

To: Plenipotentiary Representative of the Romanian Government to JINR

## JINR-RO Project Report for 2020 and 2021

### JINR-RO Project Report for 2020

JINR Laboratory: Frank Laboratory of Neutron Physics

Romanian Partner Institution(s): National Institute for Research and Development of Isotopic and Molecular Technologies, Cluj-Napoca

1) Title of the proposal: The impact assessment of metal/metal salts on crops

2) Type of the proposal: - Follow-up: Number 28 (Order 267/20.05.2020), number 62 (Order 268/20.05.2020), number 65 (Order 269/ 20.05.2020)

*If the proposed project is correlated with a simultaneous grant proposal, the title of the respective grant proposal shall be indicated:* The impact assessment of metal/metal salts on crops

3) Theme and activity from the JINR Topical Plan:

Theme code: 03-4-1128-2017/2022

Priority: 1

Theme name: Investigations of Neutron Nuclear Interactions and Properties of the Neutron

Activity or experiment: Applied and methodological research

The research teams from the two institutions (INCDTIM and JINR) have participated together in the project, entitled: **“The impact assessment of metal / metal oxide nanoparticles on wheat”**, in the year 2020, Number 28 (Order 267/20.05.2020), number 62 (Order 268/20.05.2020), number 65 (Order 269/ 20.05.2020) (according to the list approved by JINR).

Within the project was followed:

Each 50 grains of wheat were sown in 0.81 L pots, with a diameter of 13.5 cm, which contained 400 g of soil mixed with:

- 75 mL water – control sample
- 60 mg CuSO<sub>4</sub> dissolved in 75 mL water
- 120 mg CuSO<sub>4</sub> dissolved in 75 mL water
- 60 mg Cu(NO<sub>3</sub>)<sub>2</sub> dissolved in 75 mL water
- 120 mg Cu(NO<sub>3</sub>)<sub>2</sub> dissolved in 75 mL water

The plants watering were done once every 3 days with 75 mL of ultrapure water. All plants were grown under controlled conditions of temperature and humidity. Plant samples were taken three weeks and six weeks after sowing, respectively.

## 1. Extracts obtaining and characterization of pigments

### 1.1. Extracts obtaining

0.5 g of freshly ground wheat was mixed with 20 mL of acetone and centrifuged for 10 minutes at 7000 rpm. 20 mL was added to the remaining plant and stirred on a shaker for 30 minutes at 300 rpm, at room temperature, after which it was centrifuged for 10 minutes and the supernatant was decanted. The operation was repeated with another 10 mL of acetone until the plant material discolored. The solutions were combined in the same bottle and analyzed. The pigment extraction was performed in duplicate.

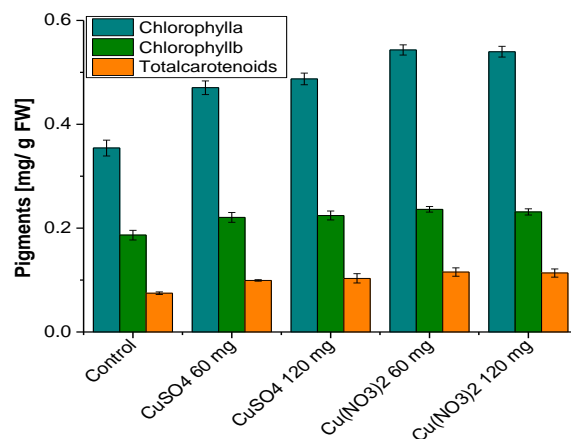
### 1.2. Characterization of extracts

The study of the literature has shown that an elegant and precise way of qualitative, but also quantitative, analysis of chlorophyll a, chlorophyll b and carotenoids in an extract of plant tissue can be done by UV-VIS spectroscopy. In this regard, the absorption spectra of the extracts in the wavelength range 400-750 nm were recorded using a T80 UV-VIS Spectrophotometer (PG Instruments Limited), and the concentrations for chlorophyll a ( $c_a$ ), chlorophyll b ( $c_b$ ) and total carotenoids ( $c_{(x+c)}$ ) were calculated from the following formulas (Lichtenthaler and Buschmann, 2001):

$$c_a \text{ (mg/mL)} = 11.24 * A_{661.6} - 2.04 * A_{644.8}$$

$$c_b \text{ (mg/mL)} = 20.13 * A_{644.8} - 4.19 * A_{661.6}$$

$$c_{(x+c)} \text{ (mg/mL)} = (1000 * A_{470} - 1.90 * c_a - 63.14 * c_b) / 214$$



**Figure 1.** Comparative diagram of the pigment content.

The amount of chlorophyll a, chlorophyll b and total carotenoids were lower in the control plants than in the treated ones. Comparing the plants treated with the two copper salts, it was observed that the amount of pigments was lower in the case of plants treated with CuSO<sub>4</sub> than in those treated with Cu(NO<sub>3</sub>)<sub>2</sub>.

