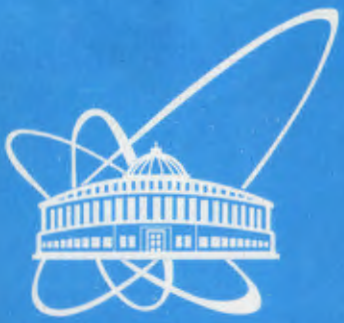

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# EPITHERMAL NEUTRON ACTIVATION ANALYSIS OF BLUE-GREEN ALGAE *SPIRULINA PLATENSIS* AS A MATRIX FOR SELENIUM-CONTAINING PHARMACEUTICALS

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## Introduction

Blue-green microalgae *Spirulina Platensis* (spirulina) is widely used in fundamental as well as applied biotechnology. It is one of the most concentrated natural sources of nutrition known in the world. Spirulina acts as a functional food. Its extracts are extensively employed in food, in pharmaceutical as well as perfume and cosmetics industries, and in medicine.

This microalgae is 60-70% vegetable protein, well-balanced in amino acids, rich in beta carotene and iron, and the world's richest natural source of vitamin *B-12* and essential fatty acids. It contains an extensive list of vitamins, minerals, phytonutrients and other biologically active beneficial substances. A complete bibliography documenting its health benefits can be found at the website http://www.Spirulina.com/ as well as in [1].

The biological essentiality of selenium for humans has been recognized more than 40 years ago. The understanding of the role of selenium in biology and medicine has significantly advanced over the last two decades [2]. Selenium is known to be a normal component of several enzymes, proteins and some aminoacryl transfer nucleic acids. Low levels of selenium intake are reported to cause diseases such as cardiomyopathy (Keshan disease), cancer and endemic osteoarthropathy characterized by necrosis of cartilage and dystrophy of skeletal muscles (Kaschin-Beck disease), anemia, whereas about two orders of magnitude higher levels of selenium are known to cause toxic effects.

Certain selenium compounds used as dietary supplements have been claimed to prevent carcinoma, acquired immunodeficiency syndrome (AIDS), to slow ageing process, to enhance sexual activities, *etc.* [3-7].

Assuming that positive biological effects in man are stimulated by the algae *Spirulina Platensis* and selenium compounds, the use of these algae as a matrix for the production of selenium-containing pharmaceuticals seems to be of great potential. To verify this working hypothesis, it is necessary to determine:

- the background level of chemical composition of blue-green algae *Spirulina Platensis ;*
- the level of selenium accumulation in spirulina matrix at different selenium loadings in the nutrient medium;
- the levels of toxic elements intake by the spirulina cells.

To cultivate spirulina, a standard mineral nutrient medium was used. Mercury was selected as a representative of toxic element intake by spirulina biomass.

Instrumental neutron activation analysis (INAA) using epithermal neutrons has previously been shown to be feasible for multi-element determination in biological samples [8]. In order to study simultaneously a large number of elements in blue-green algae *Spirulina Platensis,* epithermal neutron activation analysis (ENAA) was carried out at the IBR-2 pulsed fast reactor in Dubna which has a very high ratio of epithermal to thermal neutrons.

## Experimental

*Sample preparation*

Spirulina was cultivated in a bioreactor of 20 *L* volume at a standard nutrient medium at optimal conditions (pH, temperature, light, etc.). After cultivating for 4-5 days, the cell suspension was subjected to centrifugation. The precipitated biomass was well-rinsed in distilled de-ionized water for three times up to neutral *pH* values. This wet biomass was then placed in an adsorption-condensation lyophilizer (designed by the authors and described earlier

[9]) and dried according to the procedure reported in details elsewhere [10]. The native dry biomass was finally pelletized to 5 mm pellets using a special titanium press-form.

Three experiments were carried out. In the first experiment, spirulina was cultivated in a standard nutrient medium and using Tbilisi ordinary drinking water as a solvent. For control, spirulina was also cultivated in distilled de-ionized water containing growth medium.

In the second experiment, spirulina was cultivated in the same nutrient medium with additional loading of selenium or mercury. In experiments with selenium, seleneous acid $H_2SeO_3$ (certified as «chemically pure») was used to provide selenium concentration within the range of 0.016 ÷ 2770 *μg/L*. In case of mercury, mercuric nitrate $Hg(NO_3)_2 \cdot 0.5H_2O$ (certified as «pure») was added to give mercury concentration within the range of 0.0033 ÷ 33 *μg/L*.

