycotoxins determination via LC-MS/MS. In this paper, we present the preliminary results on evaluating ME toward 8 mycotoxins before and after the clean-up of blackcurrant extract employing microspheres of 4-vinylpyridine crosslinked with trimethylolpropane trimethacrylate (poly(4VP-*co*-TRIM) or 1,4-dimethacryloyloxybenzene (poly(4VP-*co*-14DMB).

Introduction: Mycotoxins are secondary metabolites produced by various species of filamentous fungi. They are formed on various agricultural commodities both before and after harvest. Fruits are especially susceptible to being infected since they can be easily mechanically damaged by insects and animal bites, during unfavorable weather conditions or harvesting and postharvest handling. Mycotoxins exhibit high toxicity toward living organisms [1,2]. They can cause cancer, allergies, and organ toxicity [3] as well as many other abnormalities and malfunctions in humans and animals, e.g. hepatocellular carcinoma, abnormalities in fetal development, renal failure, interstitial nephropathy, and urothelial tumors [4]. Various mycotoxins exhibit hepatotoxic, neurotoxic, teratogenic, and nephrotoxic properties [4]. Due to their hazardousness, the content of mycotoxins in food and feed should be strictly monitored. Extremely high restrictions are applied to baby food [5-7]. Therefore, the relevant authorities constantly update the acceptable levels of toxins in various types of food [5-7]. Due to the high toxicity of mycotoxins as well as the restriction imposed by the relevant institutions and consequently the regulatory recommendations, new LC-MS/MS methods characterized by lower limits of detection and quantification as well as lower ME are in demand. Therefore, considering our previous findings [8], we propose polymeric materials that could be applied in the dispersive solid phase extraction (dSPE) step during the sample preparation protocol before the LC-MS/MS determination of mycotoxins in soft and highly pigmented fruits. We found that the polymeric materials effectively adsorbed polyphenolic compounds (especially flavonoids and anthocyanins) [8]. Their effectiveness was even greater than primary secondary amine and graphitized carbon black toward flavonoids removal [8]. Thus, studied polymers have the potential to be used to purify extracts of highly pigmented fruits before LC-MS/MS analysis.

Experimental: Microspheres were washed with acetonitrile (ACN, hypergrade) (4 times x 4 min (shaken), the ratio of polymeric material to ACN was 10 mg: 1 mL. Each time the mixture was centrifuged after shaking, and the organic solvent was discarded. Before further use, polymeric material was evaporated to dryness in the Genevac EZ-2 Elite Personal Evaporator to remove ACN residue. To determine the ME, 10 g of fruit pulp (blackcurrant) was vigorously hand-shaken with 10 mL of ACN for 1 min. Then, QuEChERS salt (1 g MgSO<sub>4</sub>, 0.25 g NaCl, 0.25 g sodium citrate, 0.125 g sodium hydrogen citrate sesquihydrate) was added and the sample was mixed again for 1 min followed by centrifugation (10 min,  $8228 \times g$ ) using the 5408 Eppendorf centrifuge. Then, 1 mL of the obtained extract was transferred into a tube containing 10 mg of poly(4VP-co-TRIM) or poly(4VP-co-14DMB) (dSPE step), shaken for 2 min, and centrifuged using the 5804 Centrifuge for 10 min at 16000  $\times$  g. A 600  $\mu$ L of purified supernatant was transferred into a glass vial and evaporated until dry. The residue was dissolved in 600  $\mu$ L of H<sub>2</sub>O:0.2 mol/L NH<sub>4</sub>HCO<sub>3</sub>:CH<sub>3</sub>OH (30:5:65,  $\nu/\nu/\nu$ ), vortexed and sonicated. The obtained solution was fortified or not with a mixture of mycotoxins standards. ME was calculated using the equation:  $ME[\%] = A/B \cdot 100$ , where a is the average area of the analyte signal in the sample fortified before extraction, B is the average analyte signal in a standard solution prepared in a neat solvent [9]. The concentration levels of fortification (A and B) are presented in Table 1.

Mycotoxins	CAS	Molecular formula	Levels of sample fortification [µg/kg]
Penicillic acid	90-65-3	$C_8H_{10}O_4$	25
Fusarenon X	23255-69-8	$C_{17}H_{22}O_8$	50
Neosolaniol	36519-25-2	$C_{19}H_{26}O_8$	25
Aflatoxin B <sub>1</sub>	1162-65-8	$C_{17}H_{12}O_6$	3.125
Aflatoxin G1	1165-39-5	$C_{17}H_{12}O_{7}$	6.25
Aflatoxin G2	7241-98-7	$C_{17}H_{14}O_{7}$	3.125
Ochratoxin A	303-47-9	C <sub>20</sub> H <sub>18</sub> ClNO <sub>6</sub>	25
T-2 toxin	21259-20-1	$C_{24}H_{34}O_9$	12.5

 Table 1. Selected mycotoxins, their basic characterization, and the levels of fortifications in experimental samples.

The prepared samples were centrifuged (10 min, 16000 × g) and filtered through 0.22 µm syringe hydrophilic filters. a sample was analyzed in triplicate using the 1290 series UHPLC system (Agilent Technologies, Santa-Clara, CA, USA) consisting of a degasser, binary pump, autosampler, column thermostat and controlled by Agilent MassHunter Acquisition software v.B.08. The apparatus was coupled with a 6460 triple quadrupole mass spectrometer (Agilent Technologies) equipped with an electrospray ion source (Agilent Jet Stream). For chromatographic separation, an HPLC column Shimpack Scepter C18-120, 1.9 µm, 2.1 mm x 100 mm was used. The mobile phase components were: H<sub>2</sub>O:0.2 mol/L NH<sub>4</sub>HCO<sub>3</sub> (95:5,  $\nu/\nu$ ) (solvent A) and CH<sub>3</sub>OH:0.2 mol/L NH<sub>4</sub>HCO<sub>3</sub> (95:5,  $\nu/\nu$ ) (solvent B). The flow rate and the temperature

were set to 0.25 mL/min and 40 °C. To obtain appropriate separation of the signals, the following gradient program was used: 0-2 min 5-20% solvent B, 2-4 min 20-40% solvent B, 4-10 min 40-95% solvent B, 10-12 min 95% solvent B, and postrun 2 min. The detection was performed using dynamic Multiple Reaction Monitoring (dMRM) mode (Table 2). Data were analyzed with Agilent MassHunter Quantitative Analysis software v.B.07.

Mycotoxins	RT [min]	Transitions	F [V]	CE [eV]
Penicillic acid	3.25	171 > 153 171 > 125	60 60	4 10
Fusarenon X	5.34	372.1 > 355 372.1 > 247	80 80	8 8
Neosolaniol	5.62	400.1 > 185 400.1 > 214.9	80 80	10 24
Aflatoxin B <sub>1</sub>	7.96	313 > 285 313 > 270	100 100	24 34
Aflatoxin G <sub>1</sub>	7.38	329 > 243 329 > 311	140 140	24 24
Aflatoxin G <sub>2</sub>	7.07	331 > 313 331 > 285	80 80	30 30
Ochratoxin A	7.55	404 > 358 404 > 239	100 100	10 24
T-2 toxin	9.30	484.2 > 185 484.2 > 215	100 100	14 16

Table 2. Settings of the dMRM method applied for selected mycotoxins determination in positive polarity.

Results: Our study aimed to assess the ME during the determination of selected mycotoxins (Table 1) via LC-MS/MS analysis. We chose the blackcurrant matrix as a representative of a highly pigmented fruit. To the clean-up step, we applied two polymeric materials: poly(4VP-co-TRIM) and poly(4VP-co-14DMB). The results from the preliminary studies indicated that studied polymers decreased ME during the quantitative determination of selected mycotoxins via LC-MS/MS. The reduction of ME was observed in the case of penicillic acid, aflatoxin  $B_1$ , aflatoxin  $G_1$ , aflatoxin  $G_2$ , and ochratoxin a (Table 3). In other cases, ME remained constant or slightly increased (fusarenon X, neosolaniol, T-2 toxin) (Table 3). The results are promising. Due to the disadvantages of commercially available dSPE material, searching for new effective materials for toxins determination is an up-to-date research goal. ME can be assessed considering the following division: negligible (ME > 90 and <110%), soft (80–90% and 110-120%; medium (50-80% and 120-150%); and strong (ME < 50% and <150%) [9]. Furthermore, ME values >100% and <100% mean ion enhancement and suppression, respectively. In terms of LC-MS/MS analysis of unpurified blackcurrant extract, medium and strong suppression of mycotoxin signals is observed. However, the application of poly(4VP-co-TRIM) or poly(4VP-co14DMB) improves the studied parameter. Moreover, the color of the acetonitrile extract of the blackcurrant after dSPE with poly(4VP-co-TRIM) or poly(4VP-co-14DMB) was reduced significantly in comparison to the unpurified extract.

RT – retention time; F – fragmentor voltage; CE – collision energy

<b>Table 3.</b> The ME of determined mycotoxins in samples with and without the dSPE step ( $h=2$ ).							
$ME \pm SD [\%]$							
-co-TRIM							
$\pm 0.63$							
$\pm 0.24$							
$\pm 0.39$							
$\pm 1.84$							
$\pm 1.30$							
$\pm 2.44$							
$\pm 3.51$							
± 1.09							
$\pm 0.24$ $\pm 0.39$ $\pm 1.84$ $\pm 1.30$ $\pm 2.44$ $\pm 3.51$ $\pm 1.09$							

Science and industry – challenges and opportunities

SD - standard deviation

**Conclusions:** The application of poly(4VP-*co*-TRIM) or poly(4VP-*co*-14DMB) as sorbents in the dSPE step improves the analytical performance (especially ME) of the LC-MS/MS method for the multi-mycotoxins determination. The reduction of ME of a greater number of studied mycotoxins is significant, thus our next step will be the optimization of various sample preparation steps to obtain the best analytical parameters that will meet the current recommendations for analytical methods.

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### INNOVATIONS IN THE REMOVAL OF PER- AND POLYFLUOROALKYL SUBSTANCES FROM AQUEOUS MATRIX

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**Abstract:** Per- and polyfluoroalkyl substances (PFAS) are considered emerging pollutants. They constituted a large group of water-soluble and persistent organic compounds. PFAS are widely used in various branches of industry, for example, they can be applied during the production of furniture, textiles, carpets, industrial surfactants, additives, protective coatings, firefighting foams, and all kinds of packaging. From these applications, the substances are likely to end up in the environment. Their relatively high concentration in environmental samples (e.g. wastewater, river water, lake water, drinking water, stormwater, groundwater, and soil) is very concerning due to their high toxicity toward living organisms. Therefore, new, effective, environment-friendly, low-cost approaches for PFAS removal from water samples are in demand.

Introduction: Per and polyfluoroalkyl substances have been extensively exploited in various ranges of industries, manufacturing, and consumer goods. The broad range of applications results from their unique properties. This large group of fluorine-containing organic compounds is environmentally persistent. Thus they are recognized as "forever chemicals" [1]. PFAS are also characterized by thermal stability due to the presence of the C–F bound, which is the strongest covalent bond in organic chemistry [2]. They have both hydrophobic and lipophobic properties due to the low polarizability of fluorine atoms [3]. It has been reported that PFAS concentrations in aqueous medium vary from pg/L to mg/L [4]. It is very concerning because PFAS are hazardous and toxic to living organisms. PFAS could enter the human body after oral ingestion. Moreover, they are not metabolized. Their concentration in human tissue and blood serum is approximately ng/mL [3]. PFAS causes reproductive and developmental problems, endocrine disruption as well as cancers [5]. Moreover, two of the most abundant PFAS (long-chain PFOA and PFOS) are probably linked to a high incidence of thyroid disease, kidney and testicular cancer, high cholesterol, ulcerative colitis, and pregnancy-induced hypertension in humans [3]. Nowadays, there are cost-effective methods for PFAS removal that are utilized in water utilities (such as Granular Activated Carbon (GAC) filters, reverse osmosis, or ion-exchange resins) [1]. However, most drinking water treatment plants do not remove PFAS before supplying water to the public [3,6]. The applied methods were not purposed to precisely bind PFAS, thus the contaminants might pass through these systems without destruction/sorption [1]. There is a great need to design PFAS-specific adsorbents and treatment procedures for the overall destruction of PFAS. At present, researchers are searching for new approaches for capturing/removing PFAS. Amen et al.

[1] divide these techniques into four groups: adsorption, membrane separation technology, fractionation techniques, and destruction techniques. This short review is focused on the first and last ones. Among first of them, the most studied materials are biochar, activated carbon, metal-organic framework, as well as ion exchange resins [1]. Vu et al. added also minerals, polymers, and biomaterials to this list [7]. Adsorption has many advantages due to its simplicity, effectiveness, low cost, and environmental friendliness. The most widely used adsorption materials for PFAS removal are both resins and carbonaceous materials (especially activated carbon) [7]. Researchers are searching for new approaches for enhancing the adsorption capacity of carbon-based materials toward PFAS adsorption. Moreover, they want to overcome some limitations linked to the application of these materials (e.g. relatively high cost, poor regeneration, non-selectivity towards specific PFAS, and susceptibility to the effect of coexisting substances like dissolved organic matter) [7]. Whereby, they proposed some new materials, e.g. adsorbents with improved surface basicity, multi-walled carbon nanotubes engineered with carboxyl and hydroxyl groups, carbonaceous materials combined with polymeric substances (e.g. polyacrylonitrile fiber-derived activated carbon fibers) and others [7]. The predominant forces that govern the adsorption of PFAS on several adsorbent materials are electrostatic and hydrophobic interactions [3]. Other mechanisms are hydrogen bonding and covalent bonding [3]. The mechanism is also affected by PFAS molecular structure (e.g. long or short chain), the physicochemical characteristics of the adsorbent (i.e., surface functional groups, polarity, and porosity), as well as matrix composition [3]. The main goal of the application of destructive technologies towards PFAS removal is to decompose pollutants into safer by-products [1]. Amen et al. mentioned several approaches (e.g. electric field-nanofiltration, electrochemical water treatment technology, direct current plasma, plasma-based technologies, and catalytic ozonation) that are effective in degrading PFAS (efficiency ranging from 60% to 100%) [1]. To avoid secondary pollution (e.g. after PFAS adsorption, due to the slow sorption rate), various redox treatment processes like electrochemical, photocatalytic, photolytic, photochemical, sonochemical, radiochemical, and thermochemical have been used. The goal of this step is to completely mineralize PFAS compounds. Among all listed above, photocatalytic degradation is promising due to the mild required conditions as well as relatively high efficiency [8].

**Experimental:** Our study aimed to verify the effectiveness of modified bismuth tungstate (Bi<sub>2</sub>WO<sub>6</sub>) towards photocatalytic activity for the photodegradation of selected PFAS, namely perfluorohexanoic acid (PFHxA, molecular formula:  $C_5F_{11}COOH$ , CAS 307-24-4). In the studied materials, Bi<sup>3+</sup> was partially substituted for Zn<sup>2+</sup>. The doping of Zn<sup>2+</sup> in Bi<sub>2</sub>WO<sub>6</sub> was as follows: 0 at%, 1 at%, 2.5 at%, 7.5 at%, 12.5 at%, 17.5 at%, 22.5 at% [9]. After the dispersion of material in an aqueous solution of PFHxA and the achievement of the adsorption-desorption equilibrium, the mercury lamp (emitting radiation centered: 500–550 nm, intensity: 7.31–7.53 mW/cm<sup>2</sup>, photon flux: 20.83 × 1019 m<sup>2</sup>/s) was turned on [8]. During the photocatalytic reaction, samples were collected at different times (till 120 min). The analyte was quantified using a fast liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF/MS) method using a 1200 Series high-performance liquid chromatograph (Agilent Technologies) equipped with an autosampler quaternary pump with degasser, column thermostat, and tandem mass spectrometer (Agilent Technologies 6538 UHD Accurate Mass Q-TOF

LC/MS) equipped with a dual ESI ion source. The appropriate separation was achieved using analytical column Zorbax Eclipse Plus C18 rapid resolution HT (2.1 x 50 mm, 1.8  $\mu$ m) as well as aqueous 5 mmol/L ammonium formate (A) and methanol (B). Column temperature was set to 40 °C. Ionization was proceed in the negative ion polarity mode. Ion source gas parameters were as follows: temperature 280 °C, flow rate 9 L/min. Moreover, capillary potential, fragmentor, and nebulizer pressure were set to 3500 V, 80 V, and 35 psi. Ions were acquired in MS scan mode at 50 -1000 *m/z* with a scan rate of 1 scan/s. The volume of injection was 5  $\mu$ L. Data was acquired and analyzed with software Agilent Mass Hunter software versions B.06.01 and B.07.00 [8]. The ratio of PFHxA concentrations before and after the photocatalytic reactions was applied to calculate the amount of PFHxA removed from the solution. Nowadays, researchers have published the results of studies that were carried out under unrealistic conditions. Thus, we accessed the effects of various parameters, such as the initial pH of the solution, the presence of competing ions, and the water matrix on the efficiency of the photocatalytic degradation of PFHxA over studied materials [8].

**Results:** The content of the  $Zn^{2+}$  substituent influenced the photodegradation kinetics of the target PFAS over the studied materials (Table 1) [8].

The amount of Zn <sup>2+</sup>	C <sub>experimental</sub> /C <sub>initial</sub>					
substitution [at %]	0 min	45 min	120 min			
0	1	$0.76\pm0.01$	$0.63\pm0.01$			
1.0	1	$0.85\pm0.08$	$0.60\pm0.04$			
2.5	1	$0.57\pm0.02$	$0.43\pm0.02$			
7.5	1	$0.92\pm0.01$	$0.68\pm0.01$			
12.5	1	$0.85\pm0.10$	$0.74\pm0.08$			
17.5	1	$0.87\pm0.06$	$0.76\pm0.05$			
22.5	1	$0.89\pm0.04$	$0.78 \pm 0.05$			

Table 1. Kinetics of photocatalytic degradation of PFHxA over studied materials.

Although the photodegradation of the analyte was slow, the pronounced change in its concentration was noted in the first 45 min under light irradiation. The highest photodegradation rate of PFHxA was observed for sample 2.5 at% followed by 1.0 at%, 0 at%, and 7.5 at%. Material 22.5 at% was characterized by the lowest photodegradation rate of PFHxA. The results indicated that the photo-excited holes and superoxide radicals are the major reactive species involved in the photodegradation of the analyte. Moreover, the photocatalytic removal efficiency of the analyte was much higher in tap water (53.30  $\pm$  4.08%) compared to distilled water (75.95  $\pm$  5.49%) and treated wastewater (67.68  $\pm$  3.26%). The natural components of the water matrix (especially inorganic ions) are recognized as potential competitors of active sites on the surface of a photocatalyst (Table 2).

Table 2. Effects of inorganic ions on photocatalytic degradation of PFHxA over sample 2.5 at %.

Competing ion	C <sub>experimental</sub> /C <sub>initial</sub> [%]
without	$63.32 \pm 1.25$

Cl	$71.56 \pm 1.46$
NO <sub>3</sub> -	$99.53\pm9.10$
PO4 <sup>3-</sup>	$87.25\pm2.34$

The photocatalytic removal efficiency of PFHxA was significantly reduced in the presence of studied inorganic anions (Table 2) [8].

**Conclusions:** The material with 2.5 at%  $Zn^{2+}$  substitution was favorable to enhancing the adsorption and photodegradation of PFHxA over the studied photocatalysts due to the optimal concentration of oxygen vacancies. The presence of inorganic ions decreased the photocatalytic efficiency of the analyte. The photocatalytic removal efficiency of the analyte was much higher in tap water compared to that of distilled water and treated wastewater.

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## TARGETING THE KYNURENINE PATHWAY IN DIGESTIVE SYSTEM CANCERS

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Abstract: Despite advances in surgery, chemotherapy, and immunotherapy, digestive system tumors remain among the most prevalent malignancies and the leading cause of cancer-related mortality. Early diagnosis and effective treatment are crucial to improve clinical outcomes, especially in the case of highly invasive liver, gastric and pancreatic cancers. Since metabolic reprogramming is a hallmark of cancer, an intensive search is underway for molecular indicators that reflect physiological changes in the functioning of the human body as the disease progresses. Growing evidence suggests that tryptophan (TRP) metabolites from the kynurenine pathway (KP) are cancer discriminants in case-control studies. Liquid chromatography coupled with mass spectrometry (LC-MS) enables the identification of differentially expressed TRP metabolites, which is crucial for discovering novel cancer biomarkers and gaining a better understanding of disease etiology. In this work, methodological challenges in terms of LC-MS quantification of TRP metabolites to support the discovery of serum biomarkers for digestive system tumors are discussed.

Introduction: Dysregulation of TRP metabolism is suspected to influence the development of digestive system tumors such as gastric, liver, colorectal, and pancreatic cancers [1]. Depletion of TRP via KP plays a crucial role in the adaption microenvironment of cancer to support its progression. Several studies underlined the role of KP metabolites as biomarkers and therapeutic targets in gastric and pancreatic cancer. Choi et al. demonstrated that serum concentration of anthranilic acid (AA) and kynurenic acid (KYNA) increases significantly in the gastric cancer group, while level of kynurenine (KYN) significantly decreases [2]. Geca et al. have found a positive correlation between KYN serum level and stage cT of the disease. Furthermore, serum concentrations of 3-hydroxykynurenine (3HKYN) and xanthurenic acid (XA) negatively correlate with cM [3]. Engin et al. have reported a significant increase of [KYN]/[TRP] ratio in Helicobacter pylori seropositive cancer patients in comparison to uninfected control [4]. In the case of pancreatic cancer, an elevated serum level of 3-hydroxyanthranilic acid (3HAA) has been associated with a significantly lower risk of the disease [5]. Botwinick et al. have reported a positive correlation between the plasma level of KYN and burden of metastatic disease to the lymph nodes, and a negative correlation between the maximum diameter of the primary lesion and level of this metabolite [6]. To evaluate changes in TRP metabolism and discover diagnostic biomarkers for digestive system tumors, quantitative methods based on LC-MS have been recognized as a powerful analytical tool in the profiling of human serum and have found increasing clinical application in the area of biomarker discovery.

**Experimental:** Serum from healthy controls was obtained from the Regional Blood Donation and Hemotherapy Centre in Lublin (Poland) under approval of the Bioethical Committee of the John Paul II Catholic University of Lublin (Ethic Code:2/2023 from 18.04.2023). The determination of TRP and KP metabolites in serum was carried out using the Agilent Technologies 1200 Infinity LC system connected to a 6120 single quadrupole mass spectrometer equipped with API-ESI source. The target compounds were separated using XTerra MC C18 (3.5  $\mu$ m, 4.6×150 mm) analytical column (Waters, Ireland) and the mobile phase composed of 0.1% ( $\nu/\nu$ ) acetic acid in water (solvent A) and 0.1% ( $\nu/\nu$ ) acetic acid in methanol (solvent B) at a flow rate of 0.5 mL/min. The column temperature and injection were 25°C and 10  $\mu$ L, respectively. The ions were monitored in a Single Ion Monitoring (SIM) mode and in positive polarity. Detailed settings of the applied LC-MS methods were collected in Table 1.

Analyte	SIM	Ion type	SIM time	Retention time	Fragmentor			
	ion $(m/z)$		segment [min] [min]		voltage [V]			
Settings for tryptophan determination								
3NT	227.0	$[M+H]^+$	0-15	100				
TRP	205.0	$[M+H]^+$	0-15	8.4	80			
Gradient pro	gram of the mobile phase	: 0-12 min 15-50% solve	nt B; 12-15 min	50-15% solvent H	B (post run: 5			
		min)						
Ν	MS settings: nebulizer pressure: 55 psi; DGT: 300°C; DGF: 10 L/min; Vcap: 4500 V							
Settings for the determination of kynurenine pathway metabolites								
3HKYN	281.0	$[M+C_4H_9]^+$	0-10	8.0	80			
KYN	265.0	$[M+C_4H_9]^+$	10-17	12.0	100			
3NT	283.0	$[M+C_4H_9]^+$		13.2	60			
KYNA	246.0	$[M+C_4H_9]^+$	17-25	23.8	60			
QA	224.0	$[M+C_4H_9]^+$	25-42	29.5	80			
NA	180.0	$[M+C_4H_9]^+$		32.5	100			
PIC	180.0	$[M+C_4H_9]^+$		100				
XA	262.0	$[M+C_4H_9]^+$	42-48	43.5	60			
Gradient program of the mobile phase: 0-15 min 28% solvent B; 15-17 min 28-52% solvent B; 17-44 min 52% solvent B; 44-48 min 52% solvent B (post run: 4 min)								
MS settings were as follows: nebulizer pressure: 50 psi; DGT: 350°C; DGF: 11 L/min; Vcap: 3000 V								

 Table 1. Instrument settings for LC-MS methods.

Abbreviations: 3HKYN - 3-hydroxykynurenine; 3NT - 3-nitro-L-tyrosine; DGF - drying gas flow; DGT - drying gas temperature; KYN - kynurenine; KYNA - kynurenic acid; NA - nicotinic acid; QA - quinolinic acid; PIC - picolinic acid; SIM - Single Ion Monitoring; TRP - tryptophan; Vcap - capillary voltage; XA - xanthurenic acid;

For the determination of KP metabolites, serum (100  $\mu$ L) was mixed with 3NT (4.42  $\mu$ M), and then with 50  $\mu$ L of 15% (*w*/*v*) trichloroacetic acid (TCA). Next, the sample was centrifuged (13 200 ×g, 5 min, 4 °C), and the collected supernatant was evaporated to dryness (EZ-2 Elite Personal Evaporator, Genevac Ltd., Ipswich, UK). The residue was mixed with 140  $\mu$ L of a mixture of *n*-butanol-acetyl chloride (9:1, *v*/*v*)

(derivatization strep) and incubated for 2 h at 57 °C in a thermoblock (Eppendorf, Hamburg, Germany). Next, the sample was dried again, dissolved in 500  $\mu$ L of water and subjected to purification by solid phase extraction (SPE) using a Gilson GX-271 ASPEC system (Middleton, WI, USA). Serum was purified using the CHROMABOND® HR-X cartridge. SPE conditions were as follows: conditioning, 1 mL of methanol / 1 mL of the mixture of water:methanol:acetic acid (95:5:0.1,  $\nu/\nu/\nu$ ); loading, 500  $\mu$ L of the sample; washing, 1 mL of mixture of water:methanol:acetic acid (95:5:0.1,  $\nu/\nu/\nu$ ); elution, 1 mL of the mixture of methanol:acetonitrile (50:50,  $\nu/\nu$ ) / 500  $\mu$ L of acetonitrile. After SPE, the collected eluate was dried and dissolved in 150  $\mu$ L of 0.1% ( $\nu/\nu$ ) acetic acid in water and analyzed by the LC-MS method. In order to determine TRP, serum (50  $\mu$ L) was fortified with 3NT (0.24 mM), mixed with 50  $\mu$ L of 15% ( $w/\nu$ ) TCA, vortexed and centrifuged (13 200×g, 5min, 4 °C). Before LC-MS analysis, the samples were diluted 100-times with 0.1% ( $\nu/\nu$ ) acetic acid in water.

**Results:** Table 2 summarized data on the concentration of TRP and KP metabolites in serum from healthy donors and patients with digestive system cancers.

		0.011			B						
Analyte	TRP [µM]	KYN [µM]	3HKYN [µM]	3HAA [µM]	ΑΑ [μM]	KYNA [µM]	PIC [µM]	ΧΑ [μM]	ΝΑ [μM]	QA [μM]	Ref.
Healthy (men, $n = 3$ )	29.22 - 35.21	2.09 - 2.91	0.31 - 0.32	-	-	0.64 - 1.54	0.98 - 1.75	<0.02 - 0.023	0.08 - 1.61	0.09 - 0.86	This work
Healthy (women, $n = 3$ )	21.61 - 32.13	2.96 - 11.19	0.40 - 1.73	-	-	0.30 - 0.64	<0.12 - 0.87	<0.007 - 0.021	0.14 - 0.26	<0.04 - 0.07	This work
Patients with gastric cancer (n = 18)	5.15 - 109.56	0.20 - 1.41	0.02 - 1.80	-	-	0.01 - 0.12	-	0.03 - 0.27	-	0.07 - 0.15	[7]
Patients with gastric cancer (n = 35)	55.1*	0.89*	-	-	0.05*	0.04*	-	-	0.17*	-	[2]
Patients with pancreatic cancer (n = 187)	46.5 - 105.7	1.02 - 2.19	0.03 - 0.09	0.02 - 0.08	0.01 - 0.09	0.02- 0.16	-	0.006 - 0.04	-	-	[5]

 Table 2. Comparison of available LC-MS data on serum levels of tryptophan and KP metabolites in healthy controls and patients with digestive system cancers.

\*mean value

Abbreviations: 3HAA - 3-hydroxyanthranilic acid; 3HKYN - 3-hydroxykynurenine; AA -anthranilic acid; KYN - kynurenine; KYNA - kynurenic acid; NA - nicotinic acid; QA - quinolinic acid; PIC - picolinic acid; TRP - tryptophan; XA - xanthurenic acid;

In each case quantitative analysis was carried out using LC-MS method employing different protocols for sample preparation. They also differ in the number of monitored KP metabolites. Table 2 shows that the serum level of TRP is much higher compared to its metabolites, which usually requires dilution of the sample before analysis [7].

The analysis of blood serum is preceded by the removal of proteins from the sample using TCA [7] or acidified acetonitrile [2]. As demonstrated here, protein removal might be followed by further purification by solid phase extraction (SPE). Furthermore, by analysing of serum samples from healthy donors, we demonstrated the benefits of introducing the derivatization step into the sample preparation protocol. KP metabolites contain carboxyl group in the structure and undergo an esterification reaction in the presence of alcohol (*n*-butanol) and a catalyst to form esters. This strategy allows for improving method selectivity towards two structural isotopes – picolinic acid (PIC) and nicotinic acid (NA). Another advantage of introducing the derivatization stage is shift of 3HKYN, quinolinic acid (QA), PIC and NA signals to more favorable retention times. The analytical protocol described in experimental section allowed us to detect differences in serum levels of some KP metabolites between healthy individuals and patients with gastric cancer or pancreatic cancers (unpublished data). Furthermore, the introduction of the derivatization step into a sample preparation protocol improved LC-MS allowing for a more comprehensive evaluation of KP modulation in digestive system cancers. Data normalization on an internal standard (isotope stable labeled or structural analogs) is especially useful for improving the accuracy of quantitation of KP metabolites in complex matrices such as serum. Internal standards, added at the beginning of sample preparation, correct variations and errors in extraction, injection, chromatography, ionization, and detection between samples Availability and cost of stable labeled internal standards are limited primarily by reagent availability. In this situation, structural analogs such as 3NT might be used for data correction. 3NT is not endogenously present in serum, and shows analytical behavior similar to KP metabolites [7]. Furthermore, as demonstrated here, 3NT can be used as an internal standard in protocols based on the derivatization reaction since it produces ester similarly to KP metabolites (Table 1).

**Conclusions:** Tryptophan metabolism *via* KP is a promising molecular target for digestive system tumors such as gastric cancer. Despite limited reports on this topic, available data encourage the expansion of research in this direction. New achievements in analytical methods provide promise for better understanding of KP modulation in digestive system tumors and might translate into improved patient diagnosis or personalized therapies.

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# EVALUATION OF THE GREENNESS AND SUSTAINABILITY OF ANALYTICAL METHODS FOR THE DETERMINATION OF KYNURENINE PATHWAY METABOLITES

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Abstract: The kynurenine pathway is a major metabolic route of tryptophan involved in the regulation of the immune system and presents a potential molecular target for the treatment of many human diseases. Methodological achievements in the field of sample preparation and analytes detection allow simultaneous measurement of multiple metabolites of the kynurenine pathway from a small amount of biological sample. Since environmental awareness has increased in analytical chemistry, the demand for green and sustainable alternatives to current methodological solutions in terms of measurement of tryptophan metabolites is suspected. This work aims to assess of environmental and occupational hazards associated with different analytical methodologies proposed for the determination of kynurenine pathway metabolites. The assessment was conducted using an Analytical GREEnnes (AGREE) calculator, which allows evaluating the greenness of a particular analytical method based on the 12 principles of Green Analytical Chemistry.

**Introduction:** The kynurenine pathway (KP) is a major metabolic route of essential amino acid - tryptophan initiated *in vivo* by the activity of indoleamine 2,3-dioxygenase or tryptophan 2,3-dioxygenase. KP is involved in the regulation of the immune system, neuronal function, intestinal homeostasis and is a potential target for cancer immunotherapy. Furthermore, tryptophan and its metabolites play a role in the gut-brain axis, which links the gastrointestinal tract and the central nervous system. Imbalances in KP, resulting in enhancement of metabolites with neuroactive properties such as 3-hydroxykynurenine and quinolinic acid, contribute to the development of various neuropsychiatric diseases [1]. Many studies have also demonstrated that tryptophan metabolism *via* KP facilitates cancer progression by suppressing of immune response, facilitating of angiogenesis, increasing drug tolerance, and enhancing the resistance of chemo/radiotherapy [2,3]. Some KP metabolites show promise as biomarkers of gastric cancer [4].

Trucking KP modulation in pathogenic states requires input of large number of reliable quantitative data on their concentration in different biological matrixes such as blood, serum, urine, brain tissue, and cells. To address this demand, numerous analytical methods have been proposed for the quantification of KP metabolites. The developed approaches vary considering the detection method used, sample handling, number of target analytes and type of biological matrix subjected for analysis [5, 6]. The utility of already developed analytical methods might be scaled using various criteria. Methods should be primarily fit-for-purpose and show a high analytical performance and

practicability. Validation of the method and the determination of parameters such as linearity, accuracy, precision, and recovery allow the evaluation of its analytical performance. Recent trends point to the evaluation of environmental and occupational hazards associated with a particular analytical method. For these assumptions, recommendations of Green Analytical Chemistry (GAC) should be taken into consideration. One of the available metrics established to quantify the greenness of analytical methods is the Analytical GREEnness Metric Approach (AGREE). The AGREE software allows a straightforward assessment of analytical methodology's greenness. The input criteria refer to 12 GAC principles. Each of the input variables is further transformed into a common scale in 0-1 range, while the output is a clock-like graph [7].

Experimental: Analytical methods developed for the determination of KP metabolites in biological samples were evaluated using the Analytical GREEnness calculator to assess their compatibility with GAC requirements [7]. Methods employing different detection methods and methodological solutions applied in the sample preparation step were selected. During the evaluation of the greenness of analytical methodologies, the following GAC principles were taken into consideration: *Principle 1*: direct analytical techniques should be applied to avoid sample treatment; *Principle 2*: minimal sample size and minimal number of samples are goals; Principle 3: in situ measurements should be performed; *Principle* 4: integration of analytical processes and operations saves energy and reduces the use of reagents; Principle 5: automated and miniaturized methods should be selected; Principle 6: derivatization should be avoided; Principle 7: generation of a large volume of analytical waste should be avoided and proper management of analytical waste should be provided; Principle 8: multi-analyte or multiparameter methods are preferred; *Principle 9*: the use of energy should be minimized; Principle 10: reagents obtained from renewable source should be preferred; Principle 11: toxic reagents should be eliminated or replaced; *Principle 12*: the operator's safety should be increased.

**Results:** A total of 4 different analytical approaches for the determination of KP metabolites (Table 1) were evaluated using the Analytical GREEnness calculator. Two methods were based on liquid chromatography-tandem mass spectrometry (LC-MS/MS), one on liquid chromatography-mass spectrometry (LC-MS), and one on differential pulse adsorptive stripping voltammetry (DP-AdSV). Overall score with values close to 1 indicating that particular procedure meets GAC requirements. The analyzed methods obtained scores in the range from 0.46 to 0.59. Examples of graphs generated with the Analytical GREEnness calculator were presented in Fig.1. For each analytical method, the scores corresponding to GAC principles 3 and 10 are unsatisfied, while, in the case of principles 2 and 6, their performance was acceptable (Table 2).

Method	Number of target compounds (number of KP metabolites)*	Major steps of sample preparation step	Time of analysis	Method of detection	Application	AGREE score	Ref.
1	7(6)	Protein	14 min	LC-MS/MS	Serum	0.55	[8]

 Table 1. General comparison of different analytical methods developed for quantification of KP metabolites

 with a score generated using the Analytical GREEnness calculator.

		precipitation internal standard addition centrifugation					
2	9(8)	Protein precipitation internal standard addition SPE centrifugation concentration	12 min	LC-MS/MS	Peritoneal fluid	0.47	[8]
3	4(4)	Protein precipitation internal standard addition centrifugation incubation concentration	45 min	LC-MS	Cancer cell culture medium	0.46	[9]
4	1(1)	Direct analysis	<1.5 min	DP AdSV	Cancer cell culture medium	0.59	[10]

\*number of analytes, which can be determined during a single analytical run; DP AdSV - differential pulse adsorptive stripping voltammetry; LC-MS – liquid chromatography – mass spectrometry; LC-MS/MS – liquid chromatography – tandem mass spectrometry; SPE – solid phase extraction;



Fig.1. Examples of graphs with final scores generated using the Analytical GREEnness calculator during the evaluation of environmental and occupational hazards associated with Method 1 (A) and Method 2 (B).

Despite a limited number of detected KP metabolites, voltammetric protocol (Method 4) has been consider greener than LC-MS or LC-MS/MS methods that allow multi-target analysis. The voltammetric-based method obtained the best scores for GAC principles 1 (minimal sample handling), 4 (integration of analytical processes and operations), 9 (energy consumption), 11 (reduced use of toxic reagents) and 12 (operator safety). However, LC-based methods benefit from principles 2 (minimal sample size), 5 (degree of automation and miniaturization), and 7 (reduced amount of analytical waste). These results are in agreement with previous conclusions [11].

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Evaluated analytical	GAC principle											
method	1	2	3	4	5	6	7	8	9	10	11	12
Method 1	0.3	0.98	0.0	0.8	0.75	1.0	0.51	0.76	0.58	0.0	0.36	0.6
Method 2	0.0	0.78	0.0	0.2	0.75	1.0	0.45	0.87	0.66	0.0	0.32	0.6
Method 3	0.0	0.92	0.0	0.4	0.75	1.0	0.43	0.29	0.75	0.0	0.32	0.6
Method 4	0.48	0.75	0.0	1.0	0.5	1.0	0.2	0.61	0.93	0.0	0.8	0.8

 Table 2. Individual results obtained during the assessment of the fulfillment of a particular analytical method with GAC principles. Scores were generated using Analytical GREEnness calculator.

**Conclusions:** The Analytical GREEnness calculator allows for comparison of various approaches in order to select those with the lowest environmental impact and to identify possible "green" trouble spots during conceptualization and development of new analytical procedures. The study indicated that new developments in the field of quantitative analysis of KP metabolites should focus on the possible use of materials obtained from renewable sources in different stages of the analytical protocol. New solutions that allow *in situ* measurements are also desirable to improve greenness and sustainability.

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# ANTIOXIDANT PROPERTIES OF EXTRACTS FROM MINI KIWI (ACTINIDIA ARGUTA)

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**Abstract:** Plant products are rich in antioxidants, which protect organisms from the negative effects of reactive oxygen species. This paper concerns the determination of antioxidant properties of extracts obtained from mini kiwi (*Actinidia arguta*) using the ABTS and FRAP methods. Additionally, the content of polyphenols was determined in each of the extracts. The results of the presented studies prove that the examined extracts exhibit antioxidant properties. Owing to these properties, they are able to protect the living body against oxidative stress and thus prevent various diseases.

**Introduction:** Oxygen has an ambivalent effect on living organisms [1]. On the one hand, it is an essential substrate for cellular respiration, on the other hand, it can also be toxic. Reactive oxygen species are responsible for its negative effects. These species include free radicals that have unpaired electrons in their structure (for example, the superoxide anion radical, the hydroxyl radical or the peroxide radical) or non-radical species whose reactivity is higher than that of oxygen in the triplet state (such as hydrogen peroxide or ozone) [2,3]. At the physiological level, reactive species play a beneficial role. In excess, they cause harmful oxidation (including proteins, sugars, nucleic acids, lipids), which leads to many unfavorable changes in the body and, consequently, to many serious diseases [4,5]. Antioxidants help in the fight against the unfavorable oxidation caused by reactive forms. Every cell of the human body, through evolution, has been equipped with these substances. However, this endogenous antioxidant defense is not always sufficient, and the body struggles with so-called oxidative stress. In such a situation, antioxidants supplied to the body so-called exogenous antioxidants, which the body absorbs with food, are helpful [6,7]. Plant products are rich in antioxidants, especially polyphenolic antioxidants. Therefore, research is being conducted worldwide to find new sources of plant antioxidants. This paper is part of this research trend, as its main goal is to determine the antioxidant properties of extracts obtained from mini kiwi fruit (Actinidia arguta), which contain various health-promoting compounds, including the above-mentioned polyphenols. The content of these compounds makes them extremely valuable, and some even classify them as so-called superfood. The antioxidant properties of extracts obtained from mini kiwi fruit were measured using various tests, including ABTS and FRAP. Additionally, the content of polyphenols was determined in each of the extracts.

**Experimental:** The mini kiwi extracts were prepared by maceration. For this purpose, 1 g of freeze-dried and ground fruits was poured with 10 cm<sup>3</sup> of 70% MeOH. After 72 h, the extract was filtered and stored in the fridge at  $-18^{\circ}$ C. The antioxidant properties of

the extracts were evaluated spectrophotometrically using ABTS and FRAP methods. The polyphenols content was determined by the Folin-Ciocalteau reagent method.

*ABTS assay:* The ABTS<sup>•+</sup> used in the study was formed by the reaction of 2,2'-azinobis(3-ethylbenzenethiazoline-6-sulfonate) (ABTS) with potassium persulfate. For this purpose, 5 cm<sup>3</sup> of ABTS solution at a concentration of 7 mmol/dm<sup>3</sup> was mixed with 0.088 cm<sup>3</sup> of potassium persulfate at a concentration of 140 mmol/dm<sup>3</sup>. he mixture was incubated in the dark for 16 h and diluted with methanol to obtain an absorbance value of  $0.7 \pm 0.05$  [8]. 2.9 cm<sup>3</sup> of methanolic ABTS<sup>•+</sup> solution was mixed with 0.1 cm<sup>3</sup> of antioxidant solution. After one hour of incubation, the absorbance value in the tested solutions was read and converted to percentage of inhibition (%) according to the following equation:

#### $I(\%) = (1 - A_{60}/A_0) \times 100\%$ (1)

where:  $A_{60}$  and  $A_0$  are the absorbance values of ABTS<sup>•+</sup> in 60 and 0 min of the radical neutralization reaction, respectively. Antioxidant activity was expressed as Trolox equivalents.

*FRAP assay:* The FRAP test solution containing: FeCl<sub>3</sub>•6H<sub>2</sub>O in distilled water (final Fe(III) concentration in the solution was 20 mmol/dm<sup>3</sup>), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl (final TPTZ concentration was 10 mmol/dm<sup>3</sup>) and 0.3 mol/dm<sup>3</sup> CH<sub>3</sub>COOH/CH<sub>3</sub>COONa buffer solution at pH = 3.6 was prepared according to the procedure of Benzie and Strain [9]. The solutions were mixed in the ratio 1:1:10, respectively. The same volume proportions were maintained in the measurements as in the ABTS method (2.9 cm<sup>3</sup> of test solution and 0.1 cm<sup>3</sup> of extracts). The mixture was left in the dark for 60 min at 37 °C. After an hour, the absorbance of the formed complex was measured at 593 nm. The final results were expressed as Trolox equivalents.

*Polyphenol content*: Total polyphenol concentration in extracts was determined using the Folin-Ciocalteu (F-C) method [10]. For this purpose,  $0.1 \text{ cm}^3$  of extract was added to  $1.58 \text{ cm}^3$  of water and mixed with  $0.1 \text{ cm}^3$  of F-C reagent. After 5 minutes,  $0.3 \text{ cm}^3$  of 20% w/v aqueous sodium carbonate solution was added to the total. After 2 hours of incubation, absorbance was measured at 765 nm. Total polyphenol content was calculated from the calibration curve for gallic acid was expressed as acid equivalents in mg per g of sample.

**Results:** Antioxidant activity was determined for ten extracts obtained from freeze-dried fruits of different varieties of *Actinidia arguta*, which are marked with appropriate numbers (Table 1). The sample numbers correspond to the individual extract numbers.

No of sample	Name
1	Actinidia arguta Rogów
2	Actinidia arguta Lucy
3	Actinidia arguta Geneva
4	Actinidia arguta Bingo
5	Actinidia arguta Weiki F
6	Actinidia arguta Mirzan Scarlet
7	Actinidia arguta Vitikiwi
8	Actinidia arguta Jumbo

Table 1. Names of examined cultivars

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9	Actinidia arguta Ananasnaya
10	Actinidia arguta Chang Bai Giant

Antioxidant activity was determined using the ability of the tested sample to neutralize the color radical (ABTS method, data in Fig.1) and their ability to reduce iron ions (FRAP method, data in Fig.2). In both methods, antioxidant properties were expressed as milligram of Trolox (treated as a standard antioxidant) per gram of the tested sample (dry weight used to prepare the extract).



Fig.1. Antioxidant activity of the examined extracts – ABTS method.



Fig.2. Antioxidant activity of the examined extracts - FRAP method.

As can be seen from the presented data, all the examined cultivars have antioxidant activity. Moreover, regardless of the method used, the greatest antioxidant properties are shown by the Vitikiwi extract (sample no. 7), as well as the weakest by the extracts from Lucy (sample no. 2) and Jumbo (sample no. 8). As mentioned in the Introduction, polyphenolic compounds may be largely responsible for the antioxidant properties of extracts. Therefore, in the following studies it was decided to determine the content of these compounds in the extracts studied. The results are presented in Fig.3.



Fig.3. Polyphenolic content determined for the examined extracts, expressed in mg of gallic acid per g of a given sample.

As can be seen from the presented data, the highest polyphenol content is found in the Vitikiwi fruit extract (sample no. 8), and the lowest in the Jumbo (sample no. 8) and Lucy (sample 2). The results show the correlation between polyphenol content and antioxidant properties (the higher polyphenol content the higher antioxidant properties). This may confirm that polyphenols are responsible for antioxidants properties.

**Conclusions:** The results of the presented studies prove that the extracts obtained from *Actinidia arguta* fruits exhibit antioxidant properties. Thanks to these properties, they are able to protect the living organism from oxidative stress, which can cause adverse changes in the body, including many diseases.

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# TLC-DIRECT BIOAUTOGRAPHY OF ACTINIDIA ARGUTA CULTIVARS

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Abstract: The growing interest in traditional medicinal plants is driven by the need for novel bioactive compounds to treat infectious and chronic diseases without dangerous side effects. Actinidia arguta (mini kiwi) is a functional food with antioxidant, antidiabetic, anticancer, antimicrobial, and anti-inflammatory properties. The aim of this study was to bioprofile ten cultivars of A. arguta (fruit extracts) using thin-layer chromatography (TLC) combined with bioassays to detect bioactive constituents like antioxidants and enzyme inhibitors. Freeze-dried fruits were extracted with methanol:water (70:30, v/v) and analyzed using TLC. Antioxidant activity was assessed via the DPPH test, while  $\alpha$ -glucosidase and lipase inhibition were evaluated using direct bioautography. The presence of organic acids, including ascorbic, citric, malic, oxalic, quinic, and lactic, was confirmed by derivatization with phosphomolybdic acid. Antioxidant activity was detected in all samples, with the highest in A. arguta Vitikiwi PBR. It also showed the strongest  $\alpha$ -glucosidase and lipase inhibitory activity, suggesting that it has the highest therapeutic potential among the other cultivars. These findings highlight A. arguta as a promising source of bioactive compounds with health benefits, particularly in metabolic disorder prevention.

Introduction: Interest in plants used in traditional medicine is on the rise. This fact is driven, among others, by many serious side effects of synthetic drugs and the increasing resistance of pathogenic microorganisms to antibiotics. Consequently, there is a rising demand for discovering new natural sources of bioactive compounds with therapeutic potential against chronic and infectious diseases [1,2].Kiwi (Actinidia), a fruit widely available on the European market, has been utilized for centuries in Traditional Chinese Medicine due to its pharmacological properties. Currently, it is primarily valued mainly for its sensory attributes, antioxidant activity, and application as a food additive. Moreover, its enzymatic properties contribute to supporting digestive processes [3,4]. A. arguta, commonly known as mini or baby kiwi, is a species that has been introduced to the market on a larger scale relatively recently. Due to their bioactive properties, which include antioxidant, anti-diabetic, anti-cancer, anti-microbial and antiinflammatory effects, the fruits of A. arguta are considered a nutritionally valuable functional food. Additionally, their small size and thin, edible skin enhance their convenience as a health-promoting snack [5,6]. Plant extracts are a rich source of bioactive compounds and phytochemicals. Among the most popular methods of analyzing plant extracts are high-performance liquid chromatography (HPLC) and thinlayer chromatography (TLC) [1,7]. The latter has a major advantage over HPLC due to the possibility of analyzing many samples in one run, easy evaporation of a mobile phase, which facilitates detection, as well as the relative simplicity of the method. What is also important TLC can be hyphenated with spectroscopic methods and bioassays performed directly on TLC plates. In the presented study, bioprofiling of ten different *Actinidia arguta* varieties was conducted using thin-layer chromatography combined with biological assays performed directly on the TLC plate. This effect-directed approach enabled the detection of bioactive zones in fruit extracts, indicating the presence of antioxidants as well as inhibitors of glucosidase and lipase.

**Experimental:** Freeze-dried Actinidia arguta fruits (1 – A. arguta Ananasnaya, 2 – A. arguta Bingo PBR, 3 – A. arguta Chang Bai Giant, 4 – A. arguta Geneva, 5 -A. arguta Jumbo, 6 – A. arguta Lucy, 7 – A. arguta Rogów, 8 – A. arguta Vitikiwi PBR, 9 – A. arguta Weiki F, 10 – A. arguta Mirzan Scarlet September Kiwi) were extracted with the methanol: water (70:30, v/v) solution in a ratio of 5 ml per 0.5 g of a given sample. Maceration was carried out for 72 h in the dark, then the extracts were filtered and stored at -18 °C. TLC analysis was performed on 10×20 cm TLC Si60 F254 plates (Merck, Darmstadt, Germany); the extracts were applied with the automatic Linomat 5 applicator (CAMAG, Muttenz, Switzerland). Chromatography was performed using ethyl acetate : methanol : water mixture (70:20:10, v/v/v) in the DS chamber (Chromdes, Lublin, Poland). Documentation was done using TLC Visualiser 2 and WinCATS (CAMAG), followed by chemical derivatization or bioautography. DPPH test was performed by spraving 0.2% DPPH solution in methanol onto dried TLC plates. Antioxidants were visible as yellow spots on a purple background. Dot-blot test was performed by manually applying extracts to TLC plates (10×10 cm or 10×20cm, Merck) using 10 µl Hamilton syringe (Bonaduz, Switzerland). Derivatization with phosphomolibdic acid (PMA) solution was performed by spraying chromatograms with 5 ml PMA solution (20 g PMA in 20 ml ethanol) and then heating for 5 min at 110 °C. Compounds appeared as bluish zones against white background. The  $\alpha$ -glucosidase inhibition assay was performed by spraying the chromatograms with a substrate solution  $(2-naphthyl-\alpha-D-glucopyranoside in ethanol)$ , then with  $\alpha$ -glucosidase solution (100 U in 10 ml of acetate buffer, pH 7.5) followed by incubation for 20 min at 37 °C. Visualization was performed with Fast Blue B solution, where inhibitors appeared as white zones, against a purple background. Lipase inhibition was tested by spraying the chromatogram with 1-naphthyl acetate followed by lipase (100 U in 10 ml of 0.05 M TRIS buffer with addition of 5 mg of BSA, pH 7.4) and incubating for 30 min at 37°C. Fast Blue B visualization revealed inhibitors as white zones against a purple background.

**Results:** TLC-DPPH test revealed the highest antioxidant properties for cultivars numbered 3 (*A. arguta* Chang Bai Giant), 4 (*A. arguta* Geneva), 7 (*A. arguta* Rogów) and especially 8 (*A. arguta* Vitikiwi PBR). The strongest antioxidants were found at  $R_F$  0.27 (A),  $R_F$  0.11 (B),  $R_F$  0.05 (C) and  $R_F$  0.18 (D) (Fig.1a). The unknown compound in zone a is responsible for glucosidase inhibition of all tested cultivars as well as for lipase inhibition of sample 8 (Vitikiwi). Inhibition zones (C) of glucosidase and lipase are seen near the start for all samples (Fig.1b,c).

The zone C corresponds to very polar component(s) of all varieties which are retained strongly on polar silica gel, probably organic acid(s), as is described in the literature [5]. To confirm this statement, derivatization with PMA was done (Fig.1d). Most of organic acid standards are close to the start, while the dark blue spot of ascorbic acid ( $R_F$  about 0.25, probably zone A) is seen in the Vitikiwi PBR sample.



Fig.1. Chromatograms/bioautograms of ten various cultivars of *A. arguta* (fruit extracts, VIS):
a) TLC-DPPH, b) α-glucosidase inhibition. c) lipase inhibition, d) derivatization with PMA reagent.
1. *A. arguta* Bingo PBR (5 μl), 2. *A. arguta* Vitikiwi PBR (5 μl), standards of : 3. ascorbic acid, 4. malic acid, 5. citric acid, 6. quinic acid, 7. oxalic acid, 8. lactic acid.

Additionally, dot-blot test of lipase inhibition for whole (not separated) extracts of various cultivars was performed. The results pointed to Vitikiwi as the strongest lipase inhibitor among other extract (Fig.2,8a). As shown earlier, the Vitikiwi fruits possess also the highest antioxidant properties. The next most important lipase inhibitors appear to be *A. arguta* Geneva (Fig.2,4a) and *A. arguta* Mirzan Scarlet September Kiwi (Fig.2,10a).



Fig.2 Dot-blot test of A. arguta fruit extracts (1a-10a) in visible light – lipase inhibition.
1a – Ananasnaya, 2a – Bingo PBR, 3a – Chang Bai Giant, 4a – Geneva, 5a – Jumbo, 6a – Lucy, 7a – Rogów, 8a – Vitikiwi PBR, 9a –Weiki F, 10a – Mirzan Scarlet September Kiwi.

**Conclusion:** As known from the literature, *Actinidia arguta* has valuable pharmacological properties, including strong antioxidant activity. In order to confirm this claim and to find the most active variety, bioprofiling was carried out on ten extracts from different varieties of *A. arguta* fruits. TLC-direct bioautography proved to be the method of choice. This method made it possible to compare the ten samples in a single run, i.e. in parallel on the same TLC plate developed with the appropriate mobile phase, dried to evaporate solvents and subjected to the given bioassay. As shown, the extracts of different varieties differed in biological activities: antioxidant and inhibition of enzymes, i.e.  $\alpha$ -glucosidase and lipase. The best therapeutic properties could be attributed to the extract of *A. arguta* Vitikiwi PBR. In addition, mini kiwi has been shown to be a rich source of organic acids, such as ascorbic, citric, malic, oxalic, quinic and lactic.

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# STUDY OF THE MOLECULAR MECHANISMS OF STATIN-INDUCED MYALGIA WITH UNTARGETED METABOLOMICS

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**Abstract:** Statins are the most commonly used drugs worldwide. Their use may be connected with adverse effects of the therapy. The most commonly observed are muscle symptoms. The mechanism of myalgia (one of the statin-connected side effects) is still unclear. An untargeted metabolomics study was applied to explain the molecular mechanisms of myalgia.

**Introduction:** Statins, by blocking the internal production of cholesterol, help to control low-density lipoprotein (LDL) levels [1]. High LDL level is an important risk factor for cardiovascular diseases. In general, statins are well tolerated by patients. However, since the introduction of the therapy at the turn of the 80' and 90' of the twentieth century, both: the side and the pleiotropic effects, of statin-based treatment have been revealed. The most commonly observed adverse effects are muscle symptoms (broad range from myalgia to rhabdomyolysis). The prevalence of these effects is still discussed. The healthcare practitioners indicate a quite high risk (even up to 25% of treated patients) of statin-induced side effects, but these data are not confirmed with randomized trials [2-5]. The common patients' reaction, when muscle symptoms appear, is an abandonment of therapy. Uncontrolled hypercholesterolemia significantly increases the risk of CVD episodes. The mechanism of myalgia is still unclear. In the literature, several potential mechanisms are described. It is worth mentioning, among others: the inhibition of cholesterol synthesis in skeletal muscle in patients with high serum statin levels [6], disturbances in the prenylation of proteins [7], the influence of statin on the mitochondrial function [8] or oxidative stress [9]. The metabolomics approach focuses on small molecules (below 1000 Da) analysis. This is the reason, why it is a good tool for observing molecular mechanisms. In the presented study, an untargeted approach was applied to obtain as much information as possible. The main aim of the presented study was an attempt to explain the potential mechanism of myalgia observed by statintreated patients based on the untargeted metabolomics analysis of plasma samples.

**Experimental:** The study consists of two metabolomics platforms: LC-MS and GC-MS. Both of them were used to collect untargeted metabolomics data. In both parts, the same sample was analyzed. Patients were divided into three groups: the first one was people who were statin-treated and who observed myalgia during therapy, the second one was also patients with statin treatment, but without side effects, the third group (control group) consisted of patients at the moment of the diagnosis of the need for LDL-

decreasing therapy, but before starting statin treatment. Statin-treated patients received a 40 mg daily dose of simvastatin. From each patient plasma samples were collected. Details of the study group parameters are presented in Table 1.