## 2. Obtaining and characterizing of alcoholic extracts

### 2.1. Obtaining of alcoholic extracts

Over 0.5 g of fresh plant (FW) was added 7.5 mL 60% ethanol. The mixture was subjected to ultrasonic-assisted extraction using an Elma Transsonic T ultrasonic bath for 30 minutes at room temperature, and then centrifuged at 7000 rpm for 10 minutes. The supernatant was decanted and stored in the refrigerator at 4°C until analysis. All extracts were obtained in duplicate.

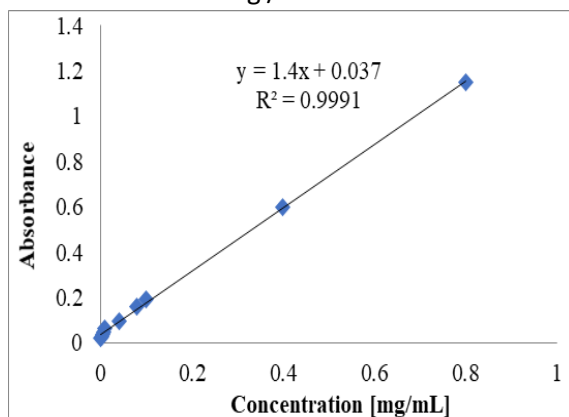
### 2.2. Characterization of alcoholic extracts

#### 2.2.1. Determination of total polyphenol content

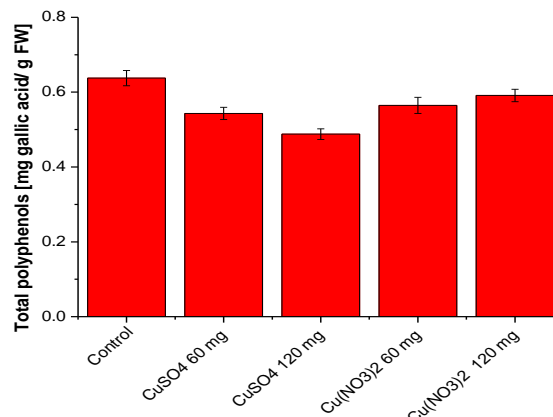
The content of total polyphenols was determined by the Folin-Ciocalteu method according to the protocol presented by Ivanova et al. (2010).

For this purpose, 1 mL of extract was added to a 10 mL volumetric flask containing 5 mL of double distilled water. To this mixture was added 0.5 mL of Folin-Ciocalteu reagent, the contents were mixed, and after 3 minutes of standing, 1.5 mL of Na<sub>2</sub>CO<sub>3</sub> (5 g / L) was added. The volume of the flask was adjusted with double-distilled water. After keeping the samples at 50°C (in a water bath) for 16 minutes in closed flasks, followed by cooling to room temperature, the absorbances were read in relation to the blank sample (double-distilled water) at a wavelength of 765 nm.

The calibration curve was constructed using standard gallic acid solutions in the range 0.001 - 0.800 mg / mL, obtained by successive dilutions with double-distilled water starting from a solution of concentration 1 mg / mL.



**Figure 3.** Gallic acid calibration curve at 765 nm.



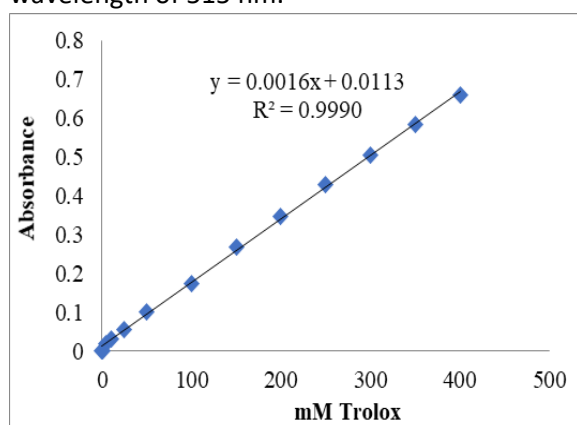
**Figure 4.** Comparative diagram of the total amount of polyphenolic compounds.