In the third experiment, globular protein C-phycocyanin was extracted from the freshly prepared spirulina biomass, without any additional loading in the nutrient medium.

Reagent blank is an important factor that adversely influences the reliability of analytical results. Contamination with selenium and mercury from the chemical reagents used was considered negligible.

*Analysis*

Spirulina samples of about 0.5 *g* were packed in aluminum cups for long-term irradiations and heat-sealed in polyethylene foil bags for short-term irradiation. Neutron flux density characteristics and the temperature in the irradiation channels equipped with a pneumatic system are given in **Table 1**.

**Table 1**. The main characteristics of the irradiation channels

| Irradiation channel | Neutron flux density ($n/cm^2 s$)×$10^{12}$ | | | T° C [11] | Channel diam., mm | Channel length, mm |
|---|---|---|---|---|---|---|
| | Thermal | Resonance | Fast | | | |
| Ch1 | Cd-coated | 3.31 | 4.32 | 70 | 28 | 260 |
| Ch2 | 1.23 | 2.96 | 4.10 | 60 | 28 | 260 |

Long-lived isotopes were determined using irradiation cannel Ch1. Samples were irradiated for 5 d, repacked and then counted twice after decays of 4 and 20 d. The counting time varied from 1.5 to 10 h. To determine the short-lived isotopes of Mg, Al, Cl, Ca, V, Mn, and I, irradiation cannel Ch2 (conventional NAA) was used. Samples were irradiated for 3 min and measured twice after 3-5 and 20 min. decay for 5-8 and 20 min, respectively.

Gamma-ray spectra were recorded using a large volume Ge(Li) detector with a resolution of 1.96 keV at the 1332.4 keV line of $^{60}Co$, with an efficiency of 30% relative to a 3x3" NaI detector for the same line. Data processing and element concentration determinations were performed on the basis of certified reference materials and comparators using software developed in our laboratory [12].

Since selenium and mercury belong to the group of volatile elements, the temperature in the irradiation site is of great importance. The critical temperature for the correct determination of selenium and mercury in biological samples has been reported to be about 90-100 °C [13]. The temperature in the irradiation channels (Ch1 Cd-coated и Ch2) was no higher than 60-70° C [11]. Still a certain loss of mercury during the irradiation cannon be excluded. For selenium such loss appears less probable. The interfering effects of $^{75}Se$ and $^{182}Ta$ gamma lines with the 279.1 keV line of $^{203}Hg$ were taken into consideration.

*Analytical Quality Assurance*

Three certified reference materials (CRMs), namely IAEA Lichen-336 , IAEA Bottom Sediments SDM-2T and Nordic Moss DK-1 were used for quality assurance purposes.

Along with these international CRMs, home-made synthetic multi-element standard samples for neutron activation of biological materials called SSB-1 and SSB-2 based on phenol-formaldehyde resin were used [14]. As seen from **Table 2**, the results of ENAA are in good agreement with the recommended values.

The accuracy was generally within 10÷15 %.

**Table 2**. Comparison of recommended and obtained NAA results of home-made synthetic multi-element standards SSB-1 and SSB-2

| Elements | Amount of element introduced [14] | Amount of element determined by NAA [14] | Amount of element determined by ENAA in the present work |
|---|---|---|---|
| SSB-1 | | | |
| Se | 2.84 | 2.80 ± 0.07 | 2.26 ±0.33 |
| Cr | 1.80 | 1.78 ± 0.05 | 1.85 ± 0.20 |
| Au | 0.028 | 0.026 ± 0.0006 | 0.034 ± 0.0008 |
| Sb | 0.47 | 0.45 ± 0.01 | 0.44 ± 0.09 |
| Ag | 1.89 | 1.72 ± 0.05 | 1.92 ± 0.12 |
| Rb | 9.45 | 9.05 ± 0.26 | 9.12 ± 1.09 |
| Fe | 284 | 296 ± 6 | 315 ± 40 |
| Zn | 18.9 | 19.5 ± 0.5 | 22.05 ± 3 .52 |
| Co | 0.66 | 0.63 ± 0.05 | 0.75 ± 0.07 |
| SSB-2 | | | |
| Ca | 5210 | 3740 ± 160 | 5400 ± 500 |
| Ba | 10.3 | 10.6 ± 0.3 | 10.2 ± 1.2 |
| Hg | 0.50 | 0.10 ± 0.01 | 0.14 ±0.04 |
| Sn | 30.8 | 24.0 ± 1.9 | 28.0 ± 4.0 |
| Br | 20.8 | 20.7 ± 0.6 | 19.4 ± 2 |
| Cs | 0.50 | 0.48 ± 0.01 | 0.4 ±0.1 |
| Ni | 10.3 | 9.5 ± 0.6 | 9.83 ±1 |
| Sc | 37 | 37.2 ± 0.8 | 37.8 ± 2 |
| Na | 3070 | 3660± 590 | 3500 ± 320 |