Parameter	Control	Myalgia	No-myalgia	<i>p</i> -value
n	18	23	39	-
Gender (F/M)	9/9	9/14	17/22	-
Age [year] <sup>a</sup>	59±7	61±6	63±5	0.118
BMI <sup>a</sup>	$28.0{\pm}2.8$	28.9±4.1	27.9±3.5	0.529
Cholesterol [mM] <sup>a</sup>	$5.86 \pm 0.91$	4.27±0.59	4.14±0.56	9.38E-9 (0.405)
HDL [mM] <sup>a</sup>	$1.45\pm0.38$	1.49±0.42	1.5±0.42	0.902
LDL [mM] <sup>a</sup>	4.16±0.85	$2.48 \pm 0.64$	2.43±0.51	8.51E-9 (0.754)
TG [mM] <sup>a</sup>	$1.53 \pm 0.81$	1.37±1.04	1.02±0.37	0.036 (0.07)
SIM in plasma [ng/mL] <sup>a</sup>	<loq< td=""><td>1.55±1.27</td><td><math>1.52\pm0.96</math></td><td>0.914</td></loq<>	1.55±1.27	$1.52\pm0.96$	0.914
SIMA in plasma [ng/mL] <sup>a</sup>	<loq< td=""><td>3.61±3.00</td><td>3.36±2.57</td><td>0.767</td></loq<>	3.61±3.00	3.36±2.57	0.767

 Table 1. The study group characteristics.

a) Mean value  $\pm$  SD; SIM – simvastatin, SIMA – simvastatin hydroxy acid form.

An LC-MS system consists of a 1290 Infinity UHPLC system combined with a 6545 high-resolution QTOF analyzer (both Agilent Technologies). Details of LC-MS methods are presented in Table 2.

	LC	MS			
Column type	Zorbax Extend-C18		Ion source	AJS ESI	
	(2.1 × 50 mm,	, 1.8 μm)			
Column	60 °C		Ion modes	ESI+,	ESI-
temperature					
Injection volume	1 µL		Operating mode	e full scan	
Flow rate	0.6 mL/min		Acquisition m/z range	uisition <i>m/z</i> range 50-100	
Solvent A	Water with 0.1% formic acid		Scan rate	1.5 scan/sec	
Solvent B	Acetonitrile with 0.1% formic		Nebulizer	52 psig	
	acid				
Gradient	0.0 – 1.0 min	5% B	Nozzle voltage	1000 V	
	7.0 min	80% B	Capillary voltage	positive	3000 V
11.5 min 1		100% B		negative	4000 V
	12.0 min 5% B				
	15.0 min 5% B				

 Table 2. Details of LC-MS method parameters.

On the day of analysis, samples were thawed on ice. Protein precipitation and metabolite extraction were performed by vortex mixing (for 1 minute) one volume of the plasma sample with three volumes of freeze-cold (-20 °C) methanol/ethanol (1:1) mixture (containing internal standard (IS) – zomepirac 1  $\mu$ g/mL). After the extraction, the samples were incubated on ice for 10 minutes and then centrifuged at 21 000×g for 10 minutes at 4 °C. The supernatant was filtered through a 0.22- $\mu$ m nylon filter into glass vials. Quality control (QC) samples were prepared by mixing equal volumes of all samples. The obtained mixture was prepared following the same procedure as the remainder of the samples.

A GC-MS-based metabolic fingerprinting was performed using a 7890B gas chromatograph connected to a 7000D mass selective detector (Agilent Technologies). Details of GC-MS methods are presented in Table 3.

GC a	nd MS	Temperatures			
Column	DB-5MS (30 m ×	Injection port	Injection port 250 °C		
	0.25 mm, 0.25 µm)	temperature			
Injection volume	1 µL	Temperature	0.0-1.0 min	60 °C	
Split	1:10	program (oven)	Increase	10 °C/min	
Carrier gas	Helium		Final temp.	320 °C	
Gas flow rate	1 mL/min	Transfer line	280 °C		
		temperature			
Ion source	EI (70eV)	Ion source	300 °C		
		temperature			
Acquisition m/z	50-600	Quadrupole	15	0 °C	
range		temperature			
Scan rate	1.38 scan/sec				

Table 3. Details of GC-MS method parameters.

An aliquot of 50  $\mu$ L plasma and 150  $\mu$ L cold acetonitrile containing IS1 4-nitrobenzoic acid (25  $\mu$ g/mL) were mixed for metabolite extraction. The mixture was vortex-mixed for 2 minutes and centrifuged at 15 000 × g for 10 min at 4 °C. Finally, obtained supernatant of each sample (150  $\mu$ L) was collected to a GC vial equipped with an insert and evaporated to complete dryness using a vacuum concentrator. All analyzed samples were subjected to a two-step derivatization process. First, methoxymation was performed by adding 20  $\mu$ L of methoxylamine hydrochloride in pyridine solution (15 mg/mL) and then incubating at room temperature in the dart for 16 h. Following this, 20  $\mu$ L MSTFA + 1% TMCS was added to each sample and placed in the oven to react for 1 h at 70 °C. At last, 60  $\mu$ L of heptane containing methyl stearate (10  $\mu$ g/mL) as instrumental IS2 was added.

**Results:** Clear sample separation in comparison myalgic group vs non-myalgic was not observed in any part of the experiment (LC part, both polarization mode and GC part). Only the comparison of myalgia with control (Fig.1A,B) and no-myalgia with control (Fig.1C,D) show clear separation. A domination of decreasing effect was observed (Fig.2). Mainly metabolites from the lipids group are responsible for observed group separation (Table 4).



**Fig.1.** The OPLS-DA plots show the differences between data obtained from the myalgic group and control (A: positive mode, B: negative mode), and from the non-myalgic group and control (C: positive mode, D: negative mode). Green: control, blue: myalgia, orange: no myalgia groups.

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**Fig.2.** Volcano plots show domination of decreasing effect, as a result of statin therapy in the comparison of both data sets: myalgia (A: positive mode, B: negative mode), and no-myalgia (C: positive mode, D: negative mode) versus control.

Table 4. Groups of statistical significant identified metabolites in comparison with control group.

Comparison	Lyso PC	Lyso PE	PC	PE	SM	Other
Myalgia vs control	6	1	11	1	5	13
No myalgia vs control	8	-	16	-	7	11

**Conclusions:** Plasma metabolites do not directly differentiate patients with and without myalgia.

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### MITOCHONDRIAL AND CHLOROPLAST DNA MAINTENANCE BY RECA HOMOLOGUES IN A. THALIANA

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**Abstract:** DNA repair is an essential process for the cell, enabling it to protect itself against both external and internal DNA damaging factors. This process has been well studied in nucleus, however, there is DNA present in mitochondria and chloroplast, as well, and not a lot of information gives us an insight into repair processes of their genomes. RecA protein, responsible for homologous recombination in DNA repair, is first identified in bacteria, and its homologues have been identified in *A. thaliana*. This study implements bioinformatics methods in order to shed light on the functioning of both RecA homologous and DNA repair mechanism in mitochondria and chloroplast. The results indicate the interactions between RecA homologues and many important proteins involved in homologous recombination and DNA repair, putting RecA proteins in the center of this process – after the excision of damaged DNA and before the addition of new nucleotides. Additionally, there are also regulatory proteins involved, whose task is to initiate and monitor the process of DNA repair, and our results indicate that they act specifically through RecA protein.

**Introduction:** DNA repair tries to correct mistakes that have been caused during DNA synthesis, or normal operation of DNA [1]. The importance of this mechanismis seen through observation of cellular processes - the regular human DNA polymerase makes approximately one mistake in 100,000 nucleotides[1]. This might seem low, however, human genome has  $\sim 3.2$  billion base pairs [2], meaning that DNA polymerase would make more than 30,000 mistakes per one duplication of genetic material. Thankfully, there are DNA proofreading and repair mechanisms which decrease the final number of errors of DNA polymerase 100,000 times, from one mistake in10<sup>5</sup>nucleotides to one mistake in10<sup>10</sup>nucleotides [1].Sometimes, DNA damage occurs in such a way that both of the DNA strands are broken which can lead to the breakdown of chromosomes. Nonhomologous end joining (NHEJ) and homologous recombination (HR) are mechanisms which can fix double stranded DNA breakage [1]. HR is more accurate than NHEJ but more complicated at the same time. It is based on the exchange of a portion of the DNA sequence between two sister chromatids – damaged part of the sequence from one DNA strand is replaced with the unchanged part from homologous strand. This type of mechanism is extremely important for the cell, which is the reason why all of the proteins performing it in different organisms share similar features [1]. Some of these are RAD proteins [3], DMC1 [4], UvsX [5] and RecA [6]. RecA, originally discovered in E. coli, is a 38 kilo Dalton protein with the length of ~350 amino acids [7], it has three domains in its structure [8], and it participates in the repair of double-strand brakes on the DNA [6]. RecA promotes recognition of homology and strand exchange between the two homologs during both of the functions: HR and DNA repair [9]. DNA repair occurs in a way that RecA forms a bundle of many protein molecules without which the repair couldn't happen, which is the case in mutated RecA proteins [10]. It also has the ability to induce cell-cycle arrest if it detects that the damage has been made to the DNA [11]. It has been shown that RecA expression is low in cells working under normal conditions, but increases drastically after the cell has been exposed to DNA-damaging factors [8]. It is similar in structure to other proteins responsible for the meiotic recombination and DNA repair, like Rad and DMC1 proteins [9,12]. Mitochondria and Chloroplast contain their own genomes and they also contain mechanisms for DNA repair, especially since they are more prone to damage due to ROS (reactive oxygen species) produced during photosynthesis, or in larger amounts during respiration (which is one of the reasons why mitochondrial DNA is less stable than chloroplast DNA) [13]. They share some similarities with nuclear DNA repair mechanisms, but they also have differences. Genomes of these organelles do not encode proteins for DNA repair on their own; instead, they rely on the ones encoded by the nucleus DNA [14]. Unfortunately, most of the knowledge of DNA repair in organelles comes from the studies on yeast and there is not a lot of information on DNA repair mechanisms in plant organelles [15]. The main aim of this research is to better understand the function of four RecA homologous proteins found in A. thaliana. Additionally, we want to understand the structure of proteins themselves and the mechanism of DNA repair in plant organelles, namely mitochondria and chloroplast, by using the tools of computational biology, thus contributing to the overall knowledge of DNA repair systems in general.

**Experimental:** There are several major steps in the analysis of protein(s). The first one is the analysis of the sequence itself and looking for homologues. The second major one is the analysis of the structure and the function of the protein. And the last one (in our study, at least) is the analysis of interactions between our protein of interest and other proteins. All of this is accompanied by the visualization of results, most notably the construction of phylogenetic tree, representation of protein's 3D structure and representation of the interactome. Retrieval of Sequences: This study focuses on four RecA homologous proteins: RecA-like 1, RecA homolog 2, RecA homolog 3 and RecA homolog 4 (At1g79050; At2g19490; At3g10140; At3g32920). They will be referred to as RecA1, RecA2, RecA3 and RecA4, respectively, in the later part of this study. Sequences of proteins were retrieved from TAIR (The Arabidopsis Information Resource) database and NCBI (National Center for Biotechnology Research). TAIR is a central genome database for A. thaliana, annotating gene function and expression patterns, and giving us information about proteins, biochemical pathways, genome organization, etc. [16-17]. NCBI was developed as information systems for molecular biology [18]. In total, it contains sequences of genomes, transcripts and proteins, which can be viewed, analyzed and edited with additional software [19]. Multiple Sequence Alignment: Retrieved sequences from TAIR and NCBI databases were aligned using online Clustal Omega tool, available at the EMBL-EBI (European Bioinformatics Institute) website. Multiple sequence alignment is an important step in the analysis of proteins, as it helps us analyze the structure and the function of proteins, phylogeny connections and other characteristics [20]. It is especially useful when aligning the uncharacterized sequence of protein (or nucleic acid) with already known ones in popular databases, thus being able to infer the function of the unknown sequence [21]. Clustal Omega is relatively recent, and it is both precise and fast, making it better option over its predecessors which showed a decrease in precision with the increase in the

sequence length [22]. Phylogenetic Tree Construction: After the multiple sequence alignment, protein sequences were submitted to Phylogeny.fr for the construction of phylogenetic tree. By constructing a phylogenetic tree, one can infer a lot of information about two or more organisms (genes, or proteins), the most important one being: how closely they are related to one another [23]. Phylogeny, fr is a popular online tool for the analysis of phylogenetic relations. It is very useful since it produces simple and fast results and it targets a wide range of users [24]. Since the quality of phylogenetic tree results is better if there are more proteins/organisms compared, we have inserted six other proteins (ten in total) from six other organisms; Solanum tuberosum, Spinacia oleracea, Glycine max, Musa acuminate, Solanum pennellii and Brassica napus. The sequences of organisms were found through BLAST (Basic Local Alignment Search Tool) and chosen randomly, by finding similar ones to our proteins of interest, in nonredundant protein database in NCBI. BLAST allows a user to find regions of different sequences that are similar on a local level [25]. Protein 3D Structure Prediction and Refinement: Since the structures of the proteins have not been determined by any experimental method yet, Phyre2 software was used for the prediction of the 3D structure of the protein, using homology modelling. Phyre2 is on online server with a number of tools available at user's disposal to both predict protein's structure and analyze it at the same time [26]. After the prediction, .pdb files produced were submitted to the further refinement of the protein structure using 3D refine. This is an online tool for the improvement of protein's predicted 3D structure, trying to bring the quality of the model closer to those that were experimentally determined, with small computational requirements [27,28]. Since this tool produces several models, each of these models was validated in several different tools for the quality of the protein model in order to pick the best one that will be used in the later parts of this study. 3D Structure Validation and Visualization: Even though software for 3D structure prediction is reliable, especially since it predicts the structure using homology modelling, and even though predicted structure has been further refined in accordance with stereochemical laws, it still hasn't been confirmed experimentally, which is the reason why it is necessary to validate the 3D structure using the available tools: RAMPAGE, Verify3D, OMEAN, PROCHECK and DFIRE. RAMPAGE (Ramachandran plot assessment) is a tool which checks the quality of the protein model by checking phi and psi angles which can have only certain values in a properly folded protein [29]. Verify3D is a software that compares protein's 3D model with its amino acid sequence by checking each amino acid specifically and its viability to hold its place in protein's 3D model [30]. QMEAN is a tool which measures the absolute quality of protein models and gives the probability that their quality is similar to experimental models [31]. PROCHECK is a tool which the overall stereochemical quality of protein structures [32]. Lastly, DFIRE (distance-scaled finite ideal-gas reference state) is a software that assess non-bonded atomic interactions in the protein model, giving a final score as a result [33]. After final modelling of the atoms in 3D space and their validation has been completed, proteins were visualized using two available software: PyMOL and DeepView - Swiss PDB Viewer; the latter one being available at ExPASy Bioinformatics Resource Portal. PyMOL was used for the visualization of the protein's structure, while PDB Viewer was used for the visualization of electrostatic potential of our proteins of interest. Identification of Protein Domains: Domains are very important when learning about the protein, which is the reason why they were identified in our proteins of interest, using SMART (Simple Modular
Architecture Research Tool) available at EMBL (European Molecular Biology Laboratory). SMART is used for the identification of protein domains, as well as for the analysis of their architectures. It contains more than 1200 protein domain models, and it is connected with other databases, having at its disposal information about more than 100 million protein features, including annotated domains [34]. Since SMART looks through more databases, there is a possibility of detecting overlapping domains in a protein. In order to confirm which one is the "dominant" one, CDD (Conserved Domain Database) is used. CDD is a software (and a database) which solves the problem of overlapping domains in a protein model by grouping them in a homologous domain models, thus being able to properly annotate the protein of interest [35]. Localization of Proteins: Protein localization prediction was the next step in our research. Several Online tools were used for this, namely: PSI-predictor (Plant Subcellular Localization integrative predictor), SUBA3 (SUBcellular localization database for Arabidopsis proteins) and LocTree3. All three of these software perform localization prediction based on collective collaboration of multiple predictors using several databases, together with the incorporation of machine learning methods [36-38]. Prediction of the Interactome: After the identification of domains present in our proteins, Interactome was analyzed in order to find out which proteins can interact with our proteins of interest. This was accomplished using STRING database, which contains information about protein-protein interactions, both physical as well as functional ones, covering more than 2000 organisms [39]. The significance and reliability of the search was kept at high level by increasing the confidence level to 0.9 (highest confidence) and by limiting the number of interacting proteins to 10 per each RecA homologue – meaning only 10 best predictions were taken into consideration. Prediction of Docking Sites: Prediction of docking sites was done using ClusPro online server. It is a first fully automated server for protein docking prediction, founded in 2004 and currently, it is the best, most reliable way of predicting docking sites of protein-protein interactions [40-41]. ClusPro uses two .pdb files (from both the protein and the ligand) and it performs computations for billions of different complexes based on surface complementarity. The best ones are selected based on good electrostatic and desolvation free energies [42]. In order to get the most out of the results, all of the proteins from the predicted interactome have been taken into consideration. Since the tool requires PDB input for both our protein of interest and its interacting protein, we have predicted and refined the structures of all interacting proteins using the same methodology like before – for our proteins of interest; we have used phyre2 server to model the protein in 3D, we have used 3D refine in order to improve the structure and we have used RAMPAGE and Verify3D in order to choose the model with the highest quality. In the end, all relevant produced .pdb files from interacting proteins have been checked for docking against all four of our proteins of interest.

**Results:** MSA results from Clustal Omega are visualized in two main forms. The first one is the Percent Identity Matrix, shown in Table 1, and the second one is the actual alignment of sequences together with some similarities/differences, According to MSA, all proteins share relatively good percentages of similarity, while the highest similarity, which stands out from the other ones, is shared by the RecA2 and RecA4.

Table 1. luc	Tuble 1. Identity Matrix percentage of four Reers homologues from Mors anglinent.						
Protein	RecA2	RecA1	RecA3	RecA4			
RecA2	100.00	43.54	44.94	78.12			
RecA1	43.54	100.00	36.70	45.70			
RecA3	44.94	36.70	100.00	49.11			
RecA4	78.12	45.70	49.11	100.00			

Table 1. Identity Matrix percentage of four RecA homologues from MSA alignment.

Phylogenetic Tree Construction: Constructed phylogenetic tree is shown in Fig.1. According to results, there seem to be three groups of proteins, with respect to their similarity/divergence, which will be further elaborated in the discussion part of this study.



Fig.1. Phylogenetic Tree of RecA homologues and some plant species.

Protein 3D Structure Prediction and Refinement: As it is expected, the 3D structure of protein is the least reliable immediately after the 3D prediction, using Phyre2 server. Refinement of the protein structure results in several models, all of which were validated to choose the best one. Validation results after the first step of refinement for one protein can be seen in Table 2. Refined models 4 and 5 have the highest quality when checked with RAMPAGE (they show an increase of 3.1% of residues in favored region.

 Table 2. Ramachandran plot assessment- RecA2 initial 3D structure, and several refined structures using 3D

 Refine

Refine.						
Residues in	Phyre2 model	Refined model 1	Refined models 4 & 5			
Favored region	94.9%	97.2%	98.0%			
Allowed region	4.0%	2.0%	1.7%			
Outlier region	1.1%	0.9%	0.3%			

It is, however, also clear that there were cases when two refined models share the same percentage of residues in all three regions. In order not to rely on chance, additional validation software is used to further improve the quality of the final model by choosing the best one among those with the same scores. Verify 3D software was used next to assess refined models 4 and 5 of protein RecA2, and the results can be seen in Table 3.

 Table 3. Validation of 3-D structure models with similar scores after the first validation.

Model	Score
Model 4	88.86% of the residues had an averaged 3D-1D score $\geq 0.2$
Model 5	88.58% of the residues had an averaged 3D-1D score $\geq 0.2$

This additional validation showed us that model 4, refined using 3DRefine tool, was the best one to continue with. The same methodology was used for other proteins, which allowed us to pick the best models to work with in the later parts of this study.

3D Structure Validation and Visualization: Once the best protein models were chosen for all four RecA homologues, final validation of the structure was performed, which also

included results from the dFire server. Final validation results can be seen in Table 4. RAMPAGE gives the results in three categories: amino acid residues in favored region, in allowed region and in outlier (non-favorable) region. The first two have been combined together in our results. Verify 3D gives one result, showing the amount of residues in good position, with respect to their linear amino acid sequence. DFire is the server which calculates total negative energy, so the more negative the result is, the closer the protein model is to its native state.

	RAMPAGE (residues in allowed region)	Verify 3D (3D-1D score >= 0.2)	QMEAN6	Z-score	G-factor	dFire
RecA1	99.1%	79.14%	0.689	-1.00	-0.41	-743.45
RecA2	99.7%	88.86%	0.707	-0.77	-0.92	-778.95
RecA3	98.9%	79.38%	0.682	-1.05	-0.57	-769.07
RecA4	99.5%	72.35%	0.574	-2.11	-0.83	-435.11

 Table 4. Structure validation of four RecA protein models - predicted with Phyre2 and refined with 3D Refine.

Validation results indicate that all protein models have high quality. RAMPAGE shows that most of the proteins' residues are in the allowed region, while only small percentage of them is in the non-favored region.



Fig.2. 3D structural prediction of RecA homologues and refined structure. (a) RecA2; (b) RecA1; (d) RecA3; (e) RecA4.

Also, Verify 3D software shows that all four proteins have amino acids in favorable locations with respect to their position in primary structure of the protein, with RecA2 having the best model. QMEAN server results, QMEAN6 and Z-score also show the good quality of proteins, same as like PROCHECK results, namely the G-factor. DFire software is the last one used, and it also confirms the good quality of our protein models, showing relatively high total negative energy for the proteins that are medium in length/size. Visualized protein structures can be seen in Fig.2. All four protein models show similar core domain, having both alpha helices and beta sheets, with the RecA4

model having the most dissimilar 3D structure. We can see from 3D models that the dominant secondary structure element is alpha helix, followed by beta sheets which are also composed from substantial number of residues. Electrostatic potential of RecA homologues, computed by Swiss-PDB viewer, can be seen in Fig.3. RecA2 and RecA4 have the highest amount of positive charge, but they do contain patches of negative charge, while RecA1 and RecA3 have roughly the same amount of positive and negative charge on their surfaces.



Fig.3. Electrostatic potential of RecA homologues. (a) RecA2; (b) RecA1; (c) RecA3, (d) RecA4. Blue clouds are positive and red ones are negative.

Identification of Protein Domains: SMART tool, used for identification of domains present in RecA family of proteins, detect three overlapping domains present, which are shown in Table 5, with the exception of RecA4, which does not contain Rad51 domain. CDD software annotated all four RecA homologues as having RecA domain.

	Domains						
Protein	RecA		AAA (overlapping)		Rad51 (overlapping)		
	Start	End	Start	End	Start	End	
RecA1	85	347	134	305	111	316	
RecA2	60	325	111	285	79	319	
RecA3	64	329	115	311	79	301	
RecA4	1	203	33	186	-	-	

 Table 5. Domains analysis of RecA homologues in A. Thaliana.

Localization of Proteins: Three tools in total were used to predict the localization of our proteins of interest, and the results are summarized in Table 6. LocTree3 software showed structures confidence in the percentages, PSI in the values from 0 to 1 (one being the highest confidence) and SUBA3 simply proposes the possible location of query proteins. When observing the results in general, RecA2, RecA3 and RecA4 are expected in mitochondria, while RecA1 is expected in chloroplast. If we go into details, there is a probability that RecA2 is also present in the cytosol and chloroplast (apart from mitochondria), and RecA4 might be present in both mitochondria and chloroplast. These results will be further elaborated in the discussion.

			r		
Protein	LocTree3		PSI		SUBA3
RecA1	Chloroplast	99%	Chloroplast	78.8%	Plastid
RecA2	Mitochondrion	89%	Cytosol Mitochondrion Chloroplast	40.9% 11.4% 23.1%	Mitochondrion
RecA3	Mitochondrion	99%	Mitochondrion	75.8%	Mitochondrion
RecA4	Mitochondrion	89%	Mitochondrion Chloroplast	20.4% 23.1%	Mitochondrion

Table 6. Subcellular localization of RecA proteins obtained from different bioinformatics tools.

Prediction of the Interactome: The interactions were also checked/confirmed with STRING, and 12 most important interacting proteins, according to smart, are shown in Table 7. Confidence value (CV) is high for all of them, making the results reliable. In general, most proteins have similar interactions, with the exception of RecA1, who alone interacts with THI1. MSH1 is also an exception, interacting only with RecA3, but it actually interacts with RecA2, RecA3 and RecA4. This is due to the fact that only 10 results with highest confidence are shown in the table specific for each RecA protein, while the total sum of interactions between already chosen proteins is shown in Fig.4, including also the results with confidence values of 0.9 and higher. The same methodology used for the localization prediction of RecA homologues has been implemented for the localization of the interacting proteins, after which the results have also been summarized in Table 7. The interactions are also available in graphical representation, shown in Fig.4.

Protein	ID	Function	Location	RecA Protein
SCE1	AT3G57870.1	SUMO-conjugating enzyme; catalyzes covalent attachment of SUMO proteins	Nucleus Cytosol Mitochondria Vacuole Peroxisome	All four
RAD51C	AT2G45280.2	DNA repair protein RAD51-like 3	Cytosol Nucleus Mitochondria Chloroplast	All four
SRS2	AT4G25120.1	Suppressor of RAD Six-screen mutant 2	Nucleus Cytosol Mitochondria Chloroplast	All four
XRCC3	AT5G57450.1	Plays essential roles in DNA damage repair in both somatic and meiotic cells.	Nucleus Cytosol Mitochondria	All four
MRE11	AT5G54260.1	Involved in DNA double-strand break repair (DSBR)	Nucleus Mitochondria	All four
RAD50	AT2G31970.1	DNA repair protein in double-strand breaks (DSBs) by non- homologous end joining (NHEJ)	Nucleus Cytosol Mitochondria	All four
UVH3	AT3G28030.1	Involved in nucleotide excision repair (NER) of UV- and oxidative damaged DNA	Nucleus Mitochondria	All four

Table 7. List of interactions between RecA homologues and other proteins from.

ATR	AT5G40820.1	Plays a central role in cell-cycle regulation by transmitting DNA damage signals to downstream effectors of cell-cycle progression	Nucleus Membrane Mitochondria	All four
DMC1	AT3G22880.1	May participate in meiotic recombination	Cytosol Mitochondria	All four
REV3	AT1G67500.2	DNA polymerase zeta catalytic subunit	Nucleus Mitochondria Chloroplast Cytosol	RecA 2 and 4
THI1	AT5G54770.1	Thiazole biosynthetic enzyme; Involved in biosynthesis of the thiamine precursor thiazole	Chloroplast	RecA1
MSH1	AT3G24320.1	MUTL protein homolog 1; DNA mismatch repair protein specifically involved in maintenance of mitochondrial genome configuration	Mitochondria Chloroplast	RecA3

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Fig.4. Graphical representation of the interactome of RecA homologues.

Prediction of Docking Sites: Due to the fact that there are a lot of interactors and our proteins of interest (RecA2, RecA1 and RecA3) show identical binding with interacting proteins, only specific docking predictions have been shown; namely all ten interactions with RecA2 and two additional interactions with RecA1 and THI1, and RecA3 and MSH1. The exception is RecA4, which exhibits different structure and docking patterns, so all docking predictions from RecA4 have been included as well. MSA shows that our proteins are homologous to each other. Proteins containing at least hundred residues and sharing 30% similarity are considered to be homologous, even though there are exceptions [43]. The highest similarity is between RecA2 and RecA4, almost 80%. which is also confirmed by phylogenetic tree. There seem to be three "types" of recA proteins: RecA2 and RecA4 together, RecA1 and RecA3, which has been confirmed by previous research [44]. Plants generally do not have four RecA genes, unlike A. Thaliana [45]. 3D models of proteins show high similarity between one another, which further confirms the homology predicted by MSA. All proteins share almost identical core regions with side regions having greater degree of variability. RecA4 shows the lowest structural similarities to RecA2even though it had the highest sequence similarity which confirms the fact that inferring structural similarity from sequence alone does not have to provide good results, the same way two seemingly distant proteins (less than 20%) sequence similarity) can be homologous [43,46]. Electrostatic potential plays an important role protein-protein and protein-ligand binding and recognition [47]. Since the DNA is negatively charged due to phosphate groups [2], proteins which interact with it have positive charge including RecA homologues.RecA1 and RecA3 show different electrostatic potential, which indicates that they might need additional help to work properly like the change in pH, since it can fluctuate in mitochondria and chloroplast due to their activities [48,49], and it has been shown that repair of DNA double-stranded breaks is sensitive to pH fluctuations pH [50]. PH does increase to optimal level for DNA repair during photosynthesis and cellular respiration [48,49] which suggests that it might help in the initiation of RecA activity. All four proteins are mainly predicted to be localized in mitochondria and chloroplast, with the exception of RecA2, which can also be in cytosol. These results are expected since RecA protein possess the ability of cellcycle arrest in order to prevent the transmission of the damage [51]. RecA2 being predicted in the cytoplasm in our results indicates that it also has the ability of preventing cell cycle progression after the damage has been detected. Interacting proteins mostly interact with all four RecA homologues, with some exceptions. MRE11 is involved in DSBR and its primary role is that of a nuclease [52]. It works in collaboration with RecA proteins, first excising the damaged part of the DNA, after which RecA proteins search for the homology between two DNA strands. Recent studies suggest that MRE11 is the one influencing which type of DNA repair will occur – either NHEJ, or HR [53], which indicates that MRE11 might recruit RecA to the site of DNA damage. MRE11 works in close coordination with RAD50, which also participates in the repair of dsDNA. Proposed role of RAD50 is that it binds the DNA strands and holds them in close proximity. Moreover, it can act as a regulator of MRE11, preventing it to excise nucleotides past a given point [54,52]. MRE11 and RAD50 make a complex in order to perform DNA repair [55], which also indicates that they work together in recruiting RecA protein for the strand exchange, after they have finished with the excision. RAD51C has two important functions - it participates in the HR of doublestranded DNA breaks [56] and in the cell cycle regulation [57]. Regarding DNA repair itself, it contributes to formation and stability of Holliday structure [58]. This suggests that RAD51C comes after RecA has been incorporated into the repair mechanism. More precisely, RecA protein scans for strand homology and initiates the strand exchange after which RAD51C stablizies the whole structure. Recent study shows the increase of RAD51C presence in mitochondria after induced stress and decrease in mitochondria numbers in cases of mutated RAD51C [59]. Additionally, since RAD51C also participates in the pathway responsible for cell cycle arrest after the detection of DNA damage [57], it is possible that RecA2 itself participates in the triggering of the pathway, binding to RAD51C and transmitting the signal further.

RAD51C works by forming two types of complexes during DNA repair, one of which is complex CX3, composed of RAD51C and XRCC3 protein [60]. XRCC3 also participates in HR by binding to the intersection point of the four duplex arms of the Holliday junction [61]. Together with RAD51C, it comes after the RecA has initiated the strand invasion and helps in the completion of the DNA repair process in mitochondria. This is also supported with the evidence of XRCC3 participating in the maintenance of mitochondrial genome [59]. UVH3 is involved in nucleotide excision repair of DNA as

a response to oxidative stress, UV radiation and ionizing radiation. The mode of action is unclear, but the research suggests that it possesses endonuclease activity, which puts it before RecA protein - excising the damaged nucleotides after which the RecA protein can come and initiate the strand invasion [62]. MSH1 is involved in mtDNA stability and repair by binding to DNA mismatchesand protecting against oxidative stress [63-64]. The exact mechanism is unkown, however, studies on yeast suggest that it has proofreading ability [65] and can induce heteroduplex rejection if the two DNA strands contain mismatches [66]. This indicates that MSH1 is regulator of HR and RecA proteins. MSH1 also participates in mtDNA maintenance [63-64], but our study suggests that it can perform the same functions in chloroplast as well. Similarly to MSH1, SRS2 is also involved in DNA repair through proof reading ability. It is DNA-dependent ATPase and DNA helicase [67] and it can also block recombination by disrupting RAD51 [68]. It also participates in the rescue of recombination intersections and forks by blocking the protein which promotes strand invasion (RAD51) and by recruiting endonucleases which will excise the damaged DNA, after which the recombination (or replication) is able to continue [69]. We found out that SRS2 can also be localized in both mitochondria and chloroplast, indicating that it can help in the repair of mitochondrial and chloroplast DNA by proofreading the recombination process. SCE1 (SUMO-conjugating enzyme) helps in the attachment of SUMO (small ubiquitin-like modifiers) proteins, which play important roles in many cell functions, including DNA repair, by covalently attaching to proteins and altering their function [70]. Cells with mutated SUMO proteins are, among other things, hypersensitive to DNA damaging agents [71]. In the case of double-stranded brakes in the DNA, RAD52 can become SUMOvlated which induces HR [72]. In the case of mutated RecA proteins which are unable to bind to SUMO, mitochondria and chloroplast might be more sensitive to DNA damage [71], possibly because ubiquitin acts as an antagonist to SUMO [72], which means that RecA protein will become degraded and thus unable to participate in the DNA repair. RecA proteins can also induce cell-cycle arrest in the case of DNA damage. This occurs most likely through interaction with ATR –a protein which has been shown to induce G2 arrest in response to double-strand brakes induced by gamma radiation [73]. Our results indicate that ATR is also present in mitochondria where it can interact with RecA homologues. This further confirms that RecA homologues in mitochondria can also induce cell cycle arrest in the case of DNA damage caused by oxidative stress by communicating with ATR protein and initiating a signal cascade. Apart from DNA repair, recombination also happens in meiosis. RecA/RAD51 homologue, DMC1 [74], participates in the pairing of homologous chromosomes during HR [75]. Unfortunately, there is not a lot of information on DMC1 protein in A. thaliana, and the research from Yeast suggests that DMC1 is meiosis-specific. Together with this, our results indicate that RecA2 might also participate in DNA recombination during meiosis, since it could be localized in the cytosol and it interacts with DMC1. THI1 protein has only one interaction - with RecA1. Both of these proteins are localized only in chloroplast. THI1 thiazole biosynthetic enzyme – is involved in the biosynthesis of thiamine, but it can also participate in stress response and DNA damage tolerance and it can be localized in the chloroplast [76,77]. Interaction with RecA1 indicates that it is directly involved in the repair of the DNA, however, the exact mechanism of action remains unclear. After the excision of damaged DNA and strand invasion, replacement of excised nucleotides is needed. This can be performed by REV3 which functions as the catalytic subunit of DNA polymerase zeta [78]. This suggests that, once the strand invasion has completed, REV3 works in collaboration with RecA homologues in order to synthesize the missing part of the DNA. Additionally, REV3 is important since it is involved in translesion synthesis – which represents the mechanism of DNA replication that can ignore DNA lesions and continue past them [79]. Moreover, recent studies suggest that REV3 also plays a role in recombination, since REV3-deficient cells showed decrease in recombination [80]. This indicates the possible dual crosstalk between RecA and REV3 – both for the strand invasion and polymerization.

**Conclusions:** Cellular processes are very complex and even though they are of great importance to us and we put so much effort into their study, a lot of mechanisms still remain at least a partial mystery to us. DNA repair is one such mechanism, especially the one present in mitochondria and chloroplast. Based on the interactome analysis and results in general, we conclude that the following mechanism occurs while repairing double-strand DNA breaks in mitochondria and chloroplast: after the DNA damage is detected, MRE11 nuclease excises the damaged nucleotides while RAD50 controls the excision keeps the strands in the close proximity for the RecA protein to initiate strand invasion. This indicates that mechanisms in mitochondria and chloroplast require additional attention, as there are a lot of mechanisms which still remain unclear to us. focusing on RecA proteins, we will be able to better understand not only the mechanism of DNA repair, but also the regulation of the cell cycle in general, in the case of DNA damage which is important for many areas of science, including cell culture, genetic recombination and the modern medicine.

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## THE INFLUENCE OF BETULIN AND ITS DERIVATIVES ON EXPRESSION OF APOPTOSIS AND AUTOPHAGY RELATED GENES IN COLORECTAL CANCER CELL LINE

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**Abstract:** Colorectal cancer (CRC) is a prevalent malignancy with rising incidence rates among young people. Despite advances in diagnostics, treatment options remain limited. Treatment-resistant CRC is a significant challenge, necessitating the exploration of novel anticancer compounds. In this context, betulin (BET) has emerged as a promising candidate. However, its poor bioavailability has led to the development of derivatives with enhanced pharmacological profiles. The effectiveness of these compounds can vary based on their chemical structure and the cancer type. Autophagy, a form of programmed cell death, is crucial in carcinogenesis and drug resistance and is interconnected with apoptosis. This study aims to assess the impact of betulin and its derivatives on the expression of apoptosis and autophagy related genes in colorectal cancer cell line RKO.

Introduction: Colorectal cancer (CRC) is one of the most significant healthcare challenges worldwide. It is one of the most common cancers, being the second most frequently diagnosed cancer in women and the third in men, and is one of the leading causes of cancer-related mortality. Additionally, it is projected that the number of cases among individuals aged 20 to 30 will increase dramatically. Despite advances in diagnostic methods, treatment options for CRC remain relatively limited, often requiring surgical resection, administration of chemotherapeutic agents such as 5-fluorouracil, cisplatin, and oxaliplatin, or a combination of these approaches. In some cases, immunotherapy may also be considered [1,2]. Treatment-resistant CRC poses a significant oncological challenge. Limited treatment options have prompted the search for new anticancer compounds, among which betulin (BET) plays an important role. Betulin has numerous biological properties, including anti-inflammatory, antibacterial, antiviral, antioxidant, hepatoprotective, neuroprotective, and anticancer activities. BET, known chemically as lup-20(29)-ene-3,28-diol, is classified as a lupane-type pentacyclic triterpene, and is a naturally occurring compound primarily found in the outer bark of birch trees (Betula spp.). Substances with a triterpene structure are most commonly obtained through the extraction of crushed birch bark using various organic solvents or mixtures. The obtained extracts are recrystallized or purified using their chromatographic methods. High-purity betulin can be obtained with high yields using ionic liquids, supercritical fluid technology, or thermal sublimation [3]. The poor solubility of betulin and betulinic acid may pose limitations in using these compounds as therapeutic agents. Attempts to overcome these limitations include introducing new functional groups into the molecule, forming complexes with cyclodextrins, liposomes, carbon nanotubes, or gold nanoparticles [4]. Due to its poor bioavailability, betulin undergoes chemical modifications, resulting in derivatives with proven enhanced pharmacological and pharmacokinetic properties. The synthesis method and substituents significantly influence the compound's effects on cells, including cancer cells. Moreover, the cytotoxic activity may depend on both the derivative and the type of cancer. New (28-propynoylbetulin) betulin derivatives include EB5 and ECH147 (29 diethoxyphosphoryl-28-propynoylbetulin). EB5 was obtained from betulin via the Steglich reaction using propynoic acid, DCC (N,N'-dicyclohexylcarbodiimide), and DMAP (4-dimethylaminopyridine). A multi-step synthesis pathway facilitated the conversion of betulin into the phosphonate derivative ECH147 (Fig.1) [5,6].



Fig.1. Chemical structure of betulin, EB5 and ECH147 [3].

Induction of cell cycle arrest and programmed cell death in cancer cells are effective cancer treatment strategies. Among the types of programmed cell death, apoptosis and autophagy can be distinguished. The roles that apoptosis and autophagy play in cancer may depend on many factors, such as the tissue and cell types, tumor stage, type of oncogenic mutation, extent of damage or stress, and intratumoral oxygen or nutrient levels. There are strong relationships between autophagy and apoptosis; these processes often interact and can inhibit or promote each other. Disturbance of this dynamic balance may be associated with tumorigenesis [7,8]. Betulin and its derivatives may regulate both types of programmed cell death. BET induces apoptosis in colorectal cancer, cervical cancer, and inhibits the viability of gastric cancer cells through ROS-dependent apoptosis. mTOR activity, a key modulator, is suppressed by betulin in some cancer cells, leading to the induction of autophagy, which acts as an apoptosis inducer in cell lines [9]. The aim of this study was to assess the changes in the expression profile of selected genes associated with apoptosis (BAX, BCL2, CASP3 and CASP7) and autophagy (BECN1, P62) under the influence of betulin and its two new derivatives-EB5 and ECH147 in colorectal cancer line RKO.

**Experimental:** Cell Culture Conditions: the colorectal cancer cell line RKO (CRL-2577; ATCC, USA) was maintained at 37 °C in a 5% CO<sub>2</sub> incubator in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (EuroClone, Milan, Italy) and 50 mg/mL gentamicin (BioWhittaker, Lonza, Basel, Switzerland). To investigate the effect of betulin and its derivatives on selected gene expression levels, cells were treated with betulin, EB5, ECH147, 5-fluorouracil (5-FU), and cisplatin (CIS) at a concentration of 10  $\mu$ g/mL. This concentration was selected based on previous cytotoxicity assay data [5]. To assess the influence of tested compounds, cells were incubated with them for 24 hours. Untreated cells served as controls. Ribonucleic Acid

Extraction and RT-qPCR: total RNA was extracted using TRIzol reagent according to the manufacturer's protocol. cDNA syntesis was performed with smART First Strand cDNA Synthesis kit (EurX, Poland). Changes in the expression of apoptosis and autophagy related genes: *BAX, BCL2, CASP3, CASP7, BECN1* and *P62* were assessed by real-time qPCR using a LightCycler® 480 (Roche, Switzerland) and Fast SG qPCR Master Mix (EurX, Poland).

**Results:** The expression of genes related to apoptosis – *BAX, BCL2, CASP3* and *CASP7* changed significantly under the influence of 5-FU and cisplatin (*CASP7* being significantly activated only by CIS). In the case of betulin and its derivatives, no significant changes in the expression of the analyzed genes were observed compared to the control cells (Fig.2A-D). In the case of genes associated with autophagy, changes in the expression profile were also observed after the application of the tested compounds. In the case of the *BECN1* gene, an increase in expression was observed solely under the influence of cisplatin. However, the expression of the *P62* gene was significantly higher following treatment of the cells with all tested compounds. Betulin and its two derivatives influenced the expression of *P62* to a similar extent as 5-FU and cisplatin (Fig.3A-B).



**Fig.2.** Relative gene expression of selected apoptosis related genes – *BAX* (A), *BCL2* (B), *CASP3* (C), *CASP7* (D), where: c-control, 5FU-cells treated with 5-fluorouracil; CIS-cells treated with cisplatin; BET-cells treated with EB5 derivative, ECH147-cells treated with ECH147 derivative; \*p < 0.05 vs control, betulin and-its derivatives, # - p < 0.05 vs CIS, ^ p < 0.05 vs EB5.



**Fig.3.** Relative gene expression of selected autophagy related genes – *BECN1* (A) *and P62* (B), where: c-control, 5FU-cells treated with 5-fluorouracil; CIS-cells treated with cisplatin; BET-cells treated with betulin; EB5-cells treated with EB5 derivative, ECH147-cells treated with ECH147 derivative; \*p < 0.05 vs control, betulin and its derivatives.

**Conclusions:** Betulin and its derivatives EB5 and ECH147 do not affect the profile of selected genes associated with the apoptosis process. In the case of autophagy, no significant difference was observed for the *BECN1* gene, however a significant expression increase was noted for the autophagy marker P62. This may indicate that betulin could influence the regulation of this process. It is worth conducting further studies to analyse other genetic markers of both processes and to extend them to other colorectal cancer cell lines. The betulin derivatives EB5 and ECH147 act on the tested cells similarly to betulin.

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# FTIR, RAMAN SPECTROSCOPY AND TGA-FTIR STUDY TO DETECT HYDROCORTISONE INCOMPATIBILITY

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Abstract: Hydrocortisone, a natural corticosteroid hormone, is secreted by the adrenal cortex during times of stress. Its synthetic equivalent is used to treat inflammatory and rheumatoid diseases, allergic conditions, and autoimmune diseases such as Addison's disease (adrenal insufficiency). It is available commercially as the unchanged hormone or as acetate, cypionate, sodium phosphate, butyrate, valerate, and sodium succinate in the form of pharmaceutical preparations such as tablets, capsules, ointments, creams and injections. It is practically insoluble in water, therefore research is necessary to select excipients that contribute to increasing its solubility and thus bioavailability. These studies use thermal and spectroscopic methods as well as thermal methods coupled with spectroscopic methods. The most commonly used spectroscopic methods in these studies are Fourier transform infrared (FTIR) and Ramana spectroscopy, and thermal methods coupled with spectroscopic methods, include thermogravimetry (TGA) coupled with FTIR (TGA-FTIR). In this work, FTIR, Raman spectroscopy and TGA-FTIR were used to study the incompatibility in the mixtures of hydrocortisone with hypromellose and sodium carboxymethylcellulose. Differential scanning calorimetry (DSC) was used to confirm the results. The results show that mixing hydrocortisone with these excipients can contribute to the increase of its solubility.

Introduction: FTIR and Raman spectroscopy are the most commonly used non-thermal techniques, which together with thermal techniques are recommended for screening active substance-excipient compatibility [1]. They use the so-called fingerprint area of the active substance and the excipient, based on the chemical and physical attributes of these substances. The high sensitivity of these techniques to the chemical structure and environment means that any subtle deviations in the physicochemical properties of the active substance due to the interaction with excipients are easily detected [2]. The observed physicochemical changes of the drug substance are caused by interactions with polymorphic excipients that lead to changes, dehvdration. formation of hydrates/solvates, changes in the deformation behavior of powders, etc. By comparing the spectra of mixtures with the spectra of the components, potential incompatibilities between components can be detected [2, 3]. Incompatibilities between mixture ingredients are indicated by band shifts, changes in their intensity and shape, as well as the disappearance of bands or the appearance of new ones. The coupling of thermal and spectroscopic techniques enables an in-depth understanding of the incompatibilities occurring and contributes to the insight into excipients and their matching to the active substance. At the same time, the use of thermal and spectroscopic methods ensures the

absence of costly material losses and accelerates the development of a suitable preformulation [1].

**Experimental:** Hydrocortisone, 98,00% was obtained from Thermo Scientific (Mundelein, IL, USA), carboxymethylcellulose sodium from Chemat (Gdansk, Poland) and hypromellose from Sigma-Aldrich (Steinhem, Germany). Hydrocortisone mixtures with excipients were homogenized using a spatula in a porcelain mortar. Samples for FTIR measurements were prepared in the form of KBr tablets and then spectra were recorded in the wavenumber range of 4000–400 cm<sup>-1</sup> every 4 cm<sup>-1</sup> using a Nicolet 380 spectrometer (Thermo Fischer Scientific, Madison, USA). Raman spectra were recorded at a DXR laser wavelength of 780 nm and a power of 15 mW using a DXR SmartRaman spectrometer (Thermo Fisher Scientific, Madison, USA) in the range of 3414–400 cm<sup>-1</sup> every 2 cm<sup>-1</sup>. TGA-FTIR analyses were performed in the temperature range of 25–700 °C using a TG Q5000 thermogravimetric analyzer (TA Instruments, New Castle, Delaware, USA) coupled to a Nicolet 6700 spectrometer (Thermo Fischer Scientific, Madison, USA) under nitrogen atmosphere and at a heating rate of 20 °C/min. DSC was used to confirm the spectroscopic and TGA-FTIR results. Thermoanalytical analyses were performed with a differential scanning calorimeter 822e (Mettler Toledo, Schwerzenbach, Switzerland) in a nitrogen atmosphere, ranging from ambient to 350°C at a heating rate of 10°C/min.

**Results:** Figure 1A,B shows the FTIR and Raman spectra of hydrocortisone, its mixtures with hypromellose and sodium carboxymethylcellulose, and those of these two excipients. In the FTIR spectrum of hydrocortisone (Fig.1A) one can find very strong OH stretching vibrations at 3437 cm<sup>-1</sup>, aromatic CH stretching at 3018 cm<sup>-1</sup>. In the range of 2970–2894 cm<sup>-1</sup> there are vibrations assigned to  $CH_3$  asm. stretching,  $CH_2$  asym. stretching, CH<sub>3</sub> sym. stretching, CH<sub>2</sub> sym. stretching. The next vibrations at 1713 cm<sup>-1</sup> belong to CO stretching, at 1642 cm<sup>-1</sup>, 1610 cm<sup>-1</sup> and 1570 cm<sup>-1</sup> aromatic C=C stretching, at 1432 cm<sup>-1</sup> CH<sub>3</sub> asym bending, at 1391 cm<sup>-1</sup> COH in plane bending, at 1322 cm<sup>-1</sup> CC stretching, at 1281 cm<sup>-1</sup> CO stretching, at 1237 cm<sup>-1</sup> CC stretching [4]. In the Raman spectrum of hydrocortisone (Fig.1B), the following can be distinguished: at 2956 cm<sup>-1</sup> and at 2918 cm<sup>-1</sup> CH<sub>3</sub> asymmetric stretching, at 1714 cm<sup>-1</sup> CO stretching, at 1647 cm<sup>-1</sup> and 1615 cm<sup>-1</sup> aromatic CC stretching, at 1439 cm<sup>-1</sup> CH<sub>3</sub> asymmetric bending, at 1337 cm<sup>-1</sup> CH<sub>3</sub> symmetric bending, at 1286 cm<sup>-1</sup> C-O stretching, and at 1230 cm<sup>-1</sup> C-C stretching [4]. The spectra of hypromellose and sodium carboxymethylcellulose, which are carbohydrates, show the following vibrations, OH and CH stretching in the range of  $3600-2800 \text{ cm}^{-1}$ , and in the range of  $1500-1200 \text{ cm}^{-1}$  for HCH and CH<sub>2</sub>OH, followed by CO stretching in the range of 1200–950 cm<sup>-1</sup>. In turn, deformation vibrations: COH, CCH and OCH occur in the range of 950–700 cm<sup>-1</sup>, and in the range of 700–500 cm<sup>-1</sup> CCO and below 500 cm<sup>-1</sup> CCO and CCC [5-7]. In the spectrum of the mixture of hydrocortisone with hypromellose, there is no clear change in the hydrocortisone bands. This spectrum represents the sum of the bands of hydrocortisone and hypromellose. In the case of the spectrum of mixture of hydrocortisone with sodium carboxymethylcellulose, there is a significant decrease in the intensity of the hydrocortisone bands, which therefore suggests the occurrence of incompatibility between hydrocortisone and sodium carboxymethylcellulose. In turn, in the Raman spectra of mixtures of hydrocortisone with hypromellose and carboxymethylcellulose,

the presence of hydroxycortisone bands is observed, but their intensity is much lower than in the case of the bands of pure hydrocortisone. TGA-FTIR analyses for hydrocortisone and its mixtures with hypromellose and sodium carboxymethylcellulose are presented in Fig.1C-E. Figure 2a,b shows TGA-FTIR analyses performed for excipients. As a result of the decomposition of hydrocortisone, its mixtures with excipients and sodium carboxymethylcellulose, characteristic bands for methane can be identified. In the case of the decomposition of hypromellose, these will be bands of mainly low-molecular oxygen-containing compounds such as: alcohols (e.g. methanol), hydroxy ethers (methoxyethanol) and carbonyl compounds (e.g. valeraldehyde). These products are also present in the case of the mixture of hydrocortisone with hypromellose.



Fig.1. A) FTIR spectra and B) Raman spectra and 3D surface plot for TGA-FTIR spectra of the evolved gaseous products for C) hydrocortisone, D) hydrocortisone - hypromellose mixture, E) hydrocortisone - carboxymethylcellulose sodium mixture, F) DSC curves.



Fig.2. 3D surface plot for TGA-FTIR spectra of the evolved gaseous products for a) hypromellose and b) carboxymetylcellulose sodium.

As a result of further decomposition of hydrocortisone, its mixtures with excipients and the excipients themselves, bands associated with the presence of water, carbon dioxide and carbon monoxide and carbonyl compounds are recorded. DSC results reveal a significant reduction in the height of characteristic melting peak of hydroxycortisone (Fig.1F), confirming the spectroscopic results of incompatibility between hydrocortisone and sodium carboxymethylcellulose. However, in the case of mixing hydrocortisone with hypromellose, incompatibilities occur after a longer time after mixing, therefore FTIR did not show any changes in the hydrocortisone bands in the spectrum of the mixture.

**Conclusions:** The use of spectroscopic methods such as FTIR and Raman spectroscopy and thermal methods coupled with spectroscopic methods such as TGA-FTIR enabled the identification of incompatibilities in mixtures of hydrocortisone with sodium carboxymethylcellulose and hypromellose. These results were confirmed by DSC analyses.

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## USE OF FTIR AND PXRD ANALYSIS TO PREDICT THE INCOMPATIBILITY BETWEEN METHYLXANTHINES AND SELECTED EXCIPIENTS

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Abstract: Detection of incompatibilities between active substance and excipient is an essential step in drug development prior to formulation. This allows for obtaining appropriate stability and bioavailability of the active substance and the possibility of manufacturing solid dosage forms. The advent of spectroscopic methods, in addition to thermal methods, has significantly influenced the early prediction, monitoring and characterization of incompatibilities between components of a pharmaceutical mixture, resulting in avoiding costly waste of materials and enormously reducing the time required to obtain an appropriate formulation. The use of spectroscopic techniques helps to identify incompatibilities between components based on changes in the spectra of mixtures associated with the shift, change in intensity or disappearance of characteristic bands of the components or the appearance of other new bands. Sometimes there are difficulties in interpreting the spectra of mixtures due to the overlap of component bands. In this work, FTIR spectroscopy and PXRD were used to predict the incompatibility in the mixtures of caffeine, theobromine and theophylline with selected excipients (aminoacetic acid, glucitol and sucrose), which were mixed at the ratios 4:1, 1:1 and 1:4. The results of spectroscopic studies showed the incompatibility of these methylxanthines with glucitol and sucrose, confirmed by differential scanning calorimetry (DSC).

Introduction: Spectroscopic methods, along with thermal analysis methods, are the most suitable for assessing incompatibilities between the active substance and the proposed excipients. Commonly used spectroscopic methods in incompatibility investigation are Fourier transform infrared spectroscopy (FTIR), Raman spectroscopy and powder X-ray diffraction (PXRD) [1]. Spectroscopic methods typically investigate the structure and environment of organic compounds. These techniques take into account the solid-state behavior of the active substance and its formulations, making it possible to detect potential incompatibilities since vibrational changes indicate the existence of possible intermolecular interactions between components. Using these techniques, it is possible to detect pharmaceutical interactions that result from dehydration, hydrate/solvate formation, desalting, transformations from crystalline to amorphous form and vice versa, or polymorphic changes during pharmaceutical processing [2]. The advantage of spectroscopic techniques is that by using the physicochemical properties of active substances and excipients it is possible to obtain an individual "fingerprint" for them. The relatively high sensitivity of these techniques contributes to the rapid detection of even minimal deviations in the physicochemical properties of the active substance, which may result from interactions with excipients. Hence, these techniques enable rapid detection of incompatibilities manifested by changes in the spectra of mixtures of the active substance with the excipient, i.e. shifts and changes in the intensity of the component bands or the disappearance of the component bands, as well

as the appearance of new bands not belonging to the components [3]. Additionally, thermal analysis, especially differential thermal analysis (DSC), is typically used to confirm spectroscopic results. Therefore, spectroscopic techniques (FTIR and PXRD) were used to investigate the incompatibility in methylxanthines mixtures (caffeine, theobromine and theophylline) with selected excipients (amino-acetic acid, glucitol and sucrose) at the ratio of 4:1, 1:1 and 1:4, the results of which were then confirmed using a thermal method, i.e. DSC.

Experimental: Methylxanthines (caffeine, theobromine and theophylline) were obtained from Fluka (Buchs, Switzerland), amino-acetic acid and sucrose from Thermo Fisher Scientific (Mundelein, IL, USA), glucitol from AmBeed (Arlington Hts, IL, USA). Mixtures of mentioned above methylxanthines with selected excipients were prepared by mixing appropriate amounts of the ingredients in a porcelain mortar using a plastic spatula. FTIR spectra of the mixture samples and pure components prepared as KBr pellets were recorded using a Nicolet 380 FTIR spectrophotometer from Thermo Fischer Scientific (Madison, USA) in the mid-infrared spectral range at an instrument resolution of 4 cm<sup>-1</sup>. PXRD patterns of the studied samples were obtained with a D2 Phaser (Bruker, Karlsruhe, Germany) with a CuK  $\alpha$  lamp (k = 0.154060 nm), a current of 10 mA and a voltage of 30 kV and an exposure time of 0.10 s using a step size of  $0.02^{\circ}$ . The measurements were performed in the diffraction angle range (2 $\theta$ ) of 7–55° using Diffrac.suite software. The DSC 822<sup>e</sup> calorimeter (Mettler Toledo, Schwerzenbach, Switzerland) was used to perform thermal analyses of 4-5 mg samples at a heating rate of 10 °C/min, with a nitrogen flow of 70 ml/min and in the range of 25-300 °C. The calorimeter was previously calibrated using indium and zinc.