It was also found that in the plants treated with copper salts, the amount of polyphenols was higher than in the control plants. Comparing the plants treated with the two copper salts, it was found that in those treated with Cu(NO<sub>3</sub>)<sub>2</sub> the total amount of polyphenols was higher than those treated with CuSO<sub>4</sub>. This is due to the fact that plants treated with Cu(NO<sub>3</sub>)<sub>2</sub> are more stressed than those treated with CuSO<sub>4</sub>, and in response to stress produce a higher amount of polyphenols.

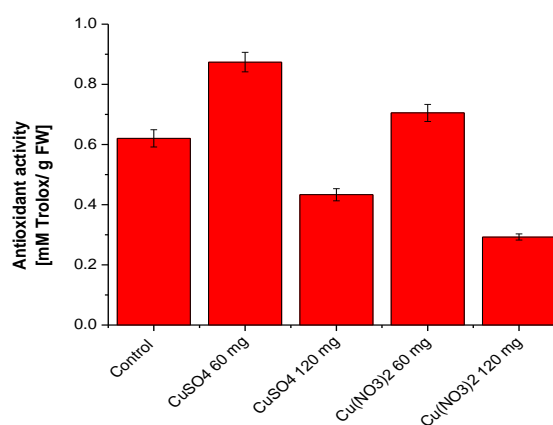
### 2.2.2. Determination of antioxidant activity by DPPH assay

The antioxidant capacity was evaluated after a slightly modified procedure, reported by Brand-Williams and collaborators (Brand-Williams et al., 1995). Thus, 0.01 mL of extract was added to 3.9 mL of DPPH – 2,2 diphenyl-picryl-hydrazyl radical solution (0.0025 g / 100 mL methanol) and after 10 minutes left in the dark the absorbance of the mixture was measured at 515 nm compared to the control sample (0.01 mL extract added to 3.9 mL methanol). Results were calculated from the calibration curve and expressed in mM Trolox / 1 g plant.

The calibration curve was plotted for different concentrations of Trolox (0 - 400 µM) at a wavelength of 515 nm.



**Figure 5.** Calibration curve for Trolox at 517 nm.



**Figure 6.** The antioxidant activity of the analyzed extracts.

The plants treated with 60 mg copper salts had a higher antioxidant activity than the control plants, and those treated with 120 mg of copper salt had a lower DPPH than the control.

### 3. Multielemental investigation of wheat and soil substrate by NAA

To determine the elemental content of the wheat biomass and soil substrate, neutron activation analysis (NAA) at the pulsed fast reactor IBR-2 (FLNP JINR, Dubna) was used. To determine short-lived isotopes, biological (about 0.3 g) and substrate samples (about 0.1 g) were irradiated for 3 min and 1 min respectively under a thermal neutron fluency rate of approximately  $1.6 \times 10^{13} \text{ n cm}^{-2} \text{ s}^{-1}$ . Both types of samples were measured for 15 min. In the case of long-lived isotopes substrate (about 0.1 g) samples were irradiated for 3 days under a resonance neutron fluency rate of approximately  $3.31 \times 10^{12} \text{ n cm}^{-2} \text{ s}^{-1}$ , repacked and measured using high purity germanium detectors twice (after 4–5 days and 20–23 days of decay). The irradiation of wheat biomass for determination of long-lived isotopes is planned for December 2020, the gamma spectra will be collected by the end of January 2021. The complex data processing and interpretation will be performed in the first quarter of 2021.

## References

Brand-Williams W., Cuvelier M.E., Berset C., LWT—Food Science and Technology 1995; 28:25-30  
Ivanova V., Stefova M., Chinnici F., Journal of the Serbian Chemical Society 2010; 75:45-59  
Lichtenthaler H. K., Buschmann C., 2001. Current Protocols in Food Analytical Chemistry (Units: F4.3.1-F4.3.8, John Wiley & Sons Inc., New York

The results obtained within the project were communicated at:

1. The 6<sup>th</sup> International Conference „Advances in engineering & management – ADEM 2020” which will take place between 10-11 December 2020 in Drobeta-Turnu Severin, Romania, Comparison of the effect of soil amendment of biogenic and chemical obtained CuO NPs on bioactive compounds and elemental accumulation in wheat, authors: I. Lung, O. Opriş, M.L. Soran, O.A. Culicov, A. Ciorîţă, A. Stegărescu, I. Zinicovscaia, N. Yushin, K Vergel, I. Kacso, G. Borodi, M. Parvu.
2. EUROASIA CONGRESS ON SCIENTIFIC RESEARCHES AND RECENT TRENDS-VII December 7-8, 2020 / Baku, AZERBAIJAN, The influence of CuO nanoparticles and copper sulphate and nitrate salt on composition and ultrastructure of wheat, authors: M.L. Soran, O.A. Culicov, I. Lung, O. Opriş, A. Stegărescu

Also, the results obtained are presented in the manuscript entitled: “The impact assessment of CuO nanoparticles on the composition and ultrastructure of *Triticum aestivum* L.”, sent for publication at RSC Advances. Moreover, an article entitled "The impact of two copper salts on composition and ultrastructure of wheat" is currently being prepared.