The discrepancy between the introduced and determined amounts of Hg in the columns 1 and 2 of **Table 2** is due to the evaporation of mercury during the technological process of phenol-formaldehyde resins synthesis [14].

## Results and Discussion

A total of 31 major, minor and trace elements were determined in the spirulina biomass from the first experiment, i.e. cultivated in standard nutrient medium dissolved in distilled de-ionized and in Tbilisi ordinary drinking water. The results are shown in **Fig. 1** where they are arranged in the «hierarchic» descending order of their concentrations. As follows from this figure, the concentrations range over 8 orders of magnitude, from major to

ultra-trace elements. It is evident that the levels of elemental content in biomass cultivated in drinking water are higher or equal to concentrations obtained for the samples cultivated in distilled de-ionized water. Exceptions are Ba, Sb, As, Tm, Ta exhibiting low concentrations in case of drinking water. However, this alogic, at the first sight, result could be due to the reagents certified as «chemically pure» or «pure» which might have contained the above mentioned elements as impurities.

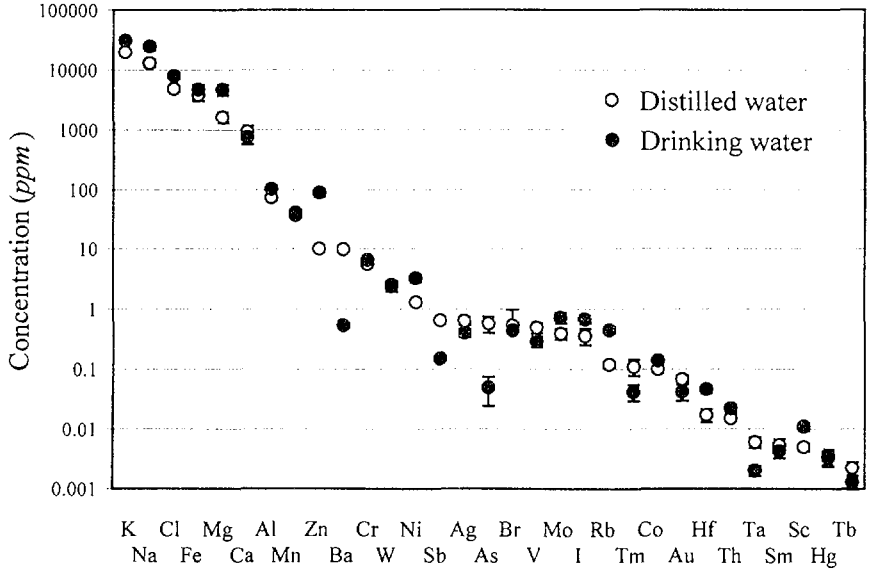


**Fig. 1.** Elemental distribution in lyophilized samples of *Spirulina Platensis* cultivated in distilled and drinking water

In future, the elemental data of the present work could be supplemented by the data for Pb, Cd, Cu, etc. which could be obtained using other analytical techniques such as AAS and ICP-MS. It is interesting to note that there is a good agreement between results for K, Na, Ca, Mg, Mn, and Zn obtained by NAA in the present work and those obtained using AAS by the authors in 1996 [15]. Iron concentration by ENAA is twice as high as the AAS data, which could be explained by possible incomplete dissolution of iron compounds in case of AAS.

Results obtained in experiments with Se and Hg loadings are given in **Table 3** and are shown in **Figs. 2** and **3**. In both cases, the best approximation corresponds to a polynomial of the 2nd order: $y = -0.00008\,x^2 + 0.3\,x - 1$ and $y = 0.0005\,x^2 + 0.1\,x + 0.16$ for Se and Hg, respectively. Selenium concentration in the nutrient medium of the order of ~ 100 $\mu g/L$ seems to be critical; above this value the intensive accumulation of Se in spirulina matrix is observed with a possible maximum at 1100–1200 $\mu g/L$. This range of concentrations is to be examined more carefully in the future experiments.