**Results:** Figure 1A presents the FTIR spectra of caffeine, theobromine and theophylline and excipients. The FTIR spectra of methylxanthines are very similar and can be divided into two regions: 4000-1500 cm<sup>-1</sup> and below 1500 cm<sup>-1</sup> [4,5]. The latter region is the fingerprint region, which is extremely difficult to interpret, as it contains both valence vibrations of single C–C and C–N bonds and deformation vibrations of C–H bonds. In the caffeine spectrum, in the range up to 2000 cm<sup>-1</sup>, there are two bands that reflect valence vibrations of C–H groups, corresponding to wave numbers 3125 and 3000 cm<sup>-1</sup>. These vibrations are associated with two methyl substituents in the pyrimidine ring and one methyl substituent in the imidazole structure, as well as with the C-H bond in the imidazole ring. In the spectra of theophylline and theobromine, more bands are observed in this range, which is related to the presence of a secondary amine N-H group in their molecules, with the stretching vibrations originating from this group forming a doublet. The bands in the range from 1708 to 1666 cm<sup>-1</sup> correspond to the valence vibrations of the carbonyl groups in the 1,6-pyrimidine position. In the range of 1800–1500 cm<sup>-1</sup>, valence vibrations of the C=N double bonds of the imidazole ring or C=C bonds of the fused xanthine ring may also occur. In the spectrum of caffeine, these vibrations correspond to wave numbers 1582 or 1556 cm<sup>-1</sup>, while in the spectra of theophylline and theobromine there is no band at 1582  $\text{cm}^{-1}$ , and only a band at 1556  $\text{cm}^{-1}$  occurs. The spectrum of amino-acetic acid shows bands characteristic of amines and carboxylic acids [6]. The amine group frequencies occur in the wave number range of 3500–3200 cm<sup>-1</sup>, the N–H stretching and bending vibration bands in the region of 1650-1560 cm<sup>-1</sup> and 900–650 cm<sup>-1</sup>, the C–N stretching vibration band in the region of 1230–1030 cm<sup>-1</sup>.



Fig.1. A) FTIR spectra of methylxanthines and excipients, B) PXRD patterns of methylxanthines and excipients, C) FTIR spectra of mixtures and ingredients, D) PXRD patterns of mixtures and ingredients.

In contrast, the intense, broad O–H stretching vibration band characteristic of carboxylic acids occurs in the region of 3000-2500 cm<sup>-1</sup>, and the O-H bending vibration bands at approx. 1400 and 920 cm<sup>-1</sup>. In turn, the C=O stretching vibration band of carboxylic acids is present at about 1700 cm<sup>-1</sup>, and the C-H stretching vibration bands are in the region of 2960–2850 cm<sup>-1</sup> and 1470–1350 cm<sup>-1</sup>. In the spectra of glucitol and sucrose the following vibrations are present: OH and CH stretching in the wavenumber range of  $3600-2800 \text{ cm}^{-1}$  and HCH and CH<sub>2</sub>OH in the range of  $1500-1200 \text{ cm}^{-1}$ , as well as CO stretching in the range of 1200–950 cm<sup>-1</sup>. In turn, the vibrations of COH, CCH and OCH deformation bands are observed in the range of 950-700 cm<sup>-1</sup>, and exocyclic deformation bands in the range of 700-500 cm<sup>-1</sup>, and endocyclic deformation bands below 500 cm<sup>-1</sup> [7,8]. The diffractograms of methylxanthines and excipients are shown in Fig. 1B. The highest diffraction line intensities of caffeine were observed at  $2\theta$ : 11.96; 12.50; 23.69; 24.03; 26.38; 27.00; 28.34°, and theobromine at 20: 10.78; 13.60; 16.63; 19.28; 19.72; 23.77 and 27.27°. In turn, the diffraction reflections of theophylline was observed at 20: 7.26; 12.75; 14.51; 21.79; 24.20; 25.57; 26.60; 27.51; 27.81; 29.43°. The diffraction patterns of excipients reflect also their crystalline nature. In the case of amino-acetic acid, the highest reflection intensity was obtained for diffraction angles  $2\theta$ equal to 14.83; 19.02; 20.11; 23.97; 28.46; 29.90; 35.44; 36.60°. In turn, the most intense diffraction lines for glucitol occur at  $2\theta$  of 11.94; 18.88; 22.09; 22.80; 23.63;  $25.63^{\circ}$ , and for sucrose at  $2\theta$  of 8.54; 11.88; 12.95; 13.35; 15.68; 16.87; 19.04; 19.70; 20.57; 21.08; 24.82; 25.29; 40.42°. Comparing the spectra of mixtures of methylxanthines with those of ingredients (Fig.1C), changes suggesting incompatibilities can be observed, i.e. changes in the intensity of some characteristic methylxanthine bands or splitting of these bands in the range of 1850-1300 cm<sup>-1</sup>, i.e. alterations in the

bands assigned to stretching C=O, C–N, C=N vibrations. These changes are observed after mixing methylxanthines with glucitol and sucrose. In the case of the spectra of mixtures of methylxanthines with amino-acetic acid, the component bands did not change. In turn, the PXRD patterns of mixtures of methylxanthines with excipients (Fig.1D) rather show diffraction lines similar to the lines of the components themselves. DSC results (Fig.2A,B) show exemplary mixtures of one of the methylxanthines with sucrose and glucitol and confirm the FTIR and PXRD outcomes. The characteristic melting peak of methylxanthine in mixtures with these excipients disappeared.



Fig.2. A) DSC curves of caffeine mixtures with sucrose and ingredients, B) DSC curves of caffeine mixtures with glucitol and ingredients.

**Conclusions:** Spectroscopic results (FTIR and PXRD) for mixtures of selected methylxanthines (caffeine, theobromine and theophylline) with excipients such as aminoacetic acid, glucitol and sucrose at the ratios of 4:1, 1:1 and 1:4 demonstrated that two excipients glucitol and sucrose were incompatible with methylxanthines. Thermal analysis as DSC confirmed these results.

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### APPLICATION OF RAMAN SPECTROSCOPY AND PXRD IN COMPATIBILITY STUDY OF PHARMACEUTICAL MIXTURES

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Abstract: Both Raman spectroscopy and powder X-ray diffraction (PXRD) are widely used in the qualitative analysis of pharmaceutical preparations. Furthermore, Raman spectroscopy is one of the vibrational spectroscopy techniques that, together with Fourier transform infrared spectroscopy (FTIR), complement each other. In turn, PXRD provides a direct measure of the crystalline form of a substance. A crystalline form exhibits a unique set of diffraction lines, and the absence of a crystalline line from the active substance during analysis of a dosage form can indicate that the substance has become amorphous. Raman spectroscopy and PXRD analysis are extremely helpful in the case of incompatibility studies between components of pharmaceutical preparations. These techniques allow to see changes in the active substance, which are the result of changes in crystallinity/amorphousness and polymorphic forms of the active substance in the presence of excipients and with/without adsorbed moisture. Therefore, the abovementioned methods were used to study the compatibility of naproxen sodium with selected excipients (hydroxypropylcellulose and hydroxyethylcellulose). The results of these methods were confirmed by thermal methods such as differential scanning calorimetry (DSC). The obtained results revealed the incompatibility of naproxen sodium with hydroxyethylcellulose.

Introduction: Spectroscopic techniques, including Raman spectroscopy and powder X-ray diffraction (PXRD), are essential tools in pharmaceutical analysis, used in assessing the compatibility of formulation ingredients as well as in quality control [1]. Raman spectroscopy as a vibrational spectroscopy technique is often used together with Fourier transform infrared spectroscopy (FTIR). However, Raman spectroscopy is a fast and sensitive method for the structure of organic compounds, does not require sample preparation for analysis and is non-destructive to the sample. PXRD, in turn, is a sensitive method for determining the crystalline form of a substance. In the case of both Raman Spectroscopy and PXRD, they present a unique pattern of peaks for a tested substance, defining its structure and degree of crystallization [2]. Both methods are also vastly important for testing incompatibilities between components of pharmaceutical preparations. Identification of incompatibilities between components of pharmaceutical mixtures by these methods is performed by comparing the spectra/diffractograms of the mixtures with those of the components [3]. Incompatibility between ingredients in the spectra/diffraction patterns of mixtures are manifested by shifts, spectral/diffraction changes, the appearance of new peaks due to component interactions, or the disappearance of component peaks. Incompatibility detected by both methods are confirmed by thermal analyses, most often by differential scanning calorimetry (DSC).

**Experimental:** Naproxen sodium was purchased from AmBeed (Arlington Hts, IL, USA), hydroxypropylcellulose and hydroxyethylcellulose from Sigma-Aldrich (Steinhem, Germany). Naproxen sodium was mixed with excipients in a porcelain mortar with a plastic spatula. Raman spectra were recorded using a DXR SmartRaman spectrometer (Thermo Fisher Scientific, Madison, USA) at a DXR laser wavelength of 780 nm and a power of 15 mW in the range of 3414–400 cm<sup>-1</sup> with resolution of 2 cm<sup>-1</sup>. PXRD patterns of naproxen, its mixtures and excipients were made in the diffraction angle range of 7–55° with using a D2 Phaser (Bruker, Karlsruhe, Germany) with Diffrac.suite software, at a CuK  $\alpha$  lamp (k = 0.154060 nm), a current of 10 mA and a voltage of 30 kV and an exposure time of 0.10 s using a step size of 0.02°. DSC was used to verify the Raman and PXRD results. Thermal analyzes were carried out using an 822e differential scanning calorimeter (Mettler Toledo, Schwerzenbach, Switzerland) in a nitrogen atmosphere and in the temperature range of 25–400°C with a heating rate of 10°C/min.

**Results:** The Raman spectrum of naproxen sodium (Fig.1A) reveals Raman shift bands at 3011 cm<sup>-1</sup> for CH<sub>3</sub> stretching vibrations and 2859 cm<sup>-1</sup> for CH<sub>2</sub> stretching vibrations. as well as at 1617 cm<sup>-1</sup> for C–C stretching vibrations and C–H bending vibrations, both in the ring. Further, at 1465 cm<sup>-1</sup> the CH<sub>3</sub> bending vibrations are observed, at 1422 cm<sup>-1</sup> the CH<sub>3</sub> and CH<sub>2</sub> bending vibrations, as well as at 1388 cm<sup>-1</sup> and 1341 cm<sup>-1</sup> the CH<sub>2</sub> bending vibrations [4]. Raman analysis was enhanced by recording an FTIR spectrum for naproxen sodium (Fig.2). In the FTIR spectrum of sodium naproxen, O–H stretching vibrations occur at 3188 cm<sup>-1</sup>, C=O stretching vibrations unbonded with hydrogen and bonded with hydrogen at 1726  $\text{cm}^{-1}$  and 1684  $\text{cm}^{-1}$ , respectively. In addition, C–O stretching vibrations can be found at 1090 cm<sup>-1</sup>, symmetrical aryl-O stretching vibrations at 1264 cm<sup>-1</sup> and 1028 cm<sup>-1</sup>, and aromatic C=C stretching vibrations at 1604 cm<sup>-1</sup> and 1481 cm<sup>-1</sup> [5,6]. In the case of Raman spectra of excipients (Fig.1A), there is a band or overlapping bands at  $\sim 2900$  cm<sup>-1</sup> of CH/CH<sub>2</sub> stretching vibrations, and in the range of 1500–1200 cm<sup>-1</sup> C–H deformation vibrations, in the range of 1200–950 cm<sup>-1</sup> symmetric C-C and C-O stretching vibrations. There are also marker bands resulting from symmetric CC and CO stretching vibrations in the region of 1200–1000 cm<sup>-1</sup> and in the region below 500  $cm^{-1}$  of skeletal respiration modes [7,8]. Raman spectra of the mixtures were compared with the spectra of the components. A significant decrease in the intensity of the absorption bands of naproxen sodium in the mixtures, changes in the shape and shifts of the bands were observed, which indicates the existence of incompatibility between naproxen sodium and excipients. Diffraction patterns of naproxen sodium and their mixtures are presented in Fig.1B. The most intense lines of naproxen sodium were observed at  $2\theta$  of 6.45, 12.42, 13.14, 16.63, 17.81, 18.76, 20.11, 22.14, 23.48, 26.97 and 28.21. In turn, the diffraction patterns of the excipients reveal their amorphous form. The diffraction patterns of the mixtures after comparison with those components showed the presence of diffraction lines of naproxen sodium. In turn, the DSC analysis of the mixtures of naproxen sodium with hydroxyethylcellulose and hydroxypropylcellulose, carried out additionally to confirm the Raman and PXRD results, showed the presence of a melting peak of naproxen sodium, but in the case of the mixture of naproxen sodium with hydroxyethylcellulose, this peak was significantly reduced. Hence, it can be assumed that hydroxyethylcellulose was incompatible with naproxen sodium.



Fig.1. A) Raman spectra and B) PXRD patterns of naproxen sodium, excipients and their mixtures.



Fig.2. FTIR spectra of naproxen sodium, hydroxyethylcellulose and their mixtures.

**Conclusions:** The use of Raman spectroscopy and PXRD allowed to observe significant decreases in the intensity of the bands belonging to sodium naproxen in the mixtures, which suggested incompatibilities. DSC results confirm that mixing sodium naproxen with hydroxyethylcellulose has a much greater effect on the physicochemical changes of sodium naproxen than mixing with hydroxypropylcellulose.

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# MANAGEMENT OF WASTE FROM SEMI-DRY FLUE GAS DESULFURIZATION – PRELIMINARY STUDIES

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**Abstract:** This study investigates the potential transformation of semi-dry FGD waste into useful gypsum through simple oxidation with atmospheric oxygen and manganese(II) ions as catalyst.

Introduction: Sulfur dioxide removal processes play a crucial role in environmental protection. There are three main types of flue gas desulfurization (FGD): wet, dry, and semi-dry, each with specific advantages and disadvantages [1]. Consequently, Polish power plants and combined heat and power plants utilize different variants of these processes. However, the semi-dry FGD process generates a byproduct that is difficult to manage, unlike the solid waste from wet FGD, which produces synthetic gypsum widely used in construction. The solid byproduct of semi-dry FGD consists mainly of calcium sulfite and contains unreacted calcium hydroxide, calcium carbonate, and potentially calcium chloride along with some amounts of gypsum. Its storage requires continuous monitoring due to its alkaline pH and the chemical instability of calcium sulfite, which undergoes slow oxidation. This oxidation leads to oxygen depletion in and around the storage sites, causing land degradation. Efforts have been made to repurpose this waste, for example, as an additive in concrete [2] or, after modification, as a soil amendment for pH regulation [3]. A promising and relatively simple utilization method is its use as a partial substitute for limestone in the wet FGD process [1]. However, with ongoing shifts away from fossil fuels, influenced in part by the current global political situation, it remains uncertain whether the balance between semi-dry and wet FGD installations will allow for such waste management. Moreover, the potential decommissioning of power plants using wet FGD could lead to a significant shortage of gypsum in the construction market. Therefore, this study investigates the potential transformation of semi-dry FGD waste into useful gypsum through simple oxidation with atmospheric oxygen.

**Experimental:** The waste used in this study, a light gray powder, was obtained from a combined heat and power plant utilizing the semi-dry FGD process for flue gas purification. Samples of 3, 6, or 15 g of waste were weighed into a 500 ml beaker, followed by the addition of 300 ml of water. The mixture was stirred using a magnetic stirrer, with temperature and pH monitored, and compressed air supplied. A suitable amount of 1 M sulfuric acid was added to adjust the pH to 4, and in some cases, a catalyst (0.25 M MnSO<sub>4</sub> solution) was introduced. During air bubbling, suspension samples were collected, and sulfite content was determined using an iodometric method. Once all calcium sulfite had reacted, air bubbling was halted, and sediment samples were

taken for SEM analysis (scanning electron microscope Phenom Pro Desktop SEM, Phenom–World, Netherlands) to assess crystal size. Factors considered during postoxidation sampling included crystallization time (0, 2, 5, 20 h), suspension mixing, neutralization to pH 7, catalyst presence, and the introduction of crystallization nuclei (10 wt.% gypsum from previous experiments relative to the waste). To identify both the waste and the resulting product, XRD analysis was performed using an X-ray diffractometer (Seifert 3003TT powder, RICH. SEIFERT & CO. GmbH & Co. KG, Ahrensburg, Germany).

**Results:** The XRD analysis of the investigated waste confirmed the presence of calcium sulfite and gypsum (Fig.1). In the 2-theta range of  $5-28^{\circ}$ , an amorphous phase was also observed, likely originating from other sample components (mainly unreacted calcium hydroxide, as well as some calcium carbonate and calcium chloride). The high pH (~12) of the prepared slurry indicated the presence of alkaline components. To neutralize the slurry and convert these compounds into the desired gypsum, 1M sulfuric acid was added dropwise (a sudden addition or the use of a more concentrated acid would increase the risk of sulfur dioxide release).



Fig.1. XRD diffractogram of the waste used in the study.

Once the pH was adjusted to 4, the oxidation of sulfite to sulfate was initiated by air bubbling. Various concentrations of the waste slurry in water (10, 20, and 50 g/l) were used, along with different concentrations of  $Mn^{2+}$  ions as catalyst (0, 50, and 100 mg/l). The process was carried out until 99% of the initial sulfite content was removed. The results (Fig.2) indicate that increasing the slurry concentration from 10 g/l to 20 g/l is beneficial, but further increasing it to 50 g/l is detrimental, as the reaction time extends disproportionately (3.5 times longer vs. a 2.5-fold increase in slurry concentration). The addition of  $Mn^{2+}$  (50 mg/l) significantly reduces reaction time, and for a 20 g/l slurry, doubling the catalyst concentration further decreases the reaction time by approximately 20%. ICP-AES analysis confirmed that the catalyst concentration in the solution after gypsum crystallization and filtration remains unchanged within the method's error margin (±1.5%). The catalyst was added after acidification; if introduced into the alkaline mixture, it reacts to form brown manganese oxide. Further studies are needed to develop a catalyst recycling procedure.



Fig.2. Amount of CaCO<sub>3</sub> (as a percentage of the initial content) as a function of time during oxidation.

During the process, the solution pH decreases to 2.2-2.5 (Fig.3) due to sulfite oxidation, suggesting that pH monitoring can be used to determine the reaction endpoint.



XRD analysis confirmed that the reaction product is gypsum (Fig.4). Selected SEM micrographs of gypsum obtained under different conditions are shown in Fig.5. Regardless of the applied conditions (crystallization time, slurry stirring, neutralization to pH 7, presence of catalyst, or introduction of crystallization seeds), the resulting gypsum crystals exhibit similar sizes, appearing as platelets and needles ranging from a few to several micrometers, with crystals exceeding 20 µm being rare.



Fig.4. XRD diffractogram of the obtained product.



**Fig.5.** SEM micrographs of the obtained product (slurry: 20 g/l): a, b – samples filtered immediately after CaSO<sub>3</sub> oxidation, c, d – samples filtered 20 h after CaSO<sub>3</sub> oxidation, a, c – reaction without catalyst, b, d – reaction with catalyst (Mn: 100 mg/L), magnification: 5000×.

**Conclusions:** Preliminary studies confirm the feasibility of producing gypsum from semi-dry FGD waste through simple oxidation with atmospheric oxygen using manganese ions as a catalyst. Further research is necessary to establish a catalyst recycling procedure and assess the suitability of the obtained gypsum for construction applications.

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### VERIFICATION OF THE METHOD OF DETERMINATION SELECTED ANTIOXIDANTS IN FEED BY HPLC METHOD WITH SPECTROPHOTOMETRIC DETECTION

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Abstract: The aim of the paper was to verify the HPLC method with spectrophotometric detection of the determination of antioxidant contents (propyl galusate, ethoxyquine EQ, butylated hydroxytoluene BHT, octyl galusate and butylated hydroxyanizole BHA) in feeds according to the procedure VDLUFA FG-VI [1]. The following validation parameters were determined: the limit of quantifications LOQ for individual antioxidants were set at 4 - 7 mg/kg, the upper range for individual antioxidants at 152 - 169 mg/kg; repeatability from 2.9% to 3.9%, average 3.58% at the LOQ and from 0.9% to 2.4%, average 1.72% at the upper range; recovery rate from 99.1% to 99.7%, average 99.46% at the LOO, average 99.3%, and from 99.3% to 100%, average 99.6% at the upper range, uncertainty extended calculated by the GUM model method from 9% to 16%, on average 12.2% at the LOO and from 4% to 5%, on average 4.4% at the upper range [2]. The obtained validation parameters were assessed as satisfactory. The proven method of testing selected antioxidants in feed can be recommended for the purposes of official supervision. The verified method has been tested by participation in the international proficiency testings, organized by the Austrian Food Safety Agency, Institute of Animal Nutrition and Feed, Vienna, Austria.

Introduction: Antioxidants play an important role in animal feed by preventing fat oxidation processes and improving the stability of feed additives, especially vitamin A. Their use is to prolong feed suitability, protect the health of the animals and improve their breeding performance. The antioxidants used in feed are divided into natural and synthetic. Natural ones include: vitamin E (tocopherol), vitamin C (ascorbic acid), carotenoids (beta carotene, lutein), polyphenols and vegetable oils (e.g. rosemary extracts). Synthetic antioxidants are propyl galusate (PG), ethoxyquine (EQ), butylated hydroxytoluene (BHT), octyl galusate (OG) and butylated hydroxyanisole (BHA). They are used for feed and premixes, for the stabilisation of feed oils and fats and for the protection of feed raw materials. Antioxidants are authorised as feed additives for use in all animal production groups, in the category "technological additives' and in the functional group "antioxidants' in accordance with Regulation (EC) No 1831/2003 [3]. The maximum content of 100 mg/kg for the antioxidant in feed has been established for propyl galusate [4]. Octyl galusate is currently not authorised for use in feed in the European Union, it was withdrawn in 2018 due to the lack of sufficient data confirming its safety [5]. In 2017, the European Medicines Agency (EMA) The EFSA banned ethoxyquine in feed due to a lack of scientific certainty about its safety. The reason was the risk of the presence of p-phenetidine, which is formed in the process of production of ethoxyquine. P-phenetidine has carcinogenic effects and can cause mutations in genetic material in both humans and animals [6-8]. For the antioxidants BHA and BHT, the maximum content in feed has been set at 150 mg/kg (together or separately for each of them) [7,9]. It is appropriate for laboratories to have methods of marking all antioxidants in order to confirm that unauthorised antioxidants are not used in the manufacture of premixes and feed mixtures.

The aim of the work was to verify, by means of validation procedure, the HPLC method with spectrophotometric detection for identification and quantification of selected antioxidants according to the method VDLUFA FG-VI [1] in fortified feed samples according to the current requirements in the field of validation methods of feed testing [10,11].

**Experimental:** a grinding sample weighing about 5 g containing selected antioxidants (PG, EO, BHA, OG and BHT) was weighed into a 50 ml centrifugal tube. 10 ml of deionized water, 10 ml of acetonitrile and 10 µl of sodium ascorbate solution were then added. The tube was shaken for about 6 minutes on a shaker, a portion of a salt mixture (disodium citrate, trisodium citrate, sodium chloride, magnesium sulfate) was added and shaken again for 6 minutes. Then, 6 ml of supernatant was taken, transferred to a dSPE tube, shaken by hand for about 2 minutes and spun for 3 minutes at 4 000 rpm. The resulting extraction solution was transferred through a membrane filter to a chromatographic vial, possibly diluting with acetonitrile. The test solution was analyzed against calibration curves of individual antioxidants, which were prepared by dissolving approximately 250 mg of each pattern, obtaining a base solution used to prepare a series of calibration levels by subsequent dilutions. The analysis was performed using an HPLC kit equipped with a C18, 3.8  $\mu$ m, 4.6  $\times$  100 mm column and a DAD detector. A gradient form of eluent delivery and different wavelengths characteristic of the labeled antioxidants (PG 286 nm, EQ 257 nm, BHA 286 nm, OG 275 nm, BHT 275 nm) were used. The results were statistically evaluated in accordance with current requirements for validation of test methods.

Results: The results of the HPLC method for determination antioxidants according to the VDLUFA FG-VI method in feed are presented in Table 1. The tested antioxidants were verified at two content levels. The studies showed very good calibration linearity for all the antioxidants analyzed. The correlation coefficient of R2 for all substances was above 0.9999, which means an almost perfect match of the linear model to the experimental data. The low coefficient of variation (CV) of the slope confirms the high precision of the measurements (from 0.7% to 3.6%). The calculated LOQ values for the five antioxidants range from 4.00 mg/kg (BHA, EQ, PG, OG) to 7.00 mg/kg (BHT). This means that the method allows the detection of low levels of these substances in feed, which is crucial for controlling their contents. The validation showed very good repeatability of the method (from 2.9% to 3.9% at the LOO levels and from 0.9% to 2.4% in the upper ranges of the method for selected antioxidants). The recovery values for all antioxidants ranged from 99.1% to 100%, which is a positive indicator of the accuracy of the analytical method, confirming its reliability and applicability for routine testing. The calculated uncertainties (U) for the individual antioxidants ranged from 4% to 16% with lower levels having higher uncertainty. The highest uncertainty was obtained for PG at 3.95 mg/kg (16%) and the lowest for BHA, EQ and OG at 152 - 167 mg/kg (4%). The method was tested in the available international studies of IAG Austria organized by the Austrian Food Safety Agency, Institute of Animal Nutrition and Feed, Vienna, Austria. The laboratory obtained satisfactory results (Table 2). The Table shows the results of the determination of selected antioxidants in different years. The assigned values, the test results and the z-score, which indicates how far the obtained result deviates from the reference value, were analyzed. According to the PN-EN-ISO/IEC 17043 standard, the acceptable result is  $|z-score| \le 2.0$ . The National Laboratory for Feedingstuffs obtained all results consistent with the assigned values.

Material tested	Validation parameters, unit	Lower range (LOQ)	Upper range
	Propyl gallate (PG	i) – E 310	
	Range of quantification, mg/kg	3.95	169
Fortified feed	Repeatibility (%)	3.8	2.1
sample	Recovery (%)	99.7	100
	Expanded uncertainty (%)	16	5
	Ethoxyquin (EQ)	– E 324	
	Range of quantification, mg/kg	4.0	167
Fortified feed	Repeatibility (%)	2.9	1.1
sample	Recovery (%)	99.1	99.9
	Expanded uncertainty (%)	13	4
	Butylated hydroxyanisole	(BHA) – E 320	
Fortified feed	Range of quantification, mg/kg	3.78	159
sample	Repeatibility (%)	3.9	0.9
	Recovery (%)	99.5	99.6
	Expanded uncertainty (%)	9	4
	Butylated hydroxytoluen	e (BHT) – E 321	
Fortified feed	Range of quantification, mg/kg	7.23	152
sample	Repeatibility (%)	3.9	2.1
	Recovery (%)	99.5	99.3
	Expanded uncertainty (%)	9	5
	Octyl gallate(OG)	) – E 311	
Fortified feed	Range of quantification, mg/kg	4.12	152
sample	Repeatibility (%)	3.9	2.4
	Recovery (%)	99.5	99.4
	Expanded uncertainty (%)	14	4

Table 1. Selected validation parameters for determination antioxidants by HPLC method.

Tabela 2. Results of analyses of selected antioxidants on proficiency testing.

Antioxidant, unit	PT Austria	n PT	Assigned value	Result	Standard deviation for PT	z-score
Ethoxyquin EQ, mg/kg	2020	8	13.86	13.5	1.97	-0.18
Ethoxyquin EQ, mg/kg	2021	8	33.5	35.3	2.41	0.76
Butylated hydroxyanisole BHA, mg/kg	2024	6	106	88.8	32.2	-0.60
Butylated hydroxytoluene BHT, mg/kg	2024	6	51.7	54.8	13.3	0.19

n PT - number of PT laboratories

**Conclusions:** In the summary of the conducted research the following conclusions can be formulated: (a) HPLC method with spectrophotometric detection for determination antioxidants in feed was characterized by very good precision, linearity of the calibration curve and low measurement uncertainty; (b) recoveries close to 100% indicated good accuracy of the analytical method for determination selected antioxidants in feed samples; (c) LOQ values on the levels of several mg/kg are sufficient to monitor the

antioxidant content of feed in accordance with the provisions of "feed law"; (d) the validation results confirm that the method is suitable for routine determination of antioxidant content in feed; (e) the method has been tested in international proficiency tests in which all acceptable results were obtained.

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### POSSIBILITIES OF ACCREDITATION OF THE NIRS METHOD IN THE FIELD OF BASIC FEED NUTRIENTS BY THE POLISH CENTRE FOR ACCREDITATION

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**Abstrakt:** Validation parameters for determination moisture, crude protein, crude fat, crude fibre and crude ash in feed materials and mixtures using near infrared reflectance spectrometry (NIRS) according to the PN-EN ISO 12099:2017 standard [1] were presented. Validation tests were carried out on poultry mixture, pig mixture and wheat. Participation in proficiency tests organised by Bipea, an authorised organiser of proficiency tests from France, enabled the assessment of the method bias and its statistical significance. Expanded uncertainties for the tested matrices were adopted based on PT data from 2016-2025 [2,3]. These parameters are continuously updated based on current results. The specified validation parameters and the method of their control were accepted by the Polish Centre for Accreditation (PCA). As a result, the NIRS method was included in the scope of accreditation AB 856 of the National Laboratory for Feedindstuffs (NLF).

Introduction: Near infrared reflectance spectroscopy (NIRS) is commonly used to predict the content of organic compounds, e.g. in plant and animal feed materials and their mixtures. This technique uses interactions occurring in a sample irradiated with a beam of electromagnetic waves in the range from 770 nm to 2500 nm. The resulting NIR spectra are the result of light absorption by organic molecules. In this wave range, the energy generated causes excitation of bending, stretching and rotation vibrations of molecules. The test equipment allows for determining the content of compounds containing characteristic functional groups, such as CH-, OH-, NH. The generated wave beam, after reflection from the sample, is directed to the NIR device detector, which then records important information about the chemical compounds in the tested material. The spectrometer calibration software allows for the identification of characteristic functional groups and their quantitative assessment [4,5]. In the field of feed testing, NIRS technique enables prediction of such nutrients as moisture, crude protein, crude ash, crude fat, crude fiber, NDF fiber, ADF starch and amino acids. It also enables direct estimation of the energy value of the scanned feed mixture or determination of adulteration of the feed product. The reliability of the results predicted by the NIR analyzer requires continuous supervision and confirmation of compliance with the assumptions of the Standard [1]. Validation of the method for feed materials and mixtures was carried out in the scope of basic tests. The work also used the results obtained in comparative tests of feeds, in which both European laboratories and national laboratories of domestic producers of feed mixtures and research institutes participated. This enabled the calculation of the method bias depending on tested parameter and matrix. The developed validation data and the proposed method of control of test results obtained using the NIRS analyzer were assessed positively during the audit by auditors
of the Polish Center for Accreditation. The scope of accreditation AB 856 of the National Laboratory for Feedindstuffs [6] was extended to include the near infrared reflectance spectrometry method in relation to basic feed materials and compound feeds for poultry, pigs and laying hens.

The aim of the work was to validate the method and check the validation parameters specified in the PN-EN ISO 12099:2017 standard. PCA requirements for method accreditation were also taken into account.

Experimental: Validation of the near infrared reflectance spectrometry (NIRS) method was performed using a FOSS InfraXact 7500 spectrometer operating in the spectral range from 570 nm to 1848 nm. The instrument was equipped with calibration software provided by the manufacturer for feed materials and mixtures in the range of parameters such as: moisture, crude ash, total crude protein, crude fat, crude fiber. The method was verified for the above-mentioned content of ingredients on feed mixtures for poultry and pigs and wheat. The precision parameters assessed were interlaboratory repeatability and reproducibility. The obtained results were checked using the Grubbs test for detection of outliers. The root mean square error of prediction RMSEP, expanded uncertainties, with the coverage factor k=2, and the method bias for the considered matrices were calculated based on the results of proficiency tests from 2016-2025 according to the PN-EN ISO 12099 standard. The predicted values using the NIRS technique were compared in PT with the results of analyses obtained with reference methods according to the Commission Regulation (EC) No. 152/2009 [7,8]. These data are updated on an ongoing basis based on the results obtained from PT tests. The obtained data enabled the inclusion of the NIRS method in the scope of accredited methods of the National Laboratory for Feedindstuffs in Lublin (accreditation certificate AB 856), in the scope of basic nutrients, in accordance with the PN-ISO 12099:2017 standard.

**Results**: The NIRS method was validated for basic nutrients using the FOSS InfraXact 7500. Table 1 shows the results of the repeatability test for two operators scanning the poultry feed mixture.

					F	J.						
			Operato	r 1	Operator 2							
	Protein	Ash	Fat	Moisture	Fiber	Protein	Ash	Fat	Moisture	Fiber		
	20.60	4.57	5.40	11.54	3.14	20.85	4.58	5.38	11.30	3.07		
	20.73	4.57	5.32	11.57	3.13	20.97	4.58	5.48	11.34	3.09		
	20,76	4.56	5.32	11.52	3.15	20.98	4.60	5.48	11.34	3.10		
	20.93	4.60	5.23	11.46	3.07	21.10	4.58	5.46	11.27	3.09		
	20.68	4.54	5.40	11.56	3.10	21.13	4.61	5.46	11.23	3.10		
	20.98	4.61	5.46	11.49	3.15	21.25	4.63	5.48	11.24	3.14		
	20.86	4.60	5.33	11.36	3.11	20.77	4.55	5.27	11.17	3.07		
	20.97	4.59	5.38	11.43	3.16	21.17	4.65	5.48	11.13	3.09		
	20.95	4.64	5.38	11.39	3.15	20.69	4.53	5.29	11.15	3.05		
	20.68	4.59	5.46	11.40	3.17	20.93	4.58	5.34	11.13	3.09		
Х	20.81	4.59	5.37	11.47	3.13	20.98	4.59	5.41	11.23	3.09		
SD	0.13	0.03	0.07	0.07	0.03	0.17	0.03	0.08	0.08	0.02		
CV	0.64	0.60	1.23	0.63	0.97	0.82	0.70	1.45	0.68	0.77		

Table 1. Testing the repeatability of the NIRS method on the InfraXact 7500 device, % m/m - compound feed for poultry

X-mean value, SD-standard deviation, CV-coefficient of variation

The values do not differ significantly and are characterized by a small scatter of results, which indicates the appropriate competence of the operators and the high measurement accuracy of the NIR analyzer used. Participation in multi-year comparative studies allowed for the calculation of the NIRS method bias for basic parameters and its statistical significance according to the PN-EN ISO 12099 standard. Data for the matrices checked during validation are presented in Table 2. They are updated with new values from proficiency tests. Each time, the result predicted by the spectrometer is corrected for bias and its uncertainty is given.

		70 III.	m, iast up	uale rebruary.	2025.		
Matrix	Parameter	Min	Max	Bias	Significance	RMSEP*	U**
	Moisture	9.8	11.4	-0.25	Yes	0.39	0.78
	Protein	17.3	19.4	-0.35	Yes	0.70	1.40
	Ash	4.5	6.8	-0.51	Yes	0.64	1.28
	Fat	3.8	8.1	0.32	Yes	0.37	0.74
	Fiber	2.1	3.5	0.23	Yes	0.36	0.72
Compound	Moisture	9.3	10.8	-0.36	Yes	0.43	0.86
feed for	Protein	16.5	21.1	-0.46	Yes	0.67	1.34
poultry	Ash	4.6	5.4	0.11	Yes	0.64	1.28
	Fat	3.0	5.5	0.18	No	0.49	0.98
	Fiber	2.9	5.0	0.43	Yes	0.47	0.94
Wheat	Moisture	10.9	12.5	-0.26	Yes	0.32	0.64
	Protein	9.7	12.5	0.78	Yes	0.87	1.74
	Ash	1.3	1.6	0.05	Yes	0.12	0.24
	Fiber	1.8	2.5	0.52	Yes	0.60	1.30

Table 2. Assessment of the NIRS method bias and its statistical significance according to PN-EN ISO 12099,% m/m, last update February 2025.

\*RMSEP – mean square error of prediction, \*\*U – Expended uncertainty with coefficient of expanding k=2.

The scope of application of the method is for: moisture from 7.00% to 14.0%, crude ash from 1.10% to 7.20%, total protein from 6.00% to 49.0%, crude fat from 1.20% to 24.0%, crude fibre from 2.20% to 12.6%. The method has been included in the scope of KLP accreditation and is used for wheat, barley, corn, soybean meal and compound feed for pigs and poultry - excluding crude ash in mixtures for layers.

**Conclusions:** Validation of the method for determining moisture, crude protein, crude fat, crude fibre and crude ash in feed materials and mixtures using near infrared reflectance spectrometry (NIRS) was carried out according to the PN-EN ISO 12099:2017 standard. The obtained validation parameters were accepted by the Polish Centre of Accreditation and the NIRS method was included in the scope of accredited methods of NLF. Participation in interlaboratory comparative studies allowed confirmation of the accuracy of the method and at the same time enabled continuous supervision of the method and updating of the bias and uncertainty of the predicted results.

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# POLYCYCLIC MUSKS AND THEIR DEGRADATION IN THE ENVIRONMENT

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**Abstract:** Polycyclic musks (PCMs), such as tonalide (AHTN), are synthetic fragrance compounds widely used in the cosmetics and detergent industries. Due to their hydrophobic nature and high chemical stability, they exhibit a strong tendency for bioaccumulation and long-term contamination of aquatic environments. Conventional wastewater treatment processes are ineffective in eliminating them, leading to their accumulation in surface waters, sediments, and soil. This study focuses on two key degradation processes of tonalide in the environment – hydrolysis and photodegradation. Both mechanisms play a significant role in the environmental fate of AHTN in aquatic ecosystems.

**Introduction:** Musks are fragrance compounds commonly used in the perfumery, cosmetics, and chemical industries. They are classified into natural and synthetic types. Synthetic musks dominate the fragrance market, including nitro, polycyclic, macrocyclic, and acyclic compounds. Among these, polycyclic musks such as tonalide (AHTN) and galaxolide (HHCB) are especially popular due to their pleasant, musky scent [1]. Owing to their widespread use and high stability, the presence of polycyclic musks in the environment has become a significant ecological concern. These compounds are characterized by octanol-water partition coefficient (log Kow = 4.5-6.3), indicating their hydrophobic nature and potential for bioaccumulation in living organisms. Polycyclic musks have been detected in human breast milk, blood, and adipose tissue [2–4]. One of the leading environmental challenges PCMs pose is their low susceptibility to conventional wastewater treatment methods. The efficiency of treatment processes is crucial in determining the amount of polycyclic musks released into aquatic ecosystems. Municipal wastewater treatment plants are not fully effective in removing these substances, resulting in their continuous discharge into the aquatic environment [5]. The concentration of these compounds in surface waters ranges from several nanograms to several hundred micrograms per liter, with galaxolide and tonalide being the predominant substances [6]. Their low biodegradability allows them to persist in the environment for extended periods, accumulating in sediments, surface waters, and aquatic organisms [7]. Effective removal strategies are urgently needed given, the potential risks associated with PCM contamination. Increasing attention is being paid to natural degradation processes, such as photodegradation and hydrolysis, which may be key in reducing PCM concentrations in aquatic ecosystems.

**Experimental:** Tonalide (Sigma-Aldrich, Germany) stock solution at a concentration of 2.6 g/L, respectively, was prepared in ethanol and stored in the dark at -4 °C for two weeks (Honeywell). Chemicals used for the preparation of the synthetic sewage medium, including peptone, meat extract, urea, EDTA, FeCl<sub>3</sub>·6H<sub>2</sub>O, H<sub>3</sub>BO<sub>3</sub>, CuSO<sub>4</sub>·2H<sub>2</sub>O, KI, MnCl<sub>2</sub>·4H<sub>2</sub>O, Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, ZnSO<sub>4</sub>·7H<sub>2</sub>O, CoCl<sub>2</sub>, CH<sub>3</sub>COONH<sub>4</sub>, NH<sub>4</sub>Cl, KH<sub>2</sub>PO<sub>4</sub>,

MgSO<sub>4</sub>, CaCl<sub>2</sub>, KCl were purchased from Sigma-Aldrich, Germany. Synthetic wastewater was prepared in sterilized 1 L flasks [8]. Other reagents included ethanol with a purity higher than 96%, concentrated acetic acid (POCh, Gliwice, Poland), dichloromethane, and hexane (Sigma-Aldrich, Germany). All glassware materials were thoroughly washed using organic solvents and water to ensure cleanliness and prevent contamination. To investigate the kinetics of hydrolysis and photodegradation, tonalide solutions at concentrations of 20 and 517  $\mu$ g/L were prepared in either water or synthetic wastewater. Photodegradation experiments under daylight exposure were conducted over 14 days under natural but controlled conditions in a phytotron. The chamber maintained a constant temperature of  $22 \pm 0.5$  °C and a day/night cycle of 16/8 hours, using fluorescent lighting with a photon flux of 50 µmol/m<sup>2</sup>/s. Simulated sunlight exposure was performed using a solar simulator (SUNTEST CPS+, ATLAS, Champaign, IL, USA), which emits radiation in the 300-800 nm range. A xenon arc lamp was used in the experiment, providing an irradiance of 750 W/m<sup>2</sup>. The temperature inside the simulator chamber was kept constant at 30 °C, and the total radiation dose reached 5400 kJ/m<sup>2</sup>. At the start of experiments and at regular intervals during exposure to simulated sunlight, appropriate volumes of water or synthetic sewage solutions were collected to determine AHTN concentration. The tests at each concentration were performed in triplicate. The collected samples were subjected to liquid-liquid extraction (LLE). 2 mL of taken solutions were quantitatively introduced into the test tube and extracted three times with 2 mL hexane each time (Rotator Multi Bio RS-24). The organic extracts were combined and evaporated to dryness in a concentrator (Concentrator plus<sup>™</sup>, Vacufuge plus Eppendorf). The residue was reconstituted with 0.5 mL mobile phase and subjected to chromatographic analysis (HPLC-FLD). The chromatographic measurements were done using a liquid chromatograph with a FLD detector. The chromatographic separations were run on C18 column Hypersil Gold (250 mm x 4.6 mm, 5µm) using a Thermo Scientific UltiMate 3000 HPLC instrument (Dionex). The HPLC system consisted of a pump, autosampler, column compartment, and fluorescence detector (Thermo Scientific Dionex) and was controlled by Thermo Scientific Dionex Chromeleon chromatography data system (CDS) software. The injection volume was 10  $\mu$ L, and the flow rate was 0.6 mL min<sup>-1</sup>. The following isocratic mobile phase composition was adopted: acetic acid 0.07 % in acetonitrile and acetic acid 0.09 % in water (4:1). The excitation wavelength of the fluorescence detector was set to 252 nm. The emission wavelength of the fluorescence detector was set to 336 nm. The total run time was 15 min. Two calibration curves were constructed: the one obtained by a given extraction procedure using water solutions and the second by diluting the AHTN solution in a synthetic sewage medium at concentrations ranging from  $0.1 - 21 \,\mu g/L$  and  $103 - 517 \mu g/L$ . The calibration curves were constructed by plotting the peak areas against the ATHN concentration (n = 3). Under the optimal detection conditions, the validation parameters of the developed method, such as linearity, detection limit, precision, repeatability, and others, were determined. The detection limit (LOD) and limit of quantification (LOQ) of the investigated compound were counted using the equations: LOD=3,3 x s/a and LOQ=10 x s/a (s- standard deviation, a-slop of the calibration curve). The elaborated chromatographic method of AHTN determination is characterized by low LOQ and LOD values equal to  $0.13 \mu g/L$  and  $0.42 \mu g/L$ , respectively. The method's precision was evaluated by analyzing six replicates of samples, obtaining a relative standard deviation of 1.18 % - aqueous samples and 2.78%

- synthetic wastewater samples for a concentration of 207  $\mu$ g/L. The reproducibility was obtained by preparing three independent calibration graphs on three different days, resulting in an average slope of 45278 and 34791. All the materials and reagents used in the analysis were proved interference-free by performing two extraction blanks, for no ATHN was detected (below LOD).

**Results:** To determine the reaction order, natural logarithms of analyte concentrations over time were calculated for hydrolysis in aqueous solutions and photodegradation under daylight conditions in both aqueous and synthetic wastewater solutions, as well as for photodegradation under simulated sunlight in model solutions. Subsequently, the dependence of the natural logarithm of concentration on reaction time was established. It was found that the hydrolysis and photodegradation reactions of the analytes in wastewater and model solutions can be described by the pseudo-first-order kinetics model [9]. The kinetic curve equations determined the reaction rate constant (k) and the half-life  $(t_{1/2})$ .

$$C_t = C_0 e^{-kt} (1)$$
  $t_{1/2} = \frac{ln2}{l_t} (2);$ 

where:  $C_t$  and  $C_0$  (µg/L) are the AHTN concentrations at time t and t=0, respectively, k (min<sup>-1</sup>) is the removal rate constant for each experiment under sunlight exposure or natural daylight, t<sub>1/2</sub> is the relevant half-life (minutes).

The rate constants (k), half-lives  $(t_{1/2})$ , and correlation coefficients  $(\mathbb{R}^2)$  characterised removal of the AHTN by the studied processes from aqueous solutions and synthetic wastewater are shown in Table 2.

		Parameter						
Studied process	Sample matrix	k [m	t <sub>1/2</sub> [min]		$\mathbf{R}^2$			
		AHTN concentration [µg/L]						
		20	517	20	517	20	517	
Hydrolysis		0.00011	0.00013	5674	5026	0.9206	0.7649	
Photodegradation under daylight	Aqueous solutions	0.00015	0.00016	4752	4464	0.8735	0.8402	
Photodegradation under simulated sunlight		0.0195	0.0221	36	31	0.9977	0.9955	
Photodegradation under daylight	Synthetic wastewater	0.00008	0.00015	8539	4709	0.9615	0.8178	

**Table 2.** Rate constants (k), half-lives $(t_{1/2})$ , and correlation coefficients  $(R^2)$  were determined during the hydrolysis and photodegradation of AHTN experiments from aqueous solutions and synthetic wastewater.

Tonalide is a photolabile compound. It undergoes hydrolysis (k = 0.00011 and 0.00013 min<sup>-1</sup>) and photodegradation under daylight exposure (k = 0.00015 and 0.00016 min<sup>-1</sup>) to a limited extent. In both cases, the reaction exhibited high half-life values-5674 min and 5026 min, respectively-indicating significant persistence of tonalide in aqueous environments. Photodegradation of tonalide in synthetic wastewater under daylight conditions was less efficient than pure aqueous solutions. The rate constants were 0.00008 min<sup>-1</sup> (20 µg/L) and 0.00015 min<sup>-1</sup> (517 µg/L), corresponding half-lives of 8539 min and 4709 min. The highest degradation efficiency was observed under

simulated sunlight conditions. The rate constants reached 0.0195 min<sup>-1</sup> (20  $\mu$ g/L) and 0.0221 min<sup>-1</sup> (517  $\mu$ g/L), and the half-lives decreased significantly to 36 and 31 minutes, respectively.

**Conclusions:** Tonalide demonstrates high stability in aqueous solutions, as confirmed by long half-lives and low reaction rate constants in hydrolysis. The degradation of synthetic wastewater proceeds more slowly than that of pure water. Studies have shown that polycyclic musks like tonalide undergo only limited photodegradation under simulated environmental conditions. Its ecological persistence and bioaccumulation potential raise significant concerns. Incomplete degradation of polycyclic musks, such as tonalide, in the environment may lead to accumulation in aquatic systems, adversely affecting organisms' growth and oxidative stress responses and posing potential genotoxic risks. Human exposure data also highlight considerable risks for vulnerable populations, such as pregnant women and infants [10]. The highest degradation efficiency of tonalide was observed under simulated sunlight, where the half-life was reduced to under 40 minutes. Photodegradation under sunlight is several hundred times more effective than under daylight exposure, underscoring the importance of considering radiation intensity in modelling tonalide degradation. The compound's high stability under low sunlight may lead to its accumulation in surface waters, posing potential toxic threats to aquatic organisms.

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# WHAT CAN RAMAN SPECTROSCOPY TELL US ABOUT THE MOTION AND ARRANGEMENT OF STEROIDAL MOLECULAR GYROSCOPES?

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**Abstract:** An analysis of different measurement methods applied to study steroidal molecular gyroscopes is presented. The application of Raman spectroscopy in predicting rotational dynamics and sample orientation is described.

**Introduction:** Molecular gyroscopes (Fig.1) are molecules created in resemblance of macroscopic gyroscopes, constructed from two parts: fast-moving rotators, and large stators, tasked with shielding the inner part of the molecule [1]. Steroidal units, which are large molecules with a carbon backbone made of four fused rings, have been commonly used as stators in such molecules since 2010 [2]. Additionally, due to their numerous biological applications, steroids are a widely studied and are easily functionalized. They are commonly used in modern hybrid materials, such as steroid-doped liquid crystal polymers [3], functionalized nanotubes [4], and gels [5].

Various methods for investigating steroidal molecular gyroscopes (SMGs) have been proposed. Structural and packing motifs have been explored through X-ray scattering experiments [6,7]. Rotator dynamics have been studied using <sup>1</sup>H NMR, <sup>13</sup>C NMR, and dielectric spectroscopy [2,8,9]. Conformation in solution and in bulk samples has been analysed using CD spectroscopy [9,10]. In this paper, we discuss Raman spectroscopy as a tool that provides insight into both the structural and dynamic properties of SMGs [9-11].



Fig.1. (A) Reinshaw InVa Raman microscope, (B) steroidal molecular rotor, (C) adapter allowing sample rotation, (D) system allowing temperature variable measurements.

**Experimental:** Raman spectroscopy measurements were conducted using Reinshaw InVia Raman microscope equipped with near IR laser working at 785 nm wavelength (Fig.1(A)). All spectra were recorded in 3200-100 cm<sup>-1</sup> spectral range, with spectral resolution better than 2 cm<sup>-1</sup>. The spectral parameters of the bands were determined using fitting package of Wire 3.4 software. Polarized Raman spectra were registered using a microscope stage adapter allowing sample rotation in the 0-360° range, with a 5° step (Fig.1(C)). Temperature measurements were performed using a Linkam THMS 600 cooling/heating stage, with a temperature range of 350–140 K and a step size of 10 K (Fig.1(D)).

Results: Raman spectroscopy is a widely used tool for identifying compounds and gaining insights into their structural properties. In the case of SMGs, Raman spectral analysis allows the isolation of bands corresponding to oscillations of different parts of the molecule-namely, the stator, rotator, and axle of rotation. For SMGs with a 1,4-diethynylphenylene group acting as both the rotator and axle of rotation, two prominent Raman bands appear around 2220 and 1600 cm<sup>-1</sup>. These bands are associated with the stretching of triple CC bonds in the axle of rotation and double CC bonds in the rotator, respectively [9-11]. The positioning of these bands can provide valuable information about the structural behaviour and dynamics of these molecular structures. In the paper published in 2018, we analysed the influence of linking stators with additional bridging chains, with the aim of shielding the rotator and allowing it more freedom for rotation [11]. Variable temperature measurements showed the splitting of bands assigned to stretching vibrations of CC double and triple bonds at 170 and 260 K, observed only for SMGs with linked stators (Fig.2(A)), which was later assigned to the more rigid structure of the whole molecule. In the paper published in 2020, we analysed the shift of bands assigned to the same vibrations due to deuteration of the rotator [10]. The difference in the position of bands in compounds with 1,4-diethynylphenylene and 1,4-diethynylphenylene-d4 was discussed, leading to conclusions about differences in molecular dynamics (Fig.2(B)). Moreover, conclusions about the orientation of molecules in crystallites of the studied SMGs were derived based on polarized Raman spectra. Similarly, in the 2024 study, we used Raman spectroscopy to confirm the crystallinity and orientation of samples with fluorine-substituted rotators [9] (Fig.2(C)). The maximum intensity of a band assigned to the stretching of the triple CC bond in the axle of rotation likely corresponds to the projection of that bond position onto the observation plane, additionally confirming the near linearity of both triple bonds in the structure of the SMG investigated.



**Fig.2.** (A) splitting of a bands assigned to stretching vibrations of CC double and triple bonds at 170 and 260 K, proving increased rigidity of the SMGs structure after stator linkage [11], (B) comparison of Raman spectra of SMGs with 1,4-diethynylphenylene (left) and 1,4-diethynylphenylene-d4 (right) rotator [10], (C) change of the intensity of a band assigned to CC triple bond stretch in a polarized Raman spectrum of SMG with angle of rotation [9].

**Conclusions:** Raman spectroscopy provides valuable insights into the structural and dynamic properties of SMGs, enabling the analysis of key components like the stator, rotator, and axle of rotation. In studies across 2018, 2020, and 2024 Raman spectroscopy was used to analyse the influence of factors such as stator linking, deuteration, and fluorine substitution on the molecular dynamics, and, orientation, and crystallinity of these systems.

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## STRUCTURAL AND DYNAMIC PROPERTIES OF FLUORINE-SUBSTITUTED STEROIDAL MOLECULAR GYROSCOPES: SPECTROSCOPIC ANALYSIS

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**Abstract:** The positions of vibrational modes in the Raman and IR spectra of fluorinesubstituted steroidal molecular gyroscopes are analysed, leading to conclusions about rotator deformation and providing indirect evidence of rotator rotation.

**Introduction:** According to the definition provided by Skopek et al. (2007), a molecular gyroscope consists of a functional axis of rotation, a rotating domain (called the rotator), and an external part (called the stator), which is responsible for shielding the rotating moiety [1]. A molecular gyroscope should closely resemble the structure and symmetry of a toy gyroscope. Various structures have been proposed, starting with Rose's gyroscope-like porphyrins (1985) [2], followed by molecular gyroscopes featuring a rotating ML group, introduced by Gladysz et al. [3-5], molecular gyrotopes proposed by Setaka et al. [6,7], and structures with a phenylene-based rotator and trityl/triptycyl stators, developed by García-Garibay et al. [8,9]. In more recent years structures with open topology, with massive steroidal stators and phenylene based rotator have been proposed by various groups [10-12] This study examines steroidal molecular gyroscopes with 1,4-diethynylphenylene, 1,4-diethynyl-2-fluoro-phenylene, and 1,4-diethynyl-2,3-difluoro-phenylene rotators anchored to the steroidal stators at C3-C3' position.

**Experimental:** Molecular gyroscopes **1-3** (Fig.1) were synthesised according to a procedure described in [6c]. Vibrational spectra of polycrystalline samples of **1-3** were recorded using two types of equipment: a Renishaw InVia Raman microscope equipped with a thermoelectrically (TE)-cooled CCD detector and a semiconductor laser operating at 785 nm (3200-100 cm<sup>-1</sup>, spectral resolution:  $2 \text{ cm}^{-1}$ ), and Bruker Equinox 55 FT-IR spectrometer (7000-400 cm<sup>-1</sup>, spectral resolution:  $2 \text{ cm}^{-1}$ ). The FT-IR absorption spectra was recorded at room temperature using KBr pellets with dispersed compounds (c = 1:1000).



Fig.1. Structural formulas of compounds 1–3.

**Results:** The Raman and IR spectra recorded for the samples of compounds 1-3 are presented in Fig.2. a broad band due to the stretching of the O-H bond (stators, C3, C3') is visible in the IR spectrum in the  $3600-3200 \text{ cm}^{-1}$  range. The broadening of this band is due to hydrogen bonding between the stators and/or rotators of different molecules. In the  $3000-2800 \text{ cm}^{-1}$  region, multiple modes corresponding to the C-H bond stretching in the CH<sub>3</sub>, CH<sub>2</sub>, and CH groups of the stators are present. The C-H bond stretch in the phenylene ring is represented by a weak (in both IR and Raman) band above  $3000 \text{ cm}^{-1}$ , which is only visible in the spectrum of sample **2**. Around 2220 a strong band corresponding to the stretch of triple CC bond is observed in Raman spectra. The position of the band corresponding to the stretch of the double CC bond of the rotator ring (also known as quadratic ring stretch) positioned around 1600 cm<sup>-1</sup>. Below 1500 cm<sup>-1</sup>, different modes can be observed, forming a characteristic fingerprint of the compound.



Fig.2. Raman (A) and IR (B) spectra of samples 1-3.

The positions of bands assigned to the stretching vibrations of C=C double bonds in the rotator were compared to the theoretical values obtained using DFT (B3LYP, 6-31G(d)) calculations for isolated molecules of compounds 1-3 [6c], as shown in Table 1.

 Table 1. Position of the band assigned to the stretch of the CC double bond (rotator) in the experimental and theoretical spectra of samples 1-3.



1587 vw

The theoretical model predicts a blue shift of the band as the number of substituted fluorine atoms increases, which aligns with the trend observed for molecular gyroscopes 1 and 3. However, in the experimental spectrum of sample 2, a decrease in the wavenumber corresponding to the band maximum is observed. Additionally, an increase in the band intensity in the IR spectrum is noted for this sample. The unexpected shift in position can be explained by assuming an asymmetrical deformation of the rotator in molecule 2 due its rotation, as illustrated above Table 1. Such a deformation would lead to the stretching of the C31'-C32' bond, which strongly contributes to the vibration, resulting in a lower oscillation energy and a redshift of the band position. This effect would not be observed in the theoretical calculations, as they do not account for the rotator or the interactions between neighbouring molecules. Similarly, a shortening of this bond due to symmetrical deformation (as in molecular gyroscope 3) would lead to a blueshift in the band position. To confirm this notion, a larger blueshift is observed in the experimental spectrum of sample 3 compared to the one predicted by theoretical calculations.

Bands intensity: vs - very strong, s - strong, m - medium, w - weak, vw - very weak.

**Conclusions:** The assignment of bands in the Raman and IR spectra of fluorinesubstituted steroidal molecular gyroscopes was carried out. The analysis of the band position associated with the quadratic stretching of the rotator ring enabled the prediction of the type of ring deformation (asymmetrical/symmetrical) in mono- and di-substituted rings. Since the only explanation for the band positions in the vibrational spectra is linked to ring deformation, which can only result from its rotation and inertial effects, this serves as indirect evidence of ring rotation.