To: Plenipotentiary Representative of the Romanian Government to JINR

### JINR-RO Project Report for 2021

JINR Laboratory: Frank Laboratory of Neutron Physics

Romanian Partner Institution(s): National Institute for Research and Development of Isotopic and Molecular Technologies, Cluj-Napoca

1) Title of the proposal: The impact assessment of metal/metal salts on crops

2) Type of the proposal: - Follow-up: Number 72 (Order 365/11.05.2021), number 71 (Order 366/11.05.2021), number 32 (Order 367/ 11.05.2021)

*If the proposed project is correlated with a simultaneous grant proposal, the title of the respective grant proposal shall be indicated:* The impact assessment of metal/metal salts on crops

3) Theme and activity from the JINR Topical Plan:

Theme code: 03-4-1128-2017/2022

Priority: 1

Theme name: Investigations of Neutron Nuclear Interactions and Properties of the Neutron

Activity or experiment: Applied and methodological research

The research teams from the two institutions (INCDTIM and JINR) have participated together in the project, entitled: "The impact assessment of metal/metal salts on crops", in the year 2021, number number 72 (Order 365/11.05.2021), number 71 (Order 366/11.05.2021), number 32 (Order 367/ 11.05.2021) (according to the list approved by JINR).

Within the project was followed:

Lettuce seeds (10 grains) were sown at a depth of 1 cm in plastic pots (0.81 L, 13.5 cm in diameter) containing 636 g of garden substrate with active humus and fertilizer for 6 weeks (Agro, 50 L.). The heavy metal salts selected in this study were: copper(II) chloride dihydrate ( $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ ), cadmium acetate dihydrate ( $\text{Cd}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$ ), zinc acetate dihydrate ( $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$ ), manganese(II) chloride tetrahydrate ( $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ), nickel chloride ( $\text{NiCl}_2$ ) and lead(II) sulfate ( $\text{PbSO}_4$ ). The concentrations of the heavy metal salts were: Cu (15, 30, 100 mg/kg); Cd (0.75, 1.5, 3 mg/kg); Zn (75, 150, 300 mg/kg); Mn (0.675, 1.35, 1.5 g/kg); Ni (15, 30, 75 mg/kg) and Pb (15, 30, 50 mg/kg). The 3 different concentrations were: one under accepted limit, one – maximum accepted limit and one above maximum accepted limit. These salts were dissolved each in 200 ml of ultrapure water, with which the pots were watered. The plants watering was done every four days with 100 mL of ultrapure water.

All plants, including control (grown in the absence of heavy metals salts), were grown under controlled light conditions (for 12 h from 24 h), 60% humidity and a day / night temperature cycle of 20/10°C.

Three replicates of each plants were increased. Plant samples were taken six weeks after sowing.

## 1. Extracts obtaining and characterization of pigments

### 1.1. Extracts obtaining

0.5 g of fresh lettuce leaves are ground with liquid nitrogen in the presence of  $\text{CaCO}_3$  (0.1 g). 10 mL of acetone is added over the thus crushed plant and the grinding is continued for 2 minutes, after which the clear solution is decanted, and the rest is centrifuged for 10 minutes at 7000 rpm. Over the remaining plant after decantation, it is adding another 10 mL of acetone and agitated on the shaker

at 450 rpm for 30 minutes, then centrifuge and separate the supernatant. The operation is repeated with another 5 mL of acetone, at the end the plant being discolored. The solutions from the three extraction stages come together in a single bottle. The pigment extraction was performed in triplicate.

