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EDITORIAL

The most significant achievements in the field of natural sciences are reached in joint collaboration, where important roles are taken by biology and chemistry. Therefore publication of a Journal, displaying results of current studies in the field of biology and chemistry, facilitates highlighting of theoretical and practical issues and distribution of scientific discoveries.

One of the basic goals of the Journal is to promote the extensive exchange of information between the scientists from all over the world. We welcome publishing original papers and materials of Biological and Chemical Conferences, held in different countries (after the process of their subsequent selection).

Creation of special International Journal of Biology and Chemistry is of great importance, because a great amount of scientists might publish their articles and it will help to widen the geography of future collaboration. We will be glad to publish also the papers of the scientists from the other continents.

The Journal aims to publish the results of the experimental and theoretical studies in the field of biology, biotechnology, chemistry and chemical technology. Among the emphasized subjects are: modern issues of technologies for organic synthesis; scientific basis of the production of physiologically active preparations; modern issues of technologies for processing of raw materials, production of new materials and technologies; study on chemical and physical properties and structure of oil and coal; theoretical and practical issues in processing of hydrocarbons; modern achievements in the field of nanotechnology; results of studies in the fields of biology, biotechnology, genetics, nanotechnology, etc.

We hope to receive papers from a number of Scientific Centers, which are involved in the application of the scientific principles of biology, biotechnology, chemistry and chemical technology on practice and carrying out research on the subject, whether it relates to the production of new materials, technology and ecological issues.

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Behaviour of some hydrobionts in experimental conditions of accidental oil pollution

Abstract: This work presents results on behavioral reactions and sensitivity of molluscs and fish to experimental modelling of accidental oil pollution during the first 72 hours. It is shown that mobile hydrobionts, such as fish attempt to escape the contaminated area during the first 24 hours after the oil spill. Benthic animals – molluscs are more susceptible to pollution, they almost instantly lose their mobility, become inert and try to escape their shells further on, which serves as an evidence of death throes. Light crude with high content of paraffin was used for the present experiment.

Key words: oil impact, molluscs, fish, experiment, behavioral reactions.

Introduction

The problem of anthropogenic contamination is currently topical for Kazakhstan, this includes contamination of aquatic and terrestrial ecosystems by hydrocarbons. This is relevant to water reservoirs with active oil extraction sights, Caspian Sea shelf, in particular, is already showing some signs of strong oil pollution, which is known to be toxic for hydrobionts when the levels of threshold limit value (TLV) are exceeded and leads to a reduction in unique biological diversity. Gas and oil extraction activities exert polluting effects on the surroundings at all stages of the production cycle – during geological surveyence works, well-boring, extraction of oil and gas, their preparation and storing, transportation and processing. From a chemical point of view, crude oil is a complex mixture of paraffin, cycloparaffin and aromatic hydrocarbons. Apart from these basic components it contains sulfuric and nitrogenous compounds, organic acids, microelements. Oil may also contain naphthenic acids and phenolic compounds, which in turn contain polyaromatic hydrocarbons – environmentally highly dangerous compounds. In soluted form oil is 80-90% aromatic hydrocarbons (benzene, toluene, ethylbenzene, xylene and others), which are highly toxic [1, 2].

The negative impact of oil pollution on biota is observed at all fresh-water and marine ecosystem links. The highest priority hazards for living organisms are water-soluble fractions of oil that contain aromatic hydrocarbons with toxic, teratogenic and mutagenic effects. Many of oil products have an ability to accumulate, retain, metabolize in the organisms of hydrobionts and can be transmitted through food nets [3, 4].

Aside from direct impact on microorganisms, vegetation, invertebrates, fish and other aquatic animals, these toxicants influence the processes of gameto and embryogenesis, development of larvae and young fish [5, 6].

Failure of separate physiological functions occurs at organism level, behavioral changes, an increase in mortality due to direct poisoning or decreases in steadiness. Considering sensitivity, fish become vulnerable due to oil pollution directly through skin and gills. At populational level the contamination may cause changes in quantity and biomass, birth rate and mortality, sexual and size structure, type of dynamics and a number of functional properties [7].

Benthic organisms are significantly more stable to oil pollution than planktonic organisms, which quickly perish from oil concentrations of about 0.01-0.001 mg/l. Contamination of bottom deposits by oil and oil products leads to a restructure of benthic communities in marine and fresh-water ecosystems [8, 9].

Thus, oil pollution impact on hydrobionts and ecosystems as a whole is a subject of observations in many researches. Review of such researches allows noting different reactions of organisms to oil pollution in post-emergency situations. However, behavior of hydrobionts during the first 24 hours of oil spill is poorly explored. In the present work an attempt to observe sensitivity and behavioral reactions during the first three days of some benthic (molluscs) and pelagic (fish) animals for modeling accidental oil pollution.