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# ANTIOXIDANT ACTIVITY OF *MEDICAGO SATIVA* AND SOLIDAGO GIGANTEA EXTRACTS OBTAINED BY CARBON DIOXIDE IN A SUPERCRITICAL STATE

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**Abstract:** Supercritical carbon dioxide extractions of *Medicago sativa* L. (alfalfa) and *Solidago gigantea* Ait. (goldenrod) were carried out to obtain extracts enrich in antioxidants. Box – Behnken design (BBD) was used to generate the experimental plan, where the wide range of process parameters were investigated. Antioxidant activity was determined by DPPH method. All of obtain extract exhibit an antioxidant activity. According to BBD plan, the highest antioxidant activity for goldenrod extract was obtained under 60 °C, 50 MPa and 5 kg/h and for alfalfa 40 °C, 20 MPa, 5 kg/h, respectively 5.09 and 11.17  $\mu$ mol TEAC/g of dry extract. Due to the antioxidant activity, extracts from *M. sativa* and *S. gigantea* have an application potential as additives in food, supplements or cosmetics.

**Introduction:** Supercritical fluid extraction with carbon dioxide (SFE-scCO<sub>2</sub>) is a green technology widely used for plant extraction. Due to the properties of carbon dioxide in a supercritical state (properties are intermediate between the liquid and gaseous states), the efficiency of extraction is determined by two phenomena: the solubility of compounds in the solvent and their diffusion from the plant matrix. This is a great advantage because both polar and non-polar bioactive compounds are efficiently extracted from the plant matrix [1-4]. Solidago gigantea Ait. (goldenrod) and Medicago sativa L. (alfalfa) are widely spread in Poland. Goldenrod was distributed as an ornamental plant and became an invasive species. Alfalfa is cultivated mainly as feeder due to the high concentration of protein. Studies have been shown that extracts from S. gigantea and M. sativa are reach in bioactive compounds (Fig.1). Due to high content of bioactive nutrients, preparations from alfalfa and goldenrod have antifungal, antibacterial, anti-inflammatory, insecticidal and nematicidal properties [2-4]. Both of those plants are easily accessible and finding further application for them is well-based. Bioactive compounds are valuable components in human diet and serve an important role in nutraceutical potential and health-promoting effects due to their high antioxidant activity. Antioxidants are compounds that can neutralize free radicals by donating electrons, thereby preventing or reducing oxidative stress. There are a few methods that allow for rapid antioxidant activity determination like DPPH and FRAP methods. Both methods are based on color changing and measured spectroscopically. The degree of color change is proportional to the antioxidant activity of the sample. The greater the change, the stronger the antioxidant activity of the sample [5-6].



Fig.1. Composition of bioactive compounds in S.gigantea and M. sativa extracts and their properties.

**Experimental:** Supercritical fluid extractions were performed on quarter-technical plant (SITEC-Sieber Engineering AG, Switzerland) placed in Łukasiewicz Research Network - New Chemical Syntheses Institute in Puławy. Box – Behnken design (BBD) was used to generate the experiment. Influences of three input variables: temperature, **T**: 40-80 °C (313.15 – 353.15 K), pressure, **P**: 20-80 MPa and solvent flow rate, **F**: 3 – 7 kg/h were investigated. Output variables was antioxidant activity of samples. Finally, response surface methodology (RSM) was used to analyze and evaluate the influence of chosen input variables (T, P, F) on output variable (DPPH) in obtained extract. The DPPH procedure was based on the method described by Espín et al., with some modifications [5]. To 200  $\mu$ L of a 0.1 mM ethanol solution of DPPH (initial absorbance of DPPH = 0,9), 50  $\mu$ L of the extracts were added and incubated in the dark for 30 min. Absorbance was measured at a wavelength of 517 nm using a Varioskan<sup>TM</sup> LUX Multimode microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The results were expressed as micromoles of Trolox Equivalent Antioxidant Capacity (TEAC) per gram of dry extract.

**Results:** For individual feedstock fifteen extractions, at different process parameters (according to BBD), were performed using carbon dioxide in a supercritical state. Antioxidant activity for all samples were determined by DPPH method. Summary data are listed in Table 1. The highest antioxidant activity for goldenrod extract was obtain under 60 °C, 50.00 MPa and 5.00 kg/h. For alfalfa, extract with the highest value of determined variable was obtained under 40 °C, 20 MPa and 5.00 kg/h. Respectively, it was 5,09 and 11,17  $\mu$ mol TEAC/g of dry extract. Generally, alfalfa extracts have higher antioxidant activity was different for each individual feedstock. These differences are caused by a unique bioactive compounds composition in raw plant. However, fact that extracts exhibit antioxidant activity is extremely important from the point of view of their potential application as additives in the food, cosmetics or pharmaceuticals.

No.	Bo	x-Behnken design		DPPH, µmol TEAC/g of dry extract			
	T, ⁰C	P, MPa	F, kg/h	Medicago sativa L.	Solidago gigantea Ait.		
E1	60	80.00	7.00	0.36	2.99		
E2	80	80.00	5.00	8.90	4.22		
E3	80	20.00	5.00	5.90	2.63		
E4	40	50.00	3.00	8.36	4.39		
E5	60	50.00	5.00	2.88	5.09		
E6	80	50.00	3.00	8.75	3.23		
E7	60	50.00	5.00	2.69	3.98		
E8	60	50.00	5.00	5.13	3.36		
E9	40	80.00	5.00	3.08	2.65		
E10	60	20.00	3.00	5.70	3.06		
E11	40	20.00	5.00	11.17	3.63		
E12	40	50.00	7.00	3.36	4.79		
E13	60	80.00	3.00	4.48	2.82		
E14	60	20.00	7.00	3.36	2.97		
E15	80	50.00	7.00	6.67	3.13		

 Table 1. Results of DPPH analysis of obtained extracts of Solidago gigantea Ait. and Medicago sativa L.

 according to BBD.

Response Surface Methodology (RSM) was used for further SFE-scCO<sub>2</sub> optimization. The main goal was to evaluate process parameters providing extraction, where final product has the highest content of antioxidants. Generally, RSM is a statistical technique used for modeling and analyzing problems where multiple variables influence a response of interest. RSM is particularly useful for optimizing processes by understanding the relationships between inputs (factors) and outputs (responses). The quality of the adopted model fitting is expressed by the most important statistical factors like coefficient of determination ( $R^2$ ), adjusted coefficient of determination, lack of fit, model of F-value and p-values.

Outpu	t variables	$\mathbf{D}^2$	Adjusted <b>D</b> <sup>2</sup>	Lаск оf	fit, LOF	Model		
Output variables		К	Aujusteu K	F-value	p-value	F-value	p-value	
ווממת	M. sativa	0.96	0.88	0.25	0.86	12.70	0.006	
DPPH	S. gigantea	0.91	0.76	1.62	0.06	5.81	0.034	
$R^2 > 0.8$ –	statistically in	nportant; Model	p-value <0.00	01 – very h	ighly signif	icant, p <0	).01 very	
significar	nt. p <0.05 signi	ficant. $p > 0.1$ no	t statistically sig	nificant: LO	F>0.05 – sta	tistically in	portant	

Table 2. Main statistical factors obtained for optimization od SFE-scCO<sub>2</sub> of *M. sativa* and *S.gigantea*.

Coefficient of determination for chosen response - DPPH ( $\mu$ mol TEAC/g of dry extract) as output variable was of 0.96 and 0.91, which indicates that the adopted quadratic model explains 96 % and 91% dependence between the response and T, P and F for alfalfa and goldenrod respectively. That value (above 0.80) also has proven the correctness of the adopted model. Adjusted R<sup>2</sup> (0.88 and 0.76) indicate an excellent fit, especially due to optimization was carried out in quarter-technical plant, where the reproducibility may be slightly lower than in the laboratory scale. Lack of fit F-value and p-value indicate the adequacy of the description between selected model and variations in obtained results. Small lack of fit F-value – 0.25 and 1.62 and an insignificant p-value – 0.86 and 0.06 prove that the model describes properly the dependence between input and output variables. The Model F-values of 12.70 and 5.81 implies the model is significant. Model p-values less than 0.05 indicate model terms are significant.

As a final results, process parameters allowing to obtain extract with the highest antioxidant activity was generated: (a) *M. sativa*: 40.27 °C (313.42 K), 21.32 MPa, 3.91 kg/h – predicted value 11.35  $\mu$ mol TEAC/g of dry extract; (b) *S. gigantea*: 38. 12 °C (313.27 K), 76.7 MPa, 3.57 kg/h – predicted value 5.23  $\mu$ mol TEAC/g of dry extract. SFE-scCO<sub>2</sub> under generated conditions were performed and antioxidant activity in obtain extracts were measured. 11.21 and 5.30  $\mu$ mol TEAC/g of dry extract for alfalfa and goldenrod respectively were determined. The obtained results are within the confidence interval.

**Conclusions:** Optimization of supercritical carbon dioxide extraction of alfalfa and goldenrod ended successfully. Extracts exhibit different antioxidant activity due to the values of temperature, pressure and solvent flow rate. Process parameters have a directly impact on the content of antioxidants. According to Box-Behnken design plan, for goldenrod extract the highest antioxidant activity was obtained under 60 °C, 50 MPa and 5 kg/h and for alfalfa 40 °C, 20 MPa, 5 kg/h, accordingly 5.09 and 11.17 µmol TEAC/g of dry extract. However, as a result of response surface methodology, the highest antioxidant activity was obtain under 40.27 °C, 21.32 MPa, 3.91 for alfalfa and 38.12 °C, 76.70 MPa and 3.57 kg/h for goldenrod. It was 11.21 and 5.30 µmol TEAC/g of dry extract for alfalfa and goldenrod respectively. The results have been shown that the optimization of the SFE-scCO<sub>2</sub> of plant material have to be approached individually for each feedstock due to their different composition. Finally, alfalfa and goldenrod extracts from *M. sativa* and *S. gigantea* have an application potential as additives in food, supplements or cosmetics.

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# APPLICATION OF HIGH-RESOLUTION MASS SPECTROMETRY IN THE ANALYSIS OF CHERRY POLYPHENOLS (*PRUNUS CERASUS* L.)

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**Abstract:** Polyphenols constitute a diverse group of biologically active compounds commonly present in food. These compounds exhibit significant biological activities, including antioxidative, anti-inflammatory, antibacterial, antifungal, and anticancer effects. Flavonoids are particularly noted for their inverse relationships with civilisation diseases, e.g. type 2 diabetes, and inflammation-linked neurodegeneration. This paper presents the results of a study on the application of ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF) in the qualitative analysis of polyphenolic compounds present in sour cherry (*Prunus cerasus* L.) fruit. A metabolic approach was used to detect free polyphenols and their glycosides. Five compounds were identified: cyanidin hexoside, quercetin deoxyhexose-hexoside, catechin, epicatechin, and kaempferol hexoside.

Introduction: Polyphenols are a diverse group of biologically active compounds found in food. Flavonoids represent the most numerous group of polyphenolic compounds in the plant world. They are found in all plant parts: bark, flowers, seeds, leaves, roots, fruits, and woody tissues. They have garnered significant medical interest [1]. The best-known activity of polyphenolic compounds is their ability to neutralize free radicals [2], as well as their antibacterial, antifungal, and anticancer effects [3]. It has been proven that increased intake of flavonols is inversely proportional to the risk of developing coronary heart disease [4]. Moreover, an inverse relationship between flavonoid consumption and the development of type 2 diabetes has been observed [5]. It is also known that flavonoids affect the nervous system, and free aglycones and their derivatives can cross the blood-brain barrier [6]. The ability of flavonols to protect against inflammatory processes leading to nerve damage has also been demonstrated [7]. Additionally, the interest in the sour cherry fruit is due to its health-promoting effects, e.g., counteracting oxidative stress, reducing inflammation, modulating blood glucose, and enhancing cognitive function [8]. The aim of the study is to investigate the use of ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF) for the qualitative analysis of phenolic compounds found in P. cerasus fruit.

**Experimental:** The research material consisted of cherry fruits obtained from local growers in the Lublin Province. The extraction of polyphenolic compounds from the fruits was carried out using a previously described method [9]. A total of 3 g crushed fruits were extracted with

methanol/deionized water/formic acid (70:27:3, v/v/v) in a cooled water bath for 1 hour. The extract was then refrigerated for 24 h (4 °C, in the dark) followed by centrifugation (4 °C, 13,131 x g, 15 min) and filtered (PTFE 0.2  $\mu$ m) into autosampler vials. The sample was subjected to UHPLC-Q-TOF analysis using a high-performance liquid chromatograph coupled with a mass spectrometer (6550 iFunnel LC-Q-TOF, Agilent Technology) [10]. Chromatographic separation was achieved on a Zorbax Extend C18 column (2.1 x 100 mm, 1.8  $\mu$ m). The mobile phase consisted of 0.1% formic acid in deionized water (A) and acetonitrile (B). Gradient elution was performed over 32 min at a flow rate of 0.4 mL/min. Electrospray ionization (ESI+; Jet Stream, Agilent Technology) was applied. The analysis was conducted in MS scan mode (range: 100–1000 m/z; reference ions: m/z 121.0509 and m/z 922.0098) and MS/MS mode recorded at collision energies (CE): 10, 20, and 40 eV.

**Results:** In the polyphenolic profile of cherry fruit, the following classes of compounds were distinguished: anthocyanins (cyanidin hexoside), glycosidic derivatives of flavonols (quercetin deoxyhexose-hexosided and kaempferol hexoside), and flavanols (catechin and epicatechin). Cyanidin hexoside and quercetin deoxyhexose-hexoside were determined using the Metlin database by a formula based on accurate mass with an isotopic distribution score > 80%. Catechin, epicatechin and kaempferol hexoside were determined using the Metlin database based on MS/MS spectra match with a score > 80%. Detailed results are presented in Table 1.

Rt [min]	Measured $m/z$	MS/MS m/z	CE [eV]	Compound formula	Theoretical $m/z$	$\Delta$ [ppm]	Compound
4.5	291.0911	123.0438; 139.0384; 147.0434; 111.0429; 91.0535	40	C <sub>15</sub> H <sub>14</sub> O <sub>6</sub>	291.0863	-1.83	Catechin
6.3	449.1090	287.0553; 137.0223; 213.0540	40	$C_{21}H_{21}O_{11}$	449.1084	-2.48	Cyanidin hexoside
7.7	291.0911	139.0391; 123.0440; 147.0440; 207.0648; 165.0542	20	C <sub>15</sub> H <sub>14</sub> O <sub>6</sub>	291.0863	-2.29	Epicatechin
14.5	611.1623	303.0502; 229.0472; 257.0421; 153.0169; 85.0285	40	C <sub>27</sub> H <sub>30</sub> O <sub>16</sub>	611.1607	-2.24	Quercetin deoxyhexose- hexosided
17.5	449.1083	287.0547; 165.0180; 121.0272; 153.0184	40	$C_{21}H_{20}O_{11}$	449.1078	-0.86	Kaempferol hexoside

**Table 1.** Retention times, measured m/z of molecular or pseudo-molecular ions (ESI+), mass fragments<br/>(MS/MS), collision energy, compound formula, corresponding theoretical m/z and accuracy ( $\Delta$ )<br/>of polyphenolic compounds identified in cherry fruits.

Figure 1 presents the results obtained for kaempferol hexoside, used for its putative identification based on MS/MS spectrum matching. The availability of analytical standards for specific derivatives would be essential for validated identification and determination of which specific sugar derivative is present in the examined flavonoids.



**Fig.1.** MS/MS spectrum of kaempferol hexoside (A), the comparison of the MS/MS spectrum of kaempferol hexoside (B) with Metlin database, and the isotopic distribution of kaempferol hexoside (match 93.98%) (C).

**Conclusions:** The results highlight of the effectiveness of high resolution mass spectrometry in detecting secondary metabolites. The use of UHPLC-Q-TOF enables the rapid qualitative identification of secondary plant metabolites, as demonstrated by the identification of kaempferol derivative in the cherry fruit extract. The polyphenolic content of cherries is noted, however, future analysis should be continued. The study of cherry flavonoids remains crucial since civilisation diseases are complex and multifactorial conditions characterised by complex pathology, including oxidative stress and inflammation.

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### OPTIMIZATION OF TRYPSIN DIGESTION TIME FOR THE LC-MS IDENTIFICATION OF PEPTIDE MARKERS TO DISCRIMINATE CHICKEN LIVER IN PROCESSED FOODS

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**Abstract:** The adulteration of meat products with offal is a globally recognized issue, reported worldwide. The increasing production of processed meat products, their complex composition, the variety of processing techniques used in their manufacture, and the growing sophistication of adulteration methods pose significant challenges to the identification of product ingredients. Current detection methods remain insufficient. In this study, we investigate the feasibility of using liquid chromatography coupled with high-resolution mass spectrometry to identify unique peptides specific to chicken (*Gallus gallus*) liver tissue. Our objective was to optimize the in-solution trypsin digestion time for the effective LC-MS-based identification of heat-treated chicken liver peptide markers.

**Introduction:** Chicken liver is an edible animal offal that serves as a valuable source of nutrients. It has a high protein content (20.98 g per 100 g of liver), comparable to that of meat (approximately 20 g of protein per 100 g of meat) [1]. Liver is also rich in vitamins B, A, C, H, and PP, as well as essential minerals, fats, and polyunsaturated fatty acids [1]. Due to its high content of fatty acids, antioxidants, and ferritin (the primary protein responsible for iron storage in the liver) it is recommended as a dietary component for patients with anemia [2]. Since liver is significantly cheaper than meat, it is often used as a substitute for skeletal muscle tissue in highly processed products [3]. The morphological changes that occur in meat during processing make it impossible to distinguish between muscle and liver tissue. Directive 2001/101/EC of the European Parliament and the Council introduced a standardized labeling system for meatcontaining products, specifying that only skeletal muscle tissue that does not exceed the maximum allowable limits of natural fat and connective tissue content can be classified as 'meat.' Mass spectrometry-based proteomics offers a highly sensitive approach for food composition verification, enabling the identification of trace amounts of proteinderived peptides that remain stable after thermal processing, thus providing robust biomarkers for food authenticity. Efficient protein extraction is a critical step in the analysis of protein-derived peptides. Protein digestion represents the most timeconsuming stage in sample preparation protocols used for studies on heat-stable, tissuespecific peptide markers [4]. In a typical in-solution digestion protocol employed in LC-MS analysis, approximately 100  $\mu$ g of protein is digested in an extraction solution using trypsin. Initially, proteins are reduced with dithiothreitol (DTT) and subsequently alkylated using iodoacetamide (IAA). The samples are then subjected to trypsin digestion and incubated. The duration of protein digestion varies, typically ranging from 4 hours [5] to 24 hours [6], with overnight incubation (12–18 hours) at 37 °C being the most commonly used approach [4]. The aim of this study was to evaluate the effect of trypsin digestion time on the number of identified proteins and peptides in thermally processed chicken liver tissue, with a main focus on the effectiveness of the digestion of proteins from which specific peptide markers, previously identified for this tissue as liver authenticity markers, are derived [7]. The resulting tryptic digests of chicken liver proteins were analyzed using ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF).

**Experimental:** Chicken (*Gallus gallus*) livers were obtained from local meat suppliers. After visible fat was removed, the liver samples were cut and separately boiled in water at 100°C for 15 minutes. Following boiling, the samples were cooled to room temperature and processed according to a previously reported protocol [7], which included homogenization with 100 mM aqueous ammonium bicarbonate, in-solution protein digestion with trypsin, and peptide purification using Sep-Pak C18 Plus cartridges. The protocol was modified with regard to trypsin digestion time. The incubation was performed for: (I) 3 hours (n = 3), (II) 6 hours (n = 3), (III) 9 hours (n = 3), (IV) 12 hours (n = 3), (V) 15 hours (n = 3), and (VI) 18 hours (n = 3). The tryptic protein digests were analysed using an Agilent Technology 1290 Infinity series high performance liquid chromatograph coupled to an Agilent Technologies 6550 iFunnel quadrupole time-of-flight mass spectrometer (Q-TOF) operated in positive electrospray ionisation (ESI+) mode. The tryptic protein digests were separated using a Zorbax Eclipse Plus C18 RRHD (2.1x 150 mm, 1.8 µm) column and 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) as the mobile phase. The gradient program was as follows: 0–2 min, 3% B; 2–40 min, to 35% B; 40–45 min, to 40% B; 45–50 min, to 90% B; 50–55 min, 90% B; and a 5 min post-run at 3% B. The flow rate was set at 0.3 mL/min, the sample injection volume was 10  $\mu$ L, and the column temperature was maintained at 40 °C. The MS instrument was operated in the following parameters: ion source gas  $(N_2)$  temperature 250 °C with a flow rate of 14 L/min; nebulizer pressure 35 psi; sheath gas temperature 250 °C; sheath gas flow 11 L/min; and capillary voltage of 3500 V. The nozzle voltage was set at 1000 V and the fragmentor voltage at 400 V. The MS/MS spectra were extracted using Spectrum Mill MS Proteomics Workbench (Agilent Technologies). The extracted fragmentation spectra were searched against the chicken (*Gallus gallus*) NCBI protein database.

**Results:** Chicken liver proteins were subjected to tryptic digestion across six time points, and the resulting peptides were analyzed using the UHPLC-Q-TOF–MS/MS method. Protein and peptide identification was performed by searching against *Gallus gallus* protein database. The number of identified proteins and tryptic peptides is presented in Fig.1. In total, over 720 proteins and 3,850 peptides were identified across all analyzed sample groups. The number of identified proteins and peptides showed minimal variation across different digestion times. The highest number of proteins and peptides was observed in samples incubated for 18 hours (767 proteins and 4,026 peptides); however, this was only 3% higher than the numbers obtained for samples incubated for 3 hours (750 proteins and 3,908 peptides).





Fig.1. Number of proteins and tryptic peptides identified in chicken liver samples (n=3) at six different trypsin digestion times.

The difference in the protein sequence coverage obtained after 3 and 18 hour trypsin digestion is visualized for the mitochondrial enoyl-CoA delta isomerase 1 protein (NP\_001264514.2) as an example in Fig.2.

```
(a)
1 MAAVAAAAGT FARRMARSGV LFPRSSPQVW PQAARQPPGL LPAQRRAFSN NKILVELDTS SGVATMKFKS PPVNSLSLDF 80
81 LTEFCISLEK LENDRACRGL IITSAIPRVF SSGLDITEMC GKSTEHYAEF WRAVQEMWIR LYGSNLVTVA AINGSSPAGG 160
161 CLIALSCOVR INVENPKYVI GLNEAQLGIV APFWFKDTFV NAVGHRAAER SLQLGLLHSV PEAHRMGLVD EVVPEEKLQE 240
241 KAVAVMAQWL ALPDHARQLT KSMMRKAVLD HMLAHREEDI QNFVKFTSKD STOKSLSTYM ENLRKKKR
                                                                                                  308
The matched peptides cover 42% (131/308 AA's) of the protein.
Protein Name: enoyl-CoA delta isomerase 1, mitochondrial
Species: Gallus gallus
NCBlgb_Gallus_gallus_20250207_sequence.fasta Accession #: NP_001264514.2
MS Digest Index #: 129378
Masses are:
pl of Protein: 9.30
Protein MW: 34264.2 Da
                                                   (b)
1 MAAVAAAAGT FA<u>RRMAR</u>SGV LFP<u>R</u>SSPQVW PQAA<u>RQ</u>PPGL LPAQ<u>RR</u>AFSN N<u>KILVELDTS SGVATMK</u>F<u>K</u>S PPVNSLSLDF 80
81 LTEFCISLEK LENDRACRGL IITSAIPRVF SSGLDITEMC GKSTEHYAEF WRAVQEMWIR LYGSNLVTVA AINGSSPAGG 160
161 CLIALSCDYR IMVENPKYVI GLNEAQLGIV APFWFKDTFV NAVGHRAAER SLQLGLLHSV PEAHRMGLVD EVVPEEKLQE 240
241 KAVAVMAQWL ALPDHARQLT KSMMRKAVLD HMLAHREEDI QNFVKFTSKD STQKSLSTYM ENLRKKKR
                                                                                                  308
The matched peptides cover 53% (165/308 AA's) of the protein.
Protein Name: enoyl-CoA delta isomerase 1, mitochondrial
Species: Gallus gallus
NCBlgb_Gallus_gallus_20250207_sequence.fasta Accession #: NP_001264514.2
MS Digest Index #: 129378
Masses are:
pl of Protein: 9.30
Protein MW: 34264.2 Da
  Fig.2. Chicken (Gallus gallus) mitochondrial enoyl-CoA delta isomerase 1 (NCBI accession number:
     NP_001264514.2) sequence coverage, obtained after 3 hours (a) and 18 hours (b) of digestion.
```

After 3 hours of digestion, 11 peptides were identified, covering 42% of the protein sequence, whereas after 18 hours of digestion, 14 peptides were identified, covering 53% of the protein sequence. Among the peptides obtained during the digestion of mitochondrial enoyl-CoA delta isomerase 1, the peptide SLSTYMEMLR (m/z 615.7965<sup>2+</sup>) was previously identified as an authenticity marker for chicken liver tissue in highly processed food [7]. Importantly, all previously reported chicken liver authenticity markers [7] were detected regardless of digestion time. The overlaid chromatograms of six chicken liver specific peptides obtained after 3-hour (black) and 18-hour (red) digestion are shown in Fig.3.



Fig.3. The overlaid EIC LC-QTOF-MS chromatograms of six chicken liver peptide markers obtained after 3-hour (black) and 18-hour (red) digestion.

**Conclusions:** The results shows that extended digestion times have minimal effect on protein sequence coverage. Detection of chicken liver authenticity markers was achieved at all digestion times. Therefore, a 3-hour digestion appears to be sufficient for chicken liver authenticity testing and protein profiling, with marginal additional benefit from longer digestion times.

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# MASS SPECTROMETRY IMAGING IN LOCALIZATION OF METABOLITES IN MICROORGANISM CULTURES ON GEL SUBSTRATE

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**Abstract:** Mass spectrometry imaging (MSI) combined with laser ablation remote atmospheric pressure chemical ionization (LARAPPI/CI) was used to analyze metabolites of bacteria and fungi in solid culture media. The study identified organic compounds belonging to the groups of organic acids, fatty acids, sugars, and their derivatives. The obtained ion images revealed the spatial distribution of metabolites and provided insights into microbial metabolic interactions. These findings demonstrate the potential of LARAPPI/CI-MSI for studying microbial ecosystems and their biochemical dynamics.

Introduction: Mass spectrometry imaging (MSI) has gained significant popularity in microbiological research in recent years [1,2]. Currently, this technique is predominantly applied in two-dimensional (2D) MSI, posing a challenge for developing threedimensional (3D) analytical methods. Until now, 3D MSI has typically relied on specialized software that reconstructs multiple 2D datasets into volumetric representations [3]. Ruman et al. [3] introduced an innovative platform for remote laser photoionization/chemical ionization atmospheric ablation and at pressure (LARAPPI/CI), coupled with an ultrahigh-resolution quadrupole time-of-flight (QToF) mass spectrometer. Compared to conventional techniques such as desorption electrospray ionization (DESI), matrix-assisted laser desorption/ionization (MALDI), and surface-assisted laser desorption/ionization (SALDI), this technique offers several key advantages. Similar to DESI, it operates under ambient conditions. Additionally, it eliminates the need for ultrathin sample sectioning and the application of external matrix layers, allowing for the preservation of the native structure of biological specimens. This feature is particularly beneficial for samples with heterogeneous structural integrity, such as hydrated gel regions. In this study, we explore the application of the LARAPPI/CI MSI system for the spatial localization of microbial metabolites directly within gel-based culture media. By enabling high-resolution, label-free chemical imaging, this approach facilitates a more detailed understanding of metabolic distribution in bacterial and fungal cultures. The ability to perform direct 3D mass spectrometry imaging (MSI 3D) of microbial colonies provides a new tool for studying microorganism interactions, metabolic gradients, and biotechnologically relevant compound production.

**Experimental:** The study was conducted using bacterial and fungal strains: Bacillus cereus, a facultative anaerobic, spore-forming bacterium commonly found in soil and known for its ability to produce bioactive secondary metabolites, and Fusarium

graminearum, a filamentous fungus frequently associated with plant infections and mycotoxin production. These microorganisms were co-cultured on a gel-based medium to investigate their metabolic interactions and spatial metabolite distribution using LARAPPI/CI-MSI. The sample is placed on a table (50  $\times$  50 mm) equipped with a Peltier cooling plate, maintaining a temperature of  $-18^{\circ}$ C, and mounted on a motorized XY platform. A pulsed OPO laser beam (2.93 µm, 7 ns, 20 Hz, 3.5 mJ/pulse) passes through a sapphire window, is magnified  $3.75\times$ , and directed onto the sample using a gold mirror. Upon passing through a diffractive optical element, the beam acquires a square top-hat profile and is subsequently focused by a ZnSe lens with a focal length of 50 mm (ThorLabs, Mölndal, Sweden). The optical system, along with a camera featuring a lens and a distance sensor, is mounted on aluminum rails in a fixed configuration. A dedicated gas funnel, also functioning as a focusing element, is connected to a PTFE tube with an outer/inner diameter of 6/4 mm. The overpressure in the chamber drives a nitrogen flow of 10 L/min through the tube. Ablation plumes are captured and redirected to a modified ion source (Bruker VIP HESI in APCI configuration) of the Bruker Impact II mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The ion source incorporates a VUV lamp (Hamamatsu L12542) (Hamamatsu Photonics K.K., Iwata City, Japan), mounted coaxially with the MS sampling cone. A continuous flow of solvent mixture (1% toluene in methanol; 200 µL/min) to the APCI needle is maintained by an HPLC pump (Agilent G1312A) (Agilent Technologies, Santa Clara, CA, USA) [3]. Ion source parameters: APCI nebulizer pressure -3.5 bar, endplate offset - 600 V, capillary voltage - 1000 V, corona discharge current - 6000 nA, drying gas flow -0.2 L/min, drying gas temperature -250 °C, probe gas temperature -350 °C, probe gas flow – 4 L/min, exhaust enabled. Scan range: m/z 47–1300. A 2D MSI experiment was conducted with a spatial resolution of 240 µm, without oversampling. Each pixel/voxel was exposed to the laser for 1 second at a frequency of 20 Hz. The interval between successive pixels was 1200 ms, with the sample stage moving at a speed of 50 mm/s. The delay between lines was 5 seconds. Samples were trimmed with a blade, placed on a steel plate, transferred to the ablation stage inside the chamber, and frozen.

**Results:** The LARAPPI/CI-MSI 2D analysis enabled the identification of organic compounds produced by the studied bacterial and fungal strains revealing both primary and secondary metabolites with potential biological significance (Fig.1).

Among the detected organic acids, succinic acid (Fig.1B) was produced by *Bacillus cereus* and *Paenibacillus amylolyticus*, exhibiting known exhibits antimicrobial properties, while malic acid (Fig.1C) predominantly identified in *Fusarium graminearum*, demonstrated antibacterial activity against foodborne pathogens and may also stimulate the production of *Fusarium* antagonists in soil [4-6]. In the category of sugar derivatives, ribitol (Fig.1D) was detected exclusively within F. graminearum colonies, consistent with previous observations by Corona and Munday [7], but, as in our study, it accumulated only in the colonies, not in the culture medium suggesting its intracellular accumulation rather than extracellular secretion. Fatty acid analysis revealed that azelaic acid (Fig.1E) was produced by P. amylolyticus, which demonstrates a range of therapeutic properties [8].



Fig.1. Images of the analyzed agar medium (A): Bacillus cereus (1), Fusarium graminearum (2), with the area marked by a dashed line representing the imaged region. Panels (B–G) present ion images generated using the LARAPPI/CI-MSI method, showing the spatial distribution of detected organic compounds: succinic acid (B), malic acid (C), ribitol (D), azelaic acid (E), deoxyribose 5-phosphate (F), and elaidic acid (G).

It is a natural inducer of plant defense systems and a significant bio-monomer used in the synthesis of biodegradable and sustainable polymers, plasticizers, and lubricants [9,10]. Deoxyribose 5-phosphate (Fig.1F) was identified primarily in *B. cereus*, a metabolite widely used as a PCR reactions and also serves as a building block in antisense drugs and antiviral agents [11]. Meanwhile, eladic acid (Fig. 1G at the periphery of *Bacillus cereus* colonies) shows antimalarial activity [12]. Although its presence at the edges of the *Bacillus* growth area is atypical, the most plausible explanation is its production in response to high concentrations of toxic substances derived from *Fusarium graminearum* [13]. This could indicate a previously unrecognized microbial interaction between these species.

**Conclusions:** In this study, the LARAPPI/CI-MSI 2D method was employed to investigate the metabolic activity of *Bacillus cereus* and *Fusarium graminearum* in solid culture media. This method enabled assessing the spatial distribution of metabolites synthesized within microbial colonies, providing insights into their biochemical interactions. The identified compounds included succinic acid, malic acid, ribitol, azelaic acid, deoxyribose 5-phosphate, and eladic acid. These results show that the LARAPPI/CI-MSI method is useful for studying microbial metabolism in complex environments and could be applied in future biotechnological and microbiological research.

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## ICP-OES AS AN ALTERNATIVE TECHNIQUE FOR DETERMINATION OF MACROELEMENTS, MICROELEMENTS AND CONTAMINATIONS IN ORGANIC AND ORGANO-MINERAL FERTILIZERS

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**Abstract:** ICP-OES may be used as an alternative technique for determination of macroelements, microelements, and contaminations in organic and organo-mineral fertilizers. These materials showed high variation, and it is difficult to determine the full composition. In order to ensure measurement consistency and to check competence, the Analytical Laboratory participated in proficiency tests organised by the National Chemical and Agricultural Research Laboratory in Warsaw covering the determination of different elements in organic fertilizer samples.

**Introduction:** In the last decades, crop production was carried out only using the natural resources of the soils and partial return of nutrients in the form of organic products. The development of the fertilizer industry began in the 19th century with the need to increase crop production associated with economic development and rapid population growth. Plants, for proper growth and vital activity, need mineral nutrients in the right amount, chemical form and at a specific time resulting from the growth rate of their organs and the purpose of cultivation. These nutrients are found in various mineral and organic substances in the soil and in fertilizers. Soil nutrient resources can be divided into plantavailable and total resources, mostly unavailable to crop plants [1]. Fertilizers used in agricultural production increase crop yields by enriching the soil with essential nutrients and improving its physicochemical and biological properties. These include organic fertilizers, organo-mineral fertilizers and inorganic fertilizers. Organic fertilizers, according to the Law on Fertilizers and Fertilization, are defined as fertilizers made from organic matter or mixtures of organic matter, including composts, also made with earthworms. Organic-mineral fertilizers are a mixture of organic and mineral fertilizers [1]. Natural fertilizers are characterized by many advantages including: variety of natural nutrients, positive effects on soil structure, enhance of the soil microflora with organic matter, and relatively low risk of over-fertilization. By origin, organic fertilizers can be divided into: crop residues (straw, pods, hulls), by-products of crop production (leaves, grasses, sawdust), green manures, composts produced from natural waste products on farms, organic waste from industrial processing of agricultural products, sewage sludge and composts of non-agricultural origin: municipal and industrial. The last type of fertilizers is potentially dangerous because, despite composting processes, it may not meet sanitary requirements due to potential chemical and biological contamination, significant content of pathogenic microorganisms such as bacteria, viruses, fungi, moulds, eggs of human and animal intestinal parasites, as well as toxic elements (chromium, nickel, lead, cadmium, arsenic or mercury) [2]. Uncontrollably introduced into the soil with fertilizers, they cause soil contamination, which lead to contamination of the cultivated crop contamination of the crops grown. Fertilizer manufacturers are obliged to comply with the relevant legislation, which sets out, in addition to the main components and micro-nutrients, the permissible limits for the content of substances considered harmful to the environment, and consequently to humans. Legislation on organic and organic-mineral fertilizers has been under intense development for several years. The EU has established comprehensive rules for organic fertilizers under Regulation (EU) 2019/1009 [3]. This regulation sets safety, quality, and labeling standards for organic and organo-mineral fertilizers, as well as soil improvers and plant biostimulants. It also introduces limits for contaminants like cadmium and mercury to protect soil and reduce health risks. The national legislation is governed by the Regulation of the Minister of Agriculture and Rural Development of 18 June 2008 [4]. In addition, technical specifications have been developed detailed describing the method of preparing samples of organic fertilizers and the determination of their components, recommending the ICP-OES technique as an alternative to the classical techniques, gravimetric or atomic absorption spectrometry with atomization in flame [5,6]. Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) is a powerful analytical technique used to detect and quantify trace elements and also major elements in various samples. It works by exciting atoms in a plasma state, causing them to emit light at specific wavelengths. The intensity of this emitted light is measured to determine the concentration of elements in the sample. This method is widely used in fields like environmental analysis, metallurgy, geology, and pharmaceuticals due to its high sensitivity, precision, and ability to analyze multiple elements simultaneously. Determination of micronutrients, major constituents, and trace elements by the ICP-OES technique in a complex organic-mineral matrix is a major challenge for analysts. Although it is a rapid and precise analytical method, allowing routine determinations to be carried out at trace level, it requires the correct choice of sample introduction system into the plasma torch, optimisation of the parameters of the excitation source, optical system and selection of appropriate emission lines of the analysed elements. The potential for spectral and inter-elemental interference and inter-elemental interference affecting the emission signal in the ICP-OES technique, so appropriate correction methods are recommended, e.g. the use of scandium as an internal reference standard.

**Experimental:** The aim of this study was to develop a method for the simultaneous determination of several nutrients like P, S, Ca, Mg, K, and Mn, Mo, Fe, Cu, Zn and B by ICP-OES technique using a Varian 720-ES spectrometer (Mulgrave, Australia) with horizontal argon plasma alignment and an Agilent model 5900. Homogeneous solid and liquid samples of commercially available organic and organic-mineral fertilizers were used for the study. Fertilizer samples were subjected to microwave digestion in a Mars 6 closed system (CEM,USA) so that the temperature built up to 190 °C and was maintained for 10 minutes at a maximum pressure of 19.6 bar. The special nature of the matrix required optimization of the sample preparation process. Microwave assisted acid digestion was carried out using mixture of HNO<sub>3</sub> and HCl. The tested sample, weighing approximately 0.5 g, was placed on a teflon vessel. In order to ensure the accuracy and to identify analytical errors associated with the procedure of digestion and determination of macro- and micro-nutrients in organic and organic-mineral fertilizer samples, reference materials Marsep 259 (Compost, Wepal), Marsep 275 (Cow Manure, Wepal), and AgroMat CP-1 (SCP Science) were investigated. The solutions were quantitatively

transferred to 50 ml flasks. The concentrations of elements in solutions were determined by ICP-OES technique using a Varian 720-ES ICP-OES spectrometer with horizontal argon plasma viewing, equipped with a Slurry® Glass Expansion® nebuliser, an Agilent® glass cyclone chamber, and a one-piece quartz torch (Table 1). Standard solutions for making calibration curves were prepared by diluting a commercial standard solution from Inorganic Ventures with a concentration of 1000 mg·dm<sup>-3</sup> or 100 mg·dm<sup>-3</sup>. The concentrations of the calibration standard solutions covered the following working ranges for selected elements (0.50-20 mg·dm<sup>-3</sup>). Inter-elemental interference correction was carried out using a solution (3 mg·dm<sup>-3</sup>) of scandium as an internal reference standard. Analytical Laboratory participated in proficiency tests organised by the National Chemical and Agricultural Research Laboratory in Warsaw covering the determination of different elements in organic fertilizer samples.

and A	gilent model 5900.	
Parameter	Varian model 720 ES	Agilent model 5900
Power [kW]	1.20	1.10
Plasma Ar Flow [dm <sup>3</sup> ·min <sup>-1</sup> ]	15.0	12.0
Auxiliary Ar Flow [dm3 ·min-1]	1.50	0.90
Nebuliser Ar flow [dm <sup>3</sup> · min <sup>-1</sup> ]	0.75	0.70
Inetegration time [s]	12	14
Peristaltic pump rate [rpm]	11	10

 Table 1. Basic operating parameters of simultaneous spectrometers ICP-OES Varian model 720-ES and Agilent model 5900.

**Results:** Multi-elemental analyses carried out by ICP-OES in selected commercial fertilizer products confirmed that the procedure meets the requirements and can be used for the purpose specified. Important parameters characterizing the analytical procedure include precision and accuracy. A method is considered suitable if the relative standard deviation for a series of analyses is less than 12.5% and the recovery is in the range of 80 - 110% [7]. The laboratory confirmed its competence by obtaining satisfactory parameters in proficiency tests (Fig.1).



Fig.1. Recovery of elements in CRM- Marsep 259, Marsep 275, and AgroMat CP-1.

The tested fertilizer samples have a significantly different chemical composition (Table 2). Correct results of fertilizer component determinations in relation to the declared manufacturer's values were obtained.

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Type of	Р	Κ	S	Ca	Mg	Fe	Mn	Cu	Zn	В	Mo
fertilisers			[9	6]		[mg·kg <sup>-1</sup> ]					
Granulated cow manure	1.46	1.85	2.05	4.45	1.19	0.63	467	78.0	363	31.0	3.03
Granulated chicken manure	1.42	2.10	0.70	6.70	0.75	0.10	379	66.8	336	25.6	3.46
Organic NPK 5-3-3	2.87	2.45	0.04	4.45	0.70	0.06	354	45.3	326	31.5	3.77
Solid organic mineral	0.93	0.52	3.12	1.69	0.31	2.93	295	112	264	38.5	31.3
Liquid Organic- mineral	2.77	0.49	1.17	0.06	0.15	0.01	116	1.88	10.2	2.00	1.20
Granulated compost	0.77	1.02	0.18	5.90	2.26	0.78	330	37.0	205	26.2	1.40

Table 2. Macroelements and microelements in avaible organic and organo-mineral fertilizers.

**Conclusions:** The ICP-OES technique is a suitable tool for the simultaneous analysis of macro- and micronutrients of fertilizers due to the linearity of the emission signal over a wide range of concentrations. The critical element of the tests is the selection of the appropriate sample preparation, especially in the case of P and S determinations, so as not to partially lose the analyte. Moreover, simultaneous analysis of trace elements and main components requires the selection of the best analytical line and optimization of excitation source parameters due to the risk of matrix effects and spectral interference.

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### CHANGES IN THE FERTILIZER LAW AS AN OPPORTUNITY FOR THE FERTILIZER INDUSTRY AND A CHALLENGE FOR FERTILIZER QUALITY TESTING BODIES

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Abstract: Some amendments that have been made to the European Fertilizer Law, allow to circulate freely on the European market of fertilizer products composed of various ingredients, including waste origin. Since it is permitted to combine component materials into any products, provided the requirements for acceptable impurity content and minimum nutrient content are met, manufacturers offers increasingly complex fertilizer products. Complex matrices of modern fertilizers cause some difficulties during analysis. As an example, the effect of the fertilizer matrix on the ability to determine chromium(VI) using the ion chromatography (IC) is presented. For some types of fertilizers, the results based on obtained chromatograms not allow for proper interpretation. Additionally, the possibility of determining biuret by the highperformance liquid chromatography (HPLC) in some type of fertilizer where the complexity of the matrix prevents the spectrophotometric method used for urea is presented.

Introduction: New fertilizer products can be introduced to the fertilizer market in two ways: to the European market – under the rules of the European Fertilizer Law, or to the national market - under the rules of the National Fertilizer Law. Fertilizer producers coming from countries of European Union have a possibility to choose if they want to introduce their product to the European market, considering an obligation of meeting the requirements of European law or only to the national market, when the requirements of national law are generally less restrictive. Introducing new products to the European fertilizer market is currently regulated by Regulation 2019/1009 [1]. Before that, European fertilizer market was regulated by EC Regulation 2003/2003 [2]. These two legal acts differ significantly. Primarily, the previous regulation strictly defined the types of only inorganic fertilizers that comes from chemical processes, that can be placed on the European market when the requirements for the minimal nutrient contents and their solubility and availability for plants, were met. If these demands were fulfilled, fertilizer manufacturers were allowed to introduce their products into the European fertilizer market with the EC label. In accordance with the provisions of the new regulation, it is permitted to place on the European market any fertilizer product composed of ingredients belonging to various categories of component materials. The purpose of the provisions of this regulation was primarily to allow the widespread use of fertilizer products made from recycled raw materials. The ability to compose products from different types of component materials is prompting fertilizer manufacturers to develop multifunctional products. On the other hand, Regulation 2019/1009 imposes
requirements for cornering impurities in fertilizer products, which was not required by the previous *Regulation 2003/2003*. The complexity of the matrix of multifunctional fertilizers and the variety of ingredients is the reason for analytical difficulties during quality control of fertilizer products.

**Experimental:** The effect of the complexity of the tested fertilizers matrix on the results of chromium(VI) determination by the ion chromatography (IC) and biuret determination by the high-performance liquid chromatography (HPLC) is presented. Chromium content analyses were carried out by ion chromatography with post-column reaction and UV-VIS detection, using a ion chromatograph ICS-3000, Dionex (USA) with UV-Vis detector (AD-25). The study was conducted for three fertilizers, with varying degrees of matrix complexity, such as magnesium nitrate hexahydrate, guano based fertilizer and fertilizer elemental-sulfur inorganic fertilizer. Biuret content analyses were carried out by the high-performance liquid chromatography with diode-array detection (HPLC-DAD), Waters (USA). Analyses were done for some liquid fertilizer sample of unknown composition.

**Results:** Analysis of chromium(VI) content of standard fertilizers, with simple compositions, without additives and matrix impurities, show that the obtained chromatograms reveal a clear peak corresponding to the chromium(VI) content. The baseline of the chromatographic system is stable for these fertilizers, and validation studies have shown high standard recovery. An example chromatogram of a fertilizer with a simple matrix is presented in Fig.1, for magnesium nitrate hexahydrate, in solid form, with a Cr(VI) content of 0.207 mg·kg<sup>-1</sup>. A very similar chromatogram was obtained for calcium nitrate, with a Cr(VI) concentration of 0.338 mg·kg<sup>-1</sup>. For both magnesium nitrate and calcium nitrate, standard recovery was very high, at 84.3% and 100.8%, respectively.



Fig.1. Chromatogram obtained from the analysis of magnesium nitrate hexahydrate.

In the case of fertilizers with a more complex matrix, the chromatograms obtained showed an unstable baseline, with a very faint peak corresponding to chromium(VI) content that was difficult to identify from the others. Figure 2 shows an example of this kind of chromatogram, obtained from the analysis of guano fertilizer, in solid form, containing organic compounds and plant food components like calcium, magnesium, phosphorus, potassium, nitrogen in ammonium form. A chromatogram of similar complexity was also obtained for chromatographic analysis of a sulfur-containing phosphorus-potassium fertilizer (about 20%  $P_2O_5$ , about 30%  $K_2O$ ). For both types of

these fertilizers, validation of the method showed very significantly lower pattern recovery rates than for simple matrix fertilizers, at 24.2% and 25.0%, respectively.



An even stronger effect of the matrix was observed for fertilizers containing elemental sulfur (S<sup>0</sup>), which in the soil are oxidized to the plant-available  $SO_{4^{2^{-}}}$  form, and the rate at which this conversion takes place is the determining factor regarding the effectiveness of S<sup>0</sup> as a fertilizer source of sulfur [3] (Fig.3). In the case of this type of fertilizer, the chromatogram is characterized by visible drift of the baseline, a significant number of so-called "water peaks," high noise and sometimes the presence of so-called "extraneous peaks".



Fig.3. Chromatogram obtained from the analysis of fertilizer containing elemental sulfur.

Another analysis that is negatively impacted by the complex fertilizer matrix is the determination of biuret content. Most of the fertilizers used in crop cultivation are produced based on urea as a source of nitrogen. Biuret is a byproduct of urea synthesis. It percentage in fertilizers must be kept low because it is toxic to plants. The widely used spectrophotometric method for the determination of biuret is applicable to urea and simple urea-based fertilizers [4]. Since in recent years new urea fertilizers have been introduced to the market with significantly modified composition, strongly hindering spectrophotometric analysis, it became necessary to prepare an alternative method for the determination of biuret. In order to eliminate the influence of additional components of the fertilizer matrix, it seems appropriate to use a preliminary chromatographic analysis allowing to separate the determined compound from other components of the analyzed sample. The use of preliminary chromatographic analysis allows for proper separation of the sample ingredients. A method for the quantitative determination of biuret in fertilizer products using the technique of high-performance liquid chromatography coupled to UV-VIS spectrophotometric detector was used. The result of the research was a development of HPLC-DAD method for the determination of biuret in both solid and liquid fertilizer samples with modified composition. Figure 4 shows a chromatogram for the biuret standard solution, and Fig.5 shows the obtained chromatogram for liquid fertilizer sample of unknown composition. It can be seen that the analysis conditions used resulted in a clear separation of the characteristic peak for biuret from the other fertilizer components, and thus its quantification.



Fig.4. Chromatogram of analysis of biuret standard solution at 200 mg/ml.



Fig.5. Chromatogram of biuret analysis in a liquid fertilizer sample of unknown composition.

**Conclusions:** Changes to the European fertilizer law have contributed to the marketing of fertilizers with increasingly complex matrices, requiring modification of the analytical methods used to date or the development of new ones. Studies on the determination of Cr(VI) in fertilizers by IC method have shown different standard recovery values, depending on the type of matrices currently used as nutrient carriers in agriculture. In some cases, the sample matrix suppressed the analytical signal of chromate ions, making the determination impossible, and samples containing significant amounts of sulfur caused the analytical column to damage completely, forcing the purchase of a new one. The feasibility of using HPLC-DAD method for the determination of biuret in liquid fertilizer samples with a complex matrix was proven. Taking into account all the results listed above, it can be concluded that the proposed alternative HPLC-DAD method for the analysis of biuret in fertilizer samples can find application in the work of the fertilizer laboratory.

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## EVALUATION OF THE EFFECT OF THE ADDITION OF CALCIUM SULPHATE BASED WASTE MATERIALS ON THE THERMAL DECOMPOSITION OF AMMONIUM NITRATE

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**Abstract:** The influence of the addition of calcium sulphate based by-products on the phase transitions and thermal stability of ammonium nitrate (AN) has been investigated and discussed. Differential thermal analysis (DTA) coupled with thermogravimetry (TG) and differential scanning calorimetry (DSC) were used to evaluate the stability of the system analysed. It was concluded that gypsum had a neutral effect on the thermal decomposition of ammonium nitrate. Gypsum was considered to be a suitable additive to ammonium nitrate.

Introduction: Ammonium nitrate (AN) is a well-known and commonly used inorganic fertilizer. It is also used as a component of explosives due to properties such as susceptibility to spontaneous exothermic decomposition and explosion. In the fertiliser industry, AN is used in a mixture with various additives. These may (i) act as stabilisers, (ii) reduce the unfavourable properties of AN, (iii) introduce additional nutrients or (iv) be an impurity [1]. AN is a thermodynamically unstable substance and can decompose in solution, melt and solid state. The rate and direction of decomposition of AN depends on many factors such as temperature or mass and heat exchange conditions with the environment. The presence of a substance other than NH<sub>4</sub>NO<sub>3</sub> in the system can significantly affect the decomposition of AN. In real systems, four groups of substances have been identified that affect AN decomposition and process safety: catalysts (which are not consumed during the reaction); reacting chemicals (which release energy and are therefore consumed by the reaction); decomposition inhibitors; and others (inerts and substances that react without major thermal effects) [2]. In order to study the influence of various additives and impurities on the stability and the thermal decomposition of AN, methods of thermal analysis are used. A new coal-fired power plant is being built in Pulawy. A wet lime flue gas desulphurisation system will be used because of its high efficiency and relatively low sorbent consumption. Flue gas desulphurisation gypsum (FGDG) is an industrial by-product of the flue gas desulphurisation process in coal-fired power plants. The main component of FGDG is CaSO<sub>4</sub>, but other elements and compounds may also be present. The amounts of trace elements depend on the composition of the coal burnt and the limestone, lime and other additives used in the FGD process [3]. There are opportunities to use gypsum (G) as a raw material in the fertilizer industry. The presented study evaluates the effect of the addition of calcium sulphate based by-products (gypsum and phosphogypsum) on the thermal decomposition and phase transformations of ammonium nitrate. However, phosphogypsum (PG) is only

used as a comparative material for gypsum and its use as a raw material for AN is questionable due to the very high level of impurities. Phosphogypsum (PG) is a large by-product from the production of extractive phosphoric acid (EPA) and phosphorus fertilizers. It is a major environmental challenge due to the presence of toxic and radioactive elements in phosphogypsum, and for this reason it is stockpiled in large open areas, while its re-used share does not exceed 10-15% [4,5].

**Experimental:** Mixtures of ammonium nitrate (p.a. POCH, Poland) with 10 wt.% gypsum (Kozienice, Poland) - AN 10%G and phosphogypsum (Police) - AN 10%PG were prepared. The calcium sulphate based by-products were partially dehydrated before use. The XRPD diffractogram of the partially dehydrated gypsum is shown in Fig.1.



All mixtures were prepared using pure ammonium nitrate. Ammonium nitrate without additives (AN) was also tested. The mixture of AN and hemihydrates tested in this study was prepared using an agate mortar and pestle. Thermal analyses were determined using techniques such as DSC and the simaltanious TG/DTA. Netzsch STA 449 F3 thermal analyser was used. A TG-DTA sample carrier system and type S thermocouples were used. Samples of mixtures containing approximately  $50.0\pm1$  mg AN were heated in a alumina crucible without lid in a dynamic atmosphere of synthetic air at a flow rate of  $50 \text{ cm}^3 \text{ min}^{-1}$  with a heating rate set at  $10 \,^{\circ}\text{C} \text{ min}^{-1}$ . DSC measurements of pure AN and mixtures of AN with hemihydrates were carried out in a dynamic atmosphere of an nitrogen (at a flow rate of  $70 \text{ cm}^3 \text{ min}^{-1}$  with the heating rate set at  $5 \,^{\circ}\text{C} \text{ min}^{-1}$ ) in a high-pressure sample crucible.

**Results:** The DSC curves of samples analyzed in nitrogen atmosphere in high pressure crucibles are shown in Fig.2. Table 1 shows the thermal data of temperature and heat evolved during decomposition of tested samples from the DSC curves. From the DSC curve it can be concluded that AN underwent three solid-solid phase transitions, which

can be seen as three endothermic effects (IV $\rightarrow$ III around 47°C, III $\rightarrow$ II around 94°C, II $\rightarrow$ I around 128°C). The fourth endothermic effect was related to the melting of AN. On the basis of T<sub>onset</sub>, decomposition of pure AN occurs at around 300°C, visible as an exothermic peak. AN 10%G mixture starts to decompose at lower temperature (T<sub>onset</sub>-290.1°C) than the rest of the samples. However, it can be seen in Fig.2 that the peak of exothermic decomposition is not as sharp as in the case of the AN 10%PG sample, suggesting a slower decomposition. For the same sample, the heat evolved during decomposition was the lowest, which is favourable.



Fig.2. DSC curves of thermal decomposition of tested samples (pressure crucibles) in nitrogen.

 Table 1. Thermal data of temperature  $(T_{onset}, T_{max})$  and heat evolved during decomposition of tested samples obtained from DSC curves.

Sample	Temperature of phase transition $T_{max}$			Melting	Tempera exothe	ture of erm	Heat evolved during decomposition
	IV→III	III→II	II→I		Tonset	T <sub>max</sub>	$\Delta H$
	°C					$J \cdot g^{-1}$	
AN pure	47.9	94.4	128.8	166.0	299.9	322.1	1321
AN 10%G	53.1	92.4	127.1	160.4	290.1	327.9	1106
AN 10%PG	53.8	90.1	127.1	161.3	312.4	327.4	1311

In Table 2 temperatures of the phase transitions and characteristic of decomposition peak of the tested samples in air are shown. The TG/DTA curves of all samples analyzed in the synthetic air atmosphere in alumina crucibles are shown in Fig.3. AN underwent two solid-solid phase transitions, which can be seen as two endothermic effects. The endothermic transition IV $\rightarrow$ III was not observed due to the absence of water. The third endothermic effect was related to the melting of AN. The temperature of the subsequent phase transitions in the mixtures with the addition of by-products was very similar to that of the pure AN sample. On the basis of the temperature of the decomposition peak (T<sub>onset</sub>) of the AN10%PG sample, it can be seen that decomposition starts at the lowest temperature (225 °C). In the case of AN10%G sample, the differences in the

temperatures of the decomposition peaks compared to pure ammonium nitrate are smaller. The TG curve also shows that the AN10%PG sample starts to decompose as the first.

			in air.			
Sample	Temperature of phase transition T <sub>max</sub>			Melting	Charac decompo	teristic of sition peak
	IV→II III→II II→I			Tonset	T <sub>max</sub>	
				°C		
AN pure	58.3	-	133.9	172.1	237.9	252.7
AN 10%G	57.6	-	133.3	174.4	231.6	248.5
AN 10%PG	58.0	58.0 - 133.6			225.4	237.7

Table 2. Temperatures of the phase transitions and endothermic thermal decomposition of the tested samples



Fig.3. TG/DTA curves of thermal decomposition of tested samples in air.

**Conclusions:** From the results obtained it can be concluded that the calcium sulphate based by-products have a neutral effect on the thermal decomposition of AN. However, due to the high content of impurities in phosphogypsum, only gypsum can be considered as a suitable additive to ammonium nitrate.