One may assume that the source of Se binding in spirulina could be amino acid – Cystine, so far its concentration in biomass reaches ~ 1%. The possibility of Se-Cystine complex formation and its carcinoma risk chemical protection are discussed elsewhere [2].

**Table 3.** The dependence of Se and Hg accumulation in algae *Spirulina Platensis* on a nutrient medium loading of the above elements of different concentrations

| No. | Sample index | Nutrient medium, *μg/L* | *Spirulina Platensis, ppm* | |
|---|---|---|---|---|
| 1 | Control Se* | — | 0.69 ± 0.09 | Spirulina Platensis in distilled water |
| 2 | 1+SE | 0.106 | 1.37 ± 0.15 | |
| 3 | 2+SE | 0.55 | 2.07 ± 0.2 | |
| 4 | 4+SE | 5.50 | 2.06 ± 0.2 | |
| 5 | 5+SE | 11.30 | 6.35 ± 0.6 | |
| 6 | 6+SE | 113 | 12.2 ± 1.2 | |
| 7 | 7+SE | 553 | 128 ± 11 | |
| 8 | 8+SE | 2770 | 159 ± 14 | |
| 9 | Control Hg** | — | <0.050 | |
| 10 | 2+HG | 0.0033 | 0.09 ± 0.05 | |
| 11 | 3+HG | 0.033 | 0.38 ±0.11 | |
| 12 | 4+HG | 0.33 | 0.24 ± 0.07 | |
| 13 | 5+HG | 3.3 | 0.49 ± 0.15 | |
| 14 | 6+HG | 33 | 3.9 ± 1.2 | |
| 15 | 7+HG** | — | 0.61 ± 0.18 | C-phycocyanin in distilled water |

* A nutrient medium may contain Se impurities at the background concentration level
** A nutrient medium may contain Hg impurities at the background concentration level

**Table 4** presents some results on major, minor and trace elemental content in globular protein C-phycocyanin extracted from spirulina biomass according to the method described earlier [16]. Seventeen elements were quantitatively determined using ENAA. Their levels and their order of descending concentrations differ from those for the whole spirulina cells given in **Fig. 1**. Such elements as Mg, Ca, Al, Zn, Mn are not observed, at the same time the concentrations of Sr, Cr, As, Mo, Ni, Co in C-phycocyanin are much higher than those in the undamaged spirulina biomass. Being a constituent part of spirulina biomass, C-phycocyanin may lack some elements which are generally present in the cells, whereas the contents of some other elements may increase due to the process of accumulation.

The cell cultivation is accompanied by synthesis of nucleic acids, protein, hydrocarbon, pigments, lipids and other macromolecular structures. The nature and character of biomolecular interaction with different chemical elements could be different: from weak bonding of free energy of 1-7 *kcal/mol* to strong covalent ones of the energy of -50 ÷ -110 *kcal/mol*. The elements determined by INAA and ENAA in the present work are more evidently form weak bondings such as van-der Waals (1-2 *kcal/mol*) or hydrogen (3-7 *kcal/mol*). Hydrogen bonding, as well-known, plays rather substantial biological role. For this reason, our next efforts will focus on studying the nature of binding energy of double and triple complexes of the Me→DNA; Me-protein, Me→DNA→protein. The globular protein C-phycocyanin in the present experiments serves as a starting point for such investigations.