Materials and methods

Two species of hydrobionts were chosen as experimental objects: stone morocco *Pseudorasbora parva* (Temmnick et Schlegel, 1846) and pomacea bridgesii *Pomacea bridgesii* (Reeve, 1856) that belongs to gastropod (*Gastropoda*) molluscs. 6 individuals of stone morocco and 2 individuals of molluscs were used as test objects in the experiment. The objects of the observations were kept in a well-aerated fish tank with a volume of 112 L and an optimal temperature of 21°C for a long time (more than a year).

The hydrobionts received a sufficient amount of nutrients and were not exposed to external stress factors for the whole period of time. To determine the hydrobionts' sensitivity to oil pollution, crude oil from Kumkol oilfield of Kyzylorda region with the following characteristics: light (density 0.82- 0.83% g/cm^3), low-sulphur (0.33-0.55%), with a high paraffin content (11%) was added into the tank [10]. TLV of oil products for reservoirs with commercial fishing purposes equates to 0.005 kg/m^3 [5], in terms of the fish tank volume an amount of 150 ml of oil with a concentration of 0.7 mg/L was added, this exceeds TLV by 300 times. Influence of oil on the hydrobionts was observed for 72 hours.

Results and their discussion

In the beginning of the experiment a dense oil slick was dispersed over the surface, but due to the presence of an active aerator consequently disintegrated into separate stains (Figure 1A).

The fish were concentrated near the aerator, however irregularities in the behavior could be observed after 60 minutes. Active movements of the fish were noted, they were avoiding contaminated by oil surface areas of water (Figure 1B).

The molluscs stopped to be active and started to fall down to the bottom of the tank after 3 hours. Weak attempts to turn over to the abdomen were observed, their movements were reminiscent of the attempts to "crawl out of their shells" (Figure 2A). After 8-10 hours and until the end of the experiment the molluscs were laying on the bottom of the tank "without signs of life".

A B

Figure 1 – A – oil film on the water surface; B – avoiding of the contaminated surface areas of water

After 4 hours from the start of the experiment obvious changes of a dorsal fin of one individual were noted: darkening and compression of the interradial space, inert behavior.

After 19 hours several individuals showed some signs of change of the pectoral fins, an obvious reddening that was characterized by hemorrhage at the basis of the fin, darkening of the scale and its "mashing" were noted.

After 24 hours the fish stopped to swim actively, were partly concentrated on the surface of the tank, which is related to oxygen deficiency as a result of the formation of a dense oil slick (Figure 2B).

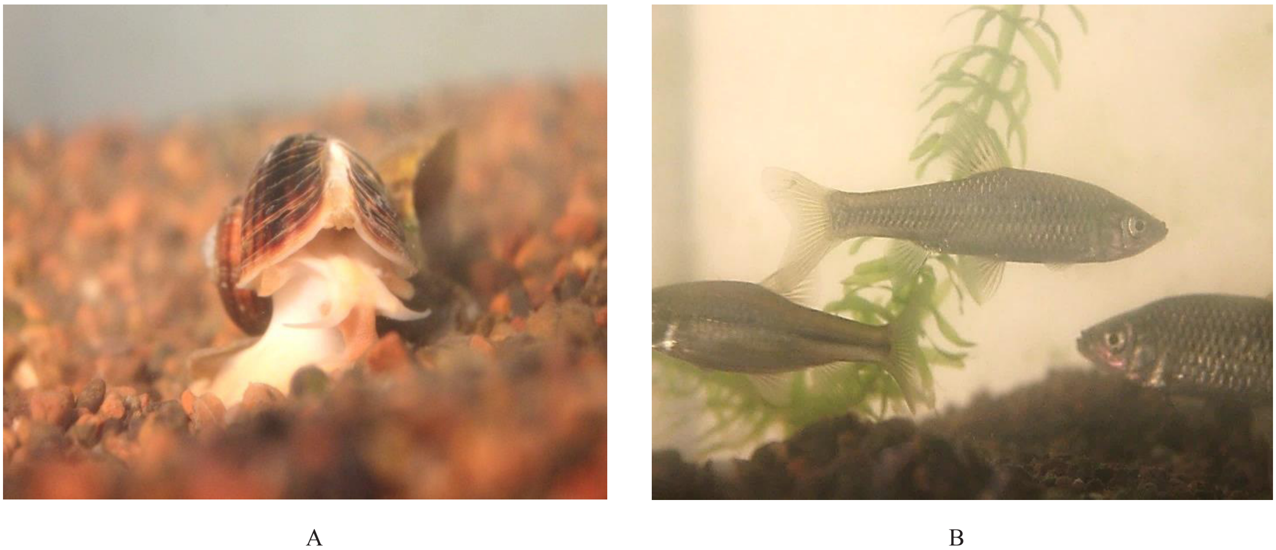

Figure 2-A – Mollusc after several hours of poisoning; B – Impairment of respiratory mechanisms

By the end of the first 48 hours the oil slick disintegrated into separate stains. The fish moved to the bottom of the tank, where they concentrated near water plants. They behaved passively and almost did not perform any swimming activities, but showed no signs of complete asphyxia.