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## EVALUATION OF THE INFLUENCE OF SELECTED FACTORS ON THE EFFICIENCY OF CHLORIDE REMOVAL FROM POLYHALITE

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**Abstract:** This paper presents a study to evaluate the effect of selected factors on the efficiency of chloride leaching from polyhalite. In none of the cases studied was sodium chloride completely removed, but the efficiency of chloride removal from polyhalite was high, up to 96%. The study concluded that the amount of water used for flushing was more influential than temperature, which is consistent with the practically unchanged solubility of sodium chloride in water as a function of temperature. However, the temperature of the water used for chloride removal influenced the presence of ions other than chloride in the aqueous solution.

**Introduction:** Along with nitrogen and phosphorus, potassium is one of the three most important macronutrients for plant growth and development. Potassium occurs in the plants as the K+ ion and does not form organic compounds. This element plays an essential role in many biochemical and physiological processes in the plants. Potassium is involved in osmotic processes, the activation of plant enzymes in various processes, the transport of ions and constituents in the xylem and the transport of organic and inorganic compounds in the phloem [1]. In the fertilizer market, potassium is mainly available in two forms: chloride and sulphate. Potassium chloride, is the most popular due to its high potassium content (up to 60% K<sub>2</sub>O) and low price. Potassium sulphate, which is available on the fertilizer market, can be an alternative to chloride salt. The sulphate form has a beneficial effect on plant growth, but its use is limited due to its higher price. With rising prices for potassium chloride and potassium sulphate based fertilizers, other potassium containing minerals may prove to be a beneficial alternative. In Table 1 various selected potassium-containing evaporite salts are shown [2].

Langbeinite	$K_2Mg_2(SO_4)_3$
Leonite	$K_2Mg(SO_4)_2$ ·4H <sub>2</sub> O
Schoenite (picromerite)	$K_2Mg(SO_4)_2$ $^{\circ}6H_2O$
Polyhalite	K <sub>2</sub> Ca <sub>2</sub> Mg(SO <sub>4</sub> ) <sub>4</sub> ·2H <sub>2</sub> O
Sylvite	KCl
Syngentite	K <sub>2</sub> Ca(SO <sub>4</sub> ) <sub>2</sub> ·H <sub>2</sub> O
Kainite	KMg(SO <sub>4</sub> )Cl·3H <sub>2</sub> O

Table 1. Various selected potassium-containing evaporite salts.

Polyhalite (K<sub>2</sub>SO<sub>4</sub>·MgSO<sub>4</sub>·2CaSO<sub>4</sub>·2H<sub>2</sub>O) is one of the most complex salt minerals found in nature. Polyhalite has a lower environmental impact than other fertilizers and

releases nutrients more slowly than conventional fertilizers. It can help to increase the efficiency of fertiliser use [3]. An important advantage of using polyhalite as a fertiliser compared to equivalent salts is the rate at which it releases minerals into the soil profile. The leaching of Ca, Mg, K and S from polyhalite appears to be slower than the leaching of these ions from commonly used soluble salts. In this respect, polyhalite has the potential for longer term effects compared to commercial fertilizers. It was assumed that the uptake efficiency of Ca, K, Mg and SO<sub>4</sub> by wheat plants would be higher if the nutrients were applied as polyhalite than as their separate sulphate salts [4]. Although polyhalite has a lower solubility limit in water than other K source fertilizers, when applied at rates below its solubility limit it should provide more than enough Ca, Mg, K and S for plant growth [5]. Today, polyhalite is mined as a primary target in only one place in the world, the Boulby mine, owned by Israel Chemical Limited (ICL), located under the North Sea off the North Yorkshire coast of the UK. Currently, ICL-UK distributes approximately 500 kt/y from the Boulby Mine as direct application fertilizer or as a bulk blend/compound NPK additive [2]. An advantage of the selected potassium minerals is their low chloride content. This is particularly important when fertilizing chloride-sensitive crops and in the manufacture of ammonium nitrate-based fertilizers because of the destabilizing effects of chloride. The presented study evaluates the effect of influence of selected factors on the efficiency of chloride removal from polyhalite. In order to be able to assess which ions have entered the solution after the chloride removal, an XRPD test was also carried out on selected samples.

**Experimental:** Ground polyhalite (Polysulphate standard - ICL) containing sodium chloride was used for the test. The particle size distribution of the test material is shown in the Fig.1.



Fig.1. The particle size distribution of polyhalite used in test.

The particle size distribution of polyhalite was expressed by these three values (  $D_v$  (10)  $-7.02 \mu m$ ;  $D_v$  (50)  $-237 \mu m$ ;  $D_v$  (90)  $-624 \mu m$ ).  $D_v$  (50)  $-237 \mu m$  means that 50% of the total particles are smaller than this size or 50% of the particles are larger than this size.

Two factors were evaluated in the chloride removal tests on polyhalite, namely the effect of water temperature and the weight ratio of polyhalite to water. Dissolution of polyhalite in water leads to its decomposition. Four samples were tested: 1. water temperature 20°C and ratio 1:5; 2. water temperature 20 °C and ratio 2:5; 3. water temperature 30°C and ratio 1:5; 4. water temperature 30 °C and ratio 2:5. A 100 g sample of polyhalite was placed on a filter in a funnel and then flooded with an appropriate volume of water at a specified temperature. The residue on the filter was dried at 105 °C and the chloride content and the calcium, potassium, magnesium and sulphur contents were determined; the water content at 400°C and the insoluble matter and silica were also determined. Water was determined by calcination of the samples at temperature 400 °C. Insoluble matter and SiO<sub>2</sub> were determined by hot dissolution of samples in 1:1 HCl, followed by draining and calcination at 1000°C. Calcium and magnesium were determined by a complexometric method. Potassium was determined by flame emission spectroscopy and chlorides by the Mohr method.

The material for XRPD measurments was prepared by slow evaporation at room temperature aqueous solution after chloride removal.

**Results:** In the Table 2 is shown chemical composition of the raw polyhalite and tested samples after chloride removal in different conditions. From the results obtained it can be concluded that in none of the cases studied was the sodium chloride completely removed, but in the case of the sample (2:5 30 °C), twice as much remained as in the other samples. In general, there is no clear effect of temperature on chloride removal, which is not particularly surprising as the solubility of sodium chloride is similar over a wide temperature range. More important is the amount of water used for rinsing.

Component	Component content, %					
	20°C	30°C	20°C	30°C	Raw	
	1:5	1:5	2:5	2:5	polyhalite	
$K_2SO_4$	25.61	25.08	25.80	25.93	24.88	
CaSO <sub>4</sub>	51.24	51.07	49.40	48.72	44.81	
$MgSO_4$	14.90	15.50	16.40	16.00	16.65	
NaCl	0.35	0.30	0.35	0.70	8.49	
H <sub>2</sub> O (400 °C)	5.58	5.55	5.57	5.64	5.35	
Insoluble mater and silica	0.35	0.40	0.41	0.22	0.39	
Total	98.03	97.89	97.92	97.20	100.57	

Table 2. Chemical composition of the raw polyhalite tested samples.

In all cases studied, the potassium content increased slightly in relation to the polyhalite before chloride removal. The same was observed for calcium sulphate content. Most magnesium migrated into the aqueous solution from samples marked as (20 °C 1:5) and (30 °C 1:5). After chloride removal, gypsum was formed in all samples, which was confirmed by other studies , not presented in this article.

In the Fig. 2 are shown XRPD diffractograms of samples after slow evaporation at room temperature (20 °C 2:5; 30 °C 2:5) of selected aqueous solution. From the analysis of the diffractograms it can be concluded that the dominant crystalline phase is the sodium chloride. However, small amounts of other compounds are also visible in the sample marked (20 °C 2:5). This is certainly related to the solubility of calcium sulphate. Unlike the solubility of most salts, the solubility of calcium sulphate decreases with increasing temperature.



Fig.2. XRPD diffractogram of selected samples (20 °C 2:5; 30 °C 2:5).

**Conclusions:** The efficiency of chloride removal from polyhalite is high, up to 96% at 30  $^{\circ}$ C at a polyhalite to water ratio of 1:5. However, the most favourable process conditions for chloride removal from polyhalite are 20  $^{\circ}$ C at a weight ratio of 2:5. The concentration of potassium and calcium ions did not decrease and magnesium ions, expressed as magnesium sulphate, decreased by only 1.5%.

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# EVALUATION OF THE FUNCTIONAL PROPERTIES OF AMMONIUM NITRATE FERTILIZERS WITH ADDITION OF POLYHALITE

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**Abstract:** This paper discusses the testing methods used to evaluate the physicochemical properties of granular and high nitrogen ammonium nitrate-based fertilizers containing at least 28% (m/m) nitrogen (N). This paper presents the results of selected physicochemical tests on ammonium nitrate-based fertilizers with polyhalite and dolomite additives. The paper discusses the issues of hygroscopicity, strength, strength after temperature cycles, oil retention and pH of the obtained fertilizers. Based on the study, it was found that the additive used had a positive effect on the physicochemical properties of the fertilizers.

Introduction: The quality of a specific mineral fertilizer, like any other chemical product, is defined by a set of characteristics that represents the functional properties of the product, distinguish it from other products of its type and determine its performance over time, from production to consumption. In order to assess the quality of fertilizers, criteria are used which are usually defined and listed in mandatory as well as nonmandatory standards and regulations for whole groups of fertilizers or individual products of the fertilizer industry and related industries. A good quality fertilizer is a fertilizer with nutrient and impurities contents in accordance with the declared values as well as with appropriate physicochemical properties. Different testing methods are used to assess physicochemical properties. The quality of fertilizers and intermediate products of various types is determined by sieve analysis, evaluation of granule shape, determination of compressive strength of granules at room temperature and under process conditions (variable temperature and humidity). It is also important to assess the tendency to caking and the effectiveness of anti-caking agents, as well as the hygroscopicity [1]. Additional testing methods are also used for a selected group of fertilizers. These are high-nitrogen fertilizers based on ammonium nitrate with a minimum nitrogen (N) content of 28% (m/m). These are tests of oil absorption (retention), durability and volume change of granules during long-term storage under laboratory conditions (approximately constant temperature) and under rapid temperature changes (25 °C and 50 °C). Thermal analysis methods are used to assess the effect of additives and impurities on the safety risk of manufacturing and storing ammonium nitrate based fertilizers under laboratory conditions. Finally, the safety of the production of these NH<sub>4</sub>NO<sub>3</sub>-based fertilizers is assessed by means of the resistance of detonation test described in Regulation (EU) 2019/1009. The technical requirements for ammonium nitrate-based fertilizers with high nitrogen content also apply to the chemical

composition. All substances other than ammonium nitrate should be chemically inert with respect to (NH<sub>4</sub>NO<sub>3</sub>). Combustible matter per carbon (C) shall not exceed 0.2% for fertilizers with a nitrogen (N) content of at least 31.5% (m/m) and 0.4% for ammonium nitrate-based fertilizers with a nitrogen (N) content of at least 28% but less than 31.5%. The production process shall not involve the addition of heavy metals. The copper (Cu) content shall not exceed 10 mg/kg and the chlorine (Cl) content shall not exceed 200 mg/kg [2]. The current Fertilizer Regulation (EU) 2019/1009 allows the use of a wide range of products from different sources for the production of inorganic, organic and organic-mineral fertilizers. The aim of this study was to investigate the effect of polyhalite on the physicochemical properties of ammonium nitrate-based fertilizers. Polyhalite is a source of additional nutrients such as potassium, calcium, sulphur and magnesium. The polyhalite that is currently available on the market also has very low levels of heavy metals and impurities [3].

**Experimental:** Four different types of ammonium nitrate based fertilizers (AN) with different levels of added polyhalite (PS) and dolomite (MD) were produced in laboratory scale. The composition of the tested fertilizer samples and the percentage content of each component are given in Table 1. Obtained fertilizers were tested to determine granular compressive strength, hygroscopicity and oil retention. The pH of fertilizers aqueous solutions was also determined. The method of carrying out the different measurements is described below.

Same la	Content, wt. %				
Sample	AN	PS	MD		
1	80	10	10		
2	80	20	-		
3	90	10	-		
4	95	5	-		

Table 1. Composition of tested fertilizers.

The compressive strength of the fertilizer granules was measured at room temperature using the Erweka THB 220 hardness tester. 30 granules with a diameter in the range of 4.5-5.0 mm were measured each time. In general, appropriate compression strength of granules, adopted in the fertilizers industry is10 N/granule for prilled nitrogen fertilizers and 20 N/granule for other fertilizer products. In interpreting the test results, the proportion of so-called "weak granules" (less than 2 N/granule) was also taken into account, which for a fertilizer of sufficient quality should be less than 5% of the number of granules tested. Durability and changes in granule strength during long-term storage were measured after exposure to two, five, ten and fifteen thermal cycles (50 °C and 25 °C). Hygroscopicity was assessed using a climatic chamber (Pol-Eko), which allows to set the temperature and humidity. Samples were measured at a temperature of 20 °C and relative humidity of 40%, 50%, 60% and 70%. The fertilizer samples were weighed once a day until the sample liquefied. Oil retention in ammonium nitrate-based fertilizers with high nitrogen content applies to granular fertilizers that do not contain oil-soluble substances. Oil retention is the amount of oil retained by a fertilizer, determined under specified conditions and expressed as a percentage by weight. The method involves fully immersing the test portion of the fertilizer in diesel oil for a specified period of time, then draining off the excess oil under specified conditions and measuring the weight gain of the test portion of the fertilizer. Before determining the oil retention in the fertilizer, the samples are subjected to two thermal cycles (50 °C and 25 °C). The product meets the test conditions if the oil retention does not exceed 4% by weight after application of the test procedure. In addition, a solution of 10 g of single-nutrient or multi-nutrient solid inorganic high nitrogen ammonium nitrate based fertilizer in 100 ml of water must have a pH of at least 4.5 [4].

**Results:** Figure 1 shows the effect of temperature cycles on the compression strength of fertilizer granules. The results show that the granules of the samples (AN 10% PS 10% MD, AN 20% PS) have the highest strength. However, even after 15 temperature cycles, the compression strength of the tested fertilizers is very high, compared to well-adopted industrial standards.



Figure 2 shows the change in sample mass as a function of relative humidity in climate chamber. It can be seen that the higher the polyhalite content in the sample, the lower the moisture absorption.



Fig.2. Sample mass change in relation to humidity.

Table 2 shows the oil retention results. In accordance with Regulation 2019/1009, the oil retention in the samples (AN 10% PS, AN 5% PS) after two temperature cycles slightly exceeds the permissible limits.

Table 2. Results of on recention.					
Sample	Oil retention, %				
AN 10% PS 10% MD	2.48				
AN 20% PS	2.64				
AN 10% PS	4.28				
AN 5% PS	4.94				

Table 2. Results of oil retention

Table 3 shows the pH results. All fertilizers received had a pH above 4.5.

	1
Sample	рН
AN 10% PS 10% MD	7.00
AN 20% PS	5.88
AN 10% PS	5.85
AN 5% PS	5.56

Table 3. Results of pH.

**Conclusions:** The granules are very durable and resistant to thermal shock. The product retains its shape and significant compression strength even after 15 thermal cycles. The strength of the granules increases with the content of polyhalite in the fertilizer. The oil retention after two temperature cycles does not exceed the acceptable value, i.e. 4% by weight for the samples AN 10% PS 10% MD and AN 20% PS. Obtained fertilizers comply with the pH requirements, with values higher than 4.5. Significant moisture absorption occurs between 60% and 70% relative humidity. An increase in the polyhalite content in the samples results in less weight gain, which is beneficial. Polyhalite has a positive effect on the hygroscopicity reduction of the tested fertilizers.

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# DETERMINTION OF VITAMIN C IN SAMPLES WITH VARIOUS MATRICES BY HPLC-DAD

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**Abstract:** Vitamin C plays a crucial role in the functioning of the human body. Most mammals are able to synthesize ascorbic acid on their own. However, human body do not have this ability, due to vitamin C deficiency must be supplemented in the diet. HPLC-DAD method was investigated in the ascorbic acid (AA) determination. The proper selection of chromatographic conditions in the HPLC-DAD method enabled the analysis of AA in real samples. Moreover, metaphosphoric acid (MPA) used in the sample preparation process proved to be an AA stabilizer.

**Introduction:** Vitamin C naturally occurs in two forms. Ascorbic acid is the reduced form of vitamin C, while the oxidized form is dihydroascorbic acid (DHA). Both forms retain the properties of vitamin C, and their interconversion occurs reversibly. It is worth to mention, that these compounds are highly unstable and under elevated temperature, presence of oxygen or metal ions (e.g. Fe, Cu) they relatively easily undergo irreversible changes [1,2]. The presence of vitamin C in living organisms is crucial for their proper functioning. It takes part in regulating the antioxidant activity in cells. It participates in the production of collagen and basic proteins throughout the body, which accelerates the healing of wounds and bone fusion. As a strong antioxidant, it supports the immune system and the body's detoxification processes. This vitamin also protects against neoplastic changes by inhibiting the formation of compounds with carcinogenic properties. It also plays an important role in the digestive system. By taking part in the metabolism of fats, cholesterol and bile acids, it protects the body against atherosclerosis, and by improving the absorption of iron, it prevents the development of anemia [3,4].

**Experimental:** The studies were carried out using two different chromatographic columns: Unisol C18 (4.6mm x 150mm),  $3\mu$ m and Luna C18 (4.6mm x 250mm),  $5\mu$ m. As a first step of the studies, analyses of ascorbic acid standard solutions (Sigma-Aldrich, USA) were performed for 5 variants. <u>Variant I</u>: column Unisol, injection 10 µl, mobile phase 0.01% H<sub>2</sub>SO<sub>4</sub> solvent 0.2% MPA, detection 240 nm; <u>Variant II</u>: column Unisol, injection 20 µl, mobile phase 0.01% H<sub>2</sub>SO<sub>4</sub> solvent 0.2% MPA, detection 240 nm; <u>Variant III</u>: column Luna, injection 10µl, mobile phase 0.2% MPA, detection 240 nm; <u>Variant III</u>: column Luna, injection 10µl, mobile phase 0.2% MPA (90%): MeOH (8%): ACN (2%), solvent 0.2% MPA (90%): MeOH (8%): ACN (2%), detection 240 nm; <u>Variant IV</u>: column Luna, injection 20 µl, mobile phase 0.2% MPA (90%): MeOH (8%): ACN (2%), solvent 0.2% MPA (90%): MeOH (8%): ACN (2%), detection 240 nm; <u>Variant V</u>: column Luna, injection 50 µl, mobile phase 0.2% MPA (90%): MeOH (8%): ACN (2%), solvent 0.2% MPA (90%): MeOH (8%): ACN (2%), detection 240 nm; <u>Variant V</u>: column Luna, injection 50 µl, mobile phase 0.2% MPA (90%): MeOH (8%): ACN (2%), solvent 0.2% MPA (90%): MeOH (8%): ACN (2%), detection 240 nm; <u>Variant V</u>: column Luna, injection 50 µl, mobile phase 0.2% MPA (90%): MeOH (8%): ACN (2%), solvent 0.2% MPA (90%): MeOH (8%): ACN (2%), detection 240 nm; <u>Variant V</u>: column Luna, injection 50 µl, mobile phase 0.2% MPA (90%): MeOH (8%): ACN (2%), detection 240 nm.

In the next stage of the research, using the previously prepared ascorbic acid calibration curve, identification and quantitative analysis of AA in real samples were carried out. Most of the analyses were conducted on blackcurrant fruit samples. Additionally, the developed methodology was used in the analysis of 100% red grapefruit juice (Tarczyn), human milk and vitamin C in drops for children (Juvit C). All presented results were obtained based on the analysis of samples in triplicate.

Blackcurrant berries were pre-ground using an IKA mill and then extracted with metaphosphoric acid solution using a homogenizer for 1 min. The remaining materials used in the tests did not require more precise processing. In order to check of the efficiency of the extraction process, analyses of blackcurrant fruits were carried out using extraction solutions with different concentrations of metaphosphoric acid (0.5 - mobile phase, 1, 3, 5 g MPA / 100 ml). In the presented study, the influence of the concentration of metaphosphoric acid solution on maintaining AA stability was investigated. For this purpose, the samples analyzed in the previous study were subjected to another analysis to verify how ascorbic acid content has changed over time (analysis after 24 h, 48 h and 72 h). After each analysis, samples were stored in a refrigerator in amber glass vessels.

**Results:** In the first stage of the research, studies have been taken to select proper chromatographic conditions for the method optimization. The change in injection volume, composition of the mobile phase and column parameters were investigated to obtain the best possible linear relationship. The greatest results, demonstrated in Table 1, were obtained for Variant V (y=163947x-14689,  $R^2=0.9995$ ). Due to for further studies the chromatographic conditions of the method corresponding to Variant V were selected.

Level	C theoretical [µg/ml]	Area [µV*sec]	$C$ according to the curve $[\mu g/ml]$	Accurancy [%]
	1.016	160128	1.066	105.0
1	1.016	162694	1.082	106.5
	1.016	161247	1.073	105.6
	5.08	839344	5.209	102.5
2	5.08	834181	5.178	101.9
	5.08	812004	5.042	99.3
	10.16	1619063	9.965	98.1
3	10.16	1604429	9.876	97.2
	10.16	1606956	9.891	97.4
	15.24	2515319	15.432	101.3
4	15.24	2515194	15.431	101.3
	15.24	2465177	15.126	99.3
5	20.32	3351771	20.534	101.1
	20.32	3299765	20.217	99.5
	20.32	3317688	20.326	100

Table 1. Analysis results of standard solutions for variant V.

In the conducted studies for blackcurrant samples, it was necessary to pre-grind the fresh fruit and then extracted them with MPA solution using a homogenizer. The best results were obtained using a two-stage sample preparation process (Table 2).

Tractad material	$AA \pm S.D. [mg/100g]$				
Tested material	homogenizer RSD grinde		grinder + homogenizer	RSD	
frozen fruit	$83.84\pm3.40$	4.05	$87.38\pm0.79$	0.90	
fresh fruit	$117.39\pm9.20$	7.83	$110.23\pm0.48$	0.43	

Table 2. AA content in blackcurrant fruit depending on the preparation method.

The change in ascorbic acid content over time in solutions with different MPA concentrations is presented in the Fig.1, while for comparison of the results of extraction of the same blackcurrant sample in water are presented in the Fig.2. In Fig.1 a gradual decrease in the AA content (from 110 to 106 mg/100 g) in tested material over time can be observed, regardless to the concentration of the metaphosphoric acid solution.



Fig.1. Decomposition of AA in solutions with different MPA concentrations.



Fig.2. Stability of AA in MPA solutions in relation to stability in water.

Based on Fig.2 a sharp decrease in AA content with the sample extraction only with water (without a stabilizing reagent) is observed over time. Using the information collected so far, the determination of ascorbic acid was carried out in different raw materials. The obtained results were compared with the literature data (Table 3). The data presented in the Table 3 confirmed the correctness of the determination method. However, low content of ascorbic acid in human milk may be caused by the fact that the analysis was carried out after 12 hours from the sample collecting. During this time,

AA may oxidize to DHA, and at this stage of the methodology development, the analysis of the total content of vitamin C was not included.

Raw material	Experin	nental data	Literature data		
Raw material	$AA \pm S.D.$	Unit	AA	Unit	
grapefruit juice (Tarczyn)	$15.45\pm0.10$	mg/100ml	10-18*	mg/100ml	
droplets (JUVIT C)	$96.89 \pm 1.68$	mg/ml	100**	mg/ml	
human milk	$1.86\pm\ 0.04$	mg/100ml	4.32***	mg/100ml	

Table 3. Determination of AA content in selected raw materials.

\*-according to the article [4]; \*\*-according to the declared content on the packaging; \*\*\*-according to the article [5];

**Conclusions:** The effect of the chromatographic parameters changes on the calibration curve quality was investigated. The most suitable conditions, that would allow obtaining the best possible linear relationship of the calibration curve (y=163947x-14689,  $R^2=0.9995$ ), were identified. The obtained results showed a significant role of the sample injection volume increasing up to 50 µl, which resulted in a much stronger and more stable ascorbic acid signal (Variant V). It is worth to mention that increasing the volume (from 10 to 50  $\mu$ l) of the sample injection into the column also improved the accuracy of the method  $(100\% \pm 10)$  in the entire range of the calibration curve (Table 1). The study of the sample preparation method yielded satisfactory results. Thanks to two- stage sample preparation procedure, it was possible to obtain results with good repeatability (Table 2). The study of the stability of ascorbic acid over time confirmed the role of metaphosphoric acid as an AA stabilizer. This is particularly noticeable in the comparison with the water extraction, where a significant decrease (about 38%) in the ascorbic acid content with time was observed (Fig.2). On the other hand, the increase in the concentration of the MPA solution does not affect the decomposition of AA over time (Fig.1). Finally, the results of the HPLC-DAD analysis of the selected materials confirmed the method accuracy in AA determination (Table 3).

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## SIMULTANEOUS DETERMINATION OF PHOSPHITE AND POLYPHOSPHATE IONS IN FERTILIZERS, FOOD AND PLANT PROTECTION PRODUCTS BY HIGH-PERFORMANCE ION CHROMATOGRAPHY

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**Abstract:** a comprehensive methodology of analytical procedures was developed, incorporating the separation and determination of the forms: ortho-, pyro-, tripoli-, trimeta- and the sum of the higher condensed polyphosphates(V) in selected samples of polyphosphoric acids and their salts with use for fertilizer (ammonium polyphosphate) or food (sodium hexametaphosphate) purposes, as well as phosphite ions (phosphonates) in plant protection products, by high-performance ion chromatography with suppressed conductivity detection (HPIC-SCD).

**Introduction:** The important role of phosphorus in supporting plant growth and yield is the basis for the production of many modern fertilizer formulations and plant protection products. The management of the phosphorus component in agriculture is a challenging task, frequently resulting in economic or ecological losses. In order to minimize these risks, numerous actions are being taken to increase the phosphorus uptake by plants. The fertilizing effect of phosphorus is only fulfilled when it is fully oxidized (H<sub>2</sub>PO<sub>4</sub><sup>-</sup>, HPO<sub>4</sub><sup>2-</sup> ). However, for fertilizer purposes, it is still commonly used in the form of phosphate(V), mainly orthophosphate. Due to their solid form, orthophosphates are poorly watersoluble substances with low migration in soil, so their availability to plants is limited. To ensure optimal nutrition of plants with phosphorus, especially during the initial cultivation phase, it is recommended to employ liquid fertilizers comprising polyphosphates with a low degree of polycondensation. These fertilizers do not exhibit the adverse characteristics of orthophosphates and ensure the availability of this element irrespective of the pH and temperature of the substrate [1]. Ammonium polyphosphates (APPs) are increasingly used for this purpose. These are mixtures of well-water-soluble ammonium salts of polyphosphoric acids. In addition to orthophosphates, they contain linear forms (pyro-, tripoly- and, in a small amount, higher condensed forms). Polyphosphoric acid salts other than APPs are added to food as stabilizers, acidity regulators, antioxidants and emulsifiers. These compounds are listed in Annex II to Regulation (EC) No 1333/2008 on food additives, which sets maximum acceptable concentration for phosphoric acid, ortho-, pyro-, tri- and higher condensed polyphosphates for different categories of raw and processed food products. Incompletely oxidized phosphorus forms phosphate(III) ions (phosphites, phosphonates)  $(H_2PO_3^-, HPO_3^{2-})$ , which, despite their better and faster assimilability, are not a source of phosphorus for plants and do not have nutritional properties. However, they are increasingly used as an additive and a supporting agent for plant protection products, e.g. fungicides [2]. This is due to the fact that an extended presence of phosphite ions within plant tissues engenders adverse conditions for the proliferation of fungi by postponing their maturation and spore formation until plant cells themselves have obtained defence mechanisms. Consequently, the utilization of phosphite-based solutions in organic production is prohibited, and maximum permissible levels have been specified for individual food products. Exceeding these limits may result in the cancellation of certification. Regrettably, fraudulent practices by fertiliser manufacturers have resulted in the placement on the market of preparations offering phosphite compounds as a source of assimilable phosphorus (phosphorus content is generally expressed as %  $P_2O_5$ ). A further complicating factor in this issue is the adulteration of plant protection products. The presence of phosphites is sufficient to qualify a product as a plant protection product; however, if phosphorus is present in another form, e.g. phosphate(V), it no longer does. Therefore, there are a variety of reasons why it is necessary to conduct research activities for the selective separation and determination of phosphate(III) and phosphate(V) ions with various degrees of polycondensation at different concentration levels. The development of modern instrumental separation techniques, particularly high-performance ion chromatography [3], has made this possible.

**Experimental:** dihydrogen NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, Ammonium orthophosphate sodium pyrophosphate  $Na_4P_2O_7 \times 10 H_2O_7$ sodium trimetaphosphate Na<sub>3</sub>O<sub>9</sub>P<sub>3</sub>. sodium tripolyphosphate  $Na_5O_{10}P_3$ , sodium phosphonate  $Na_2HPO_3 \times 5 H_2O$  and sodium hexametaphosphate  $(NaPO_3)_n$  standards were purchased from Sigma Aldrich and were of analytical grade. Test samples were selected to represent different matrices containing monophosphates and polyphosphates: PPA (polyphosphoric acid(V)),  $73 \div 82\%$  P<sub>2</sub>O<sub>5</sub>; (ammonium polyphosphate(V)),  $\geq 34.0\%$ POFAM-N  $P_2O_5;$ SHMP (sodium hexametaphosphate), 65÷70% P<sub>2</sub>O<sub>5</sub>; Delan<sup>®</sup> Pro, at least 50% K<sub>2</sub>HPO<sub>3</sub>; pork sausage homemade; sliced pork ham (Sokołów S.A.).  $0.1\div0.5 \text{ g} \pm 0.0002 \text{ g}$  of the fertilizer sample  $(10 \div 15 \text{ g} \pm 0.01 \text{ g} \text{ of the food sample})$  was weighed to and quantitatively transferred to a 100 ml volumetric flask, made up with deionized water and stirred until dissolved. Before chromatographic analysis, the solutions were filtered, preserved with 0.8 M KOH and stored at a reduced temperature (~4 °C). All separations were performed using the ion chromatography system (HPIC ICS-5000, Dionex) consisting of the ion detector set to conductivity mode, a self-regenerating anion suppressor (ADRS 600, 2 mm, Dionex) and the injection valve with a 500  $\mu$ L loop. The *high-capacity*, hydroxide-selective, anion-exchange chromatographic column designed for fast elution of multivalent anions - IonPac® AS16 (250 mm×2 mm) (Thermo Fisher Scientific) was utilized. For a fixed hydroxide gradient elution  $(55 \div 100 \div 55 \text{ mM KOH}, \text{time} = 20 \text{ min},$ flow rate = 0.25 mL/min) the order of anion elution was as follows: 1 - phosphonates (3.59 min), 2 - ortho (4.03 min), 3 - pyro (4.86 min), 4 - trimeta (5.33 min), 5 tripolyphosphate (6.03 min). The sum of the higher condensed polyphosphates(V) per  $P_2O_5(condens)$  was calculated:  $P_2O_5(condens) = P_2O_5(total) - (P_2O_5(ortho) + P_2O_5(pyro) + P_2O_5(trimeta))$  $+ P_2O_{5(tripolv)}$ .

The total phosphorus content expressed as  $P_2O_{5(total)}$  was determined by the well-known titration method. Prior to analysis, the sample containing condensed forms of phosphates had to be hydrolysed to orthophosphates.

**Results:** The method for the ion chromatographic separation and determination of: orthophosphates(V), polyphosphates(V) (pyro-, trimeta-, tripoly-) and phosphonates has been statistically evaluated in a validation process and significant method characteristics have been determined. Within the working concentration range  $(0.1 \div 10 \text{ m/L})$  the

calibration curves exhibited excellent linearity ( $r^2 \ge 0.9996$ ) and the statistical parameters of the regression lines (slope and offset) confirmed that the method was both sensitive and specific. Limit of quantification was in each case better than LOQ < 0.4 mg/L which was sufficient for the anions analysis present in the test samples (Table 1).

		Results of a	nion calibrations	
Analyte	Correlation coefficient $r^2$	Offset a	Slope b	$\frac{\text{LOQ [mg/L]}}{\frac{10 \cdot s_{y/x}}{b}}$
Phosponate HPO <sub>3</sub> <sup>2-</sup>	0.9999	$-0.0010 \pm 0.1484$ $t_a < t_{kryt}, a=0$	$1.6151 \pm 0.0244$ $t_b > t_{kryt}, \ b \neq 0$	0.33
Ortophospate PO <sub>4</sub> <sup>3-</sup>	0.9997	$0.0010 \pm 0.0870$ $t_a < t_{kryt}, a=0$	$1.3474 \pm 0.0228$ $t_b > t_{kryt}, \ b \neq 0$	0.27
Pyrophosphate P <sub>2</sub> O <sub>7</sub> <sup>4-</sup>	0.9996	$-0.0302 \pm 0.1265$ $t_a < t_{kryt}, \ a=0$	$1.6729 \pm 0.0332$ $t_b > t_{kryt}, \ b \neq 0$	0.32
$\begin{array}{c} Trimetaphosphate \\ P_{3}O_{9}{}^{3-} \end{array}$	0.9999	$-0.0124 \pm 0.0548$ $t_a < t_{kryt}, \ a=0$	$1.7582 \pm 0.0144$ $t_b > t_{kryt}, \ b \neq 0$	0.13
$\begin{array}{c} Tripolyphospate \\ P_{3}O_{10}{}^{5-} \end{array}$	0.9997	$-0.0209 \pm 0.0903$ $t_a < t_{kryt}, a=0$	$1.5202 \pm 0.0237$ $t_b > t_{kryt}, \ b \neq 0$	0.25

**Table 1.** Statistical evaluation of the linear function (y = bx + a) of anions measurement by HPIC-SCD.

 $s_{y/x}$  - residual standard deviation of the calibration curve.

Estimation of accuracy was performed by adding a known amount of analyte to the sample matrix, followed by determination of the percentage analyte recovery. The standard was added to the sample matrix prior to analysis at two concentration levels: 50% and 120% of the analyte level in the sample, respectively. The average recovery determinations obtained for the fortified samples were within the acceptable range of  $R = 90 \div 110\%$ , indicating that the method is accurate and not affected by systematic error. The small scatter of results as measured by a coefficient of variation was found to be within the accepted criteria (CV < 5%), signifying the method's precision. A comparison of the results obtained by two independent methods: HPIC-SCD (ICS 5000, Dionex) and ICP-OES (iCAP 7400+ MFC Duo, Thermo) - which differ completely in terms of sample preparation and type of detection, was carried out using the determination of dipotassium phosphonate K<sub>2</sub>HPO<sub>3</sub> in commercial plant protection product - Delan<sup>®</sup> Pro (Table 2). This confirmed the absence of statistically significant differences between the mean values of phosphonium anion concentrations obtained by the two methods. In the analysis of POFAM and PPA fertiliser samples, pyrophosphates appeared to be the predominant lower condensed polyphosphates. Trimetaphosphates were not detected, which is a direct result of the technology used to obtain these compounds, and the content of higher condensed forms was at the level of  $0.5 \div 2.5\%$  per P<sub>2</sub>O<sub>5</sub>. Analysis of sodium hexametaphosphate SHMP sample confirmed the presence of a very large number of phosphates with higher degrees of polycondensation, which could not be qualitatively and quantitatively interpreted due to the lack of suitable standards (with a total content of 67% per  $P_2O_5$ ). The total content of anionic phosphate forms in the samples of meat products did not exceed the EU limit of 5,000 mg  $P_2O_5/kg$  product. However, the commercial sausage, in contrast to the homemade product, contained the other forms, i.e. pyro-, trimeta- and tripoli-, indicating the use of polyphosphates as food additives (Table 3, Fig.1).

Test semple	HPIC-	SCD	ICP-OES			
Test sample	HPO <sub>3</sub> <sup>2-</sup> , %	K <sub>2</sub> HPO <sub>3</sub> , %	P <sub>total.</sub> , %	K <sub>2</sub> HPO <sub>3</sub> , %		
	28.6	56.5	11.1	56.6		
DELAN® DDO	27.8	54.9	10.9	55.6		
DELAN PRO	28.2	55.7	11.2	57.1		
	27.9	55.1	10.8	55.0		
Mean value, % $t(95\%,3) \times s(x)/\sqrt{n}$		55.5 ± 1.1		56.1 ± 1.5		
<i>t</i> -Student test for	Hypotheses of the test: $\mathbf{H}_0$ : $\boldsymbol{\mu}_1 = \boldsymbol{\mu}_2$ ; $\mathbf{H}_a$ : $\boldsymbol{\mu}_1 \neq \boldsymbol{\mu}_2$					
mean comparison of two series	$t_{kryt}(95\%, 6): 2.447 > t_{eksp}: 0.881$					

Table 2. Comparison of results of phosphonate determinations by two independent methods.



**Fig.1.** HPIC-SDC Chromatograms of: a - ammonium polyphosphate (APP), B – polyphosphoric acid (PPA), C – sodium hexametaphosphate (SHMP).

Test somple	% P <sub>2</sub> O <sub>5</sub>					
Test sample	orto	pyro	trimeta	tripoly	total	condens
APP POFAM-N	14.2	18.7	-	2.60	38	2.5
PPA	26.6	32.1	-	11.1	72	2.2
SHMP	0.13	0.39	2.14	0.80	70	67
PORK homemade	0.24	-	-	-	-	-
PORK Sokołów S.A.	0.34	0.037	0.0022	0.0087	-	-

Table 3. Content of condensed polyphosphates per % P<sub>2</sub>O<sub>5</sub> in tested samples.

**Conclusions:** The study confirmed the suitability of ion chromatography with suppressed conductivity detection for the determination of phosphate ions in polyphosphate(V) samples and phosphonium ions in phosphonates over a wide range of concentrations without matrix effects. The research methodology described in this document is a service offered to commercial manufacturers/users of polyphosphate fertiliser formulations, food additives and phosphonate plant protection products.

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# DEVELOPMENT OF ION CHROMATOGRAPHY -CONDUCTOMETRIC DETECTION ASSAY FOR THE DETERMINATION OF ORGANIC ACIDS IN PLANT MATERIAL

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**Abstract:** Studies about determination the content of organic acids: malic, tartaric and citric using ion chromatography with conductometric detection were conducted. The plant material used for the analyses consisted of water extracts obtained from blackcurrant seeds and freshly squeezed juices from cranberries and two types of dark grapes.

**Introduction:** Organic acids are natural compounds found in fruits and vegetables. Fruits contain mainly hydroxy acids, which, in addition to their acidity, give them a certain pleasant aftertaste and also lower the pH value [1]. These include primarily malic, tartaric and citric acids. Organic acids contained in food can also positively affect our health in certain ways [2]. The effect of extraction time on the content of organic acids was examined. For this purpose, four water extractions with different durations (1, 2, 3 and 18 h) were conducted for every of four batches (I-IV) of blackcurrant seeds from various manufacturers. The obtained results of content of acid ions were analyzed. For one batch, a two-hour extraction was also performed for different sample weights. Repeatability tests of the obtained results were performed for acid ions contained in cranberry and dark grape juice samples. The recovery of the acid standard for these samples was also examined.

**Experimental:** The ion chromatography technique with conductometric detection was used for the determinations. This is a type of high-performance liquid chromatography used for the separation and determination of anions and cations and other substances after their prior transformation into ionic form. Analyses were carried out using Dionex ICS-3000 ion chromatograph equipped with an eluent tank, a pump enabling gradient eluent flow, a suppressor to reduce the electrical conductivity of the eluent in relation to the conductivity of analyte ions, a dispenser, a pre-column (guard column), an analytical column, a conductometric detector and a computer recording. Analyses were carried out with isocratic eluent flow (Table 1).

I	0 0
Analytical column	AS11-HC 4x250mm,
+ guard column	AG11-HC 4x50mm
Eluent	NaOH 30 mM
Eluent flow rate	1.0 ml/min
Injection volume	25 µl
Column operating temperature	30 °C
Detection	Conductometric

 Table 1. Optimal chromatograph working conditions.

Each prepared solution was filtered through a 0.45  $\mu$ m filter before injection onto the column. Standard solutions were prepared by appropriate dilution of the stock standard containing 1000  $\mu$ g/ml of each acid in ionic form. Deionized water with a conductivity of 18.2 M $\Omega$  was used for dilution. Calibration was done by measuring the peak area of the ion originating from individual acids at different concentrations of the standard solution. Calibration curves were prepared in appropriate concentration ranges and were characterized by a linear course in the entire tested area and a correlation coefficient in the range of 0.9991-0.9996 depending on the acid being determined. Blackcurrant seed samples were prepared by: grinding the seeds, weighing the appropriate amount of sample, transferring to a test tube and adding water, shaking for a specified time, centrifuging, taking the appropriate amount of extract, transferring to a flask and topping up with water to the mark. Cranberry and dark grape fruit samples were prepared by: crushing the appropriate amount of fruit to obtain juice, centrifuging, taking the appropriate amount of the stract of the mark.

**Results:** In the tested samples of blackcurrant seed extracts, the presence of malic and citric acids and a small content of tartaric acid were found [3] (Fig.1).



Fig.1. Overlayed chromatograms of blackcurrant seed extract samples depending on extraction time (a) and sample weight (b).

For four batches of seeds from different producers, four water extractions were performed, differing in duration (1, 2, 3 and 18 h). In the analyzed samples of batch I, no increase in the content of malic acid was observed with the extension of extraction time, in contrast to citric acid, where such an increase occurred. In the case of batch II, an increase in content was observed for both acids with the time extension of the extraction process. Samples from batch III were also characterized by a significant increase in the content of acids with the increase of extraction time. In the case of batch IV, the extraction time above 2 h significantly increased the content of citric acid in the sample (the content of malic acid increased slightly) (Table 2).

Table 2. Organic acid ion content in analyzed samples of blackcurrant seed extract depending on extraction

		time.				
Batch		Ι	II	III	IV	
Time of extraction [h]	Acid	Acid content [mg/l]				
1	Malia	208.7	197.2	149.9	273.1	
2	Manc	217.2	208.5	201.3	292.9	

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3		217.2	248.2	208.9	300.9
18		211.9	251.8	204.4	308.1
1		422.6	518.1	367.4	410.9
2	Citric	462.9	524.5	462.4	483.4
3		471.7	602.9	491.9	492.6
18		512.3	632.6	665.8	612.8

The last batch of blackcurrant seed extract was also subjected to a 2-hour extraction with different sample weights (while maintaining the same volume of solution used for extraction) - the acid content increased proportionally to the increase in the sample weight (Table 3).

 Table 3. Organic acid ion content in analysed blackcurrant seed extract samples depending on sample weight (extraction time - 2h).

Batch	Sample weight [a]	Acid content [mg/l]			
	Sample weight [g]	Malic acid	Citric acid		
	5	119.9	193.5		
	10	238.8	382.0		
	15	379.2	580.9		

In the juice samples from two types of dark grapes differing in diameter (1.2 cm and 0.7 cm), three tested acids were identified: tartaric, malic and citric acids [2] (Fig.2), while in the cranberry juice the presence of malic and tartaric acids was found [3] (Fig.3). The average content of organic acid ions in the analyzed samples is presented in Table 4.



Fig.2. Chromatograms of dark grape juice sample: diameter 1.2 cm (c) and 0.7 cm (d).



Fig.3. Chromatogram of cranberry juice sample.

### Science and industry - challenges and opportunities

Tuble in Average content of organic dela fons in the unaryzed sumples.						
Sample	Zawartość [mg/l]					
	Malic acid	Citric acid				
Dark grape juice (diameter 1.2 cm)	749.2	6042.6	337.5			
Dark grape juice (diameter 0.7 cm)	10258.2	6787.1	195.9			
Cranberry juice	4084.5	464.6	-			

Table 4. Average content of organic acid ions in the analyzed samples.

In order to check the repeatability of the results for organic acid ions, 10 measurements were performed for a sample of juice squeezed from both types of dark grapes and for a juice sample obtained from cranberries. The results were characterized by very good repeatability. The values of standard deviations (SD) and

relative standard deviations (RSD) were low. A test of the recovery of the standard from the juice samples of both grapes and cranberries was performed. Measurements were performed for three juice samples and three juice samples with standard addition. The samples were prepared so that each of them contained the addition of the acid ion standard at three concentration levels. The amounts of the standard addition corresponded to half of the acid ion content in the sample, the total content and the double content. The recovery of the standard for grapes with a diameter of 1.2 cm was in the range of 95-102.5% (malic acid), 96-101.3% (tartaric acid), 94.2-102.3% (citric acid); for grapes with a diameter of 0.7 cm in the range of 101.2-102.4% (malic acid), 98.3-103.2% (tartaric acid), 96.1-104.1% (citric acid); and for cranberries in the range of 101.9-105.8% (malic acid).

**Conclusions:** Method for the determination of organic acids in plant material was presented. Ion chromatography with conductometric detection used in this study enabled identification and quantification of organic acids in black currant seeds water extract, cranberries and dark grapes juices. Determining the organic acid profile of juices can be used to detect adulteration (e.g. whether a more expensive juice has been replaced with a cheaper one)[4].

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### POLYETHOXYLATED DERIVATIVES AS COSMETIC RAW MATERIALS - SYNTHESIS, PROPERTIES AND FUNCTIONS IN COSMETICS

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**Abstract:** Polyethoxylated derivatives (PEG X) are the products of ethylene oxide polymerization and the corresponding raw materials (compounds or mixtures). PEG-type products are usually more hydrophilic than the original substrates; they are more soluble in water and have different surface activity. They are widely used in the production of cosmetics, including as surfactants, emulsifiers, cleansers, humectants, and skin conditioners. The study focuses on the ethoxylated derivatives of glycerides, waxes, silicones, and tweens.

**Introduction:** Growing consumer awareness and, consequently, expectations increase the demand for novel cosmetic raw materials that are safe for both humans and the environment and combine multiple functions [1, 2]. Lipophilic molecules can be modified through the introduction of oxyethylene groups to obtain more hydrophilic products. By modifying the degree of ethoxylation, various raw materials with different degrees of hydrophilicity, including water-soluble products, can be fabricated from the original substances. Ethoxylates are a mixture of homologs with polyoxyethylene chains of similar length and an average degree of ethoxylation. Most of them are surface-active compounds with multiple applications in various branches of the economy, including the cosmetics industry.

**Synthesis:** Ethylene oxide (oxirane) is used as the basic reagent in the ethoxylation process. It is a highly reactive compound due to the easy opening of the highly strained three-membered ring. Ring cleavage occurs with the participation of an acid or a base catalyst according to the  $S_N2$  reaction mechanism (Fig.1). The process proceeds in a similar manner in a basic environment.



Fig.1. Alkoxylation reaction with acid-catalyzed ethylene oxide ring cleavage according to the S<sub>N</sub>2 mechanism [3].

The lower reactivity of unprotonated epoxide is compensated by the higher basicity of the nucleophilic agent, such as alkoxide or phenoxide groups. The reaction produces a mixture of homologs with different degrees of ethoxylation. These variations can be attributed to differences in the reactivity of the raw material and homologs that participate in parallel reactions with ethylene oxide and the propagation of the oxyethylene chain. The applied catalyst has the greatest influence on the course of the reaction. The most commonly used catalysts are alkaline compounds such as NaOH or KOH and sodium or potassium alkoxides [1,3].

Ethoxylated glycerides: PEG-20 glyceryl triisostearate is one of the most widely studied ethoxylated glycerides. It is used as an emulsifier, dispersant, and solubilizer in the production of cosmetics and body care products. It is also applied in the formulations of leave-in cosmetics, such as creams and lotions, as well as rinse-off cleansing products such as face and body washes. Rinse-off and leave-in products contain up to 20% and 5% of PEG-20 glyceryl triisostearate, respectively. Due to its high molecular weight (greater than 1000 u), this compound is unlikely to bioaccumulate or cross biological membranes [4]. Its acute toxicity (LD<sub>50</sub>) exceeds 2000 mg/kg b.w. In addition, PEG-20 glyceryl triisostearate does not cause dermal irritation or sensitization, or the observed effects are negligible. This compound does not lead to eye irritation or mutagenicity. No sensitization was observed in the repeat insult patch test. Its high molecular weight and poor water solubility hinder its absorption by various routes, including through the skin. This raw material is probably removed from water in up to 90% because it is partitioned into solids during water treatment processes. There is no evidence to suggest that PEG-20 poses a health hazard or is toxic to consumers. PEG-35 hydrogenated castor oil is used as an o/w emulsifier, w/o co-emulsifier, solubilizer, and dispersant. It appears in skin creams, lotions, bath products, shower gels/body washes, liquid soaps, and face washes [2,4].

**Polyethoxylated waxes:** Polyoxyethylene derivatives of lanolin and its modified forms, such as hydrogenated lanolin, as well as beeswax are widely used as cosmetic raw materials. Lanolin analogs with 6 to 75 ethylene oxide molecules per each lanolin molecule are most commonly used as cosmetic ingredients. The solubility in water and in EtOH and the surface activity of these compounds, increase with increasing oxyethylene chain length. Completely water-soluble products are formed by attaching 75 ethylene oxide molecules to one lanolin molecule. Water-soluble lanolin can be effectively used in systems where unmodified raw materials cannot be applied because they are insoluble in water or EtOH. Unfortunately, the above leads to reduced skin substantivity. Ethoxylated lanolins have the properties of non-ionic surfactants and are most often used as emulsifiers in o/w emulsions and solubilizers, or as agents that mitigate the degreasing effect of detergents in washing products. Alkoxylated derivatives are used in creams, shampoos, hair conditioners, and shaving products. PEG-75 lanolin is the most commonly used alkoxylated lanolin derivative in commercial products. PEG-75 lanolin is an odorless liquid with a light-yellow color. This ingredient is used in cosmetics as an emollient that helps moisturize and soften the skin. PEG-75 lanolin is also an excellent surfactant that acts as an emulsifier. It is used in the production of personal care products because it nourishes and protects the skin by creating a protective barrier on its surface [5]. PEG-24 hydrogenated lanolin acts as a superfatting agent and a solubilizer. This raw material is used as an auxiliary agent in microemulsions and as a gelling agent in transparent gels. It is used in color cosmetics, creams, and balms [2].

PEG-8 beeswax is used in creams, lotions, color cosmetics, hair care products, mascaras, lipsticks, lip-balms, and sunscreen products. Polyglyceryl-3 beeswax is a hydrophilic derivative of natural beeswax that is used in cosmetic emulsions (o/w and w/o), oil-based gels, and decorative cosmetics. It improves oil gelling and oil retention capability [2]. **Tweens:** Tweens are polyethoxylated sorbitan esters that are commonly used as emulsifiers with a wide range of HLB values (Fig.2).



R is the alkyl group of a fatty acid, and (x+y+z) is the total number of ethylene oxide moles

Fig.2. Chemical structure of tweens.

Tweens are hydrophilic and are dispersible or soluble in water and solutions of electrolytes. Their solubility in aqueous solutions increases with the degree of ethoxylation. At a fixed degree of ethoxylation, aqueous solubility decreases with an increase in the number of ester groups as well as an increase in the molecular weight of the fatty acid (Table 1). The HLB of the corresponding Spans ranges from 4.3 to 8.6. Combinations of Spans and Tweens are used to prepare stable systems of o/w and w/o emulsions of various materials.

solu	itions in water a	and ons at 25 C (S – soluble, $FS$ –	partially sol	uble, O -	- ger formeu,	I = IIIsoluble).
No.	Product name	Chemical identity	HLB	Water	Mineral oil	Ethyl oleate
1.	Tween 20	PEG-20 sorbitan monolaurate	16.7	PS	Ι	Ι
2.	Tween 40	PEG-4 sorbitan monolaurate	15.6	S	Ι	Ι
3.	Tween 60	PEG-20 sorbitan monopalmitate	14.9	PS	Ι	Ι
4.	Tween 65	EG-20 sorbitan tristearate	10.5	G	PS	S
5.	Tween 80	EG-20 sorbitan monooleate	14.9	S	Ι	PS

**Table 1.** Characteristics of Tweens: chemical identity, HLB value, the solubility of 10% (w/w) Tween solutions in water and oils at  $25^{\circ}$ C (S – soluble; PS – partially soluble; G – gel formed; I – insoluble).

Tweens are extremely versatile as solubilizers for all types of fragrances and perfumes in air fresheners and other household products. They are used in a wide range of household consumer products as emulsifiers, solubilizers, wetting agents, and dispersants. Tweens are stable over a wide pH range and are electrolyte tolerant. They are considered to be biodegradable [6].

**Ethoxylated silicones:** Some silicones, including dimethicone (poly-dimethylsiloxane, PDMS), modified silicones such as amodimethicone, and silicone waxes (such as behenoxy dimethicone), are also subjected to the ethoxylation process. In addition to the ethoxy chain, these compounds may also contain a polyoxypropylene system. Their solubility and surface activity can be modified by changing the molecular weight of individual segments and the number of oxyethylene (PEG) and oxypropylene (PPG) groups per polysiloxane segment (Fig.3). The introduction of PEG groups improves the compound's solubility in water. An increased number of PPG groups improves solubility in non-polar media. This modification significantly alters the properties of the resulting products and significantly broadens the range of silicone applications.



Fig.3. Structural formula of comb type polyether-modified polysiloxanes [7].

Most of these raw materials belong to the group of non-ionic surfactants. They are capable of forming micelles in solutions, but they are characterized by greater surface activity than organic surfactants. Ethoxylated silicones are harmless to health even at high concentrations. Their safety has been confirmed by the Scientific Committee on Consumer Safety (opinions No. SCCS/1241/10 of 22 June 2010 and SCCS/1549/15 of 29 July 2016). PEG-3 dimethicone is a water-soluble surfactant that is used as a plasticizer and solubilizer in cosmetics, including hair, skin, and body care products. PEG-7 amodimethicone is used mainly in products that contain water, including rinse-out and leave-in conditioners, shampoos, hair styling products, liquid soaps, shower and bath gels. Dimethicone PEG-8 succinate reduces viscosity and acts as a foaming agent. It is used in creams, balms, stick deodorants, fragrance oils, and hair conditioners [7,8].

**Conclusions:** Ethoxylated derivatives are synthetic ingredients with numerous applications in the cosmetic industry, and research on selective and efficient catalysts for ethoxylation is being done to improve the composition of the resulting products. Due to the wide range of ethoxylates available on the market, numerous modifications, and the introduction of novel derivatives, their safety for consumers and the risks associated with the use of ethoxylates in cosmetics and other products should be continuously monitored.

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### HPLC-UV CHROMATOGRAPHIC ANALYSIS OF RETIGABINE ON A CORE-SHELL COLUMN

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Abstract: a simple and rapid high-performance liquid chromatographic method with ultraviolet detection was developed for the quantitative determination of retigabine in pharmaceutical preparations. Chromatographic separation was achieved on a C18 Kinetex column (Phenomenex,  $100 \times 2.1$  mm,  $2.6 \mu$ m.) using an acetonitrile–water mixture (38:62 v/v) with 0.01 mL of acetic acid as the mobile phase. Isocratic elution was conducted at a flow rate of 0.25 mL/min. The total duration of the chromatographic run was 7 min. Diazepam was used as an internal standard. The calibration curves were linear over a concentration range of  $0.05 - 3.0 \mu$ g/mL. The LOD and the LOQ were 0.02 µg/mL and 0.05 µg/mL, respectively. The relative standard deviations for intra- and interday precision were below 2%. The developed method can be used in quality control analyses of retigabine.

**Introduction:** Retigabine (RTG) belongs to a group of third-generation antiepileptic drugs. Epilepsy is one of the most prevalent neurological diseases worldwide, affecting nearly 1-2% of the population. More than 25 antiepileptic drugs (AEDs) have been approved for clinical use to date, and they are usually divided into three different generations. Third-generation AEDs have a novel chemical structures that introduces new mechanisms of action, promotes interactions with various therapeutic targets, and modulates different pathways of neuronal excitability that were not targeted by first- and second-generation AEDs [1]. The chemical structure of retigabine is depicted in Fig.1. Retigabine acts as a potassium channel opener by activating a specific group of voltagegated potassium channels in the brain. The drug enhances GABAergic transmission. Retigabine demonstrates linear pharmacokinetics after oral administration. It is rapidly absorbed, and its bioavailability is estimated at 60%. The maximum concentration of retigabine is achieved within 0.5 to 2 hours after administration. The degree of retigabine binding to plasma proteins is approximately 80%. Retigabine is metabolized in the liver, which leads to the formation of inactive N-glucuronides and an N-acylated metabolite with antiepileptic activity. The drug has a half-life of 6-10 hours. Retigabine and its metabolites are eliminated mainly in urine (approximately 84%), and small amounts are also excreted in feces (approximately 14%) [2,3].

To date, two HPLC methods [4,5] have been proposed for quantifying retigabine in pharmaceutical formulations. In the present article, we describe a novel, sensitive and fast high-performance liquid chromatography (HPLC) assay with ultraviolet (UV) detection for routine analysis of retigabine in laboratories.



Fig.1. Chemical structure of retigabine

**Experimental:** The experiment was conducted with the use of the Waters Alliance 2695 Separations Module HPLC system equipped with an autosampler, a Photodiode Array 2998 detector, a thermostat, and the Empower Pro v.2 computer program (Waters, USA). UV detection was performed at 225 nm. The separation parameters were optimized; the linearity of the method was evaluated, and the distribution of residuals was analyzed. The precision, accuracy, specificity, and flexibility of the method, as well as the limits of detection and quantification of the analyte were determined.