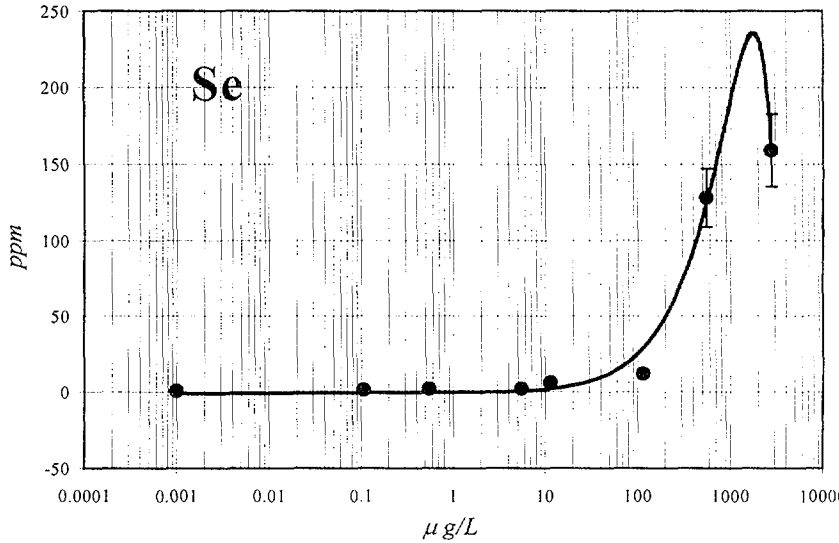


**Fig. 2.** Se in *Spirulina Platensis* biomass *versus* its content in a nutrient medium.

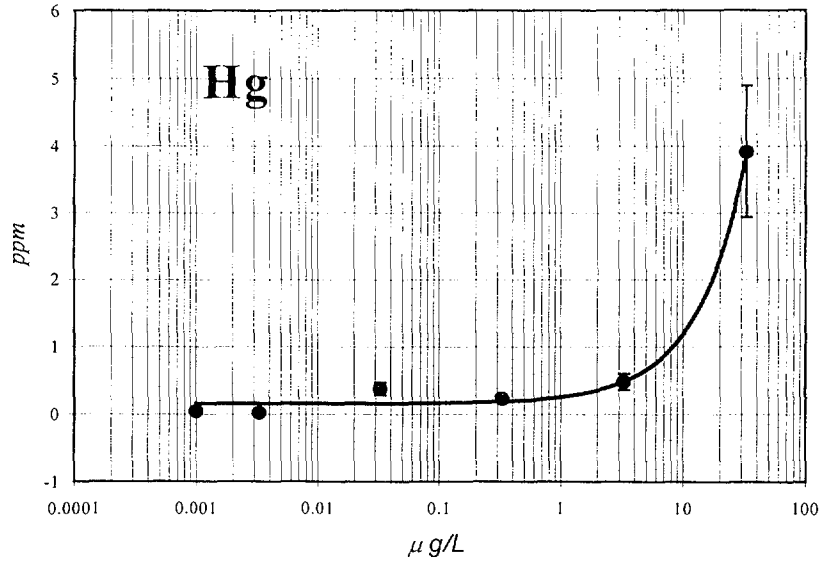


**Fig. 3.** Hg in *Spirulina Platensis* biomass *versus* its content in a nutrient medium.

**Table 4.** Content of some major and trace elements in globular protein C-phycocyanin determined by ENAA

| № | Element | Content, *ppm* | Error, % |
|---|---|---|---|
| 1 | K | 287 800 | 10 |
| 2 | Na | 42 770 | 15 |
| 3 | Fe | 2 200 | 10 |
| 4 | Sr | 77 | 30 |
| 5 | Cr | 48 | 15 |
| 6 | Mo | 11 | 4 |
| 7 | As | 10 | 11 |
| 8 | Ni | 9 | 20 |
| 9 | Au | 7 | 20 |
| 10 | Ag | 6.7 | 20 |
| 11 | Ba | 5.9 | 15 |
| 12 | Rb | 4.0 | 12 |
| 13 | Co | 3.1 | 10 |
| 14 | Br | 0.7 | 5 |
| 15 | Hg | 0.61 | 30 |
| 16 | Sb | 0.55 | 16 |
| 17 | Se | 0.08 | 27 |

## Conclusions

1. The major, minor and trace element content of *Spirulina Platensis* biomass and their distributions show that the main impurities are present at *μg/L* Spirulina biomass cultivated in a standard nutrient medium does not contain toxic element concentrations which exceed the permissible levels. (Permissible levels are assumed to be the doses, which could be accepted by man organism in principle. For example, the USA data for different elements can be found at the website http://www.spirulina.com/SPBNutrition.html). *Spirulina Platensis* could serve as the matrix for the production of pharmaceuticals, even when reagents certified as «chemically pure» and «pure» are used.
2. The possibility of the purpose-oriented incorporation of Se in spirulina biomass was demonstrated. The polynomial dependence of Se accumulation in algae *Spirulina Platensis* on a nutrient medium loading at different levels was established. This dependence may serve as a recipe to produce pharmaceuticals (curve, **Fig. 2**).
3. The dependence of Hg accumulation on its nutrient medium loading was determined. The necessity of using high purity chemical reagents free of toxic elements for the production of pharmaceuticals based on *Spirulina Platensis* matrix was emphasized.
4. The elemental composition of C-phycocyanin showed the possibility to examine separate macromolecular biological complexes comprising Spirulina cells for a better understanding of the processes involved in the bonding of the elements.