The process of adaptation to unusual, extreme (severe) conditions occurs in several stages or phases: at first decompensation events (impairment of functions) are prevalent, then events of partial adaptation – the organism is actively searching for stable states, which correspond to the new conditions and finally phase of relatively stable adaptation takes place [11].

After 48 hours molluscs and fish were transferred into a clean, well-aerated aquarium, in which they slowly started to regain their activeness. Notably, the molluscs stayed immobile for more than 48 hours and reanimated for significantly longer than the fish. The molluscs like many benthic sedentary hydrobionts are more vulnerable during accidental oil pollution. Precipitation of the oil slick, aggregation of its components in the bottom sediments during prolonged exposure in natural settings may lead to their death.

The fish that belong to a pelago-benthic ecological group do not tend to perish during the first 24 hours after the oil spill and attempt to escape the affected area such that, they express behavioral reactions by trying to escape from polluted areas [12].

It is known that depending on duration and scale of pollution a wide range of damaging effects can be observed – from behavioral anomalies, which was observed in our experiment, death of organisms at early stages of the spill and to structural and functional changes in response to chronicle influence.

Remote consequences of the pollution were also observed in our experiment. In 2-3 months after the experiment all the fish have died. Autopsy of the fish has showed disfunctions in the organs of the digestive and respiratory systems: decay of the liver tissue, formation of tumors on digestive organs and gills.

Conclusion

Thus, results of the experiment on the influence of Kumkol oil, allow us to make the following assumption: during an accidental oil spill in natural surroundings mobile pelago-benthic hydrobionts are practically not prone to dying during the first several days. Sedimentary benthic animals – molluscs, worms, and some crustaceans are more sensitive and possibility of their death in the first 24 hours is considerably high.

At the same time, oil from different oilfields is different in chemical composition. For instance, upper Paleozoic oil of Tengiz and Kashagan oilfields is characterized by aggressive features, due to a significantly large content of hydrogen sulfide (up to 20-25%) [13].

Impact of such oil on behavioral reactions of aquatic organisms immediately after its spill requires additional experimental research. In case of spills and catastrophic releases of such sulfur oil, it will probably disastrously affect the biodiversity of Caspian Sea.

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Identification of spring wheat germplasm resistant to heavy metals for create clean wheat growing technology

Abstract: The aim of this study was to identify wheat germplasm resistant to heavy metals (zinc, cadmium), which are priority pollutants in eastern Kazakhstan region, and identification donors for breeding and promising forms of wheat that are resistance to heavy metals and destined for agricultural production. The objects of research are different genotypes of spring wheat from the collection of the East Kazakhstan Research Institute of Agriculture. In field experiment plants were grown on scientific test site, under natural environmental pollution, in the suburban area of Ust-Kamenogorsk city, East Kazakhstan region. The content of heavy metals in plants was determined by atomic absorption on the device AAnalyst 300 of "Perkin Elmer". Experiments and determination of physiological parameters were conducted by the method of field experiment. The study of the accumulation of heavy metals in components of different genotypes of winter wheat in conditions of natural pollution has shown the genotypic differences in the accumulation of zinc and cadmium in plant seeds. The study of investigated heavy metals accumulation in the spring wheat seeds of East-Kazakhstan Agricultural Research Institute collection showed, that in terms of cadmium accumulation, the most resistant varieties of spring wheat are Ulbinka, Zaulbinka and Omskaya-18. Upon indicator of accumulation of zinc, the most resistant varieties are Samal and Kutulukskaya. Spring wheat varieties Ulbinka, Zaulbinka and Omskaya-18 can be recommended for further use in breeding for resistance to the accumulation of cadmium in the grain. Samal spring wheat varieties can be recommended for further use in breeding for resistance to the accumulation of zinc in the grain. The most harvest from plots has spring wheat varieties: Ulbinka-25, Glubochanka, Erythrospermum-616 and Altai. It is connected to a large number of grains per ear, high grain weight per ear, high productive tillering and good survival during the spring-summer growing season. Varieties Ulbinka-25 and Omskaya-18 can be recommended for cultivation on soils contaminated with cadmium, as these varieties of spring wheat accumulate least of cadmium in the seeds and at the same time are characterized by high yields and good survival during the spring-summer growing season in a polymetallic contamination of soil.

Key words: heavy metals, wheat, metal resistant germplasm, physiological parameters, promising forms.

Introduction

In Kazakhstan, the development of a powerful industry was based on its rich natural resources. However, the industrial centers are the areas of highest contamination by heavy metals [1].