**Results:** The chromatographic analysis of retigabine was achieved with a Kinetex coreshell column packed with superficially porous particles. Unlike standard particles with a uniform structure across the diameter, superficially porous particles have a solid silica core with a porous outer shell. This structure of the column bed improves analytical parameters by increasing sensitivity, producing narrower and higher peaks, shortening analysis time, and considerably reducing solvents use. The HPLC assay was performed using a 2.6 µm Kinetex C18 column thermostatted at 22 °C as the stationary phase and an acetonitrile–water mixture (38:62 v/v) with 0.01 mL of acetic acid as the eluent at a flow rate of 0.25 mL/min. Detection was performed at a wavelength of 225 nm using a Photodiode Array (PDA) detector. Diazepam was used as the internal standard. These conditions supported the optimal separation of retigabine and diazepam with retention times of 4.31 min and 6.56 min, respectively. The analytes were characterized by satisfactory resolution (RS>6.5). They generated single, symmetrical peaks that were well separated from the solvent peak, with a baseline between the peaks. The asymmetry factor  $(A_s)$  was 1.34 for retigabine and 1.00 for diazepam. Diazepam was used as an internal standard in the developed chromatographic procedure to minimize the influence of fluctuations in the volume of dosed samples and to correct for the loss of the analyte due to matrix effects. The method was linear over a concentration range of 0.05 - $3.0 \,\mu$ g/mL. The correlation coefficient (r) reached 0.9994. The calibration curve presenting the peak area ratio of retigabine to diazepam was plotted against retigabine concentration using the following regression equation:  $y = 1.285290 (\pm 0.009099) x$  - $0.039366 \ (\pm 0.015348)$ . The assessment of the normality of residuals revealed that data distributions did not deviate from the assumed normal distribution. This result was confirmed by the Shapiro-Wilk test, p = 0.25442 > 0.05.

The limits of detection (LOD) and quantification (LOQ) of retigabine were determined at 0.02  $\mu$ g/mL and 0.05  $\mu$ g/mL, respectively. The intra- and interday precision of the HPLC system was assessed by measuring retigabine peak areas at three concentrations: 0.12  $\mu$ g/mL, 1.3  $\mu$ g/mL, and 2.8  $\mu$ g/mL, and a diazepam concentration of 0.3  $\mu$ g/mL. The coefficient of variation denoting intraday precision ranged from 0.29% for the highest to 0.74% for the lowest concentration of RTG (n=5), and it was determined at 0.93% for diazepam (n=5). The HPLC system was characterized by satisfactory interday precision with CV values of 0.42% to 1.23% for RTG (n=5) and 1.14% for diazepam (n=5). The system's satisfactory precision was confirmed by low CV values for the retention times of retigabine and diazepam. Intraday precision was 0.02% for RTG (n=15) and 0.05% for diazepam (n=15), and interday precision was 0.02% for RTG and 0.39% for diazepam (n=9). The specificity of the method and potential interference from other antiepileptic drugs were examined. A chromatogram of the AEDs analyzed together with retigabine and diazepam under the described chromatographic conditions is presented in Fig.2. The following drugs did not interfere with the assay: rufinamide, zonisamide, moclobemide, phenobarbital, oxcarbazepine, eslicarbazepine, phenytoin, and nitrazepam. Tiagabine was eluted as a separate, distinct peak, but it was not resolved to the baseline with the retigabine peak (peaks 6 and 7 in Fig.2).



Fig.2. Chromatogram of rufinamide (1), oxcarbazepine (2), eslicarbazepine (3), phenytoin (4), nitrazepam (5), retigabine (6), tiagabine (7), and diazepam (8); Kinetex C18 column, 2.6 μm; eluent: acetonitrile – water (38:62 v/v) with 0.01 mL of acetic acid; eluent flow rate: 0.25 mL/min

The influence of small, planned changes in the separation parameters of the developed HPLC method on the measurement results was analyzed. The flexibility of the method was tested by changing the content of the modifier and acetic acid in the eluent, the flow rate of the mobile phase, and detector wavelength. The coefficient of variation for the average peak areas obtained from nine injections (three for each modifier content in the range of 36-40%) was 5.27% for RTG and 2.23% for diazepam. The coefficients of variation for the peak areas of RTG and diazepam reached 6.55% and 5.36%, respectively, when the eluent flow rate was changed by  $\pm 8\%$  relative to the original conditions. The peak areas of RTG and diazepam were not affected when the content of acetic acid in the eluent was increased from 0.005 mL to 0.015 mL / 100 mL, and CV was below 2.6% for both analytes. Changes in detector wavelength in the range of 223–227 nm did not alter the retention times of the analyzed substances, and the CV of RTG and diazepam peak areas was 6.44% and 3.03%, respectively. The CV of RTG was twice higher due to a decrease in RTG peak area with increasing wavelength. The maximum absorbance of RTG was observed at  $\lambda max = 221$  nm, and it was 35% lower at 227 nm.

The developed and validated RP-HPLC method was used to quantify retigabine in Trobalt tablets. The active ingredient was extracted from Trobalt tablets with methanol. The analysis did not reveal any peaks associated with excipients. The content of retigabine per one Trobalt tablet was determined at 50.38845±0.597216 mg. No statistically significant differences were found between the average content of retigabine per tablet determined in the analysis and the value declared by the manufacturer. The analysis was performed using Student's t-test; t<sub>calc</sub>=1.593219 < t<sub>95%,5</sub>=2.571. The satisfactory precision of the quantitative analysis of retigabine in Trobalt tablets was confirmed by the values of standard deviation (0.243812) and CV (1.18%). The accuracy of the developed HPLC method, expressed as the recovery of retigabine

from fortified samples, was determined at  $100.89\pm0.48\%$ , with CV of 0.47% to  $101.60\pm2.16\%$ , CV=2.13%; n=3. The average recovery from fortified samples was  $101.2456\pm1.83\%$ , CV=1.81%; n=9.

**Conclusions:** The developed HPLC method for quantifying retigabine on a Kinetex C18 column is simple, selective, repeatable, and characterized by satisfactory precision and high recovery of analytes. The proposed method can be applied to control the quality of pharmaceutical preparations containing retigabine and to quantify retigabine in other matrices. The core-shell technology is characterized by high efficiency and short analysis time, and it reduces reagent consumption and minimizes the reagents' harmful effects on the environment. This technology can also be applied to identify retigabine in the presence of other antiepileptic drugs, including during polytherapy. Core-shell columns with a porous layer covering the rigid core do not generate excessive back pressures. As a result, these columns can be used in both classic HPLC and ultra-high performance liquid chromatography (UHPLC) systems. The proposed analytical procedure fills the gaps in the literature on retigabine.

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# WASTE BATTERIES AND ACCUMULATORS AS A SOURCE OF CRITICAL METALS

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**Abstract:** This paper presents the characteristics of the most common types of primary and secondary electrochemical cells, commonly used as batteries, in terms of their potential use as secondary raw material for the extraction of valuable critical metals.

**Introduction:** The development of modern civilisation is linked to the necessity of producing a wide range of materials and goods used in everyday life and modern technologies. Therefore, ensuring an adequate supply of raw materials is crucial for the development of the economy of every country. Reliable and unlimited access to certain raw materials is becoming an increasing problem in the EU and worldwide. To draw attention to this problem and to encourage individual countries to use materials rationally, the European Commission has drawn up a list of critical raw materials for the EU. Similar measures have also been taken in other countries, such as the United States and Japan. On 11 April 2024, Regulation (EU) 2024/1252 of the European Parliament and the Council on establishing a framework to ensure safe and sustainable supply of critical raw materials [1] was published, which, among others:

- established the European Critical Raw Materials Board, which consists of representatives from all member states and the Commission,

- a List of Strategic Raw Materials (Annex 1) was announced, including: bauxite/alumina/aluminium, bismuth, metallurgical-grade boron, cobalt, copper, gallium, germanium, lithium – in the standard required for batteries, magnesium metal, manganese – in the standard required for batteries, graphite – in the standard required for batteries, nickel – in the standard required for batteries, platinum group metals, rare earth metals for the production of permanent magnets (Nd, Pr, Tb, Dy, Gd, Sm and Ce), metallic silicon, metallic titanium and tungsten;

- the List of Critical Raw Materials (Appendix 2) has been announced, including: antimony, arsenic, bauxite/alumina/aluminium, barite, beryllium, bismuth, boron, cobalt, coking coal, copper, calcium fluoride, fluorspar, gallium, germanium, hafnium, helium, heavy rare earth metals, light rare earth metals, lithium, magnesium, manganese, graphite, nickel – in battery-grade quality, niobium, rock phosphate, phosphorus, platinum group metals, scandium, silicon, strontium, tantalum, titanium, tungsten and vanadium. Strategic importance is determined based on the importance of raw material in terms of ecological and digital transformation, as well as defence, aerospace applications, taking into account the following criteria: the number of strategic technologies that use the raw material as an input, the amount of raw material needed to produce the respective strategic technologies, and the expected global demand for the respective strategic technologies.

Two criteria are currently used to determine the 'criticality for the EU': economic importance and supply risk. Economic importance includes a detailed analysis of the use
of raw materials based on industrial applications. The assessment of supply risks takes into account the concentration of global primary raw material production in individual countries and their supply to the European Union, as well as the governance conditions in the raw material-supplying countries, and also considers environmental aspects, the share of recycling (i.e. secondary raw materials), the substitutability of original raw materials, and restrictions on trade in supplier countries. In many cases, the risks to supply associated with the concentration of production are compounded by the low level of substitution (the ability to replace some raw materials with others) and the low level of recycling. Both previous activities of the European Union and the regulations of the aforementioned Regulation indicate the necessity of recycling, especially of materials containing strategic or critical raw materials. In this respect, an interesting recyclable material is consumer batteries. Considering the level of production of electrochemical power sources and the prospects for their further development on the one hand and the diversity of their construction and chemical composition on the other [2,3], they should be considered a relatively easy source of many critical metals. As an example of the possibilities in this area, a brief description of the most popular consumer batteries is provided below. Among primary cells, zinc-carbon and alkaline zinc-manganese batteries are popular and widely used, which is why a detailed description of their construction can be found in many sources [2,3]. A zinc-carbon cell (Leclanche dry cell) consists of a zinc can (which serves as the cell container and anode), manganese(IV) oxide and an electrolyte consisting of ammonium chloride and/or zinc chloride dissolved in water. Carbon (acetylene black/graphite) is mixed with manganese(IV) oxide to improve conductivity and retain moisture, and then compressed under pressure to form a cylindrical or cuboid shape. This mass is called the positive electrode and is also called the black mixture, the depolariser or the cathode. A graphite rod is placed in the cathode mass, which serves as a current collector for the positive electrode. The cell is placed in a steel housing with a plastic separator. During the discharge (operation) of the cell, zinc is oxidised, and manganese dioxide is reduced. The simplified overall reaction of the cell can be written as:

### $Zn + 2 MnO_2 \rightarrow ZnO \cdot Mn_2O_3$

Although the alkaline zinc-manganese battery uses the same anode and cathode material, its design is completely different. The active materials in these cells are manganese dioxide, alkaline electrolyte, and powdered metallic zinc. The electrolyte is a concentrated KOH solution (usually 35 to 52%). Powdered zinc is used as an anode to provide a large surface area to reduce current density and to mix the solid and liquid phases more homogeneously. A current collector in the form of a steel or brass rod is placed in the moist mass. Components are placed in a steel casing. The reaction of the working cell can be written as:

### $2 \operatorname{Zn} + 3 \operatorname{MnO}_2 \rightarrow 2 \operatorname{ZnO} + \operatorname{Mn}_3 \operatorname{O}_4$

Examples of reversible cells (batteries) are nickel-cadmium, nickel-metal hydride, and lithium-ion batteries. The nickel-cadmium battery (NiCd or Ni-Cd) is a type of secondary battery in which the cathode is made of basic nickel(III) oxide NiO(OH) and the anode is made of metallic cadmium [2,3]. The electrolyte is an aqueous solution of potassium hydroxide. Ni-Cd batteries have a relatively high current output, but their disadvantage is the so-called memory effect when they are not fully discharged. Until recently, these batteries were widely used in various portable devices (including

modelling, amateur radio, and power tools). The reaction that takes place during the operation of such a cell can be represented by the following equation:

 $2\text{NiO(OH)} + \text{Cd} + 2\text{H}_2\text{O} \rightarrow 2\text{Ni(OH)}_2 + \text{Cd(OH)}_2$ 

The reaction is reversed during the charging process. Ni-Cd batteries have been withdrawn from general use in the EU due to the recognised toxicity of cadmium. However, they are still present in the waste battery stream due to their use in other regions of the world, e.g. Asian countries. In addition, these batteries are still used in industrial equipment.

The nickel metal hydride battery (NiMH or Ni-MH) was introduced as an alternative to the previously discussed Ni-Cd [3,4]. The chemical reaction on the positive electrode is similar to that of the nickel-cadmium cell, with nickel oxide hydroxide (NiOOH) being used in both cases. However, in the case of NiMH, a hydrogen-absorbing alloy is used as the negative electrode instead of cadmium. The general reaction that takes place in the NiMH cell during discharging can be represented by the following equation:

 $MH + NiO(OH) \rightarrow M + Ni(OH)_2$ 

where: M – metal or alloy that absorbs hydrogen; MH – metal hydride M.

During charge, the reaction proceeds in the reverse direction. Metal M in the negative electrode of the NiMH cell is usually an intermetallic compound. Many different compounds have been developed for this application, but those currently in use fall into two classes. The most common is  $AB_5$  where a is a mixture of rare earth metals: lanthanum, cerium, neodymium, praseodymium (in the form of a so-called misch metal), and B is nickel, cobalt, manganese, or aluminium. Some cells use higher capacity negative electrode materials based on compounds of the type  $AB_2$ , where a is titanium or vanadium and B is zirconium or nickel, modified with chromium, cobalt, iron, or manganese. The electrolyte is a potassium hydroxide solution. Lithium-ion cells are currently the most widely used and are produced in different sizes and shapes depending on their application [5,6]. A single cell generally consists of four main components: anode, cathode, separator, and electrolyte. The anode is a carbon material (usually graphite) that does not contain lithium, which must be sourced from the cathode material. Lithium intercalated compounds, e.g. LiCoO<sub>2</sub> layered oxide, are used for this purpose. The principle of a lithium-ion cell is based on the migration of lithium ions through an electrolyte between two electrodes separated by a separator during reversible charging and discharging processes. During the operation of a typical LiCoO<sub>2</sub>/graphite cell, the following general reaction takes place:

$$\text{LiC}_6 + \text{CoO}_2 \rightarrow \text{C}_6 + \text{LiCoO}_2$$

There are five types of lithium-ion batteries available on the market, depending on the cathode material used. Lithium cobalt oxide (LCO) batteries are commonly used in electronic devices such as laptops, mobile phones, and cameras. Lithium manganese oxide (LMO) batteries, on the other hand, which are cheaper and safer than LCO batteries, are most commonly used in hybrid vehicles, plug-in hybrid electric vehicles and electric vehicles. Lithium iron phosphate (LFP) batteries are also used in the latter. Due to their relatively high energy density, lithium nickel-cobalt-aluminium oxide (NCA) and lithium-nickel-cobalt-manganese oxide (NMC) batteries are being intensively developed.

**Conclusions:** In light of the current regulations on ensuring access to strategic and critical raw materials, it is recommended to treat waste batteries and accumulators as

valuable raw material, not only for the production of new batteries, but also for the separation and extraction of many critical metals (Table 1).

Battery type	Recoverable critical metals
Zinc-carbon	Mn/MnO <sub>2</sub>
Alkaline zinc-manganese	Mn/MnO <sub>2</sub>
Nickel-cadmium	Ni
Nickel-metal hydride	Ni, Co, La, Ce, Pr, Nd, Mn
Lithium-ion	Li, Ni, Co, Mn

Table 1. Summary of the recovery potential of critical metals from the most common batteries [7,8].

One of the basic conditions for achieving this goal is to ensure an adequate level of collection of used batteries, but also to develop existing and new effective technologies for the recovery of these metals.

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## CATALYTICALLY ACTIVE BIO-INSPIRED PLATINUM-BASED NANOMATERIALS: SEARCH FOR Pt SUPPORTS AND MATERIALS CHARACTERIZATION

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**Abstract:** The most favorable system for platinum nanoparticles (Pt-NPs) precipitation in bio-inspired conditions was identified using *Quillaja bark* saponin and ascorbic acid, with pH adjusted to approximately 7. DLS and AFM analysis confirmed that the nanoparticles remained within the nanometric range, with an average diameter of 31.35 nm and an average height of 6.38 nm. The obtained material was successfully separated by centrifugation, yielding a dry powder. Its catalytic properties were confirmed through the model reduction reaction of 4-nitrophenol to 4-aminophenol in the presence of sodium borohydride. The synthesized Pt-NPs will be further immobilized onto supports to enhance their stability and broaden their applications in catalytic degradation processes, such as pharmaceutical and gas decomposition.

Introduction: Platinum Group Metals (PGMs), including platinum, palladium, rhodium, iridium, ruthenium and osmium, play a critical role in various industrial applications due to their exceptional catalytic, electrical and physical properties [1]. Traditional methods of extracting and purifying PGMs from primary and secondary sources are often based on complex chemical techniques such as pyrometallurgy. However, these processes are energy intensive, environmentally hazardous and economically unsustainable. As a result, the search for environmentally friendly alternatives for PGM recovery and recycling has become increasingly important [2]. In [3], our research team proposed a hydrometallurgical process for the treatment of spent automotive converters (SACs) containing Pt, Pd and Rh. This process involves leaching, extraction and stripping steps, ultimately producing a PGM-rich leachate which is purified and used to enrich the stripping solution. This research focuses on developing an environmentally friendly approach to obtaining platinum nanoparticles (Pt-NPs) and dispersing them onto various supports for safer applications. To achieve this, Pt-NPs are precipitated in bio-friendly test systems using saponins – naturally derived reducing-stabilizing agents - and ascorbic acid (vitamin C) with pH regulation. These nanoparticles will be then deposited on supports such as titanium(IV) oxide ( $TiO_2$ ), silicon(IV) oxide ( $SiO_2$ ), perovskite and magnetic materials to enhance their stability and applicability.



Fig.1. Structure of a - TiO<sub>2</sub>, B - SiO<sub>2</sub>, C - perovskites.

 $TiO_2$ ,  $SiO_2$ , perovskites, and magnetic supports are promising materials for Pt-NPs immobilization (Fig.1).  $TiO_2$  is distinguished by its high chemical resistance, porous structure, and photocatalytic properties, which, in combination with Pt-NPs, effectively enhance the degradation of organic pollutants.  $SiO_2$ , although not catalytically active itself, has a large specific surface area, allowing better dispersion of Pt-NPs and increasing their efficiency. Perovskites, due to their crystalline structure, enable efficient nanoparticle dispersion, while their ability to transport charge improves the catalytic properties of the system. Magnetic supports allow easy separation of the catalyst from the solution, enabling its recovery and reuse.

**Experimental:** In order to identify the optimal system for the precipitation of Pt-NPs, ensuring the formation of nanoparticles with catalytic properties, different conditions were analyzed. The analyzed conditions included: precursor type, saponin type, saponin concentration, presence of ascorbic acid (AA), AA concentration, pH regulation. Samples were analyzed using atomic absorption spectroscopy (AAS) and UV-VIS spectroscopy (UV-VIS) to monitor changes in platinum concentration over time (1, 24, 48, 168 h for AAS and 1, 168 h for UV-VIS) relative to the initial precursor solution, which was platinum(IV) chloride. Particle size distribution was evaluated using Dynamic Light Scattering (DLS) and Atomic Force Microscopy (AFM).

**Results:** The most promising results for platinum were obtained in the system with the conditions listed in Table 1.

Precursor	Type of saponin	Concentration of saponin [g/dm <sup>3</sup> ]	Presence of AA	Concentration of AA [mol/dm <sup>3</sup> ]	pH regulation
PtCl <sub>4</sub>	Quillaja bark	1	AA	0.00625	Na <sub>2</sub> CO <sub>3</sub> (~7)

Table 1. Precipitation conditions for platinum in the most favorable system.

The particle size distribution graph obtained after DLS analysis is presented in Fig.2, while the AFM images are shown in Fig.3. The material obtained could be separated by using centrifugation to obtain a dry powder. This enabled the confirmation of its catalytic properties through a model reaction of 4-nitrophenol (4-NPh) reduction to 4-aminophenol (4-APh) in the presence of sodium borohydride (Fig.4). The band at 400 nm originates from the dissociated form of 4-NPh in an alkaline environment at pH 11. As the reduction reaction progresses over time, a decreasing peak from 4-NPh is

observed, along with an increasing peak at 260 nm, corresponding to the dissociated form of 4-APh being formed. This indicates the proper course of the reaction and confirms the catalytic properties of the platinum nanoparticles present in this reaction [4].



Fig.2. Percentage distribution of platinum particles by size after precipitation in most favorable system.



Fig.3. AFM images of platinum particles after precipitation in most favorable system.



Fig.4. UV-VIS spectrum showing changes occurring during the reduction reaction of 4-NPh to 4-APh with platinum nanoparticles.

**Conclusions:** After conducting numerous analyses, the most favorable system for Pt-NPs precipitation in bio-inspired conditions was identified. This system utilized *Quillaja bark* saponin and AA, with the pH adjusted to approximately 7. The DLS technique confirmed that the particle size distribution remained within the nanometric range. The average diameter of particles obtained in this system, analyzed using AFM, was 31.35 nm, while the average particle height was 6.38 nm. The model reaction of 4-NPh reduction to 4-APh confirmed the catalytic properties of the synthesized platinum-based material. In the next stages, this material will be deposited on various previously mentioned supports to enhance the safety of its use and improve its applicability in different catalytic degradation processes, such as the decomposition of pharmaceuticals or gases.

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# SYNTHESIS AND CHARACTERIZATION OF PLATINUM GROUP METAL NANOPARTICLES: a COMPARATIVE STUDY OF PRECIPITATION CONDITIONS

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**Abstract:** Platinum group metals (PGMs) are critical raw materials widely used in industry due to their unique catalytic and physicochemical properties. This study explores new precipitation conditions for platinum nanoparticles (Pt-NPs) using naturally derived saponins as reducing and stabilizing agents. The research examines the impact of different stabilizers (PVP, PEG, and coco-glucoside), stabilizer concentrations, and pH control on precipitation efficiency, particle size, and catalytic properties. Preliminary results indicate that pH regulation significantly enhances the Pt-NPs formation, with efficiencies reaching approximately 90% under optimized conditions. Further analysis using AAS, UV-VIS, DLS and AFM will provide deeper insight into the nanoparticle characteristics and their catalytic potential. These findings contribute to the development of environmentally friendly and efficient methods for PGM synthesis, aligning with circular economy principles.

Introduction: PGMs, such as platinum, palladium, and rhodium, play a crucial role in industry due to their unique catalytic, electrical, and physical properties. The high demand for these metals, combined with depleting natural sources, classifies them as critical raw materials. One of the largest secondary sources of PGMs is the automotive industry, where these metals are used in catalytic converters to neutralize volatile organic compounds and nitrogen oxides (NOx). From a circular economy and sustainable development perspective, it is essential to develop methods for the safe and environmentally responsible reuse of these materials. This study represents a preliminary studies of new conditions for the precipitation of PGM nanoparticles (mainly platinum), which represents a sustainable and renewable resource-based strategy in line with future technologies of bio-inspired systems [1,2]. In this study, saponins – naturally derived compounds belonging to the glycoside group were used. Due to their unique structure, they exhibit both reducing properties, enabling the precipitation of platinum particles, and stabilizing properties that control particle size. The reducing properties are attributed to the saccharide head (glycone), while the triterpenoid backbone (aglycone) is responsible for stabilization (Fig.1) [3].



Fig.1. Saponin structure.

To achieve the most homogeneous particle size distribution, three different compounds with potential stabilizing properties were used: polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), and coco-glucoside (Fig.2).



Fig.2. Structure of a – PVP, B – PEG, C – coco-glucoside.

**Experimental:** The aim of the study is to examine the impact of various precipitation conditions on precipitation efficiency, particle size, and catalytic properties of Pt-NPs. The research considers the following factors: type of precursor, type of stabilizer (PVP, PEG, coco-glucoside), stabilizer concentration, type of natural reducing-stabilizing compound (saponins, ascorbic acid), concentration of the natural compound, pH control (Na<sub>2</sub>CO<sub>3</sub>). Following the precipitation reactions of Pt-NPs, each sample was analyzed using atomic absorption spectrometry (AAS) to monitor changes in Pt(IV) concentration in the solutions at specified time intervals (1, 24, 48, 168 h) relative to the model solution of PtCl<sub>4</sub>. At 1 and 168 hours, the samples were also examined using a UV-VIS spectrophotometer to compare the spectrum and band intensity associated with platinum. The particle size distribution was determined using the DLS technique (Dynamic Light Scattering), and selected samples were further analyzed with an atomic force microscope (AFM) to confirm particle size. In the systems where Pt-NPs were obtained, a model

reaction of 4-nitrophenol reduction to 4-aminophenol in the presence of sodium borohydride was conducted to verify and confirm the catalytic properties of NPs.

**Results:** Preliminary analyses provided results on the efficiency of platinum precipitation in different test systems using PVP or PEG stabilizers. In these cases, *Quillaja bark* (Qb) saponin was used as a natural reducing-stabilizing compound. For systems with 1 g/dm<sup>3</sup> saponin and pH control, higher efficiency values were achieved using PVP compared to PEG. Thus, the NPs precipitation efficiency is greatly influenced by pH control. For example, in systems 1 and 4, with 1 g/dm<sup>3</sup> saponin and 0.0129 mol/dm<sup>3</sup> PVP, the efficiency increased from 53.43% without pH control to 89.88% with pH control. A higher stabilizer concentration does not significantly affect the precipitation efficiency; however, is believed to play a key role in the determining the particle size obtained in the individual test systems. The highest efficiency in the systems with PVP, reaching 94.30%, was achieved in system 2 with 1 g/dm<sup>3</sup> saponin and a higher stabilizer concentration of 5 g/dm<sup>3</sup> and a lower stabilizer concentration.

No.	Test system	Conc. of saponin [g/dm <sup>3</sup> ]	Stabilizer	Conc. of stabilizer [mol/dm <sup>3</sup> ]	pH regulation	Efficiency [%]
1	1	1	PVP	000645	pH	89.88
2	2	1	PVP	0.01290	pH	94.30
3	4	1	PVP	0.00645	-	53.43
4	5	1	PVP	0.01290	-	73.86
5	13	5	PVP	0.00645	pH	90.33
6	14	5	PVP	0.01290	pН	90.10
7	16	5	PVP	0.00645	-	65.60
8	17	5	PVP	0.01290	-	79.96
9	19	1	PEG	0.00645	pH	87.16
10	20	1	PEG	0.01290	pН	88.45
11	22	1	PEG	0.00645	-	58.30
12	23	1	PEG	0.01290	-	60.94
13	31	5	PEG	0.00645	pН	91.48
14	32	5	PEG	0.01290	pН	88.99
15	34	5	PEG	0.00645	-	71.19
16	35	5	PEG	0.01290	-	75.40

Table 1. Efficiency of Pt-NP precipitation in different test systems (precursor - PtCl<sub>4</sub>, saponin - Qb).

**Conclusions:** In summary, for the preliminary studies presented, the efficiency of the pH controlled systems fluctuates around 90% and pH control is a significant variable influencing the precipitation results. The study will also be extended to include UV-VIS analysis, followed by DLS and AMF to determine the particle size distribution, which is crucial to characterize the material obtained and test its catalytic properties.

Acknowledgements: This research was financed by the Ministry of Science and Higher Education, Poland (grant No. 0912/SBAD/2510) and by the 'PhDBoost' Program for doctoral students of the Doctoral School of Poznan University of Technology (in 2024) from the University's subsidy financed from the funds of Ministry of Science and Higher Education (grant No. 0912/SPHD/2522).

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# STUDY OF THE EFFECT OF METHANESULFONIC ACID ON METAL LEACHING FROM SPENT AUTOMOTIVE CATALYSTS

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Abstract: Methanesulfonic acid (MSA) was studied for the recovery of non-precious metals from spent automotive catalysts (SACs). A Box-Behnken design was applied for three factors at three levels, i.e., concentration of MSA, presence of  $H_2O_2$  and temperature. Atomic absorption spectroscopy was applied to determine the concentrations of non-precious metals in the aqueous solutions. The use of MSA as a leaching agent resulted in zinc, iron and magnesium leaching efficiency of 100, 95, and 100% from SACs, respectively. Box plots were made to evaluate the influence of the variables analysed. Analysis of the leaching efficiency obtained showed that leaching at higher temperature is more efficient than leaching at ambient temperature. The highest Fe ions and Mg(II) leaching efficiency were obtained at 77 °C. Irrespective of the leaching conditions used, MSA completely leached the Zn present in the SAC.

Introduction: The current environmental situation requires innovative technological solutions in the spirit of sustainable development, due to issues such as the depletion of primary raw materials, pollution and the huge amount of solid waste. For this reason, the recycling of resources and the creation of circular economy and technologies are strongly emphasised [1]. The automotive industry, a foundational sector of contemporary transportation, has witnessed unparalleled expansion over the past few decades. Based on current projections, the total vehicle stock is expected to increase from approximately 800 million in 2002 to over two billion units by 2030 [2]. This expansion has resulted in a concomitant increase in the utilization of catalytic converters. Management of SACs is necessary not only due to its negative environmental impact but also because it contains many elements of very high economic value. SACs depending on the type of vehicle engine are typically comprise platinum group metals (PGMs) such as platinum (3-3980 ppm), palladium (<0.5-11500 ppm), and rhodium (<0.1-2390 ppm) and also critical nonprecious metals [3]. It is worth noting that the concentration of valuable metals in SACs, is much higher than in their respective primary resources, i.e. metal ores [4]. In recent years, hydrometallurgical processes have been increasingly used to separate metals from SACs [5]. Although a wide range of leaching agents have been used, including organic acids such as oxalic acid and lactic acid [6], DESs [7] and inorganic acids such as HCl with 0.3 M copper(II) chloride as an oxidant [8], the search for new, effective, selective, and environmentally friendly metal ion leaching agents continues. A relatively recent development in the field of metal ion recovery is the use of methanesulfonic acid as a leaching agent. This acid, with its unique ability to form highly soluble salts/complexes, is considered to be a sustainable alternative to noxious or highly corrosive leaching solutions and responds to the demands of green chemistry [9]. The objective of this study is to investigate the effect of leaching conditions, such as MSA

concentration, the oxidant addition  $(H_2O_2)$ , and temperature, on the efficiency of metal leaching from SACs.

**Experimental:** The spent automotive catalytic converter (SAC) was supplied by a Polish waste treatment company. The SAC was ground and sieved before use. Only the fraction with particle size below  $63 \mu m$  was used for experiments (Fig.1).



**Fig.1.** The SAC applied for the studies: a) before grounding b) fraction with particle size  $\phi < 63 \mu m$ .

MSA and a 30% hydrogen peroxide solution (Avantor, Gliwice, Poland) were used as a leaching agent and an oxidant, respectively. A Box-Behnken design was applied for three factors at three levels, i.e., concentration of MSA, presence of  $H_2O_2$  and temperature expressed as A, B and C, respectively. A Box-Behnken design was generated using Statistica 13.3 software ("TIBC Statistica software"). The mass of metals obtained after leaching was the response value. The parameter level of the experiments carried out is shown in Table 1.

Tuble 101 detois and levels in the Bost Benniten design				
Variables Factors -		Factor levels		
		-1	0	1
А	Concentration of MSA [M]	1	2	3
В	Presence of H <sub>2</sub> O <sub>2</sub> [cm <sup>3</sup> ]	0	1	2
C	Temperature [°C]	23	50	77

Table 1. Factors and levels in the Box-Behnken design.

Leaching was carried out in a reactor of 50 cm<sup>3</sup>. Portions of 0.1 g of the ground material were placed in the reactor. The solid to liquid ratio (S/L) used was 1/50 g/cm<sup>3</sup>. 3 cm<sup>3</sup> of a MSA solution and 2 cm<sup>3</sup> of deionised water or in some cases mixtures of the deionised water and H<sub>2</sub>O<sub>2</sub> were used as leaching agents. The experiments were carried out at 23, 50, 77  $\pm$  2 °C for 3 h using a magnetic stirrer (Velp Scientifica, MULTI-HS 6 Digital, Italy). The leachates were separated from the leached material using a centrifuge (5804, Eppendorf, Germany) and diluted appropriately with 1.5% HNO<sub>3</sub> solution to determine the concentrations of metal ions. The leaching efficiency (L) of metals was calculated from Eq.(1) as the percentage of the amount of metal leached from 1 g of solid sample (m<sub>leached</sub>). The total amount of metals present in the SAC powder (m<sub>0</sub>) was determined after mineralisation of the SAC sample with aqua regia (ambient temperature, 72 h).

$$L = m_{\text{leached}} / m_{\text{Cu0}} \cdot 100\% \tag{1}$$

Atomic absorption spectroscopy (ContrAA300, Analytik Jena, Germany) was applied to determine the concentrations of metals in the aqueous solutions.

**Results:** The results show that the highest Fe ions and Mg(II) leaching efficiency, approximately 95 and 100%, respectively, were obtained at 77 °C with the addition of 2 cm<sup>3</sup> of H<sub>2</sub>O<sub>2</sub> and for the MSA concentration of 2 M. The lowest leaching efficiency of Fe ions and Mg(II), approximately 26 and 18%, respectively, were achieved for the 2 M concentration of MSA at 23 °C with no or 2 cm<sup>3</sup> of the oxidising agent added (Table 2).

<b>Table 2.</b> Maximum and minimum values of leaching efficiency of non-precious metals.				
L[%]	Metals	Presence of H <sub>2</sub> O <sub>2</sub> [cm <sup>3</sup> ]	Temperature [°C]	Concentration of MSA [M]
95	Fe	2	77	2
100	Mg	2	77	2
26	Fe	2	23	2
18	Mg	0	23	2

\* eaching efficiency of Fe ions [%] Leaching efficiency of Mg(II) [%] Temperature [°C] Temperature [°C] (a) **(b)** Leaching efficiency of Fe ions [%] eaching efficiency of Mg(II) [%] Presence of H2O2 [cm3] Presence of H2O2 [cm3] (c) (d) Leaching efficiency of Fe ions [%] Leaching efficiency of Mg(II) [%] Concentration of MSA [M] Concentration of MSA [M] (e) (**f**)



In all leaching conditions used, MSA completely leached the Zn present in the SAC. In order to provide a broader analysis of the results obtained, box plots were made to assess the influence of the variables analysed (MSA concentration,  $H_2O_2$  addition, and temperature) on the leaching efficiency. Analysis of the effect of the temperature used on the L values showed that the highest median efficiency and the widest range of results distribution was achieved for the 77 °C both for Fe ions and Mg(II), which may indicate a greater influence of other process parameters (Fig.2a,b). Analysis of the presence of  $H_2O_2$  on leaching efficiency has indicated that the highest median efficiency and the widest range of results distribution, both for Fe ions and Mg(II) were observed with the addition of 2 cm<sup>3</sup> of  $H_2O_2$ . The absence of the oxidant yields very similar results, highlighting no impact of  $H_2O_2$  on the L values (Fig.2c,d). The results obtained for the different MSA concentrations have indicated that acid concentrations may have no effect on leaching efficiency of Fe ions and Mg(II) (Fig.2e,f)).

**Conclusions:** The highest Fe ions and Mg(II) leaching efficiency was achieved at 77 °C. The presence of  $H_2O_2$  as an oxidising agent has no effect on improving the dissolution of metals in the methanesulfonic acid solution The use of higher concentrations of MSA also does not significantly affect the level of leaching efficiency. The greatest influence on the effectiveness of leaching is the temperature used. Conducting leaching at temperatures above 70 °C gives very good results, allowing complete leaching of Zn, Fe and Mg when the other parameters are properly selected.

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### GREEN METHANESULFONIC ACID FOR VALUABLE METALS RECOVERY FROM E-WASTE

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**Abstract:** Methanesulfonic acid (MSA) was studied for the recovery of copper from e-waste (PCB). A Box-Behnken design was applied for four factors at three levels, i.e., fraction, presence of H<sub>2</sub>O<sub>2</sub>, temperature and concentration of MSA. Atomic absorption spectroscopy was applied to determine the concentrations of Cu(II) in the aqueous solutions. The use of MSA as a leaching agent resulted in a copper leaching efficiency of 98% from PCBs. Box plots were made to evaluate the influence of the variables analysed. Analysis of the leaching efficiencies obtained showed that leaching in the presence of H<sub>2</sub>O<sub>2</sub>, is more efficient than leaching without this agent. The highest copper leaching efficiency was obtained from the F2 fraction, which contained particles with diameters ranging from 63 to 355  $\mu$ m.

**Introduction:** The current environmental situation requires innovative technological solutions in the spirit of sustainable development, due to issues such as the depletion of primary raw materials, pollution and the huge amount of solid waste. For this reason, the recycling of resources and the creation of circular economy and technologies are strongly emphasised [1]. Electronic waste (e-waste) is a significant global problem that poses a threat to human health and ecosystems [2]. Globally, over 50 Mt of e-waste are generated annually, with a predicted exponential increase to 74.7 Mt by 2030. Management of e-waste is necessary not only due to its negative environmental impact but also because it contains many elements of high economic value [3]. E-waste comprises various materials, including plastics, metals and glass, some of which can be systematically recovered, making it a valuable source of raw materials [4]. It is worth noting that the concentration of valuable metals in e-waste, such as copper, silver or gold, is much higher than in their respective primary resources, i.e. metal ores [5]. In recent years, hydrometallurgical processes have been increasingly used to separate metals from e-waste [6,7]. Although a wide range of leaching agents have been used, including organic acids such as oxalic acid and citric acid [8,9], choline chloride-based DESs with organic acids [10] and inorganic acids such as  $H_2SO_4$  with  $H_2O_2$  [11], the search for new, effective, selective, and environmentally friendly metal ion leaching agents continues. A relatively recent development in the field of metal ion recovery is the use of methanesulfonic acid as a leaching agent. This acid, with its unique ability to form highly soluble salts/complexes, is considered to b e a sustainable alternative to noxious or highly corrosive leaching solutions and responds to the demands of green chemistry [12]. The objective of this study is to investigate the effect of leaching conditions, such as methanesulfonic acid concentration, oxidant addition  $(H_2O_2)$ , and temperature, on the efficiency of metal leaching from three different fractions of the e-waste under investigation.

**Experimental:** The e-waste material used in this work, i.e. printed circuit boards (PCBs) coming from various discarded desktop computers, was provided by IOK Waste Management in Belgium. The ground material was divided into three fractions depending on the particle size ( $\phi$ ): F1  $\phi$  < 63  $\mu$ m, F2 63  $\mu$ m <  $\phi$  > 355  $\mu$ m, F3  $\phi$  > 355  $\mu$ m (Fig.1).



a) b) c) **Fig.1.** The e-waste material (PCBs) applied for the studies: a) F1  $\phi < 63 \mu m$ , b) F2 63  $\mu m < \phi < 355 \mu m$ , c) F3  $\phi > 355 \mu m$ .

Methanesulfonic acid (MSA) and a 30% hydrogen peroxide solution (Avantor, Gliwice, Poland) were used as a leaching agent and an oxidant, respectively. A Box-Behnken design was applied for four factors at three levels, i.e., fraction, presence of  $H_2O_2$ , temperature and concentration of MSA, and expressed as A, B, C and D, respectively. A Box-Behnken design was generated using Statistica 13.3 software ("TIBC Statistica software"). The mass of copper obtained after leaching was the response value. The parameter level of the experiments carried out is shown in Table 1.

Variablas	Factors	Factor levels		
variables	ranables Factors		0	1
А	Fraction	F1	F2	F3
В	Presence of H <sub>2</sub> O <sub>2</sub> , [cm <sup>3</sup> ]	0	1	2
С	Temperature [°C]	23	50	77
D	Concentration of MSA [M]	1	3	5

Table 1. Factors and levels in the Box-Behnken design.

Leaching was carried out in a reactor of 50 cm<sup>3</sup>. Portions of 0.25 g of the ground material of a specific fraction (F1, F2 or F3 PCBs) were placed in the reactor. The solid to liquid ratio (S/L) used was 1/100 g/cm<sup>3</sup>. 3 cm<sup>3</sup> of a MSA solution (1, 3, 5 M) and 2 cm<sup>3</sup> of deionised water or in some cases mixtures of the deionised water and H<sub>2</sub>O<sub>2</sub> were used as leaching agents. The experiments were carried out at 23, 50,  $77 \pm 2$  °C for 3 h using a magnetic stirrer (Velp Scientifica, MULTI-HS 6 Digital). The leachates were separated from the leached material using a centrifuge (5804, Eppendorf, Germany) and diluted appropriately with 1.5% HNO<sub>3</sub> solution to determine the concentrations of Cu(II) ions. The leaching efficiency (L) of Cu(II) was calculated from Eq. (1) as the percentage of the amount of metal leached from 1 g of solid sample (m<sub>Cu</sub>).

$$L = m_{Cu}/m_{Cu0} \ 100\%$$

(1)

The total amount of copper present in the PCB powder ( $m_{Cu0}$ ) was determined after mineralisation of the PCB sample with aqua regia (ambient temperature, 72 h). Atomic absorption spectroscopy (ContrAA300, Analytik Jena, Germany) was applied to determine the concentrations of Cu(II) in the aqueous solutions at the wavelength of 324.8 nm.

**Results:** The results show that the highest leaching efficiencies, approximately 98%, were obtained for the F2 at 23 or 50 °C with the addition of 1 cm<sup>3</sup> of  $H_2O_2$ . The lowest copper leaching efficiency, less than 3%, was achieved for the F3 with no or 1 cm<sup>3</sup> of the oxidising agent added (Table 2).

L [%]	Fraction	Presence of H <sub>2</sub> O <sub>2</sub> [cm <sup>3</sup> ]	Temperature [°C]	Concentration of MSA [M]
98.80	F2	1	23	1
97.95	F2	1	50	3
0	F3	0	50	3
2.97	F3	1	50	1

**Table 2.** Maximum and minimum copper leaching efficiency.





In order to provide a broader analysis of the results obtained, box plots were made to assess the influence of the variables analysed (fraction, MSA concentration, temperature, and  $H_2O_2$  addition) on the leaching efficiency. Analysis of the effect of the fraction used on the L values showed that the highest median efficiency and the widest range of results distribution was achieved for F2, which may indicate its greater susceptibility to variations in process conditions (Fig.2a). Analysis of the presence of  $H_2O_2$  on leaching efficiency indicated that the highest median efficiency and the widest range of results distribution was observed with the addition of 1 cm<sup>3</sup> of  $H_2O_2$ . The absence of the oxidant results in the lowest median and compact distribution, highlighting the key role of  $H_2O_2$ 

as an oxidising agent (Fig.2b). The best results were achieved at 23 °C, with the highest median and a wide range of results observed, suggesting favourable conditions for effective leaching. Leaching at temperatures above 50 °C does not improve the results (Fig.2c). Analysis of the effect of acid concentration showed that the highest median and the widest range of results were obtained using 1 M MSA, suggesting that this is the best of the acid concentrations used. The results obtained for the other concentrations have indicated that higher acid concentrations may have a negative effect on leaching efficiency (Fig.2d).

**Conclusions:** The highest leaching efficiency was achieved with the addition of 1 cm<sup>3</sup> of hydrogen peroxide. The presence of  $H_2O_2$  has a significant effect as an oxidising agent which aids in the dissolution of metals in the methanesulfonic acid solution. In the presence of  $H_2O_2$ , leaching is more efficient then leaching without this agent. The highest value of copper leaching efficiency was obtained from F2, which contained particles in the range from 63 to 355  $\mu$ m. The F3 fraction, which contained particles larger than 355  $\mu$ m, showed the lowest leaching efficiency, consistent with the expectation that larger particles require more time and energy for effective leaching.

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# URBAN MINING: A SUSTAINABLE SOLUTION FOR CRITICAL METAL SUPPLY

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**Abstract:** With the rapid advancement of technology and the transition to green energy, the demand for critical metals such as lithium, cobalt, nickel, copper, platinum group metals (PGMs), and rare earth elements (REE) has increased. These materials are essential for the production of batteries, wind turbines, solar panels and various electronic devices. However, the EU's heavy reliance on imports, particularly from countries such as China, poses significant risks to supply chain stability and economic security. In response, the EU introduced the Critical Raw Materials Act (CRMA) in 2024, which aims to strengthen domestic capacity and promote sustainable practices in the supply of raw materials.

**Introduction:** Urban mining (UM) is a concept that appeared in scientific terminology as early as the 1980s, and now is one of the fastest growing trends worldwide, mainly because it involves the recovery of raw materials from areas that were previously not considered for recycling. It concerns the recycling of secondary raw materials, the recovery of valuable and critical raw materials from urban waste (called 'mines'), particularly vehicles, electronic equipment (e-waste), installations, buildings. UM approach is in line with the circular economy and sustainable development goals (especially SDG9 and SDG12) by turning waste into valuable resources, thereby reducing reliance on primary mining and mitigating environmental impacts [1]. Moreover, the strategy of urban mining addresses the growing demand for critical metals that are essential for modern technologies and strategic sectors including clean energy technologies and the digital, defence and aerospace industries. As the European Union, by Critical Raw Materials Act of 2024 [2], intensifies its efforts to ensure a sustainable and resilient supply of these materials, urban mining offers a viable solution to reduce import dependency (e.g. currently 100% of REEs used for permanent magnets are refined in China, 63% of the world's cobalt, used in batteries, is extracted in the Democratic Republic of Congo [3]). The objective of this article is to examine the challenges and opportunities of urban mining. Key Aspects of Urban Mining: UM refers to the recovering valuable materials from urban waste streams, including electronic waste (e-waste), end-of-life vehicles, industrial scraps, or batteries (Table 1). Unlike traditional mining, which involves extracting metals from natural minerals, urban mining focuses on recycling metals from products that have reached the end of their life cycle. The key aspects of this approach are as follows [4,5]: (a) resource recovery: extracting metals essential for modern technologies and renewable energy solutions such as REEs, lithium, cobalt, and indium from e-waste; (b) environmental benefits: reducing the environmental footprint associated with traditional mining, often resource-intensive and environmentally damaging; (c) economic value: maximizing the economic value of waste streams by recovering metals that are otherwise lost. Examples of metals mining from various secondary materials with a wide range of techniques including mechanical separation, pyrometallurgical (smelting, refining) and hydrometallurgical (leaching, liquid-liquid extraction) methods are shown in Table 1.

Type of secondary material	Metals mined	Ref.
Electric cables, e-waste including printed circuit boards, mobile phones, computers	Cu, Au, Ag	[6-8]
Magnet swarf and rejected magnets, permanent NdFeB magnets, compact fluorescent lamps, nickel metal hydride batteries	REE (Nd, Dy, Eu, Tb, Y)	[9]
Spent automotive converters	PGMs	[10]
Buildings, constructions	Fe, Cu, Al	[11]

Table 1. Metals recovered by urban mining from secondary resources.

Such an approach to metal production which recognises the value of residual materials for new processing stages and impact mitigation, is in line with the Waste-to-Resources (WtR) concept and the assumptions of the circular economy model [8]. A comparison of traditional and urban mining approaches is shown in Fig.1.



Fig.1. Comparison of traditional and urban mining approaches.

*Example of e-waste:* The amount of recyclable metal depends on living standards and population, as the spent products accumulate in certain areas (cities) and therefore are called 'urban mines'. It is important to indicate that as long as metal recovery from natural minerals is cheaper than recycling technologies, and natural mining resources are still available, the development of urban mines is not profitable. However, many secondary resources have higher concentrations of critical metals than natural minerals, making them an abundant and readily available resource.

The amount of e-waste generated worldwide is rising five times faster than documented e-waste recycling. It is reported that in 2022 62 mln t of e-waste was generated, and until 2030 amount of e-waste can reach even 82 mln t (Fig.2) [12].



Fig.2. Change in the global amount of e-waste [12].

There are a number of facilities working on metal recovery from secondary resources. These include Umicore's high-tech smelting and refining processes, which recover more than 95% of the copper from e-waste, or Singapore's TES-AMM, which extracts copper from waste printed circuit boards using an environmentally friendly hydrometallurgical process. An interesting example is the Tokyo 2020 Olympic medal project in Japan, which recovered metals including copper, silver and gold from over 78985 tons of old smartphones, laptops and other e-waste.

Summary – Challenges and Opportunities: Despite its potential, urban mining faces several challenges, including the need for innovative and advanced technologies to liberate and separate critical metals from complex waste matrices, as well as the creation of supportive regulations and government incentives to encourage investment in urban mining initiatives and the development of a single market for recycled materials. The economics of urban mining can be a barrier, especially when competing with primary mining, as market prices often do not reflect the environmental and social benefits of recycling. Effective policies are therefore needed to promote recycling and support urban miners. The establishment of effective e-waste collection and sorting systems is also essential to ensure a steady supply of materials for recycling processes. Conversely, the opportunities offered by UM include significant support for the EU's supply chain resilience, environmental sustainability and economic growth. By diversifying sources of critical metals, urban mining enhances the EU's ability to withstand global supply disruptions. In addition, reducing reliance on traditional mining mitigates environmental degradation and promotes the conservation of natural resources. Finally, investment in UM technologies and infrastructure can stimulate economic growth and create employment opportunities in the EU.

**Conclusions:** In conclusion, urban mining offers a sustainable solution for critical metal supply by providing environmental benefits, contributing to the circular economy, and offering economic advantages. However, several solutions are needed to improve the feasibility of urban mining, such as the development of new technologies for efficient

metal recovery and the implementation of supportive regulations that encourage the adoption of urban mining practices. This means, for example, setting standards for product design to facilitate recycling. It is also important to increase public awareness of the benefits of urban mining and to encourage people to participate in recycling programmes to improve collection rates and resource recovery.

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### PRECIPITATION OF ACTIVE PLATINUM NANOPARTICLES

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**Abstract:** The work focuses on the precipitation of Pt(IV) from acidic solutions using sodium borohydride (NaBH<sub>4</sub>, strong reducer) and ascorbic acid ( $C_6H_8O_6$ , weak reducer). Polyvinylpyrrolidone was used as a stabilizer of nanoparticles. The Pt precipitation efficiency was ~95 and ~85% for NaBH<sub>4</sub> and  $C_6H_8O_6$ , respectively. The size of the particles obtained was determined using AFM.

**Introduction:** After rhodium and iridium, platinum is the third most of the platinum group metals (PGMs) at 988 \$/troy oz [1]. Due to its unique properties (catalytic properties, chemical resistance), the demand for Pt constantly exceeds the supply. Platinum is widely used in the automotive industry, jewellery production, investment (coins, bars), chemical and petrochemical industry, electrical and medical applications) [2,3]. Platinum demand by sector in 2022 is presented in Fig.1.



Fig.1. Platinum demand by sector in 2022 [2,3].

Nanotechnology deals with the production of nanomaterials and the study of their properties. Nanomaterials are a group of materials with at least one dimension not exceeding 100 nm. Due to their very small size, they have different physical and chemical properties than their micro counterparts [4]. The application of platinum nanoparticles (Pt-NPs) is still being intensively studied. Pt-NPs can be used in electrochemistry (hydrogen or methanol fuel cell, electrochemical oxidation of ethanol), electronics (catalysts, sensors), medicine (antibacterial, antifungal, anticancer properties, radiotherapy, drug transport) [5].

The aim of this work was to investigate the effect of the type of reducer and the amount of PVP on the precipitation of platinum and to investigate the catalytic properties of the obtained Pt-NPs.

**Experimental:** Pt-NPs were precipitated by reducing platinum(IV) chloride to pure metal using two reducers: sodium borohydride (NaBH<sub>4</sub>) and ascorbic acid (C<sub>6</sub>H<sub>8</sub>O<sub>6</sub>). Polyvinylpyrrolidone (PVP) was used as a size and shape stabilizer for nanoparticles. Each experiment was performed three times. The reduction reaction of 4-nitrophenol (4-NPh) to 4-aminophenol (4-APh) was carried out at pH ~11 in the presence of the obtained catalyst (Pt-NPs) using NaBH<sub>4</sub>.Atomic absorption spectrometer (ContrAA 300, Analytik Jena, Germany) was used to determine the concentration of Pt(IV) (266.0 nm) in the solution before and after precipitation. A UV-VIS spectrophotometer (Specord 40, Analytik Jena, Jena, Germany) was used to study the course of the catalytic reduction of 4-NPh to 4-APh. An AFM microscope (NX10, Park Systems, Mannheim, Germany) was used to determine the size of the obtained Pt-NPs.

**Results:** The influence of the type of reducer and PVP concentration on the Pt(IV) precipitation efficiency was investigated. The results are presented in Fig.2.



Fig.2. Pt precipitation efficiency depending on the amount of the stabilizer (PVP) and the type of reducer: ( $\blacksquare$ ) C<sub>6</sub>H<sub>8</sub>O<sub>6</sub> or ( $\blacksquare$ ) NaBH<sub>4</sub>.

The efficiency of Pt precipitation with  $C_6H_8O_6$  was ~85%, and with NaBH<sub>4</sub> - ~95%. The amount of the PVP added has no effect on Pt precipitation with either reducing agent. However, the more PVP in the solution, the more difficult it is to separate the precipitate from the liquid phase.

To confirm the catalytic properties of the obtained material, catalytic reduction of 4-NPh to 4-APh was performed. The results are shown in Fig.3. The maximum at 400 nm corresponds to the dissociated form of 4-NPh, which occurs at alkaline pH. During the first 5 min, a decrease in absorbance was observed for 4-NPh and the appearance of a small maximum at 300 nm, which is characteristic of 4-APh in its undissociated form. The decrease in absorbance of the substrate, without the appearance of a characteristic

maximum for the product, may indicate the formation of intermediates or active complexes. After 10 min, the equilibrium state was probably established and a maximum at 260 nm can be observed, that corresponding to the dissociated form of 4-APh.



Fig.3. UV-VIS spectra of 4-NPh before reaction and after 5, 10, 15, 20 and 30 min and spectrum of pure 4-APh (pH 11).

The conversion of 4-NPh to 4-APh after 30 min of this reaction was 56%. The average size of the obtained Pt particles was determined by AFM to be 90-120 nm (reducer  $C_6H_8O_6$ ) and 80-100 nm (reducer NaBH<sub>4</sub>).

**Conclusions:** The Pt precipitation efficiency with both reducers was high, ~85 and ~95% using  $C_6H_8O_6$  and NaBH<sub>4</sub>, respectively. The catalytic properties of the obtained material were proven by 56% conversion of 4-NPh to 4-APh. Pt particle of nanometric size were obtained by precipitation of Pt with NaBH<sub>4</sub> in the presence of PVP.

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# SOLVENT EXTRACTION STRATEGIES FOR LITHIUM AND MAGNESIUM RECOVERY FROM DESALINATION CONCENTRATES: ASSESSMENT OF DEEP EUTECTIC SOLVENT POTENTIAL

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**Abstract:** The desalination industry produces significant quantities of concentrates, which contain valuable elements such as lithium and magnesium. These elements are critical and strategic raw materials within the European Union. This study evaluates the feasibility of the OA+TOPO mixture as a deep eutectic solvent (DES) for cation recovery, comparing it with DBM+TOPO and FDOD+TOPO mixtures. The results obtained confirm that OA+TOPO forms a eutectic mixture with an optimal 1:1 molar ratio. While DBM+TOPO and FDOD+TOPO effectively extract lithium, OA+TOPO demonstrates higher selectivity for magnesium, extracting up to 50% of its content. These findings suggest that selective cation recovery is a viable prospect, which would contribute to more sustainable and efficient resource extraction processes.

**Introduction:** The desalination industry plays a crucial role in the provision of drinking and industrial water supply. However, the management of the large volumes of concentrates generated remains an environmental and economic challenge [1]. In this context, a promising strategy that is emerging is the transformation of this waste into a source of valuable raw materials. Among the elements present in desalination concentrates, lithium and magnesium are of particular note due to their expanding industrial demand. Lithium is vital for battery technologies and the transition to renewable energy and electric mobility, while magnesium is essential in lightweight allovs, aerospace applications, and advanced manufacturing. The mounting demand, for these elements, coupled with inherent supply risks, has prompted their inclusion in the European Commission's Critical and Strategic Raw Materials list [2]. Solvent extraction has been shown to be an efficient and selective technique for cation recovery from complex saline matrices [3]. Its application in seawater reverse osmosis (SWRO) concentrates offers new routes for critical material recovery while improving the sustainability and profitability of desalination, taking advantage of their higher salt content compared to seawater. Prior studies have identified synergistic extractant mixtures, such as dibenzoylmethane (DBM) and heptafluorodimethyloctanedione (FDOD) combined with trioctylphosphine oxide (TOPO), as promising candidates for lithium extraction, yielding satisfactory results [3-5]. Nevertheless, the extensive utilisation of these extractants could present an environmental problem with their residues. Consequently, this highlights the potential value of comparing alternative lowtoxicity systems such as Deep Eutectic Solvents (DES). In this regard, the research

group at the Institute of Chemical Technology and Engineering in Poznan has a specialism in the development of sustainable extractants, specifically DES [6]. Concurrently, the research group at the University of Cantabria has been dedicated to the valorisation of waste streams from the water industry, specifically desalination concentrates, through the application of various advanced technologies [7]. The current study is built on these research lines, exploring the potential of a new oleic acid (OA) and TOPO extractant mixture, which may offer sustainability benefits due to the natural origin of OA. The potential classification of this mixture as a DES is also assessed. A comparative analysis of these extractants is presented to evaluate their effectiveness in recovering valuable cations from desalination concentrates, with a view to enhancing the efficiency and sustainability of resource recovery processes.