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**References:**


1. Fox D., Health Benefits of Spirulina. In *Spirulina, Algae of Life*., Bulletin No. 12. April 1993, Publ. by Institute of Oceanography, Monaco.
2. Foster H. Health, Disease and the Environment. Belhaven Press, London, 1992.
3. Combs G. F. Jr., Chemopreventive mechanisms of selenium. *Med. Klin.*, 1999 Oct.15, 94, Suppl. 3: 18-24.
4. Navarro-Alarcon M., de la Serrana H.L., Perez-Valero V., Lopez-Martinez C., Serum selenium levels as indicators of body status in cancer patients and their relationship with other nutritional and biochemical markers. *Sci. Total Environ.* 1998, Apr. 8, 212(2-3): 195-202.
5. Schumacher K., Effect of selenium on the side effect profile of adjuvant chemotherapy/radiotherapy in patients with breast carcinoma. Design for a clinical study. *Med. Klin.* 1999, Oct.15, 94, Suppl 3: 45-48.
6. Ebert-Duming R., Seufert J., Schneider D., Kohrel J., Schutze N., Jacob F., Expression of selenoproteins in monocytes and macrophages - implications for the immune system. *Med. Klin.* 1999, Oct. 15; 94, Suppl. 3: 29-34.
7. Holben D. H. and Smith A. M., The diverse role of selenium within selenoproteins: a review. *J. An. Diet. Assoc.* 1999, July; 99(7): 836-843.
8. Frontasyeva M. V., Steinnes E., Epithermal neutron activation analysis for studying the environment. Proc.*Int. Symposium on Harmonization of Health Related Environmental Mesurements Using Nuclear and Isotopic Techniques* (Hyderabad, India, 4-7 November, 1996), IAEA 1997, p. 301-311.
9. Mosulishvili L.M., Nadareishvili V.S., Kharabadze N.E., Belokobilsky A.I.. Facility for lyophilization of biological preparations. Patent USSR N779765, *Bull.* 42 (1980).
10. Mosulishvili L. M., Kharabadze N. E., Belokobylsky A. I., Gudushauri A. Sh., Application of the Absorbtion-Condensation method of lyophilization. in the Activation Analysis of Biological Materials. *Proc. Georg. AS, Biol.Ser.*, 1980, Vol. 98, N3, 685-688.
11. Nazarov V. M., Pavlov S. S., Herrera E., Frontasyeva M. V., Recent developments of radioanalytical methods at the IBR-2 pulsed fast reactor. *J. Radioanal. Nucl. Chem.*, 167 (1993) 11-21.
12. Ostrovnaya T. M., Nefedyeva L. S., Nazarov V. M., Borzakov S. B. and Streikova L. P., "Software for INAA on the Basis of Relative and Absolute Methods Using Nuclear Data Base". Activation Analysis in Environment Protection, D-14-93-325, Dubna, 1993, p. 319-326.
13. Mosulishvili L. M., Kuchava N. E., Shonia N. I., Investigation of the termostability of Synthetic Standards for instrumental neutron activation analysis of biological Samples. *Proc. Georg. AS, Biol.Ser.*, 1982, Vol.107, No.2, 501-504.
14. Mosulishvili L. M., Dundua V. Yu., Kharabadze N. E., Efremova E. Yu., Chikhladze N.V., Synthetic multielement standard samples for serial neutron activation analysis of biologycal materials, *J. Radioanal. Nucl. Chem.*, Article 83/1, 1984, pp 13-19.
15. Belokobilsky A., Gelashvili M., Mosulishvili L., Rcheulishvili A., Khizanishvili A., Investigation of the Change of Spirulina Platensis Phisical-Chemical Properties in the Growth Dynamics. *Proc. Georg. AS, Biol. Ser.*, 1996, vol. 22, N 1÷6, pp.104-109.
16. Belokobilsky A., Tsibachashvili N., Rcheulishvili A., Mosulishvili L., Accumulation of Cd(II) in C-phycocyanin from Spirulina platensis. *Bul. Georg. AS.* 1999, Vol.159, N1, 140-143.



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