Sound environmental technologies are crucial to address heavy metal pollution. Development and use of plant varieties characterized by minimal accumulation of heavy metals can provide one such environmental solution.

Plants, grown in contaminated soils, exhibit significant interspecies differences in responses to pollution.

The available literature data showed that a significant positive correlation between the concentration of heavy metals in the grain and genotypes, indicating the possibility of breeding varieties with a low potential for accumulation of heavy metals [2].

Characterization of heavy metal accumulation in wheat and identification of germplasm resistance to influence of heavy metals is an important step towards the creation and use of wheat varieties resistant to heavy metal pollutions in agricultural production systems.

This requires the study of the gene pool of cultivated plants and the selection of donors that accumulate minimum amount of heavy metals and varieties that are promising for cultivation in areas contaminated with heavy metals.

The aim of this study was to identify wheat germplasm resistant to heavy metals (zinc and cadmium), which are important to eastern Kazakhstan region, identification of donors for breeding and promising forms destined for agricultural production.

Materials and methods

Different genotypes of wheat specific to East Kazakhstan were studied. Experiments were carried out using spring wheat varieties of East Kazakhstan Agricultural Research Institute collection: Altai, Ulbinka-25, Zaulbinka, Lyazzat, Glubochanka, Nargiz, Omskaya-18, Samal, Eritorospermum-606, Kutulukskaya.

Field studies carried out for the determination of physiological parameters. Plants were grown on scientific test site, under natural environmental pollution, in the suburban area of Ust-Kamenogorsk city, East Kazakhstan region.

In the field experiment the following physiological parameters were determined: phenological observations, yield, yield structure, the content of cadmium and zinc in seeds. Conducting the experiments and determination of physiological parameters were done by the method of field experiment [3].

Heavy metals (zinc and cadmium) in soil and plant samples were determined by atomic absorption with atomization in flame and graphite furnace on the device AAnalyst 300 of "Perkin Elmer" firm.

Sample preparation was carried out using a heating unit "Hot Block" with the addition of concentrated nitric acid and hydrochloric acid at a temperature of 90 ± 5°C, in accordance with standard operating procedures.

Weighed portion of sample was placed in disposable sample cups; 5 mL of 50% nitric acid and 0.5 mL of concentrated hydrochloric acid were added. Samples were mixed well to liquid clay condition, covered with a watch glass and placed in a heating block.

The sample was heated to a temperature of 90 ± 5°C, and evaporated for 10-15 minutes without boiling. Then the sample was cooled, 5 mL concentrated HNO_3 were added and heated again for 30 minutes.

Content of capacitances were evaporated without boiling at a temperature of 90 ± 5°C for approximately up to 5mL during 2 hours, avoiding foaming. After that the sample was cooled and the volume was adjusted to 50 mL with deionized water.

To calibrate of the device calibration blank have been used, consisting of deionized water and 1% HNO_3 solution and standard samples of the company "High Purity".

After calibration of devise readings of analyzed samples were taken. Accuracy of analysis performance was checked by the screening standard of the company "Merck".

The content of heavy metal in the sample was calculated according to the formula:

$$Cmg/kg = \frac{C_{device} \times V_{samp} \times FD}{M}$$

where C_{device} – devise reading (mg/L); $V_{samp.}$ – final volume of samples (mL); *FD* – dilution factor; *M* – weighed sample (g).

Results and their discussion

The genotypes of spring wheat were studied in the field of natural soil contamination with heavy metals.

Our studies of the accumulation of heavy metals in the soil of root zone of different genotypes of winter wheat collection of East Kazakhstan Agricultural Research Institute under natural pollution showed that the content of heavy metals is different.

Zinc content exceeds the maximum permissible concentration of the metal only in the root zone soil of varieties Nargis and Samal, in the zones of other varieties such excess is not observed (Figure 1).

Thus, the wheat plants are not under stress from increased concentrations of zinc in the soil, except for varieties Samal and Nargiz, but in this case the excess is insignificant.

Investigation of cadmium in the soil of the roots zone of different genotypes of winter wheat showed that the amount of the metal exceeds the maximum permissible concentration in nearly all varieties, except variety Nargiz (Figure 2).

The scope of excess of MPC is from 1.5 to 4.5 times. The highest excess recorded in the root zone soil of variety Omskaya-18. Thus, the wheat plants are stressed by high concentrations of cadmium in the soil, and this stress is significant. It should be noted also that the study of soil contamination has polymetallic character. The excess of the MPC also observed for metals such as copper and lead.

In this connection, it was of interest to study the parameters of growth and development of these varieties to identify the tolerance to abiotic stress and agronomic resistance of varieties in the conditions of polymetallic stress. This may facilitate the identification of varieties that retain good productivity and at the same time little accumulates heavy metals in the grain.