## 1.2. Characterization of extracts

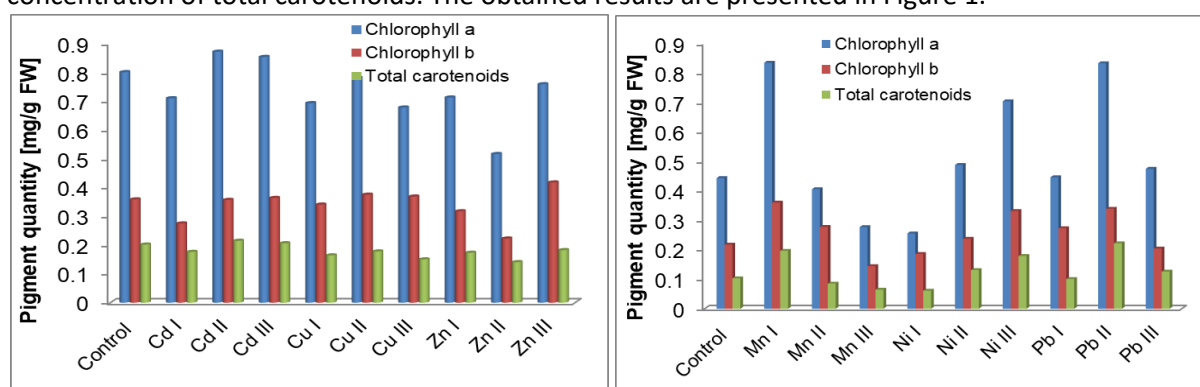
The quantitative analysis of chlorophyll a, chlorophyll b and total carotenoids from the obtained extracts was done by UV-VIS spectroscopy. In this regard, the absorption spectra of the extracts in the wavelength range 400-750 nm were recorded using a T80 UV-VIS Spectrophotometer (PG Instruments Limited). To determine the pigments concentrations, the following calculation formulas were used (Lichtenthaler and Buschmann, 2001):

$$c_a \text{ (mg/mL)} = 11.24 * A_{661.6} - 2.04 * A_{644.8}$$

$$c_b \text{ (mg/mL)} = 20.13 * A_{644.8} - 4.19 * A_{661.6}$$

$$c_{(x+c)} \text{ (mg/mL)} = (1000 * A_{470} - 1.90 * c_a - 63.14 * c_b) / 214$$

where:  $c_a$  is the concentration of chlorophyll a,  $c_b$  is the concentration of chlorophyll b and  $c_{(x+c)}$  is the concentration of total carotenoids. The obtained results are presented in Figure 1.



**Figure 1.** Comparative diagram of the pigment content.

The amount of chlorophyll a, chlorophyll b and total carotenoids depends on metal from soil and their concentration. In plants grown in the presence of Cd, Cu or Zn, the amount of pigments does not vary much from the control plants, regardless of their concentration. In contrast, in plants grown in the presence of Mn, Ni or Pb, the amount of pigments is higher than in the case of control plants if the lowest concentration of metal is added in the soil and is lower in the case of the next two concentrations of them.

## 2. Obtaining and characterizing of alcoholic extracts

### 2.1. Obtaining of alcoholic extracts

Fresh lettuce leaves (1 g) were ground in the presence of liquid nitrogen in solvent (15 mL) for 3 min after which the mixture was subjected to ultrasonic-assisted extraction using an Elma Transsonic T ultrasonic bath for 30 minutes at room temperature. the extraction solvent was 60% ethanol. After extraction, the mixture was centrifuged at 7000 rpm for 10 minutes and the supernatant was decanted and stored in the refrigerator at 4°C until analysis. All extracts were obtained in triplicate.

### 2.2. Characterization of alcoholic extracts

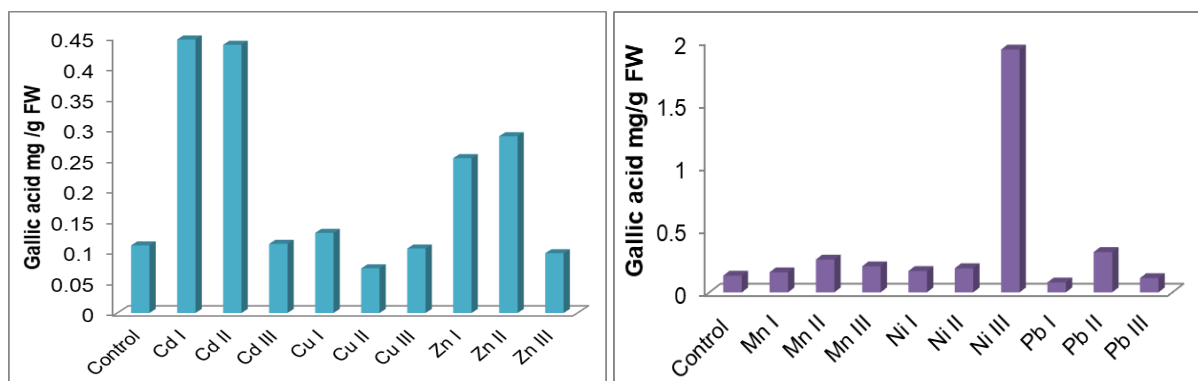
#### 2.2.1. Determination of total polyphenol content

The content of total polyphenols was determined by the Folin-Ciocalteu method (Ivanova et al. 2010). Thus, 1 mL of extract and 0.5 mL of Folin-Ciocalteu reagent was added to a 10 mL volumetric flask containing 5 ml of double distilled water. The content of the flask was mixed and after 3 minutes of standing, 1.5 mL of  $\text{Na}_2\text{CO}_3$  (5 g/L) was added and the volume of the flask was adjusted

with double distilled water. The samples were placed in a water bath at 50°C, where they were kept for 16 minutes, after which were removed and allowed to cool to room temperature. The absorbances of the samples were read in relation to the double distilled water at 765 nm.