**Experimental:** Synthesis of DESs was performed as follows: the mixtures of DBM/FDOD/OA and TOPO with different molar ratios ( $M_{DBM/FDOD/OA:TOPO} = 0:1, 1:3, 1:2, 1:1.5, 1:1, 1.5:1, 2:1, 2.5:1, 3:1, 1:0$ ) were mixed in a glass bottle with a cover, heated at 60 °C in a water bath and stirred until clear and transparent solvents were obtained. Extractants characterisation was done using the differential scanning calorimeter (DSC1 Instrument, Mettler-Toledo, Switzerland) and was utilised to ascertain the melting temperatures of the formed DESs. The samples positioned within aluminium hermetic crucibles, cooled to -70 °C and heated to 150 °C at a temperature rate of 10 °C/min. Aqueous solutions simulating SWRO concentrates were prepared by dissolving the required amounts of LiCl (2 mg·L<sup>-1</sup> of Li<sup>+</sup>) and MgCl<sub>2</sub> (1700 mg·L<sup>-1</sup> of Mg<sup>2+</sup>) in ultrapure water. Extractant mixtures of DBM/FDOD/OA and TOPO were diluted in kerosene, and mixed with the aqueous phase in glass bottles at a 1:1 volume ratio and stirred until equilibrium. Cation concentrations in the aqueous phase were measured using Atomic Absorption Spectroscopy (AAS).

**Results:** In order to assess the eutectic behaviour, it is first necessary to ascertain the capability of forming a single phase without the addition of a solvent, with a melting point (m.p.) lower than that of its individual components. After preparing different extractant mixtures at the molar ratios shown in the experimental section, a visual analysis was conducted. As demonstrated in Fig.1, illustrative examples of the ratios of the extractant mixtures are presented. While DBM+TOPO and FDOD+TOPO did not remain stable in liquid form below their melting points, the OA+TOPO mixtures formed a crystalline liquid at temperatures lower than the m.p. of the individual components, prompting further analysis via DSC.



Fig.1. Photographs of FDOD, DBM and OA mixed with TOPO in 1:1 ratios, after heating and stirring.

As demonstrated in Fig.2, the phase diagram of the OA+TOPO mixture is presented, with each melting temperature point having been obtained from the DSC analysis. As indicated, the m.p. of the mixture is lower than those of the individual components, thus confirming its classification as a eutectic mixture. The eutectic point, which corresponds to the lowest melting temperature, occurs at a value of -20.37 °C at a 1:1 molar ratio, thereby enabling a 50% reduction in TOPO usage.



Fig.2. Phase diagram of OA+TOPO mixtures with different MOA:TOPO.

Following the determination of the optimal molar ratio of the OA+TOPO mixture, a series of experiments were conducted with the objective of extracting the cations of principal interest that are present in the SWRO concentrates. The ensuing experimental results are presented in Fig.3.



Fig.3. Percentage extraction of a) Li<sup>+</sup> and b) Mg<sup>2+</sup>, for DBM+TOPO, FDOD+TOPO and OA+TOPO extractant systems at 0.014 M and 0.06 M concentrations.

As demonstrated in Fig.3, a comparison is presented of the results obtained for the OA+TOPO mixture and the DBM+TOPO and FDOD+TOPO mixtures for the extraction of Li<sup>+</sup> and Mg<sup>2+</sup>, individually. In accordance with the findings based on previous studies [4], the total extractant concentrations were established at 0.014 and 0.06 M. As illustrated in Fig.3a, in the systems comprising the  $\beta$ -diketones FDOD and DBM, the entirety of lithium present in the concentrates is extracted. Conversely, when exchanging  $\beta$ -diketone for oleic acid, it can be observed that this mixture is not lithium selective. Indeed, the extraction of the mixture is less than 10%. By contrast, in the case of Mg<sup>2+</sup> (Fig.3b), its affinity is increased with the OA+TOPO mixture, as it is able to extract between 40 and 50% of the total amount of magnesium present in the concentrates. This finding paves the way for a selective extraction of one cation or the other, with the OA+TOPO mixture being selected as the initial extraction agent for Mg<sup>2+</sup>.

**Conclusions:** The OA+TOPO mixture has been identified as a DES, with an optimal molar ratio of 1:1. Extraction tests have yielded promising results, with an notable increase in  $Mg^{2+}$  extraction from 23 to 51%. Further research is needed to determine the optimal operating conditions for this mixture, which could also offer sustainability benefits. The low affinity for Li<sup>+</sup> exhibited by 0.014 M of OA+TOPO, suggests the potential for selective separation in complex matrices. The sustainable profile and performance of the extraction process highlight the need for further research to achieve efficient and eco-friendly recovery of critical raw materials.

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## <sup>19</sup>F NMR SPECTROSCOPY FOR CHARACTERIZATION OF AZO-FLUOROSTILBENE PHOTOSWITCH

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**Abstract:** The <sup>19</sup>F NMR spectra collected for the (*E*)- and (*Z*)- isomers of an azofluorostilbene photoswitch demonstrated the effect of photoisomerization under visible light. The experimental results of the spin-spin ( $T_2$ ) and spin-lattice ( $T_1$ ) relaxation time measurements of fluorine nuclei in the aforementioned compound are presented and discussed.

**Introduction:** Azobenzenes are organic compounds characterized by the presence of two phenyl groups coupled through an azo bond. Their capability of  $E \rightarrow Z$  photoisomerisation (Fig.1) has made them the object of numerous studies on their potential use as photoswitches [1-4]. The compounds undergo  $E \rightarrow Z$  isomerisation under UV light, whilst the inverse  $Z \rightarrow E$  isomerisation is achieved through visible light irradiation or by way of thermal relaxation [5]. UV-Vis spectra of E and Z isomers show a superimposition of their absorption bands, which leads to an incomplete  $Z \rightarrow E$  conversion and the related difficulties in obtaining a single isomer. The irradiation of samples with light of an appropriate wavelength allows the achievement of a photostationary state, in which the maximum content of either the *cis*- or *trans*- isomer is 80% or 95%, respectively. The  $Z \rightarrow E$  thermal relaxation occurs quantitatively [4].



Fig.1. Photoisomerisation of azobenzene.

Inserting different substituents into the azobenzene structure resulted in shifting the maximum of absorption band towards longer wavelengths. This effect especially important in the context of photopharmacological application of azobenzene derivatives'. Specifically, photopharmacology form a way to avoid the exposure of diseased tissue to the harmful effects of UV radiation [6,7].

In our report we presented an azobenzene derivative 4-((2,4-difluorophenyl) diazenyl)benzoic acid (*p*-Azo24DF), which was not characterized previously. The modified structure leads to a separation of the absorption bands of the individual isomers, allowing an increase in isomerization selectivity. The photoisomerization of *p*-Azo24DF exhibited shift wavelength of light needed to facilitate  $Z \rightarrow E$  isomerisation

yields the best results at 535 nm light, corresponded to green light in the visible spectrum. The inverse  $(Z \rightarrow E)$  process occurs under irradiation with 400 nm light, corresponded to blue light in the visible spectrum (Fig.2) [3].



Fig.2. Photoisomerisation of *p*-Azo24DF.

**Experimental:** 2,4-difluoroaniline, DCM and oxone aqueous solution were introduced to a round-bottom flask and stirred at room temperature for 3 h. The reaction was quenched by the addition of sodium bicarbonate. The organic phase was dried over anhydrous MgSO<sub>4</sub> and evaporated under vacuum. The resulting residue was introduced to a round-bottom flask along with acetic acid and *p*-aminobenzoic acid. The reaction mixture was stirred at room temperature for 24 h and diluted with water. The resulting suspension was filtered, and the crude product recrystallised from ethyl acetate. The achieved yield of *p*-Azo24DF was 13%.

The NMR sample was prepared by dissolving the product in DMSO-*d*<sub>6</sub>. The irradiation was conducted in NMR sample tube under 530 nm green light for around 10 hours. The measurements were performed with Varian Inova 400 NMR spectrometer operated at magnetic field 9.6 T. Experimental data were collected at 298 K utilizing <sup>1</sup>H/<sup>13</sup>C/<sup>15</sup>N triple resonance probehead, with <sup>1</sup>H channel tuned to <sup>19</sup>F resonances (<sup>19</sup>F frequency 376.273 MHz).

**Results:** Synthesis of *p*-Azo24DF was performed according to a previously reported procedure based on oxidation of 2,4-difluoroaniline to a nitroso compound followed by a Baeyer-Mills condensation with *p*-aminobenzoic acid (scheme 1) [3].



Scheme 1. Synthesis of *p*-Azo24DF.

The purity and quality of synthesized compound was confirmed with <sup>1</sup>H NMR spectroscopy. <sup>19</sup>F NMR spectra of the *p*-Azo24DF product were measured before and after green light irradiation, based on which the occurrence of photoisomerization within

the sample was confirmed (Fig.3). The photostationary state yielded 47% of the (Z)-isomer.



Fig.3. <sup>19</sup>F NMR spectra of *p*-Azo24DF before (top) and after (bottom) irradiation at  $\lambda = 530$  nm, respectively.

The sample, containing both -E and Z – isomers of *p***-Azo24DF**, was used to measure the longitudinal relaxation times of <sup>19</sup>F nuclei. The fluorine atoms in the *ortho*- position (relative to the azo bond) showed longer T<sub>1</sub> relaxation times against the *para*- fluorine atoms (Table 1).

<sup>19</sup> F resonance	T <sub>1</sub> [s]
( <i>E</i> )-o	$1,780\pm 5,73\cdot 10^{-7}$
(Z)-o	$1,263 \pm 1,41 \cdot 10^{-6}$
(Z)-p	0,664 ±1,69·10 <sup>-6</sup>
( <i>E</i> )-p	$0,573 \pm 1,62 \cdot 10^{-6}$

Table 1. Longitudinal relaxation times of fluorine nuclei in p-Azo24DF.

The transverse relaxation times were also measured for the irradiated sample. The results are shown in Table 2.

Table 2. Transverse relaxation times of fluorine nuclei in p-Azo24DF.

<sup>19</sup> F nucleus	T <sub>2</sub> [s]
( <i>E</i> )-o	$0,190\pm 3,19\cdot 10^{-7}$
(Z)-o	$0,287 \pm 7,30.10^{-7}$
(Z)-p	$0,290 \pm 5,83 \cdot 10^{-7}$
( <i>E</i> )-p	0,153 ±2,17·10 <sup>-7</sup>

The described herein longitudinal relaxation time  $(T_1)$  is related to the return of the spin system magetization vector to its original position, parallel to the applied magnetic field. The  $T_2$  relaxation time is related to the decay of the transverse components of the

magnization vector in the plane perpendicular to the direction of the applied magnetic field.

**Conclusions:** The synthesised fluorine-substituted derivative of azo-stilbene was shown to be capable of photoisomerization when irradiated with 530 nm wavelength light. The maximum content of the Z-isomer in the photostationary state totals 47%. Fluorine nuclei of the individual isomers exhibit significant differences of longitudinal and transverse relaxation times.

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# UV-SHIELDING PROPERTIES OF LIGNIN-CONTAINING NANOCOMPOSITE FOILS

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Abstract: The study aimed to use technical kraft lignin of different origin (L1 - lignin from softwood and L2 - lignin from hardwood) as a functional additive for polymer foils. For comparison purposes, the reference sample without the addition of lignin was also obtained (L0). UV-shielding properties were studied using UV-VIS spectrophotometry. Surface morphology was examined using Scanning Electron Microscopy (SEM). Fourier Transform Infrared Spectroscopy (FTIR) was used to provide information about the chemical structure and functional groups present in the testing polymer.

**Introduction:** Lignin, a complex organic polymer found in the cell walls of plants, has gained significant attention as a green material for polymeric applications due to its abundance, renewability, and biodegradability. It's a byproduct of the pulp and paper industry, making it an attractive, sustainable alternative to petroleum-based materials [1-4]. This unique biopolymer is characterized by a complex, aromatic structure based on phenylpropanoid units (p-hydroxyphenyl, guaiacyl, and syringyl). Lignin exhibits unique physicochemical properties, including good thermal stability, high carbon content, the presence of various reactive functional groups as well and excellent UV-shielding properties. These attributes enable its integration into diverse polymer matrices, enhancing mechanical strength, antioxidant capacity, and environmental compatibility [5]. The water-soluble packaging industry, with polyvinyl alcohol (PVA) as its flagship material, stands at the forefront of sustainable packaging solutions, addressing environmental concerns like plastic pollution, waste accumulation, and resource depletion [6]. PVA, a water-soluble synthetic polymer, is known for its excellent filmforming ability, biodegradability, and high tensile strength. When blended with lignin, the resulting composite foils possess the properties of both materials, offering a sustainable alternative in various packaging applications [7-8].

**Experimental:** The UV-shielding properties of the foils were assessed using a Shimadzu UV-2600 UV-vis spectrophotometer (Japan). The SEM micrographs were taken using a FEI Phenom World scanning electron-ion microscope. FTIR spectra were obtained using the ATR technique with a diamond crystal using a Nicolet 8700A FTIR spectrometer. The tests were performed directly from the sample surface in the
wavenumber range of 4000-400 cm<sup>-1</sup> and with a spectral resolution of 4 cm<sup>-1</sup>. A DTGS detector was used for research. The obtained spectra were subjected to ATR correction, baseline correction, and normalization using Omnic SpectaTM software.

**Results:** The polymer foils were obtained through the casting method. The photographs of the obtained products are presented in Fig.1 (the foils with L1) and 2 (the foils with L2). The increasing amount of lignin, which was added to the polymer matrix, resulted in a slightly darker color of the foil.



Fig.1. Photograph of the foils with L1.



Fig.2. Photograph of the foils with L2.

The UV-shielding properties of the obtained materials were studied by means of UV-VIS spectrophotometry. Figure 3 presents the protective properties of the foils with L1 and L2 across different regions of the UV spectrum. The extended conjugated systems present in lignin molecules contribute to enhanced UV absorption, making lignin an effective natural UV-blocking agent when incorporated into polymer matrices.



Fig.3. Protective properties of L1 (dashed line) and L2 (solid line) across different regions of the UV spectrum.

The chemical structures of the foils were investigated using Fourier Transform Infrared Spectroscopy (FTIR), a technique widely used in polymer science. FTIR spectra of the

foils are presented in Fig.3 and Fig.4. The characteristic absorption peaks are located at  $3300 \text{ cm}^{-1}$  and  $2940 \text{ cm}^{-1}$ , corresponding to the stretching vibration of O–H and the stretching vibration of C–H bond, respectively. The peak at  $1730 \text{ cm}^{-1}$  is attributed to the stretching of carbonyl groups. The C–O–C absorption band is observed at  $1000-1250 \text{ cm}^{-1}$ .



The surface morphology of the foils was examined using Scanning Electron Microscopy (SEM). SEM micrographs are presented in Fig.4. The surface morphology can vary significantly depending on factors such as the preparation method, the concentration of lignin, and the interaction between the matrix and the additives. The foils without the addition of lignin (L0) exhibit a relatively smooth and homogeneous surface. The introduction of the lignin to the polymer system is related to changes to morphology, resulting from dispersion and compatibility within the matrix. For both types of lignin, one can observe that they are well-dispersed, and as a result, the surface remains relatively smooth with small, evenly distributed protrusions. The uniformity of this dispersion can be related to the interfacial interactions, especially hydrogen bonding, which enhances compatibility and prevents large-scale aggregations,



Fig.4. SEM micrographs of the foils.

**Conclusions:** Polymer foils with lignin were prepared through the casting method. The surface morphology was investigated using the SEM technique. Their chemical structures and the presence of appropriate functional groups were confirmed using FTIR spectroscopy. The obtained results indicate the promising use of lignin as an environmentally friendly bio-additive for polymer foils and, therefore contribute to its valorization.

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# NEW BIPHENYL-DERIVATIVE THERMOPLASTIC POLY(CARBONATE-URETHANE)S - THERMAL AND MECHANICAL PROPERTIES

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Abstract: New thermoplastic sulfur-containing poly(carbonate-urethane)s (PCURs) were obtained via a one-step melt polyaddition from biphenyl-derivative diol with six aliphatic chain. 6,6'-[[1,1'-biphenyl]-4,4'methylene groups in the i.e. divlbis(methylenesulfanedivl)]dihexan-1-ol as unconventional chain extender, 1,1'methanediylbis(4-isocyanatobenzene) and poly(hexane-1,6-diyl carbonate) diol with the molar mass of 860 g/mol. The PCURs, with hard-segment-contents ranged from 30 to 60 mass %, were examined in order to determine their thermal and mechanical properties by using differential scanning calorimetry, thermogravimetry (TG) and TG coupled with FTIR spectroscopy, as well as Shore hardness and tensile tests.

**Introduction:** The great interest in polymers containing sulfur atoms in their structure, as well as in thermoplastic polyurethanes, prompted me to continue research on the synthesis and characterization of new thermoplastic polyurethanes based on unconventional aliphatic-aromatic chain extenders with sulfide bonds and commercially available diisocyanates and polymer diols. Literature data show that the existence of sulfur atoms can improve some significant properties of such materials, for example adhesion to metals, chemical, microbiological resistance as well as refractive index [1-3]. This paper presents the results of investigations carried out on the new thermoplastic poly(carbonate-urethane)s (PCURs) from 6,6'-[[1,1'-biphenyl]-4,4'prepared divlbis(methylenesulfanedivl)]dihexan-1-ol (BMS-H diol), 1,1'-methylenebis(4isocyanatobenzene) (MDI) and poly(hexane-1,6-diyl carbonate) diol (PHCD) with the molar mass of 860 g/mol. The research focused on determining the thermal and mechanical properties of newly synthesized polymers. These properties strongly depend on applied substrates and provide many important information about materials which determine their future application.

**Experimental:** The BMS-H diol (m.p.=109-110 °C) was obtained by the reaction of ([1,1'-biphenyl]-4,4'-diyl)dimethanethiol with 6-chlorohexan-1-ol in a water-ethanolic sodium hydroxide solution and crystallized from the mixture of toluene and *N*,*N*-dimethylformamide (the ratio of 20:1 v/v). MDI (98%) from Sigma-Aldrich was used without further purification, while PHCD (Sigma-Aldrich) prior to the use was heated at 90 °C *in vacuo* for 10 h.

Reduced viscosities ( $\eta_{red}$ s) of 0.5 % PCUR solution in 1,1,2,2-tetrachloroethane were measured in an Ubbelohde viscometer at 25 °C. ATR-FTIR spectra were performed using a Perkin-Elmer 1725X spectrometer. Samples were prepared as films. Thermogravimetric (TG) measurements were done on a Netzsch STA 449 F1 Jupiter thermal analyzer in synthetic air (flow = 20 cm<sup>3</sup>/min) from 30 °C to 700 °C at the heating rate of 10 °C/min. The composition of gases evolved during the decomposition process was analyzed by a Bruker Tensor 27 FTIR spectrometer coupled on-line to a Netzsch STA instrument by the teflon transfer line heated to 200 °C. The FTIR spectra were recorded in the spectral range of 600–4000 cm<sup>-1</sup> with a 4 cm<sup>-1</sup> resolution and 16 repetitions. Differential scanning calorimetry (DSC) was carried out on a Netzsch DSC 204 instrument at a heating and cooling rate of 10 °C/min in the range of -100-200 °C. Glass-transition temperatures ( $T_g$ s) for the samples were defined as the inflection point on the curves of the heat-capacity changes. Hardness was determined using the Shore method on a 7206/H04 hardness tester from Zwick at a temperature of 23 °C. The values were read after 15 s of measurement. Tensile testing was performed on a Zwick/Roell Z010 tensile-testing machine at the speed of 100 mm/min at 23 °C; tensile test pieces of 1 mm thick and 6 mm wide (for the section measured) were cut from the pressed sheet. PCURs were synthesized according to scheme shown in Fig.1. by a one-step melt polyaddition method at  $I_{NCO}$ =1. The process was carried out in a nitrogen atmosphere at

a temperature of 140-150 °C for 2.5 hours.



Fig.1. Schematic representation of the synthesis route of PCURs.

**Results:** The PCURs were colorless rubber-like materials with relatively low molar masses, as indicated by the  $\eta_{red}$  values (Table 1). Their chemical structures were verified by ATR-FTIR spectroscopy. The spectra exhibited the following absorption bands (in cm<sup>-1</sup>): 3343–3342 (N-H stretching) and 1530-1526 cm<sup>-1</sup> (N-H bending) of the urethane group; 1735–1724 (C=O stretching of the urethane and carbonate groups); 1251–1247 (O–C–O stretching of the carbonate group); 2937–2933 (asymmetric) and 2860–2856 (symmetric) C-H stretching of the methylene group; 1597–1596 (C-C stretching of the benzene ring); 815–814 (C-H bending of *p*-disubstituted benzene ring). No bands were observed at ~2270–2260 cm<sup>-1</sup>, indicating that all the isocyanate groups were converted to the urethane ones.

The results obtained using DSC method (Fig.2 and Table 1) revealed that the PCURs had amorphous structures. Namely, the curves from the two heating cycles (see Fig. 2) did not show endothermic peaks and exhibited only the glass transition. The  $T_g$  values increased as the hard-segment content increased, from -2 °C to 22 °C (first heating cycles) or from -1 °C to 24 °C (second heating cycles). The comparison of the  $T_g$  values of the pure PHCD soft segment (-69 °C) and those of the synthesized PCURs shows that the new polymers possessed a very poor microphase separation.



Fig.2. DSC curves of the PCURs.

PCUR	Hard-segment content [mass %]	$\eta_{\rm red}$ [dL/g]	$T_5^{a}$ [°C]	$T_{10}^{a}$ [°C]	$T_{50}^{a}$ [°C]	$T_{\max}^{b}$ [°C]	<i>T</i> <sup>c</sup> <sub>g</sub> [°C]	<i>T</i> <sup>d</sup> <sub>g</sub> [°C]
PCUR-30	30	0.72	313	325	357	356, 535	-2	-1
PCUR-40	40	0.62	310	322	355	353, 544	7	7
PCUR-50	50	0.56	305	318	354	350, 547	16	17
PCUR-60	60	0.66	300	314	354	346, 547	22	24

**Table 1.** Designations,  $\eta_{red}$  values, DSC and TG-DTG data obtained for PCURs.

<sup>a</sup> the temperature of 5, 10 and 50% mass loss from the TG curve, respectively; <sup>b</sup> the temperature of the maximum rate of mass loss from the differential TG (DTG) curve; <sup>c,d</sup> first and second heating cycle, respectively.

As can be seen from the TG study (Table 1, Fig.3), the PCURs exhibited a quite good thermal stability as for polyurethanes. The  $T_{58}$  and  $T_{108}$  ranged from 300-313 °C and 314-325 °C, respectively. These temperature parameters decreased with the increase in the amount of hard segments. From this study it also follows that the PCURs decomposed in two steps. The first step, with a maximum at 346-356 °C, was associated with the decomposition of both hard segments and soft segments. This decomposition was accompanied by the release of carbon dioxide, carbon monoxide, carbonyl sulfide, sulfur dioxide, water, aliphatic aldehydes, ethers, alcohols as well as aromatic compounds (Fig.4). The second step, with a maximum at 535-547 °C, was connected to oxidative processes of previously formed products; carbon dioxide, carbon monoxide and water were only detected in the volatiles. Based on the absence of amines in the volatile decomposition products, it should be assumed that the breakdown of the urethane bonds occurred due to their dissociation into alcohols and isocyanates.

Mechanical property tests (Table 2) showed that PCURs had different tensile strength. It was also stated that with increasing hard segment content in PCUR, elongation at break decreased, while the modulus of elasticity and Shore A/D hardness increased.



Fig.4. FTIR spectra of volatile products evolved during thermal decomposition of PCUR-60: (a) 3D and (b) extracted at the maxima of decomposition.

PCUR	Shore A/D hardness	Modulus of elasticity	Tensile strength	Elongation at break
	[°Sh]	[MPa]	[MPa]	[%]
PCUR-30	58/19	0.1	7.1	600
PCUR-40	63/23	0.4	14.3	475
PCUR-50	67/24	2.0	35.3	375
PCUR-60	78/28	25.1	51.3	300

Table 2. Hardness and tensile properties of PCURs.

**Conclusions:** The new thermoplastic PCURs were amorphous colorless materials. They were characterized by relatively good thermooxidative stability, and some of them also had good or very good tensile strength. PCUR-60 exhibited a tensile strength higher than that of its commercial analogue, i.e. Biomate® 80A (46.6 MPa), in which the hard segment is composed of MDI and an aliphatic diol (butane-1,4-diol) and the soft segment is poly(hexane-1,6-diyl-ethylene carbonate)diol [4].

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## CARBOXYLATE AND PHOSPHATE ESTER EXCHANGE IN COVALENT ADAPTABLE NETWORKS

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Abstract: Vitrimers are a group of dynamic materials, i.e. covalently adaptable networks that combine the properties of thermoplastics and thermosets. Among vitrimers, networks based on carboxylate transesterification are the most widely studied, while in recent years networks containing phosphate esters have attracted attention due to the catalytic effect of phosphate groups. In previous work, we carried out a systematic analysis of the dynamic properties of photopolymerized resins with abundant pendant hydroxyls crosslinked with a phosphate diester and found that the relaxation process is complex and influenced by the occurrence of either exchange of phosphate esters or condensation where the diester is converted to a triester. Here we have shown that above a threshold temperature of 160  $^{\circ}$ C the relaxation process is further complemented by transesterification of carboxylic acid esters.

**Introduction:** Covalent adaptable networks (CANs) contain within their structure dynamic bonds capable of exchanging and reorganizing their topology under the influence of external triggers, such as temperature or light. This allows them to be processed while maintaining strength over a wide temperature range [1]. Vitrimers are one type of covalent adaptable networks. Their name comes from the dynamic epoxy networks that were first discovered by Leibler in 2011, who noticed that the viscoelastic properties of these materials were similar to those of vitreous silica [2]. Depending on the type of reversible bonds incorporated into the network structure, vitrimers can undergo reactions, e.g. transesterification, transamination or transalkylation. In recent years, CANs based on the transesterification of phosphate esters have attracted considerable interest from researchers [3]. The chemical stability of phosphate esters and their participation in transesterification make them the preferred monomers for creating sustainable dynamic materials. Strategies for designing dynamic phosphate networks have involved either the use of synthetic phosphate monomers or the development of networks with the addition of phosphate moieties to confer dynamic functionality [3]. Researchers have also described the use of mono or phosphate diesters as a catalysts for tranesterification in acrylic or thiol-acrylic networks [4,5]. In addition, the incorporation of phosphate groups into networks allows the simultaneous combination of recyclability and flame retardancy [3]. In previous work, we have studied networks consisting of a phosphate diester and a hydroxyacrylate at different molar ratios, and have demonstrated that the relaxation process is not only related to the transesterification of the phosphate hydroxyls, but is complex due to the occurrence of condensation reactions. We have shown that the condensation reaction results in an increase in network density and allows thermal control of the mechanical properties of the polymers [6].

Here we have studied the effect of the catalytic amount of phosphate monoester on the thermomechanical properties and stress relaxation in nondynamic carboxylate ester based network with pendant hydroxyls.

**Experimental:** Samples of copolymers of triethylene glycol dimethacrylate (TEGDMA) and 2-hydroxyethyl acrylate (HEA) in 1/2 ratio were analyzed both with and without the addition of phosphoric acid 2-hydroxyethyl methacrylate ester (PAME) as catalyst at 2 wt%. To prepare rectangular samples of (25/4/0.5) mm, appropriate amounts of photoinitiator in the form of monomers, catalyst and 2,2-dimethoxy-2phenylacetophenone (DMPA) 0.2 wt% were weighed into a glass beaker. After mixing, the solutions were placed between slides separated by PET spacers and photocured under UV (365 nm, I~20 mW/cm<sup>2</sup>). Conversion of C=C bonds was measured via FTIR-ATR technique. Dynamic mechanical analysis (temperature dependence of storage modulus and tangent  $\delta$ , and stress relaxation) were investigated using the DMA Q800 instrument. Samples were heated from 0 °C to 200 °C with the ramp 3 °C/min.

**Results:** The IR spectra (Fig.1) for both neat samples look the same. At a wave number of 1640 cm<sup>-1</sup>, bands for the remaining C=C bonds are visible, which disappear after heating during DMA analysis.



Fig.1. Infrared spectra of a) TEGDMA/HEA (1/2) and b) TEGDMA/HEA (1/2) with 2 wt% PAME .

The thermomechanical properties were studied by DMA analysis. Figure 2 shows the storage modulus and tangent  $\delta$  plots as a function of temperature. The TEGDMA/HEA (1/2) copolymer exhibits a higher value of storage modulus at the rubbery plateau than its analogue with PAME. However, the exact value is difficult to determine due to the continuous modulus decline in this region. Above the rubbery plateau, an increase in modulus is observed for each sample, which may be related to crosslinking by reaction of the remaining double bonds. In contrast, a more than 6-fold increase in modulus above the rubbery plateau is observed for the copolymer with PAME. Referring to previous work, this increase is related to condensation between -OH groups from PAME and pendant hydroxyls in the network [6]. The glass transition temperature determined from the maximum tangent delta is equal to 106 °C for the polymer without catalyst and 118 °C for the polymer with PAME.



Fig.2. Storage modulus and tangent  $\delta$  as a function of temperature.

Stress relaxation measurements were performed in flexure mode. Rectangular samples were heated for one hour at different temperatures. Figure 3 shows that carboxylate transestrification does not occur at 160 °C, even in the presence of PAME. At 180 °C, without catalyst, only 10% relaxation is possible, while at 220 °C the percentage of relaxation increases to more than 20%. The presence of PAME accelerates the relaxation process, reaching 20% at 180 °C and more than 30% at 220 °C.



**Fig.3.** Normalized stress relaxation of TEGDMA/HEA (1/2) copolymers with and without PAME (a transesterification catalyst) at different temperatures: a) 160 °C, b) 180 °C, c) 200 °C, d) 220 °C.

**Conclusions:** The addition of a catalytic amount of phosphate monoester has little effect on the thermo-mechanical properties of the network. At the rubbery plateau, the copolymer with PAME has a lower value of storage modulus than its reference. However, further heating causes the storage modulus to increase. This means that condensation and an increase in cross-linking density involving PAME and pendant hydroxyls can occur. Results from dynamic properties studies show that at 160 °C transesterification of carboxylate esters is not possible, even in the presence of PAME. At higher temperatures the addition of phosphate ester results in an acceleration of the carboxylate exchange.

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# PLASTICIZERS IN POLYVINYL CHLORIDE (PVC) PROCESSING: MECHANISMS, FUNCTIONALITY, AND MIGRATION

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**Abstract:** Polyvinyl chloride (PVC) is a widely used polymer in various industries due to its versatility and economic feasibility. However, in its pure form, PVC is rigid and brittle, making it unsuitable for many applications. To improve its flexibility and processability, PVC is commonly modified with plasticizers. This paper discusses the role of plasticizers in PVC, focusing on their mechanism of action, classification, and the challenges associated with their migration.

**Introduction:** Poly(vinyl chloride) (PVC) is one of the essential polymeric materials used in the modern economy. It is obtained through the radical polymerization of vinyl chloride, which is typically carried out on an industrial scale via emulsion or suspension processes, either continuously or in batch mode. The resulting polymer appears as a white powder, characterized by the K-value (Fikentscher number), which depends on the morphology of the polymer molecules and is directly related to their molecular weight. Pure PVC is inherently hard and brittle, with low flowability and poor thermal stability. Therefore, in practical applications, it is commonly modified with various additives such as plasticizers, thermal stabilizers, lubricants, and fillers. These additives enhance its processing behavior and improve its functional properties [1]. Plasticizers have a significant role in the processing of polymer materials, particularly those based on poly(vinyl chloride) (PVC). Their primary function is to modify the mechanical properties of the polymer by increasing its flexibility and improving its processability [2]. Among conventional plasticizers, phthalates are widely used due to their high efficiency in softening PVC and their low production cost. However, their tendency to migrate from finished products into the environment raises concerns, as it may lead to soil and water contamination and pose potential risks to living organisms, including humans, through endocrine disruption [3]. In response to increasing regulatory pressure and growing environmental awareness, the use of certain phthalates is being restricted, thereby driving the development of new plasticizers with improved environmental and health profiles. The definition of plasticizers adopted by the International Union of Pure and Applied Chemistry (IUPAC) in 1951 is: "A plasticizer is a substance or material added to a material (typically a plastic or elastomer) to increase its flexibility, workability, or stretchability." This definition highlights the key role of plasticizers in modifying the physical properties of polymers, primarily by reducing their glass transition temperature (Tg), making the polymer more flexible and easier to process [4]. Classification of plasticizers: Plasticizers can be classified in various ways depending on the adopted scheme. One of the most commonly used criteria is a classification based on molecular weight, which distinguishes monomeric and polymeric plasticizers. Another

criterion is the type of plasticization, dividing plasticizers into internal and external types. Additionally, plasticizers can be classified as primary and secondary, with secondary plasticizers being part of the external plasticizer group. From a practical perspective, plasticizers are typically divided into three main groups based on their chemical structure and the related characteristics that influence their performance in polymers. These groups include general-purpose plasticizers, high-efficiency plasticizers, and specialty plasticizers [5].

*Mechanism of plasticization:* In order for a plasticizer to modify the properties of a polymer, it must be introduced into the polymer matrix and thoroughly mixed. This process typically involves combining the polymer with the plasticizer at an elevated temperature, followed by shaping the resulting material into the desired form and cooling it [2]. There are three main theories explaining the mechanism of plasticizer action in a polymer (Fig.1).



The first, known as the gel theory, suggests that the plasticizer reduces the intermolecular interactions within the three-dimensional polymer network. The lubrication theory compares the action of a plasticizer to that of lubricants - its molecules, positioned on the polymer chain surfaces, enable the chains to slide relative to each other. The third, and most commonly used, theory is the free volume theory, which is widely applied to describe the plasticization mechanism. Free volume refers to the internal space available within the polymer matrix. As the free volume increases, the freedom of movement of the polymer chains also increases. A polymer in the glassy state has an internal structure with low free volume, which prevents the molecules from moving freely, making the material rigid and hard. Free volume arises due to the movement of terminal groups of the polymer, side chains, and internal motions within the polymer itself. When a plasticizer is added to the polymer matrix and the mixture is heated above the glass transition temperature, thermal energy increases, which boosts molecular movement, causing the polymer chains to separate and create more free volume. This free volume is maintained during the cooling of the polymer-plasticizer mixture, making the material more flexible and elastic [2,4,6]. Plasticizers with smaller molecules typically increase the free volume of a polymer more effectively than those with larger molecules. Additionally, molecules with branched structures are more effective as plasticizers compared to those with linear structures. Therefore, to achieve maximum free volume, the plasticizer molecule should have a relatively low molecular weight while maintaining relatively large dimensions [4,5]. Pure polyvinyl chloride (PVC) is a hard and brittle material due to the strong polar interactions between the

carbon-chlorine bonds in the polymer chains. The main purpose of using plasticizers is to improve the flexibility and processability of PVC, which is achieved by lowering the glass transition temperature of the material. The functional properties of this polymer are closely related to the interactions between the polymer chains and the plasticizer molecules. The most common intermolecular interactions between the plasticizer and the polymer chain are London dispersion forces, including van der Waals interactions, such as dipole-dipole and induced dipole interactions, as well as hydrogen bonding. Dipoledipole interactions occur between the polar groups of the plasticizer and the chlorine atoms in the PVC chain. Hydrogen bonds can form between the carbonyl group of the ester and a hydrogen atom attached to a carbon atom adjacent to a carbon atom bonded to chlorine. These interactions between the plasticizer and the polymer weaken the forces between the polymer chains, reducing their entanglement and allowing changes in the three-dimensional structure of the polymer by increasing the mobility of the chains [5,8,9].

Migration of Plasticizers: The stability of the polymer-plasticizer system under controlled conditions is influenced by various factors, including the structure, chemical composition, molecular weight, and polarity. Plasticizers are typically not chemically bonded to polymer chains, meaning they can migrate into the environment during material processing or later in the product's lifecycle. This migration leads to a gradual deterioration of the material's properties and contributes to environmental pollution and potential health risks to consumers [8,10]. Plasticizer migration from the material occurs in two phases: the migration (diffusion) of plasticizer molecules to the surface of the sample and the release (desorption) of plasticizer from the surface into the surrounding environment. The first phase is slower, so migration, expressed as the percentage loss of plasticizer, is less significant in thicker samples compared to thinner ones. The release phase is influenced by the type of medium the sample is in contact with and the volume of this medium [10]. The thickness of the material also affects migration: the thicker the material, the greater the migration, as the plasticizer has more volume to penetrate when released from the polymer. In general, larger plasticizer molecules tend to migrate less readily from the polymer matrix. Linear plasticizers migrate more easily than those with branched structures, as branching restricts the plasticizer's movement within the matrix, reducing its migration. Due to the many factors involved, predicting the degree of plasticizer migration is complex, and experimental methods are typically required. As such, organizations like the German Institute for Standardization (DIN), the International Organization for Standardization (ISO), and the American Society for Testing and Materials (ASTM) have developed various standardized methods to assess plasticizer migration, such as ISO 176, ISO 177, ASTM D2199, ASTM D1239, and DIN 75201 [5,8].

**Conclusions:** Plasticizers are indispensable in the processing and application of poly(vinyl chloride) (PVC), significantly enhancing its flexibility, processability, and usability in various industrial sectors. However, the issue of plasticizer migration remains a critical challenge, particularly due to its implications for environmental pollution and human health risks. Migrating plasticizers, especially conventional phthalates, can leach into surrounding media during the product's lifecycle, contributing to soil and water contamination, and may exhibit endocrine-disrupting activity in living organisms. In light of these concerns, it is essential to not only continue the search for

new, safer plasticizers with reduced migration potential and improved environmental profiles but also to conduct thorough testing and reevaluation of already existing compounds. Comprehensive studies on the physicochemical properties, toxicity, and long-term performance of both new and currently used plasticizers are crucial to ensure the safety of end products, maintain material quality, and support the development of sustainable solutions in PVC processing.

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## BIFUNCTIONAL CYTISINE SQUARAMIDES – SYNTHESIS AND SPECTROSCOPIC ANALYSIS

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**Abstract:** The naturally derived compounds with high specificity of action are the ones of great interest in bioorganic chemistry reports. In this study, synthetic approach was employed to construct novel conjugates of essential amino acid esters with alkaloid (-)-cytisine via a squarate linker. Recent investigations regarding squaramides showed a high diversity of applications and impacted on creativity in the development of compounds with given properties. The anion-binding tendency, together with rigidity and stability of the structure, brought to the promising possibility in causing apoptosis by regulation of the ion transport. Starting from the synthesis of novel monoamides of cytisine and squaric acid, we could expect the endow in the activity amplification. As a result, two structures of derivatives were investigated, followed by spectroscopic characterisation with the estimation of their antioxidant activity.

**Introduction:** The amide functional group plays a key role in medicinal chemistry, due to its ability to act as both a hydrogen bond donor and acceptor [1]. Structural modification of the piperidine ring C in (-)-cytisine (1, Fig.1) have yielded pharmacologically active derivatives. The chair conformation of the fused rings in the structure of 1 impacts the affinity to cholinergic nicotinic acetylcholine receptors (nAChRs), comparable to acetylcholine and nicotine. As partial agonist of specific nAChRs subtypes, cytisine (1) finds the application in nicotine-replacement therapy and also in modulating neuronal processes regulated by these receptors [2]. Its antiproliferative activity has also been linked to the group of the compounds that partially lied in apoptosis provoked by the formation of reactive oxygen species (ROS) in cancer cells [3].



Fig.1. The representation of (-)-cytisine (1) targeting position (left) and hydrogen bonding between subsequent squaramide (2) units (right).

Amide bond formation with carboxylic acid is facilitated by the high nucleophilicity of the N12 atom (traditional nomenclature) in cytisine **1**. However, the use of squaric acid derivatives, offers enhanced functionality of the resulting type of products [4]. According to the literature resources, the squaramide moiety can mimic over ten biologically relevant functional groups [5], and its planar structure allows for strong hydrogen bonding and self-assembly into stable frameworks (**2**, Fig.1) [6]. While

squaramides have been studied as specific catalysts and valve anion receptors [7-9], their primary interest lies in biomedical applications. Squaric acid diesters have been conjugated with chosen amino acids via amide bond formation [10], enhancing the structural and functional diversity of resulting conjugates. This approach is particularly valuable for improving the bioavailability of naturally derived molecules with high speficity but limited pharmacokinetic properties [11].

**Experimental:** (-)-Cytisine (1) was isolated from the seeds of golden chain (or golden rain) tree (*Laburnum anagyroides*), according to the standard procedure [12,13]. The final derivatives were obtained in subsequent substitution reactions resulting in squaramides functionalized by 1 and essential L-amino acid esters (5): alanine and phenylalanine (Fig.2). Compounds purification and preliminary MS analyses were performed using a CombiFlash Rf+Purion (Teledyne ISCO). ESI mass spectra were obtained on a Waters/Micromass (Manchester, UK) ZQ mass spectrometer (MassLynx V4.0). The FT-IR spectra of the compound were recorded in KBr tablets on an IFS 113v FT-IR spectrophotometer (Bruker, Karlsruhe) equipped with a DTGS detector. The <sup>1</sup>H and <sup>13</sup>C NMR analyses were made on a Bruker ADVANCE device (151 MHz, DMSO-d<sub>6</sub>). UV-Vis spectroscopic measurements and DPPH radical scavenging activity studies were accomplished on a spectrophotometer Jasco V-650.



Fig.2. Synthesis of bifunctional squaramides CSQ1 and CQS2 from monosquaramide-esters with 5 (alanine methyl ester hydrochloride or phenylalanine methyl ester hydrochloride).

**Results:** Based on both research publications and virtual screening [14] resources, it was found that cytisine derivatives linked through N12 atom would not possess as great affinity to nAChRs as cytisine 1 itself does. Moreover, using a squaric ring as a template, the compound can be enriched by at least 3 more hydrogen bonding centres, impacting the specificity of interaction with molecular targets. The simplicity of the synthetic procedure allowed the desired compounds to be obtained with moderate yields, but in addition, thorough purification was necessary after every step. Triethylamine as a base was used to reach the salt transformation and activation of the amino group for the effective accomplishment. The ESI-MS spectra showed signals originated from the corresponding molecular ions ( $[M^+] = 371$ ,  $[M^+] = 447$ ) with the tendency to bind metal cations (Na<sup>+</sup>, K<sup>+</sup>), forming corresponding alkali metal ion adducts. On the other hand,

the C atom from cyclobutenedione ring, which is responsible for the amide formation (C17) was detected at approximately 162 ppm for both compounds by <sup>13</sup>C NMR spectroscopy (Fig.3). Additionally, <sup>1</sup>H NMR spectra showed the presence of amide group proton ( $\delta^1 = 7.79$  ppm,  $\delta^1 = 7.93$  ppm) for the expected structures. Measured spectra FT-IR of **1**, **3** and CSQ1 or CSQ2 also confirmed obtaining the new compounds (Fig.4).



Fig.4. FT-IR overlaid spectra of (-)-cytisine (1), amidoester (3), and compound CSQ1.

Both CSQ1 and CSQ2 in methanol solution absorb radiation in preferably a UV region with almost the same maxima ( $\lambda_{max} = 293-294$  nm) and are characterized by high values of molar extinction coefficient,  $\varepsilon_{max}$  (Fig.5). The absorption peak intensity difference brought to the conclusion that benzene ring attributes to the distortion of the structure and, consequently, to the hypochromic shift, meaning that  $\varepsilon_{max} = 26570 \text{ M}^{-1} \text{cm}^{-1}$  for the alanine ester derivative **CSQ1** drops to the value  $\varepsilon_{max} = 16430 \text{ M}^{-1} \text{cm}^{-1}$  for **CSQ2**.

Free radical assay, which was made for a set of cytisine-amide compounds, showed almost negligible changes in absorbance, leading to less than 5% of scavenging, thus it is hard to consider such ester derivatives as potent antioxidants, although modification of ester groups to hydroxyl ones would probably enhance the ability to quench the model radical compound DPPH. Herein, the stereospecificity of the reaction can be confirmed by retaining the chirality of the fragments introduced.



**Fig.5.** UV-Vis overlaid spectra for CSQ1 and CSQ2 in methanol ( $c = 5 \times 10^{-5}$  M).

**Conclusions** The synthesis of novel bifunctional squaramide–cytisine conjugates was successfully accomplished via aminolysis reactions using a recently reported method [4], leading to the formation of new unsymmetrical bifunctional squaric amides. The obtained compounds were characterized and confirmed using spectroscopic techniques, including ESI-MS, FT-IR, NMR, and UV-Vis. The applied analytical methods validated the results of the study and demonstrated the potential of this approach for further modifications of squaric acid derivatives.

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## SYNTHESIS AND SPECTRAL PROPERTIES OF N-PROPARGYLCYTISINE DERIVATIVES

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**Abstract:** The synthesis and spectral characteristic of new (-)-cytisine derivative, N-propargylcytisine and its symmetrical dimer through a Glaser-Hay coupling reaction were performed. The structures were confirmed using ESI-MS, FT-IR, and NMR spectroscopy. Solvatochromic properties of both compounds were investigated using UV-VIS absorption spectroscopy in solvents of varying polarity, including protic solvents capable of hydrogen bonding. Both compounds are characterized by the formation of hydrogen bonds with protic solvents. Spectral measurements at varying concentrations confirmed the absence of molecular aggregation in solution.

**Introduction:** The presented project focuses on the synthesis and spectral characteristic of novel conjugates of a bioactive quinolizidine alkaloid, obtained via the Glaser-Hay coupling reaction. Cytisine and its derivatives are known to interact with nicotinic acetylcholine receptors (nAChRs), making them valuable model compounds for studding the mechanisms of nACh receptor modulation. Several cytisine derivatives have been synthesized and evaluated for their bioactivity across various therapeutic areas. Depending on the nature of structural modifications, some derivatives have shown promising results in preclinical studies. Notably, (-)-cytisine itself has already been approved for the treatment of nicotine addiction [1,2]. Moreover, some studies proofed that this compound has shown neuroprotective properties [3,4], prompting investigations into its potential application in neurodegenerative disorders such as Parkinson's disease. Its ability to inhibit dopamine depletion levels in the striatal tissue and to enhance dopamine release in the striatum through its interaction with  $\alpha 4\beta 2^*$  and  $\alpha 6\beta 2^*$  receptors subtypes underlines its therapeutic promise [3,4]. Additionally, cytisine acts as an iron chelator, reducing hydroxyl radicals in the brain and protecting neurons from oxidative damage [3]. To date, dimeric cytisine derivatives such as 1,2-bis-N-cytisinylethane and 1,4-bis-N-cytisinylbutyne, have exhibited high affinity for nAChRs in both in vivo and in *vitro* studies, mimicking the activity of nicotine [5].

The shape and position of the bands in the UV-VIS absorption spectra of studied compounds very often change depending on the solvent. This effect is used in solvatochromic studies. Solvent influence is usually analyzed in terms of its polarity, determining the dependence of the position of the band maxima on a solvent polarity function. However, solvatochromic studies often concern compounds with complex structures. Molecules of these compounds contain numerous heteroatoms (e.g. N, O, S) or functional groups containing a protic hydrogen atom (e.g. –OH, –NH<sub>2</sub>, –NHR). They can form hydrogen bonds with protic and aprotic solvent molecules. The energy of solute-solvent hydrogen bond changes due to electron excitation. The consequence of this is that the measured absorption spectra are affected not only by the polarity of the solvent but also by its ability to form hydrogen bonds [6].

In this project, we report the synthesis of new (-)-cytisine derivatives containing conjugated triple bonds: N-propargylcytisine (1) and the dimer of N-propargyl-cytisine (2) (Fig.1) together with their solvatochromic studies. Due to the presence of N and O heteroatoms in the structure of these compounds, the effect on the UV-VIS spectra of solvent polarity and also the ability to form intermolecular hydrogen bonds with protic solvents was determined.



**Experimental:** (-)-Cytisine was isolated from the seeds of golden chain (or golden rain) tree (*Laburnum anagyroides*), according to the standard procedure [7,8]. Compounds **1** and **2** purification and preliminary ESI-MS analyses were performed using a CombiFlash Rf+Purion (Teledyne ISCO). ESI mass spectra were obtained on a Waters/Micromass (Manchester, UK) ZQ mass spectrometer (MassLynx V4.0). The FT-IR spectra of the compound were recorded on an IFS 113v FT-IR spectrophotometer (Bruker, Karlsruhe) equipped with a DTGS detector. The <sup>1</sup>H and <sup>13</sup>C NMR analyses were made on a Bruker ADVANCE device (151 MHz, CDCl<sub>3</sub>). UV-VIS absorption spectra were recorded on a Jasco V-650 spectrophotometer in a room temperature. The solvents: n-hexane (Sigma-Aldrich,  $\geq$ 97%), 1-chloro-*n*-butane (POCH,  $\geq$ 99.8%) and acetonitrile (chemsolve,  $\geq$ 99.9%) were additionally dried over a molecular sieve A3, methanol (chemsolve,  $\geq$ 99.85%) and water (POCH for HPLC) was used as received.



Science and industry - challenges and opportunities



**Results:** N-propargylcytisine (1) was synthesized in reaction between (-)-cytisine and propargyl bromide (CH<sub>2</sub>Cl<sub>2</sub>, yield 62%, mp. 108-110 °C). The compound is soluble in most solvents, which makes it suitable for the further reaction. The dimer of N-propargyl-cytisine (2) have been obtained in condition of Glaser-Hay reaction in acetonitrile (CuCl, TMEDA) with 70% yield (Fig.1). The structures of the synthesized compounds have been confirm using ESI-MS (Fig.2), FT-IR and NMR (Fig.3) spectroscopic methods.



Fig.4. Effect of solvent properties on the UV-VIS absorption spectra of N-propargylcytisine (1)  $(c\sim 10^{-5} \text{ M}, \text{ normalized spectra}).$ 

Novel compounds **1** and **2** have been characterized by spectroscopic methods as part of preliminary analyses. The UV-VIS absorption measurements were performed for solutions of **1** and **2** in a few solvents of different polarity, including two protic ones capable of hydrogen bonding. For both compounds studied, a blue-shift of the maximum of the long wavelength band in the absorption spectrum,  $\lambda^{max}$ , was observed with solvent polarity function,  $f(\varepsilon,n^2)$ , increasing (Fig.4, Table 1). The shift of the spectra recorded in methanol and water is significantly greater than in the other solvents, indicating the

solute-solvent complexes formation by intermolecular hydrogen bonding. The shape and position of the bands in the absorption spectra of **1** and **2** do not differ significantly from each other and are similar to the absorption spectra of (-)-cytisine [9,10]. The value of the molar absorption coefficient at the maximum of the long wavelength band,  $\varepsilon^{max}$ , for both compounds increases in solvents of higher polarity. The values of  $\varepsilon^{max}$  for **1** are similar to those obtained for (-)-cytisine [9,10]. Otherwise, **2** is characterized by  $\varepsilon^{max}$  values 1.7-1.9 times higher (Table 1). Due to the low solubility, the value of the molar absorption coefficient for **2** in n-hexane was not determined. Spectral absorption studies were performed for two concentrations of **1** and **2** in solution,  $c\sim10^{-5}$  M and  $c\sim10^{-6}$  M, to exclude aggregation of compound molecules. There was no significant effect of concentration on the results obtained.

Solvent	$f(\varepsilon,n^2)$	1		2	
		$\lambda^{max}$ [nm]	$\epsilon^{max} [M^{-1}cm^{-1}]$	$\lambda^{max}$ [nm]	$\epsilon^{max} [M^{-1}cm^{-1}]$
n-hexane	-0.0003	321	7100	321	-
1-chloro-n-butane	0.209	319	6950	319	12100
acetonitrile	0.304	317	7250	317	13300
methanol	0.309	309	8300	309	15800
water	0.320	305	8200	305	13800

**Table 1.** Effect of solvent properties on the spectral characteristic of 1 and 2.

 $f(\varepsilon,n^2)$  – Lipert-Mataga solvent polarity function,  $f(\varepsilon,n^2)=((\varepsilon-1)/(2\varepsilon+1))-((n^2-1)/(2n^2+1))$ , n – refractive coefficient,  $\varepsilon$  – relative permittivity

**Conclusions:** The synthesis of (-)-cytisine's derivative, N-propargylcytisine, was successfully carried out, followed by the formation of a novel symmetrical dimer through Glaser coupling reaction. The structures of obtained compounds were confirmed using spectroscopic techniques, including ESI-MS, FT-IR, NMR and UV-VIS. These analytical methods validated the structural integrity of the cytisine derivatives. The synthesized compound containing triple C-C bonds offer promising potential for pharmacological studies, particularly regarding their interaction with nAChRs and other biological activities. Further research is required to fully evaluate their pharmacological properties.

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## COMPARISON OF ANTIOXIDANT ACTIVITY OF HOMOVANILLIC ACID AND ITS SODIUM SALT: EXPERIMENTAL AND THEORETICAL STUDY

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**Abstract:** The changes in physical, chemical and biological properties of chemical compounds decide about their biological activity. In this paper, homovanillic acid and its sodium salt were investigated for their antiradical and antioxidant potency via computational methods and DPPH assay. The molecules were theoretically modeled using the B3LYP method and the 6-311++G(d,p) basis set. Parameters such as ionization potential, electron affinity, electronegativity, chemical hardness, and chemical softness were obtained. The mechanisms underlying the antioxidant properties were described using the parameters: BDE and IP, which were obtained by computational methods and related to experimental data.

**Introduction:** Free radicals, although they are necessary to life, excess amounts can shift the balance between oxidant and antioxidant statuses, causing oxidative stress. Reactive oxygen species cause oxidative damage to biomolecules, as well as organelles, which causes many civilization diseases, e.g. heart disease, diabetes, neurodegenerative diseases or cancer [1,2]. Phenolic compounds are important antioxidants that neutralize free radicals, which can effectively prevent these diseases [3]. Homovanillic acid or 4-hydroxy-3-methoxyphenylacetic acid (HVA) has been investigated by various researches because of its biological activities (anti-inflammatory, antioxidant and antiradical activity, act as neurotransmitter) [4,5]. Homovanillic acid (HVA) is a phenolic compound found in certain foods. For example, it can be present in small amounts in olive oil (both virgin and extra virgin) and olives (black and green) [6]. Additionally, it has been detected in beer and in raw and fermented hemp seeds [7,8]. Mihali et al. found homovanillic acid in fermented teas (known as kombucha). They found the highest level of HVA in Ecuadorian horchata tea, at 74.45 mg/100g dry weight [9]. There are reports of antioxidant and antiradical activity of homovanillic acid and its derivative, homovanillyl alcohol [4]. In this paper, reactivity and the antioxidant activity of homovanillic acid (HVA) (Fig.1) and its sodium salt (NaHV) were investigated using theoretical and experimental methods. The motivation to undertake the research presented in this paper was to search for more effective antioxidants and to understand the molecular mechanisms responsible for antioxidant processes. Metal cations can modify the biological properties, including antioxidant activity, of important natural ligands [3].



Fig.1. Structures of homovanillic acid.

**Experimental:** Homovanillic acid, sodium hydroxide, 2,2-diphenyl-1-picrylhydrazyl (DPPH), methanol were purchased from Sigma-Aldrich Co. and used without purification. Sodium homovanillate was prepared by dissolving the powder of homovanillic acid in the water solution of sodium hydroxide in a stoichiometric ratio according to the procedure described in [10]. The scavenging activity of tested compounds was determined using the stable DPPH radical according to the methodology described in [11]. All calculations were carried out using Gaussian 09W software package. The initial structure for calculations was structure of homovanillic acid and sodium homovanilliate presented by Samsonowicz et al. [10]. The B3LYP/6-311++G(d,p) hybrid Density Functional Theory (DFT) method was used to determine energy of radicals, radical anions and radical cations. The selected reactivity descriptors were calculated on the basis of the equations described in the literature [12]. In addition, BDE (bond dissociation enthalpy) and IP (ionization potential) of HVA and NaHV were calculated in gas phase (for 298.15 K and 1.0 atmospheric pressure). These parameters were calculated based on formulas given in the literature [5].

**Results:** The results of the antiradical activity assay (percentage of DPPH radical inhibition, IC<sub>50</sub>) are presented in Fig.2. NaHV showed better antiradical activity than the ligand alone. The IC<sub>50</sub> value for HVA is 32.93  $\mu$ M, while for NaHV it is 24.86  $\mu$ M. A lower IC<sub>50</sub> value for homovanillic acid was obtained by Tuck and Hayball [4] who studied the activity of HVA and its derivatives against the DPPH• radical, it was 14.8  $\mu$ M, which may be due to different assay conditions. Results of carried out calculations are presented in Table 1. The lower total energy value of NaHV compared to the acid alone indicates greater stability. On the other hand, the dipole moment in the case of the NaHV is larger than for the acid. The dipole moment can be an indicator of molecular reactivity - the strength of intermolecular interactions increases with the increase of the dipole moment [13], which suggests that NaHV is more active than the acid. The energy values of HOMO and LUMO molecular orbitals can be used to assess the bioactivity of molecules [14]. The energy of the HOMO orbital characterized the electron-donating character of the compound, while the energy of the LUMO orbital was related to the ability to accept electrons [14,15].



Fig.2. A comparison of the antioxidant activities of the HVA and NaHV measured by DPPH assay: (a) percentage of inhibition of DPPH• radicals by HVA and its Na salt depending on their concentration (0.03–0.0033 mM); (b) the IC<sub>50</sub> parameter value for HVA and NaHV.