Investigation of zinc and cadmium in the seeds of winter wheat genotypes is the most important as a grain of wheat is used in the food industry. According to some researchers, the level of heavy metal cadmium in products is the problem of food security. Reduction of cadmium in the grain is one of the priorities of breeding programs [4].

Our research of accumulation of zinc in plant seeds of genotypes of East-Kazakhstan Agricultural Research Institute collection in a polymetallic soil contamination have shown that this metal accumulates in small quantities, and its content does not exceed the maximum permissible concentration in the seeds of almost all genotypes, except varieties Nargis, Altai and Glubochanka (Figure 3).

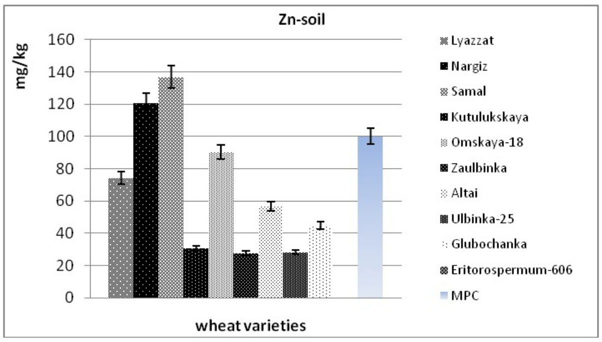

Figure 1 – Content of zinc in the root zone soil of studied genotypes (mg/kg) in relation to the maximum permissible concentration (MPC).

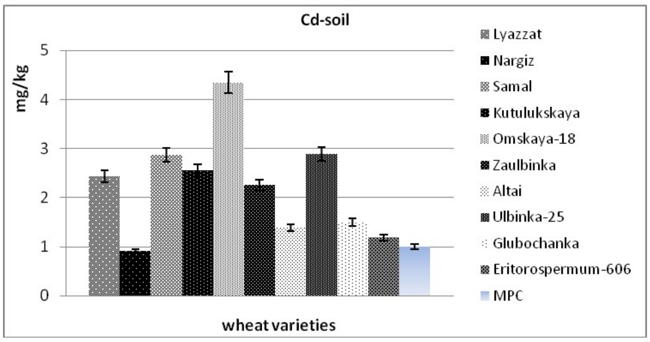

Figure 2 – Content of cadmium in the root zone soil of studied genotypes (mg/kg) in relation to the maximum permissible concentration (MPC).

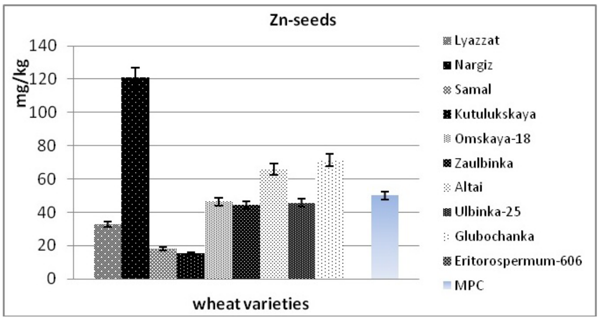

Figure 3 – Zinc content in the seeds of different spring wheat genotypes of East-Kazakhstan Agricultural Research Institute collection

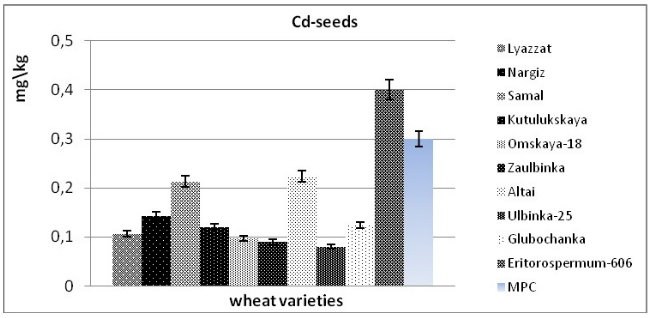

Figure 4 – Cadmium content in seeds of different genotypes of East-Kazakhstan Agricultural Research Institute spring wheat collection

This may be due to the fact that the content of the element in soil does not exceed MPC for soil or slightly exceeded. Despite this, in plant seeds of varieties Glubochanka and Altai accumulated amount of zinc is slightly above the MPC for wheat. The amount of zinc for 1.3 – 1.4 times higher than the maximum permissible concentration for grain in the seeds of varieties Altai and Zaulbinka and for 2.4 times greater than maximum permissible concentration in the seeds of variety Nargis..

The study of cadmium accumulation in plant seeds of genotypes of East-Kazakhstan Agricultural Research Institute collection in a polymetallic soil contamination have shown that this metal accumulates in small quantities, and its content is slightly higher than the MPC for the grain in case of spring wheat variety Erythrospermum (Figure 4).