To determine the total amount of polyphenols, a calibration curve was drawn using as standard a gallic acid solutions in the range of 0.001 - 0.800 mg/mL. Gallic acid concentrations were obtained by successive dilutions with double distilled water starting from a stock solution with a concentration of 1 mg/mL.

The amount of total polyphenolic compounds presented in Figure 2 was expressed as mg gallic acid/g fresh weight (FW), using the linear equation of the standard calibration curve:  $y = 0.5865x + 0.0059$  ( $R^2=0.9991$ ).



**Figure 2.** Total polyphenols quantification in lettuce extracts grown in the presence of heavy metals.

Comparing the plants treated with Cd, Cu or Zn with concentrations under accepted limit or at maximum accepted limit with the control plants, it was found that the amount of total polyphenols is greater, while for those treated with a concentration above the accepted limit is lower. For plants treated with Mn, Ni or Pb, the amount of total polyphenols does not vary significantly with that of control plants, except for plants grown in the presence of Ni at a concentration above the accepted limit. In this case, the amount of total polyphenols was much higher than in control plants.

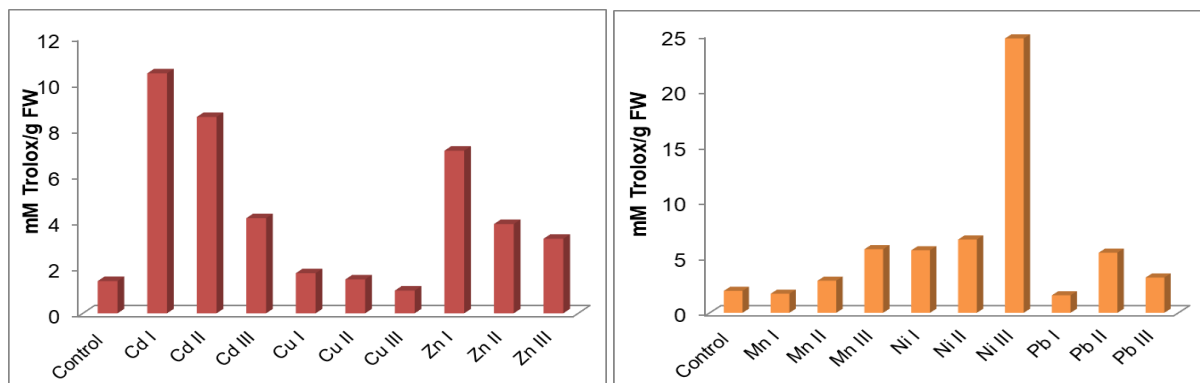
### 2.2.2. Determination of antioxidant activity by DPPH assay

A slightly modified procedure of Brand-Williams et al. (1995) was used for the antioxidant activity determination. Thus, 0.01 mL of extract was added to 3.9 mL of DPPH – 2,2 diphenyl-picryl-hydrazyl radical solution (0.0025 g / 100 mL methanol). The mixture was left in the dark for 10 min, after that the absorbance of the mixture was measured at 515 nm comparative to a mixture obtained from 0.01 mL extract added to 3.9 mL methanol.

The antioxidant activity was determined using a calibration curve drawn for different concentrations of Trolox (0 - 400 µM).

The obtained results for antioxidant capacity are presented in Figure 3 and was expressed in mM Trolox equivalents (mM Trolox/g sample), using the linear equation of the standard calibration curve:  $y=0.1755x + 0.0198$  ( $R^2=0.9924$ ).

The antioxidant activity of plants grown in the presence of heavy metals was higher than in control plants, except for plants grown in the presence of Cu at a concentration above the maximum accepted limit, Mn and Pb at a concentration under accepted limit. For these plants the antioxidant activity was lower than in the control plants.