Parameter	HVA	NaHV
Energy (Hartree *)	-650.08	-811.83
Dipole moment (Debye)	1.307	8.080
EHOMO (eV)	-6.018	-5.432
ELUMO (eV)	-0.719	-1.637
Energy gap (eV)	5.299	3.795
Ionization potential, I = -EHOMO (eV)	6.018	5.432
Electronaffinity, a = -ELUMO (eV)	0.719	1.637
Electronegativity, $\chi = \frac{1+A}{2}$ (eV)	3.369	3.534
Electronic chemical potential, $\mu = -\frac{J+A}{2}$ (eV)	-3.369	-3.534
Chemical hardness, $\eta = \frac{1-A}{2}$ (eV)	2.650	1.898
Chemical softness, $S = \frac{1}{2\pi} (eV)$	0.189	0.263
BDE (kJ/mol)	398.47	776.62
IP (kJ/mol)	374.85	671.34

Table 1. Chemical reactivity and thermodynamical parameters calculated for HVA and NaHVA.

\*1 Hartree = 2625.5 kJ/mol

Another stability parameter is the energy gap between HOMO and LUMO ( $\Delta E$ ).  $\Delta E$  is directly related to chemical softness (S) and hardness ( $\eta$ ), with soft molecules having smaller  $\Delta E$  values and hard molecules having larger  $\Delta E$  values. Soft molecules are generally less stable and more chemically reactive than harder ones [15]. NaHV shows a greater electron-accepting capacity than acid, and a smaller  $\Delta E$  value indicates a) higher salt activity b) greater softness of the molecule with respect to acid. NaHV can be classified as a soft molecule.

Some descriptors related to the ability of the tested compounds to scavenge free radicals were determined: BDE and IP. The parameter BDE (bond dissociation enthalpy) describes the ability to donate H atoms. It is used to estimate the reactivity of the molecule in the HAT mechanism. The ionization potential (related to the SET mechanism) describes the susceptibility of the molecule to ionization, and the lower the IP, the easier it is to remove an electron from the antioxidant. In general, low BDE and IP values indicate high antioxidant activity. The calculated parameters showed that NaHV has better antioxidant properties (lower BDE and IP values) than the acid.

**Conclusions:** Theoretical parameters for homovanillic acid and its sodium salt were calculated. Obtained values showed that NaHV is more reactive than acid alone and had better antioxidant properties (lower BDE and IP values) than the acid. This was confirmed by the test with the stable DPPH radical.

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# SPECTROSCOPIC AND THEORETICAL STUDIES OF ISO-VANILLIC ACID COMPLEXES WITH SODIUM, ZINC AND MANGANESE(II)

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**Abstract:** Vanillic acids are present in the plant world, they are derivatives of hydroxybenzoic acid and have a wide variety of bioactive and health-promoting properties. The aim of this study was to investigate the physicochemical properties of iso-vanillic acid and its complexes with selected metals. Metals that play a special role in biological systems, i.e.: manganese(II) and zinc, were selected for the study. The spectroscopic properties of the compounds were investigated using infrared spectroscopy (FT-IR) and electron absorption spectroscopy (UV/Vis), as well as quantum mechanical calculations using the Gaussian program.

**Introduction**: Vanillic acid (4-hydroxy-3-methoxybenzoic acid) is one of the natural derivatives of benzoic acid. It is found primarily in vanilla seeds, but its presence has also been identified in many other plants including blueberries, beetroot, onions, blackcurrant, olive, nuts, strawberries, potatoes and cereals [1]. Vanillic acid is synthesised in plants through the metabolism of acetic acid. It is responsible for the sour and bitter taste of some plant-based foods, and can also give them astringent properties [2]. Studies have reported that vanillic acid has therapeutic potential in the treatment of diabetes and obesity and against fungal and bacterial infections, and has also shown neuroprotective effects, potentially reducing cognitive impairment and oxidative stress associated with neurodegenerative diseases, and cardioprotective effects [3,4]. Besides, vanillic acid derivatives, e.g. vanillin, can be used to treat autoimmune diseases [5]. The aim of the present study was to investigate the physicochemical properties of iso-vanillic acid (3-hydroxy-4-methoxybenzoic acid) and its salts/complexes with sodium, zinc, manganese(II) using infrared spectroscopy (FT-IR), UV-Vis spectroscopy and quantum mechanical calculations. The Gaussian 09 program was used for the calculations [6].

**Experimental part:** The synthesis of iso-vanillic acid complexes with sodium, zinc and manganese(II) was carried out. 0.1g of acid each was weighed and stoichiometric amounts of a 0.1 mol/dm<sup>3</sup> NaOH solution (1:1 ratio) were added. The resulting solutions were placed in an ultrasonic bath at 60 °C to completely dissolve the acid. The sodium salt solutions were then mixed with metal chloride solutions (2:1 ratio respectively) and allowed to stand until the complexes precipitated. After precipitation, the compounds were filtered, washed and dried. The complexes were subjected to spectroscopic analysis. Samples for FT-IR spectroscopic studies were prepared in the form of KBr lozenges. The spectra were recorded on a Bruker Alpha Transmission FT-IR spectrophotometer in the range 4000-400 cm<sup>-1</sup>. Geometrical optimisation of 3-hydroxy-4-methoxybenzoic acid and its complexes with sodium, zinc and manganese(II) was performed using the DFT method. Calculations were performed using the B3LYP

method with the LANL2DZ basis in the Gaussian 09 program. Particle visualisations were performed in the Gauss View 09 program. Electron charge distribution and geometrical parameters were calculated for the optimised structures, and the shape of the HOMO and LUMO orbitals was visualised.

**Results**: Figure 1 shows the FT-IR spectra of 3-hydroxy-4-methoxybenzoic acid and its salts/complexes with selected metals.



Fig.1. FT-IR spectra of 3-hydroxy-4-methoxybenzoic acid and its complexes with sodium, zinc and manganese(II) recorded in the range 4000-400 cm<sup>-1</sup>.

The wave number values of selected bands present in the spectra are summarised in Table 1. The bands were assigned on the basis of own experiments and literature data [7]. In the spectrum of iso-vanillic acid, a broad band lying at a wave number of  $3415 \text{ cm}^{-1}$  is visible, which originates from the stretching vibration of the O-H group attached to the aromatic ring. This signal is not present in the spectrum of any of the iso-vanillic acid complexes. The signal at 2947 cm<sup>-1</sup> in the spectrum of iso-vanillic acid comes from the stretching vibration of the asymmetric C-H grouping (CH<sub>3</sub>), while in the spectra of iso-vanillates, it is present at higher wavelengths, viz. 2974 cm<sup>-1</sup> for sodium iso-vanillate, the band shifts towards lower wavelength numbers relative to iso-vanillic acid and lies at 2941 cm<sup>-1</sup>. The band originating from C-H symmetric stretching vibrations of the methyl group (CH<sub>3</sub>) in the spectrum of the acid occurs at 2849 cm<sup>-1</sup> and shifts

towards lower wavenumbers in the spectra of the complexes, in the range 2841-2843 cm<sup>-1</sup>. In the spectrum of iso-vanillic acid, there is a sharp band at 1688 cm<sup>-1</sup> originating from stretching vibrations of the carbonyl group v(C=O), which is not present in the spectra of the complexes.

Iso-vanillic acid	Sodium iso-vanillate	Zinc iso-vanillate	Manganese(II) iso-vanillate	Assignment
3415	-	-	-	v(OH) <sub>ar</sub>
2947	2974	2972	2941	vasCH (CH3)
2849	2843	2842	2841	v <sub>s</sub> CH (CH <sub>3</sub> )
1688	-	-	-	v(C=O)
1616	1610	1613	1614	8a
-	1543	1555	1553	$v_{as}(COO)$
-	1434	1427	1439	δ <sub>as</sub> CH <sub>3</sub>
1420	-	-	-	β(OH)
-	1396	1392	1358	$v_s(COO)$
1358	-	-	-	δCH <sub>3</sub>
-	1354	-	1342	$\delta_s CH_3$
1306	-	-	-	v(C-OH)
1273	1245	1272	1266	14
1225	1211	1222	1212	β(OH)
1184	1182	1181	1188	v(O-CH <sub>3</sub> )
1133	1102	1108	1107	18b
-	1025	1021	1025	δCH <sub>3</sub>
-	950	957	957	$\beta_{s}(COO^{-})$
1092	-	-	-	βOH
929	-	-	-	γOH
882	886	892	899	17b
826	841	804	816	10a
-	763	780	783	γ(COO)
-	718	723	715	1
722	-	-	-	$\beta$ (C=O)
630	-	-	-	$\gamma(C=O)$
-	636	643	636	$\beta_{as}(COO)$
-	534	564	540	6a
484	480	501	486	6b
Designations: vs - v	very strong; s - stro	ng; m - medium; w	- weak; vw - very weal	x; v - tensile vibration;

 Table 1. Wave number values [cm<sup>-1</sup>] bands in FT-IR spectra of iso-vanillic acid complexes and their salts/complexes with selected metals.

Designations: vs - very strong; s - strong; m - medium; w - weak; vw - very weak; v - tensile vibration;  $\beta$  - in-plane deformation vibration;  $\gamma$  - out-of-plane deformation vibration;  $\varphi(CC)$  - out-of-plane deformation vibration of the aromatic ring;  $\alpha(CCC)$  - in-plane deformation vibration of the aromatic ring; as - asymmetric vibration; s - symmetric vibration;

The disappearance of the band originating from the carbonyl group indicates that it is involved in the complexation reaction of iso-vanillic acid with sodium, zinc and manganese ions. In the spectra of these complexes, signals appear at 1543 cm<sup>-1</sup>, 1555 cm<sup>-1</sup> and 1553 cm<sup>-1</sup> (spectra of sodium, zinc and manganese iso-vanillinate, respectively), which originate from the asymmetric vibrations of the v<sub>as</sub>(COO<sup>-</sup>) deprotonated carboxyl group. The symmetric vibrations originating from the v<sub>s</sub>(COO<sup>-</sup>) carboxylate group in the spectra of iso-vanillic acid complexes correspond to signals lying in the range 1358-1396 cm<sup>-1</sup>. In the spectra of the complexes, bands from out-ofplane deformation vibrations v(COO<sup>-</sup>) give signals lying in the range 763-783 cm<sup>-1</sup> those originating from asymmetric out-of-plane deformation vibrations from the deprotonated carboxylate group  $\beta$ as(COO<sup>-</sup>), which lies in the wave number range 636-643 cm<sup>-1</sup>. By comparing the position of the bands originating from the asymmetric and symmetric stretching vibrations of the carboxylate group of the complexes and the sodium salt, it was possible to determine the coordination type of the metal. Zinc and manganese(II) iso-vanillates can be assigned a single-position coordination type. Figure 2 shows the UV spectroscopic spectra of iso-vanillic acid and its salts/complexes with sodium, manganese(II) and zinc.



Fig.2. UV spectra of iso-vanillic acid and complexes with Na, Mn(II) and Zn in ethanol  $(C=10^{-4} \text{ mol/dm}^3)$ .

In the UV spectra of: iso-vanillic acid the absorption maxima lie at  $\lambda_{max} = 294$ , 261, 218 and 206 nm, sodium iso-vanillinate  $\lambda_{max} = 291$ , 250 nm, with zinc  $\lambda_{max} = 293$ , 254, 207 nm, while manganese(II) iso-vanillinate  $\lambda_{max} = 292$ , 252, 207 nm. Hypsochromic shifts are observed in the spectroscopic spectrum of sodium iso-vanillate, manganese and zinc. Which may be due to disruption of conjugation or planarity and disruption of electron delocalisation in the ligand during coordination.

Using the GaussView program, the HOMO and LUMO orbitals were also visualised (Fig.3) and the energy difference  $\Delta E$  between these orbitals was calculated for each compound. A smaller energy difference between the HOMO and LUMO boundary orbitals indicates greater reactivity and stronger antioxidant activity [8].





Fig.3. Molecular orbital shapes for isovanillic acids and their complexes.

In the case of iso-vanilic acid complexes with sodium, zinc and manganese(II) ions, a lower energy difference was observed between the HOMO LUMO orbitals, relative to the free ligand (Table 2). This implies that the complexes should exhibit higher reactivity and stronger antioxidant activity.

**Table 2.** Energy differences ( $\Delta E$ ) between the HOMO and LUMO orbitals of vanillic, iso-vanillic and orthovanillic acids and their complexes with sodium, zinc and manganese.

Compound	HOMO [eV]	LUMO [eV]	$\Delta E [eV]$
Iso-vanillic acid	-0.2313	-0.0601	0.1712
Sodium iso-vanillate	-0.2020	-0.0371	0.1649
Zinc iso-vanillate	-0.2333	-0.0682	0.1651
Manganese iso-vanillate	-0.2093	-0.0588	0.1505

**Conclusions:** Spectroscopic analysis confirmed the correctness of the synthesis of the complexes. A carboxyl group was found to be involved in the coordination of the metal. The type of single-position coordination was established for zinc and manganese(II) isovanillates. Hypochromic shifts of the bands in the UV-Vis spectra were observed for all iso-vanillic acid complexes studied. Quantum chemistry calculations indicate that complexation increases reactivity and stronger antioxidant activity.

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# STRUCTURES AND REACTIVITY OF VITAMIN C, TROLOX AND GLUTATHIONE – THEORETICAL MODELING

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**Abstract:** In this work, theoretical structures of glutathione, vitamin C and trolox were modelled using the DFT method (B3LYP/6-311++G(d,p)). Modelling of structures was carried out for molecules in the gas phase, water phase and ethanol phase. The CPCM computational model was used for selected solvents. For the optimised structures, the energy values of the frontal orbitals HOMO and LUMO and reactivity descriptors (EGAP, electronegativity, softness, hardness of the molecule) were calculated. The electrostatic potential distribution was calculated and presented using ESP distribution maps. Based on the calculations performed, the reactivity of the tested molecules was assessed. All calculations were performed using Gaussian 09 software.

**Introduction:** In the antioxidant processes in the human body, an important role is played by antioxidant enzymes, vitamins and other compounds capable of removing free radicals [1]. A well-chosen and balanced diet provides the body with chemical compounds that support antioxidant processes. Vitamin C is a natural antioxidant that performs a many of protective functions in the body [2]. Trolox is a derivative of vitamin E, which has a strong antioxidant character. Glutathione is a tripeptide with antioxidant properties, composed of three amino acid residues of glutamic acid, cysteine and glycine. It occurs naturally in the body as an oxidation-reduction system that protects the -SH groups of proteins from oxidation. The study compared the reactivity of trolox, vitamin C and glutathione by theoretical modelling of the analysed molecules and calculating their reactivity parameters related to the energy of the frontal orbitals HOMO and LUMO.

**Experimental:** he structures of vitamin C, trolox and glutathione were calculated using the B3LYP/6-311++G(d,p) method. The electrostatic potential (ESP) distribution maps were calculated using the SCF method for optimised structures calculated in the B3LYP/6-311++G(d,p) method. The energy of HOMO and LUMO orbitals were calculated using the B3LYP/6-311+G(d,p) method. On the basis of the obtained HOMO and LUMO orbitals energy values, other reactivity descriptors, such as energy gap, ionization potential, electron affinity, electronegativity, chemical potential, hardness and softness, and electrophilicity index were calculated.

**Results:** The structure of vitamin C was calculated using the B3LYP/6-311++G(d,p) method in the gas phase and using 2 solvent models – water and ethanol. The energies of the frontal orbitals HOMO and LUMO and reactivity descriptors were calculated for the optimized molecules. The shapes of the HOMO and LUMO orbitals are presented in Fig.1, while the values of the frontal orbital energies and reactivity descriptors are listed in Table 1.

Energy parameters calculated for theoretically modeled molecules in the gas phase and with the solvent taken into account show differences. It was observed that the differences in the obtained values of energy parameters in the computational models in the water and ethanol phases are insignificant.



Fig.1. Shapes of frontal orbital of vitamin C calculated in gas phase, water, ethanol phase in B3LYP/6-311++G(d,p) method.

Table 1. Reactivity descriptors for vitamin C modeled by the B3LYP/6-311++G(d,p)
method in different solvents.

Reactivity descriptor	Gas phase	Water phase	Ethanol phase
Ionisation potential I=-EHOMO	8.6703	8.5560	8.5568
Electron affinity A=-ELUMO	4.4953	4.4204	4.4193
Electroegativity X=(I+A)/2	6.5828	6.4882	6.48805
Chemical potential µ=-(I+A)/2	-6.5828	-6.4882	-6.48805
Chemical hardness η=(I-A)/2	2.0875	2.0678	2.06875
Chemical softness S=1/(2n)	0.2395	0.2418	0.2416
Electrophilicity index $\omega = \mu 2/2\eta$	10.37922	10.17911	10.17397

Figure 2 shows the shapes of the frontal orbitals modeled for the trolox molecule, while Table 2 summarizes the energy values of the frontal orbitals and the values of the reactivity descriptors for trolox. Figure 3 shows the shapes of the frontal orbitals modeled for the glutathion molecule, while Table 3 summarizes the energy values of the frontal orbitals and the values of the reactivity descriptors for glutathion.



Fig.2. Shapes of frontal orbital of trolox calculated in gas phase, water, ethanol phase in B3LYP/6-311++G(d,p) method.

Reactivity descriptor	Gas phase	Water phase	Ethanol phase
Ionisation potential I=-E <sub>HOMO</sub>	8.6703	8.5291	8.5571
Electron affinity A=-E <sub>LUMO</sub>	4.9189	4.9149	4.9149
Electroegativity X=(I+A)/2	6.7946	6.7220	6.7360
Chemical potential µ=-(I+A)/2	-6.7946	-6.7220	-6.7360
Chemical hardness η=(I-A)/2	1.8757	1.8071	1.8211
Chemical softness S=1/(2))	0.266567	0.276686	0.274559
Electrophilicity index $\omega = \mu^2/2\eta$	12.3065	12.50215	12.45777

Table 2. Reactivity descriptors for trolox modeled by the B3LYP/6-311++G(d,p) method in different solvents.

The most important orbitals in a molecule are the frontier molecular orbitals (Highest Occupied Molecular Orbitals-HOMOs, and Lowest Unoccupied Molecular Orbitals-LUMOs). These orbitals determine the way the molecule interacts with other species. The frontier orbital gap helps to characterise the chemical reactivity and kinetic stability of the molecule. The soft systems have small HOMO–LUMO gap, and highly polarisable [3]. A large HOMO–LUMO gap implies high molecular stability and aromaticity low reactivity in chemical reactions while a small HOMO–LUMO gap is related to antiaromaticity [4]. On the basis of the energy values of the HOMO and LUMO orbitals, descriptors related to the reactivity of the molecules were calculated. Among the molecules tested, the glutathione molecule has the lowest energy GAP value (E<sub>LUMO</sub>-E<sub>HOMO</sub>), while the vitamin C molecule has the highest GAP energy. Glutathione is c haracterized by the highest reactivity (it has the highest softness and the lowest chemical hardness value).



Fig.3. Shapes of frontal orbital of glutathion calculated in gas phase, water, ethanol phase in B3LYP/6-311++G(d,p) method.

Table 3. Reactivity descriptors for glutathiom modeled by the B3LYP/6-311++G(d,p) method in different	nt
solvents	

Reactivity descriptor	Gas phase	Water phase	Ethanol phase
Ionisation potential I=-EHOMO	4.2567	5.2202	5.2181
Electron affinity A=-ELUMO	3.7443	3.7481	3.7492
Electroegativity X=(I+A)/2	4.0005	4.4842	4.4836
Chemical potential µ=-(I+A)/2	-4.0005	-4.4842	-4.4836
Chemical hardness η=(I-A)/2	0.2562	0.7361	0.7344
Chemical softness S=1/(2ŋ)	1.9516	0.6793	0.6808
Electrophilicity index $\omega = \mu 2/2\eta$	31.2337	13.6589	13.6859

**Conclusions:** Theoretical studies (energy of HOMO, LUMO) have shown that glutathione is a molecule characterized by higher reactivity than vitamin C and trolox. It may exhibit higher antioxidant capacity than some vitamins.

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# SYNTHESIS AND SPECTROSCOPIC ANALYSIS OF HETERONUCLEAR Pd<sup>II</sup>-Tm<sup>III</sup>-Pd<sup>II</sup> COMPLEX

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Abstract: The new heteronuclear complex of Pd<sup>II</sup>–Tm<sup>III</sup>–Pd<sup>II</sup> was obtained in the reaction of N<sub>2</sub>O<sub>4</sub>-donor Schiff base ligand (H<sub>4</sub>Sch =  $C_{17}H_{18}N_2O_4$ ) with (C<sub>6</sub>H<sub>5</sub>CN)<sub>2</sub>PdCl<sub>2</sub> and  $Tm(NO_3)_3$ ·5H<sub>2</sub>O dissolved in methanol and characterized by various physicochemical methods. The composition of the complex was determined based on spectroscopic (FTIR) and thermal analysis (TG/DSC, elemental. TG-FTIR). Additionally susceptibility measurements in the range of 2–300 K were carried out. Based on the results of elemental analysis, spectroscopic studies and thermal analysis, it found that the complex was obtained with the formula was  $[TmPd_2(H_2L)_2(NO_3)]$ ·MeOH·2H<sub>2</sub>O. It seems that all donor atoms of the ligand participate in the coordination of the metal ions. The complex is stable at room temperature and its thermal decomposition in the air proceed stepwise. Magnetic measurements reveal antiferromagnetic interactions between central ions.

Introduction: In recent years the heteronuclear complexes of salen type ligands have become one of the most popular research topics. Much of the interest stems from the fact that they are present in many biological systems and find applications in organic synthesis and chemical catalysis, modern technology, medicine, pharmaceuticals and analytics. Their structure and physicochemical properties depend on conditions of synthesis, type of metal ions and solvents, the nature of the ligands, the ratio of reagents used in the synthesis [1-5]. For example the slow relaxation of the magnetization in 3d-4f compounds mainly originates from the ligand-field effect of the lanthanide ions and the exchange couplings between lanthanide ions and transition-metal ions are used in high-density information storage devices [6]. The 3d-4f complexes are also used to promote phosphodiester hydrolysis using the RNA mimic substrate [7]. Studies have shown that they can also be used as a potential biomedical utility (as multimodal cellular probes and drug storage) [8]. In the coordination compounds formed by N,O-donor ligands d and f metal ions are captured simultaneously, and linked together through two O<sub>phenol</sub> atoms (Fig.1). Lanthanide(III) ions behave as hard acids and prefer oxygen to nitrogen donors, whereas 3d metal ions may coordinate to both nitrogens and oxygens [9-12]. As a continuation of investigation on salen type Schiff base complexes herein, we report synthesis, spectral, thermal and magnetic properties of heteronuclear Pd<sup>II</sup>–Tm<sup>III</sup>–Pd<sup>II</sup> complex.

**Experimental:** Heteronuclear  $Pd^{II}-Tm^{III}-Pd^{II}$  [TmPd<sub>2</sub>(H<sub>2</sub>L)<sub>2</sub>(NO<sub>3</sub>)]·MeOH·2H<sub>2</sub>O complex was synthesised in the manner described below. To the stirred solution of the 0.4 mmol of H<sub>4</sub>Sch = C<sub>17</sub>H<sub>18</sub>N<sub>2</sub>O<sub>4</sub> Schiff base dissolved in 40 cm<sup>3</sup> of methanol were added dropwise solutions 0.4 mmol of (C<sub>6</sub>H<sub>5</sub>CN)<sub>2</sub>PdCl<sub>2</sub> dissolved in 10 cm<sup>3</sup> of

methanol. The mixture was stirred and heated for about 30 minutes. After this time 0.2 mmol of  $Tm(NO_3)_3 \cdot 5H_2O$  in 5 cm<sup>3</sup> of methanol was added to the reagents. The resulting mixture was stirred for another 30 min (Fig.2).



Fig.1. Examples of the way how central ions are linked in heteronuclear complexes 3d-Ln<sup>III</sup>.

The contents of carbon, hydrogen and nitrogen were determined by elemental analysis using a CHN 2400 Perkin-Elmer analyzer. The amounts of Pd(II) and Tm(III) were established by X-ray fluorescence XRF method with the use of spectrophotometer of X-ray fluorescence with energy dispersion EDXRF-1510 (Canberra-Packard). The FTIR spectra of complex and the free Schiff base (H<sub>4</sub>Sch) were recorded over the range of 4000–500 cm<sup>-1</sup> using the Nicolet 6700 FTIR spectrometer equipped with a universal ATR attachment with a ZnSe crystal.



Fig.2. Scheme of the heteronuclear Pd<sup>II</sup>–Tm<sup>III</sup>–Pd<sup>II</sup> complex synthesis.

The thermal stability and decomposition of the complex was studied in an air using a Setsys 16/18 (Setaram) TG, DTG and DSC instrument. The experiments were carried out under air flow rate of 20 mL min<sup>-1</sup> in the range of 293–1173 K at a heating rate of 10 Kmin<sup>-1</sup>. The sample was heated in  $Al_2O_3$  crucibles. The TG–FTIR measurement of heteronuclear complex was performed to identify its gaseous decomposition products on the Q5000 TA apparatus coupled with the Nicolet 6700 spectrophotometer. The experiment was carried out under a dynamic nitrogen atmosphere in flowing nitrogen of 20 mL min<sup>-1</sup> in open platinum crucibles. The complex was heated up to 1073 K at a heating rate of 20 K min<sup>-1</sup>. The gaseous decomposition products were analyzed over

the range of 4000–400 cm<sup>-1</sup> using the Nicolet 6700 spectrophotometer. Magnetic susceptibility of polycrystalline sample was investigated at 2–300 K. The measurements in the range of 2–300 K were carried out with the use of Quantum Design SQUID–VSM magnetometer at magnetic field 0.1 T. The superconducting agent may generally operate at a field strength ranging from 0 to 7 T. The SQUID magnetometer was calibrated with the palladium rod sample.

**Results:** The IR spectra of the ligand (H<sub>4</sub>Sch) and its complex were recorded to confirm the complexation process (Fig.3). The strong, sharp band of the azomethine group vibration, v(C=N) is observed at 1636 cm<sup>-1</sup>. This band, due to a decrease in electron density on the nitrogen atom, is shifted to 1609 cm<sup>-1</sup> which confirms the coordination of Pd<sup>II</sup> centres *via* nitrogen atoms. In the spectrum of H<sub>4</sub>Sch, the strong band at 1213 cm<sup>-1</sup> is assigned to the phenolic stretching vibration v(C-O). This band in the spectrum of the complex is shifted towards higher frequencies (1218 cm<sup>-1</sup>), which indicates that oxygen atoms coordinate to metal ions. In addition, the new broad bands at 3400-3100 cm<sup>-1</sup> in the spectrum of the analysed complex are evidence for the presence of solvent molecules, which is in good agreement with the results of the elemental analysis and thermogravimetric data.



Fig.3. Fragment of FTIR spectrum of the complex Pd<sup>II</sup>-Tm<sup>III</sup>-Pd<sup>II</sup>.

The thermal stability of complex was studied in air at 293–1173 K. During the heating,  $[TmPd_2(H_2L)_2(NO_3)]$ ·MeOH·2H<sub>2</sub>O decomposes in a few steps. The complex is first desolvated in a single step and then gradually decomposed, finally forming the mixture of PdO and  $Tm_2O_3$  oxides. The combustion of the organic ligand is accompanied by a significant exothermic effect, as seen in the DSC curves. The TG–FTIR coupled technique was applied to identify its gaseous decomposition products. The heating of Pd<sup>II</sup>–Tm<sup>III</sup>–Pd<sup>II</sup> complex leads to the release of solvent molecules up to about 393 K. The FTIR spectrum shows characteristic bands in the regions: 4000–3600 and 1700–1400 cm<sup>-1</sup>. Next, the intensity of gases evolved during heating increases. It is connected with the great release of CO<sub>2</sub> molecules. The FTIR spectra show bands at 3800–3500

 $\rm cm^{-1},\ 2400-2300\ \rm cm^{-1}$  and at about 900  $\rm cm^{-1}$  due to stretching and deformation vibrations of carbon dioxide.

The magnetic susceptibility values decreased with increasing temperature. It suggests a weak antiferromagnetic interaction between metal centres. The dependences of magnetic susceptibility,  $\chi_m^{corr}$ , its reciprocal values and also  $\chi_m^{corr} \cdot T$  worths as a function of temperature are presented in Fig.4.



**Fig.4.** Dependence of  $\chi_m^{corr-1}$  and  $\chi_m^{corr} T$  values vs. T for Pd<sup>II</sup>–Tm<sup>III</sup>–Pd<sup>II</sup> complex.

**Conclusions:** The new heteronuclear complex  $[\text{TmPd}_2(\text{H}_2\text{L})_2(\text{NO}_3)]$ ·MeOH·2H<sub>2</sub>O crystallizes as solvate containing methanol and water molecules. The complexation process is confirmed by the shift of the band characteristic of the vibrations of the azomethine group v(C=N) in the spectrum of the complex and the appearance of the new bands, characteristic of the M–O, v(M–O) and M–N, v(M–N) vibrations, compared to the H<sub>4</sub>Sch ligand spectrum. During heating in air the complex is decomposed in few steps. It first undergoes a desolvation process, releasing all the solvent molecules and forming an anhydrous compound, which is then decomposed to form the mixture of corresponding the metal oxides. The desolvation process was confirmed by FTIR analysis of the gaseous decomposition products. The interpretation of the FTIR spectrum of the volatile components of the mixture released during the decomposition of the studied complex reveals that CO<sub>2</sub>, CO and hydrocarbons are also released during heating to 1173 K.

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# SPECTROSCOPIC STUDIES OF NOVEL COPPER(II) PHENOXYACETYLTHIOSEMICARBAZIDE DERIVATIVES

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Abstract: In the direct reaction of 1-(4-bromophenyl)-4-(phenoxy)acetylthiosemicarbazide  $(C_{15}H_{14}N_{3}O_{2}SBr)$  $(L^{1}),$ 1-(3-chlorophenyl)-4-(phenoxy)acetylthiosemicarbazide  $(C_{15}H_{14}N_{3}O_{2}SCl)$  $(L^2),$ 1-(3-bromophenyl)-4-(phenoxy)acetylthiosemicarbazide  $(C_{15}H_{14}N_3O_2SBr)$  $(L^3),$ 1-(3-iodophenyl)-4-(phenoxy)acetyl thiosemicarbazide ( $C_{15}H_{14}N_3O_2SI$ ) (L<sup>4</sup>) with copper(II) ion the four new complexes with the general formulas  $[Cu(Ac)_2L^1]$  (1),  $[Cu(Ac)_2L^2]$  (2),  $[Cu(Ac)_2L^3]$  (3) and  $[Cu(Ac)_2L^4]$  (4) were obtained. The compounds were characterized by various physicochemical measurements, such as: elemental analysis, IR spectra, X-ray fluorescence XRF method and thermal analysis. As can be seen, the compounds were obtained as solvent-free complexes. They are stable at room temperature and decompose to CuO when heated in an air atmosphere.

Introduction: The thiosemicarbazide scaffold is privileged in medicinal chemistry. Their derivatives exhibit a wide range of activities such as: anticancer [1,2], antimicrobial [3,4], antiviral [5,6] and anticonvulsant [7,8]. The progress of science and the development of modern technologies enable the search for new compounds with the desired biological activity. The only problem is the number of new chemical substances that meet strictly defined criteria of a potential drug (Lipinski's rule). Introducing additional functional groups to the basic structure, the exchange of its substituents, changes within the aromatic ring and any changes in physicochemical properties can increase the activity of the compound, reduce toxicity and improve pharmacokinetic parameters. This is exemplified by introducing a phenoxy group into the thiosemicarbazide system. The newly obtained compounds were characterized by increased anticancer activity, especially in relation to melanoma [9-11]. Coordination of metal ions to form an organometallic compound is another way of obtaining a compound with the desired properties [12]. The complexation process can significantly increase the biological activity of the compound, especially anticancer [13]. In the literature, there are examples of copper(II) compounds showing high redox activity, which makes them highly cytotoxic compared to the free ligand [14]. However, the number of works devoted to the complexation reaction of thiosemicarbazide derivatives is limited. Therefore, coordination of copper(II) ion via the thiosemicarbazide seems to be a promising modification in the perspective of searching for new therapeutic substances. Derivatives of 4-phenoxyacetylthiosemicarbazide were selected for the study, which were synthesized as compounds with promising anticancer activity against melanoma and prostate cancer cell lines [9]. The aim of the study was to optimise the synthesis of

copper(II) ion complexes with phenoxyacetylthiosemicarbazide derivatives and to characterise them using various physicochemical measurements such as: elemental analysis, IR spectra, X-ray fluorescence method XRF and thermal analysis.

**Experimental:** For the studies on the synthesis of Cu(II) complexes, 4 ligands from the 4-phenoxyacetylthiosemicarbazide derivatives were group of selected: 1 - (4 bromophenyl)-4-(phenoxy)acetylthiosemicarbazide  $(C_{15}H_{14}N_{3}O_{2}SBr)$  $(L^{1}),$ 1 - (3 - )chlorophenyl)-4-(phenoxy)acetylthiosemicarbazide  $(C_{15}H_{14}N_{3}O_{2}SCl)$  $(L^2).$ 1 - (3 - $(L^3),$ bromophenyl)-4-(phenoxy)acetylthiosemicarbazide  $(C_{15}H_{14}N_{3}O_{2}SBr)$ 1 - (3 iodophenyl)-4-(phenoxy)acetyl thiosemicarbazide ( $C_{15}H_{14}N_3O_2SI$ ) (L<sup>4</sup>). The ligands are differ in the position and type of substituent in the benzene ring, which can affect the physical and chemical properties of the resulting compounds. These compounds were obtained by the method described earlier [9].All complexes 1-4 were synthesised in the same manner described below. To the stirred solution of the appropriate ligand was added dropwise solution of copper(II) acetate in methanol (15 mL) and heated for 3.5 h. The molar ratio metal(II): ligand was equal 1:1. The contents of carbon, hydrogen and nitrogen were determined by elemental analysis using a CHN 2400 Perkin-Elmer analyser (Table 1). The amounts of Cu(II) was established by X-ray fluorescence XRF method with the use of spectrophotometer of X-ray fluorescence with energy dispersion EDXRF-1510 (Canberra-Packard). The ATR-FTIR spectra of complexes and the free ligands were recorded over the range of  $4000-500 \text{ cm}^{-1}$  using the Nicolet 6700 FTIR spectrometer equipped with a universal ATR attachment with a ZnSe crystal. The thermal stability and decomposition of the complexes were studied in an air using a Setsys 16/18 (Setaram) TG, DTG and DSC instrument. The experiments were carried out under air flow rate of 20 mL min<sup>-1</sup> in the range of 297–973 K at a heating rate of 10 K min<sup>-1</sup>. The samples were heated in Al<sub>2</sub>O<sub>3</sub> crucibles. The TG-FTIR measurements were performed to identify gaseous decomposition products on the Q5000 TA apparatus coupled with the Nicolet 6700 spectrophotometer. The experiments were carried out under a dynamic nitrogen atmosphere in flowing nitrogen of 20 mL min<sup>-1</sup> in open platinum crucibles. The complexes were heated up to 973 K at a heating rate of 20 K min<sup>-1</sup>. The gaseous decomposition products were analyzed over the range of 4000– 400 cm<sup>-1</sup> using the Nicolet 6700 spectrophotometer.

	%	С	% H		% N		% Cu	
Complex	calcd.	found	calcd.	found	calcd.	found	calcd.	found
$[Cu(Ac)_2L^1]$	40.61	40.38	3.59	2.32	7.48	9.39	11.31	15.60
$[Cu(Ac)_2L^2]$	44.10	45.04	3.90	2.70	8.12	10.41	12.28	17.20
$[Cu(Ac)_2L^3]$	40.61	40.12	3.59	2.39	7.48	9.37	11.31	15.5
$[Cu(Ac)_2L^4]$	37.48	37.00	3.31	2.18	6.90	8.61	10.44	12.8

Table 1. Results of elemental and XRF analyses for the studied complexes.

**Results:** The four novel Cu(II) complexes were obtained as a blue-green crystaline powders. Despite many attempts, it has not been possible to obtain single crystals of analyzed compounds. Solubility studies of the complexes in solutions: DMSO, DMF, MeOH and EtOH, showed that they belong to the class of compounds with very low solubility. The chemical formulas of complexes were established on the basis of elemental, X-ray fluorescence (XRF) and thermal analysis (Table 1). In the FTIR spectra

of the ligand the bands characteristic of the NH groups are observed at around 3200 cm<sup>-1</sup>. In the complexes spectra, these bands are significantly reduced and shifted. The sharp and intense bands originating from the C=O carbonyl group are at ~1700 cm<sup>-1</sup> while the C=S group vibration peaks occur at of 1219 cm<sup>-1</sup>. In the spectra of the complexes, the carbonyl group bands are less intense, while those of the C=S group remain intense but slightly shifted to 1215 cm<sup>-1</sup>. This indicates that the oxygen and nitrogen atoms of the C=O and NH groups are most likely to coordinate the copper(II) ions. Such coordination mode has already been described in the literature for a similar complexes based on Cu(II) ions [15]. The presence of the carboxylate groups COO<sup>-</sup> in the structure of the complexes is confirmed by two bands  $v_{as}(COO<sup>-</sup>)$  and  $v_s(COO<sup>-</sup>)$  at around 1540 cm<sup>-1</sup> and 1370 cm<sup>-1</sup>, 1330 cm<sup>-1</sup>, respectively. Thermal behaviour of complexes 1-4 was studied with the use of TG/DSC techniques (Fig.1).



Fig.1. TG, DTG and DCS curves of 3 in air.

The obtained compounds are stable at room temperature. The thermal decomposition of complexes takes place in several stages and is associated with defragmentation and combustion of the ligands. The decomposition process is connected with exothermic effect seen on the DSC curves. In order to identify the gaseous decomposition products of Cu(II), the coupled TG-FTIR technique was used to analyse the complexes (Fig.2). The pyrolysis of complexes is associated with the release of the following molecules: carbon oxides (CO<sub>2</sub> and CO), ammonia, hydrazine, water, phenol, sulphur oxygen(IV), aniline and carbon disulphide. The final solid product formed when the complexes decompose in air is CuO.



Fig.2. The FTIR spectrum of gaseous products evolved during the decomposition of compound 2.

**Conclusions** The complexation reaction conditions (molar ratio of ligand : metal ion, type of solvent, heating time, temperature) were optimised based on differences in ligand solubility, which may be related to the type and position of the substituent in the benzene ring. The analysed complexes show the similar solid state FTIR spectra. The coordination of copper(II) ions takes place via the oxygen and nitrogen atoms of phenoxyacetylthiosemicarbazide derivatives and the carboxylate group of acetate ions.

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# INCREASING BIOLOGICAL ACTIVITY OF LIGAND BY COMPLEXING WITH METAL IONS OR CYCLODEXTRIN – SPECTRAL, ANTIOXIDANT AND MICROBIOLOGICAL STUDIES

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**Abstract:** The effect of metal ions and the complexation with  $\beta$ -cyclodextrin ( $\beta$ CD) on the increase in the biological properties of ligand (syringic acid, Syr) were studied. Na(I) salt and Mn(II) complex with syringic acid (NaSyr; MnSyr) as well as  $\beta$ -cyclodextrin complexes with NaSyr and MnSyr (NaSyr| $\beta$ Cd; MnSyr| $\beta$ Cd) were synthetized and their structures were studied by means of FT-IR, UV/Vis methods. The antioxidant (in the ABTS assays) and antimicrobiological (against *Escherichia coli, Bacillus subtilis, Candida albicans*) properties of these compounds were discussed. The results suggested that complexation with metal ion and  $\beta$ -cyclodextrin may increase the biological properties of ligand.

**Introduction:** Syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid, Syr) is a phenolic compound found naturally in many plants, some fungi species and food product (honey, wine, olive). Syringic acid exhibits important pro-health properties i.e. antioxidant, antimicrobial, anti-inflammatory, anti-cancer, hepatoprotective, anti-diabetic, antiinflammatory, neuroprotective, cardio-protective, antiendotoxic activity which were in details described in [1]. Syringic acid is applied in industry as an ingredient of dental cements, photocatalytic ozonation agent, in bioremediation process and laccase based catalysis. The chemical structure of syringic acid determines its biological activity. The presence of two methoxy groups attached to the aromatic ring at the positions 3 and 5 as well as a hydroxy moiety at the position 4 cause strong antioxidant properties of Syr – stronger than 4-hydroxybenzoic acid and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapic acid) [2]. Therefore Syr has high application potential in the treatment of diseases associated with oxidative stress. The main problems with industrial application of Syr are to its relatively low solubility in water and sensitivity to environmental factors such as: oxygen, temperature, light, pH. For this reason, there is a need to develop new formulations of phenolic compounds with improved water solubility and even enhanced biological activity. In this paper sodium salt and manganese complex with Syr (NaSyr; MnSyr) and the  $\beta$ -cyclodextrin complex with Syr (Syr| $\beta$ Cd) and NaSyr (NaSyr| $\beta$ Cd) and MnSyr $|\beta$ Cd) were synthesized and studied for the antioxidant and antimicrobial properties.

**Experimental:** Sodium salt and mangnesium(II) complex of syringic acid were synthesized according to the method described in [3]. The formulas of the compounds were established on the basis of the elemental analysis, i.e.  $NaC_9H_9O_5 \cdot 1H_2O_5$ ;

Mn(C<sub>9</sub>H<sub>9</sub>O<sub>5</sub>) and was confirmed by the FT-IR analysis. The solid complexes of Syr, NaSyr and MnSyr with  $\beta$ -cyclodextrin were synthesized according to the method described in [3]. The correctness of the synthesis was assessed on the basis of UV/Vis (registered by the use of the spectrophotometer MACHERY-NAGEL NANOCOLOR Vis) and FT-IR spectra. The FT-IR spectra were recorded for the solid samples in KBr matrix pellets with an Alfa Bruker spectrometer (Bremen, Germany) within the range of 400–4000 cm<sup>-1</sup> with the resolution of 2 cm<sup>-1</sup>. The methodology of the antioxidant assay with 2,2'-azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS<sup>++</sup>) was described in [3]. The methodology of the antimicrobial study was presented in [4].

**Results:** In Fig.1 the exemplary spectra of FT-IR Syr and NaSyr were shown. Disappearance of selected bands in the spectra of NaSyr and MnSyr assigned to the vibrations of the carboxylic group (i.e. stretching vibrations of C=O at 1699 cm<sup>-1</sup> (v(C=O)), deforming vibration of -OH ( $\beta$ (OH)) at 1419 cm<sup>-1</sup>), which were present in the spectrum of the acid, indicated the formation of the carboxylate anion and the ability to coordinate metal ions by this group.



Fig.1. The FT-IR spectra of (a) Syr and (b) NaSyr.

Moreover, in the spectrum of NaSyr and MnSyr bands assigned to the vibrations of the carboxylate anion v(COO<sup>-</sup>) occurred at 1558 and 1535 cm<sup>-1</sup> – asymmetric stretching vibrations and 1393 and 1410 cm<sup>-1</sup> – symmetric stretching vibrations. It is also possible that metal ions were coordinated by the methoxy -OCH<sub>3</sub> and hydroxy -OH substituents attached to the aromatic ring, because the bands assigned to the vibrations of the above mentioned bands were clearly shifted comparing the spectra of Syr and metal syringates (especially bands assigned to the stretching vibrations of the -OH attached to the aromatic ring v(O-H) at 3376 cm<sup>-1</sup>, stretching vibrations v(C-OH) at 1372 cm<sup>-1</sup>, v(C-O-C) at 1322 cm<sup>-1</sup>, v(O-CH<sub>3</sub>) at 1113 cm<sup>-1</sup>). The FT-IR spectra of  $\beta$ CD, Syr| $\beta$ Cd, NaSyr| $\beta$ Cd (Fig.2) and MnSyr| $\beta$ Cd were registered to confirm the formation of inclusion complexes in the solid state.



Fig.2. The FT-IR spectra of (a)  $\beta$ Cd, (b) Syr $\beta$ Cd and (c) NaSyr $\beta$ Cd.

In the spectrum of  $\beta$ CD the characteristic bands occurred at 3386, 2925, 1643, 1157, 1079, 1028, 947, 860 cm<sup>-1</sup> assigned to the symmetric and asymmetric stretching of v(O-H),  $v(CH_2)$ , v(C-C), the bending vibration of  $\beta(OH)$  and the skeletal vibrations involving ( $\alpha$ -1,4 linkage), respectively. In the spectra of inclusion complexes the intensity of the band  $\sim$ 1460 cm<sup>-1</sup> assigned to the vibrations of the v(O-H) decreased compared to the spectrum of  $\beta$ CD, indicating the interaction of Syr, NaSyr and MnSyr with  $\beta$ CD. Additionally, the movement of the bands assigned to the v(O-H) at  $\sim$ 3420 cm<sup>-1</sup> and v(C-C) at  $\sim$ 1635 cm<sup>-1</sup> in the complexes of  $\beta$ CD compared to the spectrum of pure  $\beta$ CD proved the formation of inclusion complexes via hydrogen bonding formation. The antioxidant study revealed that all tested compounds possessed antiradical activity in the ABTS assay. Formation of the inclusion complex Syr $\beta$ Cd enhanced the antioxidant potential of Syr. The sodium salt of syringic acid NaSyr possessed higher antioxidant activity than ligand alone (Fig.2). The antimicrobial activity of tested compounds was presented in Table 1. The formation of sodium salt and manganese(II) complex with syringic acid improved the antimicrobial activity of ligand against Escherichia coli, Candida albicans and Bacillus subtilis. Whereas the formation of inclusion complexes with  $\beta$ CD didn't affect the antimicrobial potential of these compounds.





Fig.3. The percentage of ABTS<sup>++</sup> inhibition by tested compounds.

	Compounds								
Microorganism	Syr	NaSyr	MnSyr	βCD	Syr βCD	NaSyr βCD	MnSyr βCD		
	MIC (mg/mL)								
Escherichia coli	6	0.6	0.4	5	3	0.8	0.5		
Candida albicans	4	0.4	0.2	3	2	0.6	0.3		
Bacillus subtilis	3	0.3	0.1	3	2	0.6	0.2		

Table 1. MIC values for the studies compounds towards selected microorganisms.

**Conclusions:** The new inclusion complexes with  $\beta$ CD were successfully synthetized in solid state. The formation of metal complexes and inclusion complexes with  $\beta$ CD may affect the biological properties of ligand. The NaSyr and MnSyr possessed higher antimicrobial properties against selected microorganism compared to Syr. Whereas the antioxidant potential of Syr was enhanced by formation of sodium salt and inclusion complexes with  $\beta$ CD.

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# STUDIES OF THE STRUCTURE, CYTOSTATIC AND ANTIOXIDANT PROPERTIES OF VANILLIC ACIDS AND THEIR SODIUM SALTS

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**Abstract:** Vanillic acids are phenolic acids that naturally occur in nature. This study examined the correlations between the structure and cytostatic and antioxidant activities of three isomers of vanillic acid and their sodium salts. The calculations showed differences in the aromaticity of the tested ligands and their sodium salts, which may impact their different antioxidant activity and cytotoxicity towards HepG2.

**Introduction:** The current work is part of a broader topic (NCN grant: Research into the causes of an increase in the antioxidant properties of natural compounds found in food under the influence of complexation with microelements. Searching for effective antioxidants in food technology), the aim is to search for effective antioxidants and develop methods for their synthesis. Our previous work shows that the complexation of antioxidants with some metals increases their antioxidant properties [1, 2]. We also found that the antioxidant properties of ligands depend not only on the number and position of hydroxyl groups attached to the aromatic rings but also on the electronic charge distribution in the molecules and the length of the conjugated double bond system. A more balanced electronic charge distribution of antioxidants and the extension of the conjugated double bond system generally increase the antioxidant properties of the ligands. Healthy food without free radicals can contribute to the prevention of neurodegenerative diseases such as Alzheimer's or Parkinson's disease. For the above-mentioned reasons, we continue our research in the following directions: 1) searching for effective antioxidants, 2) studying the correlation between molecular structure and biological activity. We focus our research particularly on ligands of natural origin, i.e., phenolic acids [3].

**Experimental:** Sodium salts of vanillic acids were obtained by mixing the acid with sodium hydroxide solution in a 1:1 stoichiometric ratio. The resulting precipitates were dried at 100 °C. The correctness of the synthesis was checked by recording FT-IR spectra using a Bruker's Alpha Fourier Transform spectrophotometer. The optimization calculations and the selected parameters of the molecules were performed using the DFT method with the hybrid functional B3LYP and the 6-311++G(d,p) basis set, employing the Gaussian 09 program. The antioxidant activity of the compounds was determined by carrying out the direct reaction of the DPPH• radical, according to the method described in [4], and by carrying out the reaction of the ABTS•+ radical according to the method

described in [5]. The radical scavenging activity was expressed as the IC50 parameter (the concentration needed to reduce the initial concentration of the radical by 50%). In vitro cytotoxicity study was performed on human hepatocellular carcinoma Hep G2 cells (ATCC, Rockville, MD, USA). To determine the effect of phenolic acids on the proliferation of Hep G2 cells, a colorimetric method - MTS was used. Cell cultures were performed in the presence of the tested compounds at concentrations of 10 mM; 1 mM and 0.1 mM. Incubation time was 24 h. Absorbance at 492 nm was recorded after 2 h on Agilent BioTek synergy H1. For reference, a control experiment was performed to detect samples free from compounds.

**Results:** Energies and dipole moments were determined for the optimized molecular structures (Table 1). Based on the bond lengths in the aromatic ring, the aromaticity indices (HOMA, BAC and Aj) of the ligands and their sodium salts were calculated. The data show that of the tested ligands, vanillic acid has the most aromatic character, while o-vanillic acid is the least aromatic. Substitution of the sodium atom in the carboxyl group increased aromaticity of all ligands.

1			•			
Compound	Dipole moment	Energy	Energy Aromaticit		y index	
Compound	[Dy]	[a.u.]	HOMA	Aj	BAC	
O-vanillic acid	4.9602	-610.75	0.9290	0.9817	0.8182	
Vanillic acid	2.7808	-610.75	0.9672	0.9939	0.8952	
Isovanillic acid	3.9642	-610.76	0.9655	0.9923	0.8722	
O-vanillic acid sodium salt	3.2976	-772.51	0.9359	0.9850	0.8381	
Vanillic acid sodium salt	6.0157	-772.51	0.9755	0.9964	0.9180	
Isovanillic acid sodium salt	4.8358	-772.52	0.9720	0.9943	0.8871	

Table 1. Dipole moment values and aromaticity indices of tested compounds.

The energy values of the HOMO and LUMO frontier orbitals were also calculated. An electronic system with a larger energy difference between HOMO and LUMO should exhibit lower reactivity compared to a structure with a smaller value of this gap, because this parameter determines the ability to transfer charge within the molecule. The lower value of the HOMO-LUMO orbital difference, means the better the compound's antioxidant properties. Although HOMO and LUMO energies do not describe antioxidant properties, they can be correlated with the antioxidant activity of a specific molecule. The visualization of orbitals and the energy gap (Eg) value between HOMO and LUMO levels is presented in Fig.1 and 2. The HOMO and LUMO energies are also related to the global reactivity parameters: ionization potential, chemical hardness and softness, electrophilicity index (Table 2).

	0,					
	O-vanillic	Vanillic	Isovanillic	O-vanillic acid	Vanillic acid	Isovanillic acid
Molecular descriptor	acid	acid	acid	sodium salt	sodium salt	sodium salt
HOMO energy [eV]	-6.3023	-6.6615	-6.3494	-5.5221	-5.8862	-5.5853
LUMO energy [eV]	-1.5252	-1.5794	-1.4975	-1.4412	-1.5582	-1.5203
Energy gap	4.7771	5.0821	4.8519	4.0810	4.3281	4.0649
Electron affinity	1.5252	1.5794	1.4975	1.4411	1.5582	1.5203
Ionization potential	6.3023	6.6615	6.3494	5.5221	5.8862	5.5853
Chemical hardness	2.3885	2.5411	2.4260	2.0405	2.1640	2.0325

Table 2. Energy of HOMO/LUMO orbitals and other reactivity descriptors.

Chemical softness	0.2093	0.1968	0.2061	0.2450	0.2311	0.2460
Electronegativity	3.9138	4.1204	3.9234	3.4816	3.7222	3.5528
Electrophilicity index	3.2065	3.3407	3.1726	2.9703	3.2011	3.1052



Fig.1. The HOMO/LUMO (highest occupied molecular orbital/lowest unoccupied molecular orbital) electron densities in molecules of studied acids.



Fig.2. The HOMO/LUMO electron densities in molecules of studied salts.

The results of the DPPH and ABTS tests are presented in Table 3. The determined IC50 values showed that the lowest antioxidant potential was exhibited by ortho-vanillic acid and its sodium salt. Comparing the antioxidant properties of ligands to their sodium salts, it can be seen that salts exhibit better antioxidant activity than acids.

 Table 3. Summary of IC50 values [µmol/L] for vanillic, iso vanillic and orto-vanillic acids and their sodium salts obtained by the DPPH and ABTS methods.

Compound	DPPH	ABTS
O-vanillic acid	7.1184	8.2810
Vanillic acid	2.1583	2.7426

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Isovanillic acid	2.1870	2.8104
O-vanillic acid sodium salt	0.1806	7.9184
Vanillic acid sodium salt	0.3409	0.3723
Isovanillic acid sodium salt	0.5601	1.2140

The cytostatic properties of vanillic acids and their sodium salts were tested against HepG2 cancer cells. The obtained results are presented in Fig.3. Compounds with the highest ability to eliminate free radicals (according to DPPH and ABTS tests) showed a complete lack of cytopathic effect on the tested cancer cell line. However, the only compound (ortho-vanillic acid), which showed almost 20% cytotoxic activity at the highest tested concentration (10 mM) against the HepG2 cell line, is a weak antioxidant according to the conducted studies.



Fig.3. Viability (%) of HepG2 cells depending on the concentration of the tested compounds.

**Conclusions:** The conducted studies show that sodium salts have better antioxidant properties than the initial ligands; they also have a smaller electron gap and lower ionization potentials. No correlation was found between antioxidant activity and cytotoxicity towards HepG2 cancer cells.

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# FROM BINARY TO TERNARY NOWOTNY CHIMNEY-LADDER PHASES

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**Abstract:** Nowotny chimney-ladder (NCL) phases (or shorter: Nowotny phases) are a series of compounds with composite structures formed between transition metal elements (*T*) of groups IV-VIII and *p*-elements (*M*) of main groups III-V. An important feature of the chimney-ladder structures is the rule of 14 valence electrons per transition metal atom (VEC<sub>T</sub>) [1], since the 14-electron rule can be used to predict the electrical and thermoelectric properties. When the condition of 14 electrons is fulfilled, a narrow band gap is formed and the compound becomes an intrinsic semiconductor. For VEC<sub>T</sub> < 14, based on the band gap filling pattern, Nowotny chimney-ladder phases should exhibit *p*-type conduction (positive Seebeck coefficient), while *n*-type conduction is expected for VEC<sub>T</sub> > 14 [2]. For instance, to improve the thermoelectric properties of the Nowotny phase V<sub>17</sub>Ge<sub>31</sub> with a deficit of valence electrons (VEC<sub>T</sub> = 12.3) and typical *p*-type conductivity [3], the authors proposed to partially replace the *d*-block element of group V by elements of groups VI or VIII, such as Cr, Mo, Mn, or Ru, with more valence electrons.

As objects of our study, we have chosen the binary Mo–Ge and ternary Mo–Ru–Ge systems. The samples of the Mo–Ru–Ge system were synthesized by arc-melting the elements under a purified argon atmosphere. Elements of the following purities were used: Mo  $\geq$  99.8 wt.%, Ru  $\geq$  99.9 wt.%, Ge  $\geq$  99.98 wt.%. The samples were annealed at 600 °C for 70 days in evacuated quartz tubes. Phase analysis and crystal structure determinations were carried out based on X-ray powder diffraction patterns recorded with a STOE Stadi P diffractometer (Cu K $\alpha_1$  radiation), using the program package WinCSD [4]. The samples of the Mo–Ge system were synthesized by sintering powders of pure metals in vacuum-sealed quartz ampoules at 1000 °C for 4 days and at 800 °C for another 5 days, after which the ampoules were quenched in cold water. X-ray powder diffraction data from a polycrystalline sample was obtained using a PROTO AXRD Benchtop diffractometer (Cu  $K\alpha$  radiation).

Two compounds with chimney-ladder structures were observed in the Mo–Ge system:  $Mo_9Ge_{16}$  (space group  $I4_{122}$ , Pearson symbol tI100, unit-cell parameters a = 5.99324(3), c = 44.0005(3) Å and  $Mo_{22}Ge_{39}$ , (P-4c2, tP244, a = 5.99119(4), c = 107.509(2) Å). In the Mo–Ru–Ge system a ternary compound  $Mo_{2.5}Ru_{5.5}Ge_{13}$  belonging to the class of Nowotny phases was established: P-4c2, tP84, a = 5.8320(2), c = 37.616(2) Å. For the binary compounds VEC<sub>T</sub> reaches values close to 13.1, which indicates a significant deficit of valence electrons. The valence electron concentration for  $Mo_{2.5}Ru_{5.5}Ge_{13}$  is 13.9. Due to the deficit of valence electrons with respect to the 14-electron rule and previous investigations of physical properties of Nowotny phases, we expect  $Mo_{2.5}Ru_{5.5}Ge_{13}$  to be a typical p-type semiconductor with a positive Seebeck coefficient and good thermoelectric properties. In conclusion, the investigation of ternary NCL

phases with electron-rich atoms is a promising area for obtaining materials that may be used in thermoelectric devices.

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