It should be noted that in the root zone soil content of cadmium is significant and exceeds MPC almost in all genotypes, except varieties Nargiz and Altay (Figure 6). Despite this, spring wheat seeds do not accumulate significant amounts of cadmium. The greatest amounts of cadmium accumulate seeds of variety Erythrospermum. Thus, the study of investigated heavy metals accumulation in the spring wheat seeds of East-Kazakhstan Agricultural Research Institute collection showed, that in terms of cadmium accumulation, the most resistant varieties of spring wheat are Ulbinka, Zaulbinka and Omskaya-18.

In terms of accumulation of zinc, the most resistant varieties are Samal and Kutulukskaya. In respect to varieties Kutulukskaya, this fact can be seen as a disadvantage, since zinc content in soil, where the roots of this variety plants inhabit, is slight. Seeds of this wheat variety depleted of necessary for nutrition element. For variety Samal, this fact can be considered as an advantage, since the soil layer, where this variety roots inhabit, the zinc content heightened and in spite of this seeds does not accumulate significant amounts of zinc.

Spring wheat varieties Ulbinka, Zaulbinka and Omskaya-18 can be recommended for further use in breeding for resistance to the accumulation of cadmium in the grain.

Samal spring wheat varieties can be recommended for further use in breeding for resistance to the accumulation of zinc in the grain.

The number of plants before the harvest, in comparison with their number before tillering shows the survival rate during the spring and summer growing season, that is why this indicator was also determined.

The study of plants number before tillering showed that the greatest plants number observed in variety Glubochanka (Figure 5). The average number of plants, in comparison with other varieties, is observed in Zaulbinka, Omskaya 18, Altai, Ulbinka, Kutulukskaya, Samal. Smallest number of plants is in varieties – Erythrospermum 606, Lyazzat, Nargis.

Determination of the number of plants before the harvest showed the greatest number of plants observed in variety Glubochanka (Figure 5).

The average number of plants, in comparison with other varieties, observed at the varieties Zaulbinka, Omskaya 18, Altai, Ulbinka, Kutulukskaya, Samal. The smallest number of plants is in the varieties – Erythrospermum 606, Lyazzat and Nargis.

Determination of the number of plants before the harvest showed that the greatest number of plants observed at variety Glubochanka (Figure 5).

As already mentioned above, the number of plants before the harvest, relative to their number before tillering shows survival during the spring-summer growing season. Counting of the survival rate showed that the highest survival has variety Glubochanka. Varieties of spring wheat: Zaulbinka, Omskaya 18, Ulbinka 25, Kutulukskaya, Lyazzat, Nargiz and Altai demonstrated the average survival. The lowest survival rates show the varieties Erythrospermum 606 and Samal (Figure 6).

Very important indicator is productive tillering, which shows the ability of plants to form a productive lateral shoots, which make a considerable contribution to the overall productivity of the variety. The number of stems, formed on wheat plant is called coefficient of tillering. General tillering is distinguished, i.e. the number of shoots per plant, and productive tillering, by which is meant the number of shoots bearing the ear per plant. Usually productive tillering is two to three times less then general, but under favorable conditions the coefficients of general and productive tillering come closer.

For this indicator, the most productive tillering have plants of variety Glubochanka, the average among the studied genotypes – Omskaya 18, Zaulbinka, Altai, Ulbinka 25, Kutulukskaya, lower – gen-

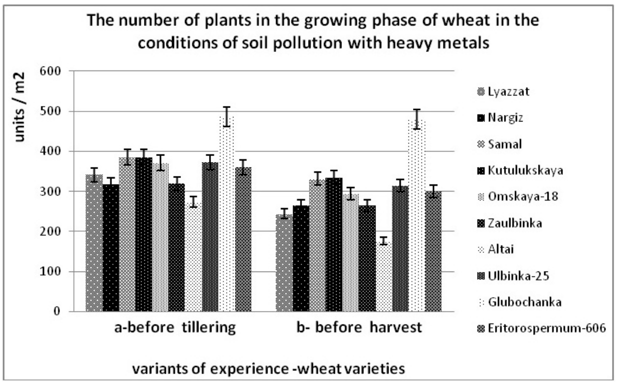

Figure 5 – The number of plants at different genotypes of spring wheat per phases of vegetation, when grown in the natural environment pollution

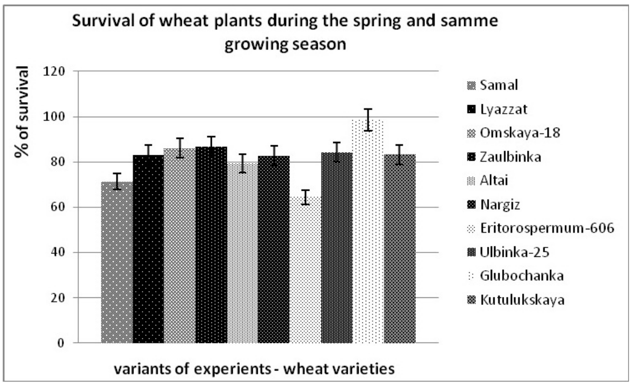

Figure 6 – Percentage of the surviving before harvesting plants of different spring wheat genotypes, when grown in the natural environment pollution

otypes Nargis, Samal, Lyazzat, the lowest – Eritrospetmum 606 (Figure 7).