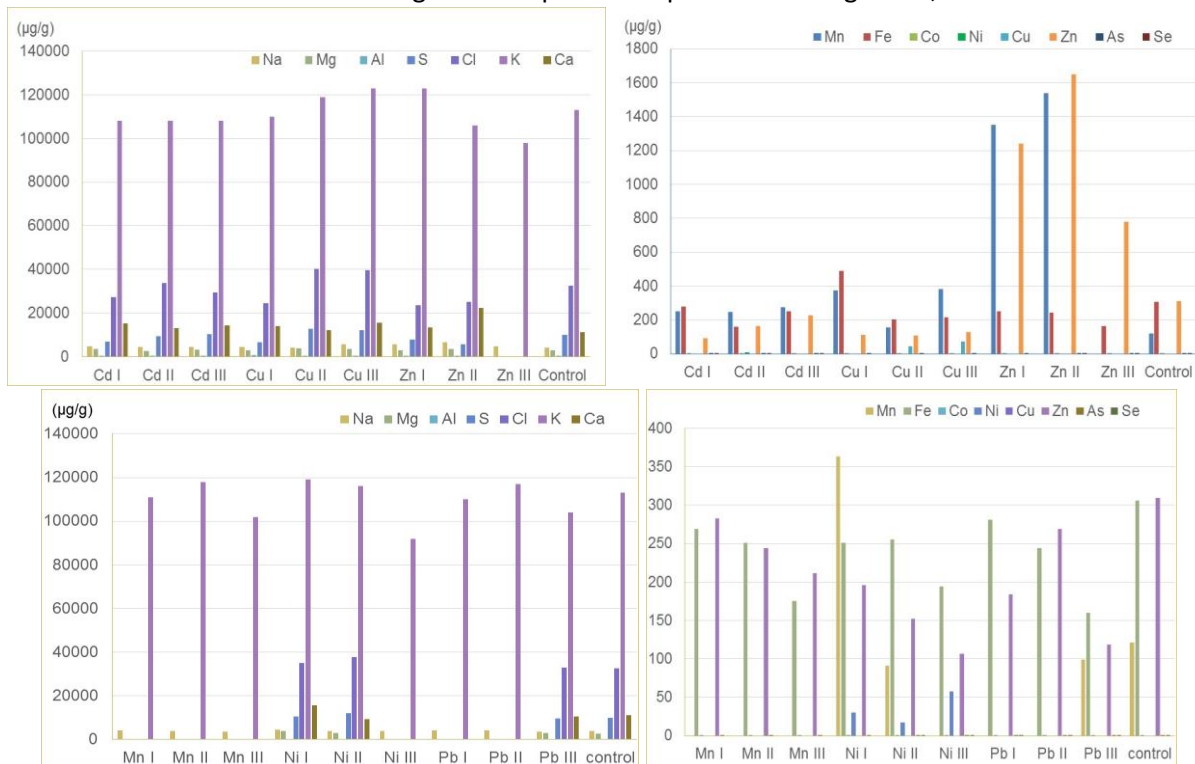


**Figure 3.** Antioxidant activity of the lettuce extracts.

### 3. Multielemental investigation of wheat and soil substrate by NAA

To determine the elemental content of the wheat biomass and soil substrate, neutron activation analysis (NAA) at the pulsed fast reactor IBR-2 (FLNP JINR, Dubna) was used. A total number of 55 plant samples and 28 soil samples were analyzed. To determine short-lived isotopes, biological (about 0.3 g) and substrate samples (about 0.1 g) were irradiated for 3 min and 1 min respectively under a thermal neutron fluency rate of approximately  $1.6 \times 10^{13} \text{ n cm}^{-2} \text{ s}^{-1}$ . Both types of samples were measured for 15 min. In the case of long-lived isotopes substrate (about 0.1 g) samples were irradiated for 3 days under a resonance neutron fluency rate of approximately  $3.31 \times 10^{12} \text{ n cm}^{-2} \text{ s}^{-1}$ , repacked and measured using high purity germanium detectors twice (after 4–5 days and 20–23 days of decay).