Metal-resistant wheat varieties should be characterized not only survival during the spring and summer growing season, but also a good productivity in conditions of soil contamination with heavy metals. So is determined such an important valuable economic feature as yield of wheat plants. Among the varieties of spring wheat of East-Kazakhstan Agricultural Research Institute collection the harvest from the plots is largest at spring wheat Ulbinka-25, Glubochanka, Erythrospermum-606 and Altay (Figure 8).

In the case of the variety Ulbinka-25 together with high yield, this variety is also characterized by high indicators, as grain weight of main ear and as well as grain weight of lateral stems. In varieties Erythrospermum 606 and Altai, along with high yields also the highest values of grain weight of main ear and average indicators weight of grain of lateral stems revealed.

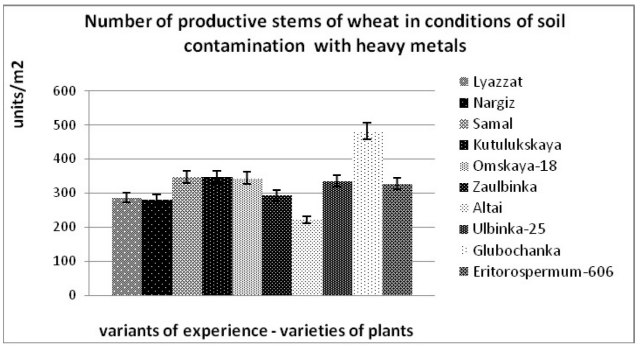

Figure 7 – The productive tillering of plants of different genotypes of winter wheat of world collection, when grown in the natural environment pollution.

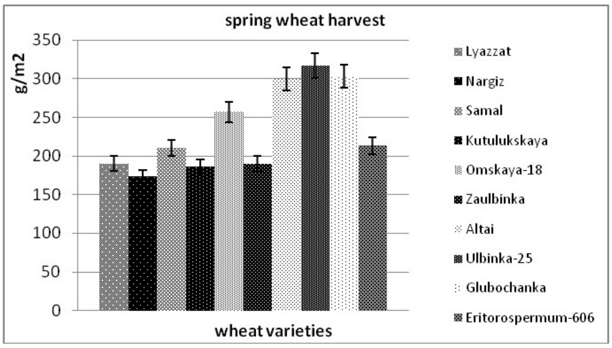

Figure 8 – The harvest of different spring wheat genotypes of the East Kazakhstan Agricultural Research Institute collection, when grown in natural conditions of environmental pollution

Variety Glubochanka, although has an average the grain mass of the main ear and lateral stems, however, characterized by the highest rates of productive tillering, i.e. the number of productive lateral stems is big and probably therefore this variety has a higher overall crop yields.

The lowest yields in conditions of soil contamination with heavy metals demonstrate varieties: Lyazzat, Zaulbinka, Samal, Nargiz. The average yield compared with other spring wheat genotypes detected in varieties Kutulukskaya and Omskaya-18 (Figure 7).

Thus, the most harvest from plots was obtained from spring wheat varieties: Ulbinka-25, Glubochanka, Erythrospermum-606 and Altai.

It is connected probably to a large number of grains per ear, high grain weight per ear, high productive tillering and good survival during the spring-summer growing season.

Varieties Ulbinka-25 and Omskaya-18 can be recommended for cultivation on soils contaminated with cadmium, as these varieties of spring wheat accumulate least of all cadmium in the seeds and at the same time are characterized by high yields and good survival during the spring-summer growing season in a polymetallic contamination of soil.

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The genetic potential of spring wheat resistance to cadmium and zinc

Abstract: The problem of obtaining safe foodstuff has remained relevant for a very long time. Long period of soil self-purification and the expensiveness of its artificial treatment forced mankind to look for new ways to solve problems related to soil pollution with heavy metals. The most perspective direction in this field is the identification of plant facilities, characterized by minimal accumulation of heavy metals. The purpose of our research is to identify wheat germplasm, resistant to heavy metals (zinc, cadmium) primarily in Eastern Kazakhstan, and discover donors for selection for metal resistance and perspective wheat forms, intended to implement into production. The research subject is various genotypes of spring wheat from the collection of Eastern Kazakhstan Agricultural Scientific Research Institute. The experiments were held in condition of modeling environmental pollution on 10-day-old sprouts of different wheat genotypes, grown on nutrient mixture, containing $CaSO_4$ 0.1mm and Cd ions at a concentration of 40 mg/L (as $CdSO_4$ salt) or Zn at a concentration of 400 mg/L (as salt $ZnSO_4$). Since a direct negative impact of heavy metals on plants is displayed in inhibiting of their growth and development (root and sprout growth weakening), indicators of wheat genotypes growth processes were examined during the experiment and Wilkins coefficient was defined. The study revealed sensitive and metal resistant samples. Studies of membrane permeability for electrolytes in terms of environmental pollution by cadmium and zinc, have led to the conclusion that plant resistance can generally be caused by cell membranes' resistance to the action of the stressor. Membrane permeability for electrolytes in the most resistant wheat variety Altai changed less comparing to a more sensitive variety of spring wheat Erythrospermum-606 during the action of both cadmium and zinc. This fact proves that the overall resistance of plants may be due to stability of cell membrane to the action of the stressor.