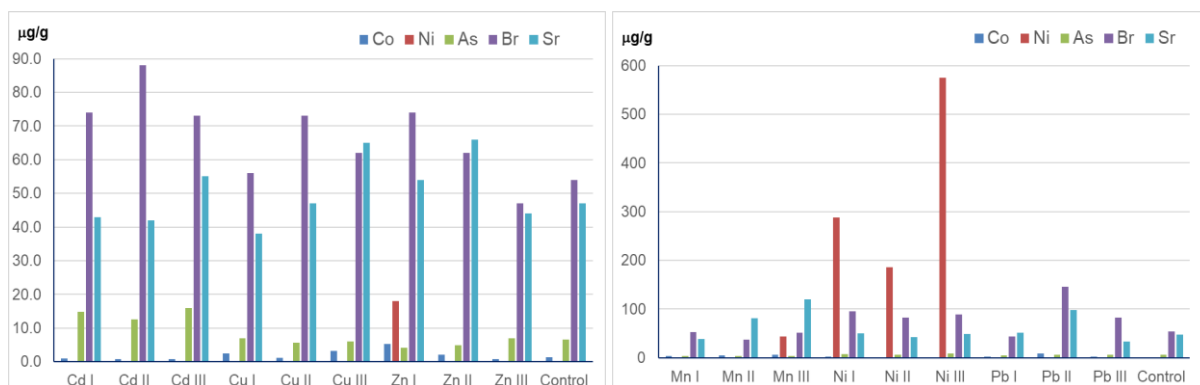
The elemental content in leaves and roots of the lettuce grown with heavy metals and also, the elemental content in soil in which grown the plants are presented in Figures 4, 5 and 6.



**Figure 4.** Variation of elemental content in leaves of the lettuce grown with heavy metals.

In the analyzed lettuce leaves were determined 34 elements (Na, Mg, Al, S, Cl, K, Ca, Sc, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Br, Rb, Sr, Mo, Sb, Cs, Ba, La, Ce, Sm, Tb, Hf, Ta, W, Th, U). Their quantity varied in plants grown in the presence of heavy metals compared to the control.





**Figure 5.** Variation of some elements content in roots of the lettuce grown with heavy metals.

In the case of lettuce roots were determined only 26 elements, namely Na, K, Sc, Cr, Fe, Co, Ni, Zn, As, Se, Br, Rb, Sr, Mo, Sb, Cs, Ba, La, Ce, Sm, Tb, Hf, Ta, W, Th and U. The concentration of the determined elements was higher or lower in the roots of the plants grown in the presence of heavy metals compared to the control, the concentration of the elements depending on the heavy metal, respectively its concentration. For example, the potassium and zinc decreased in most samples compared with control.



**Figure 6.** Variation of some elements content in soil where the lettuce was grown with heavy metals.

In the soil were determined the same elements as in the case of lettuce leaves except S, Cu, Se and Ba and in addition four new elements Ti, Zr, Eu and Yb were determined.

## References

Brand-Williams W., Cuvelier M.E., Berset C., LWT—Food Science and Technology 1995; 28:25-30  
 Ivanova V., Stefova M., Chinnici F., Journal of the Serbian Chemical Society 2010; 75:45-59  
 Lichtenthaler H. K., Buschmann C., 2001. Current Protocols in Food Analytical Chemistry(Units: F4.3.1-F4.3.8, John Wiley & Sons Inc., New York

The results obtained within the project were communicated at:

1. The 6<sup>th</sup> Green and Sustainable Chemistry Conference – Online, 16-18 November 2021, The influence of heavy metals salts on the growth of lettuce, authors: M.L. Soran, I. Lung, O. Culicov, A. Stegarescu, O. Opris, L. Strelkova, P. Nekhoroshkov, A. Sergeeva
2. 28<sup>th</sup> International Seminar on Interaction of Neutrons with Nuclei: «Fundamental Interactions & Neutrons, Nuclear Structure, Ultracold Neutrons, Related Topics», 24-28 May 2021, Dubna, Rusia, Impact assessment of copper salts on various plants, authors: I. Lung, O. Culicov, A. Stegarescu, O. Opris, A. Ciorîță, M.L. Soran, I. Zinicovscaia, N. Yushin, K. Vergel

Also, the results obtained were published in:

1. “The impact assessment of CuO nanoparticles on the composition and ultrastructure of *Triticum aestivum* L.”, authors: I. Lung, O. Opris, M.L. Soran, O. Culicov, A. Ciorîță, A. Stegarescu, I. Zinicovscaia, N. Yushin, K. Vergel, I. Kacso, G. Borodi, M. Pârvu, International Journal of Environmental Research and Public Health, 2021, 18, 6739 (**Impact Factor**: 3.390 (2020) ; 5-Year Impact Factor: 3.789 (2020))
2. “The effect of TiO<sub>2</sub> nanoparticles on the composition and ultrastructure of wheat”, authors: M.L. Soran, I. Lung, O. Opris, O. Culicov, A. Ciorîță, A. Stegarescu, I. Zinicovscaia, N. Yushin, K. Vergel, I. Kacso, G. Borodi, Nanomaterials, 2021, 11(12), 3413. <https://doi.org/10.3390/nano11123413>. (**Impact Factor**: 5.076 (2020) ; 5-Year Impact Factor: 5.346 (2020))