Key words: heavy metals, wheat, growth parameters, membrane permeability, accumulation of metals, sensitive and resistant genotypes.

Introduction

Plants, grown in contaminated soils, exhibit significant interspecies differences in responses to pollution [1]. It is known that the ability of plants to absorb, accumulate, and use chemical elements genetically determined [2].

On the basis of material obtained by screening the gene pool of wheat and by use of genetic models, Gamzikova O.I. with colleagues developed the idea of the ability to manage the edaphic stability properties by breeding method [3].

The available literature data showed that a significant positive correlation between the concentration of heavy metals in the grain and genotypes, indicating the possibility of breeding varieties with a low potential for accumulation of heavy metals [4].

Kazakhstan is characterized by the presence of mining and metallurgical industries, highly polluting by heavy metals, polluting the agricultural land in particular. Using and creating varieties that are tolerant to pollutants, especially heavy metals, is a component of environmentally clean technologies [1].

At the first stage of this process it is necessary to investigate the gene pool of cultivated and wild plants and segregate sustainable forms of plants and breeding donors, accumulating the minimum number of ecotoxicants.

Therefore, the purpose of work was the investigation of the different genotypes of winter wheat to identify the forms that are resistant to heavy metals such as zinc, cadmium, which are priority pollutants in the East-Kazakhstan region and identification of donors for breeding.

Materials and methods

Studies were carried out on various genotypes of spring wheat from the collection of Eastern Kazakhstan Agricultural Scientific Research Institute.

The following spring wheat genotypes were examined during the experiment: Altai, Ulbinka-25, Zaulbinka, Lyazzat, Glubochanka, Nargiz, Omskaya-18, Samal, Erythrospermum-606, Kutulukskaya.

Plant growth was held in terms of modeling environmental pollution on 10-day-old sprouts of different wheat genotypes, grown on nutrient mixture, which contains $CaSO_4$ 0.1mm and Cd ions at a concentration of 40 mg/L ($CdSO_4$ salt) or Zn at a concentration of 400 mg/L ($ZnSO_4$ salt). The plants were grown for 10 days in water culture with t-22^{o}C during the day and 18^{0}C at night, with a 14-hour photoperiod, light intensity – 5 thousand lx, humidity – 65%.

The measurement of biometric indicators was carried out according to common methods. The plants were dissected to an over-ground part and roots. Roots and surface organs length were measured. In order to determine the plants' dry biomass, they were put in a drying cabinet and dried at t-105^{o}C till constant weight, cooled down to room temperature and weighed.

The tolerance index or Wilkins coefficient were calculated using the formula: $I_t = I_{me} / I_c$, where I_{me} – growth of the roots on the solution of the tested metal, I_c – growth of the roots on the solution without metal [5].

The testing of contrasting by stability spring wheat genotypes for the permeability of cell membranes was conducted in laboratory conditions. To research the permeability of wheat cell membranes, identified during experiments on study of growth parameters wheat varieties contrasting by resistance were used, resistant – Altai and sensitive – Erythrospermum-606.

Membrane permeability determination for electrolytes was conducted according to Dexter conduct metric method [6]. Modified Dexter method was used to determine the release of electrolytes from plants leaves tissues, exposed to the stress effect.

A weighed sample of 500 mg leaves was immersed into 50 ml water with the known conductivity for 60 – 180 min. The exudate was drained out and electro conductivity was measured by conductivity meter at the end of the exposure.

After the counting the exudate was poured into the same cup with the sample and was boiled up and its electro conductivity was re-measured.

Calculation formula:% electrolytes elution = (a: b) x 100, where a – is the conductivity of dead tissues, b – is the conductivity of living specimens.

Results and their discussion

Our study of cadmium and zinc effect on growth parameters of wheat sprouts in the laboratory conditions showed that the ions of both cadmium and zinc inhibit plant growth.

The research on wheat varieties from the Eastern Kazakhstan Agricultural Scientific Research Institute collection showed that by the growth of surface organs at a high concentration of cadmium, genotypes can be arranged as follows: Altai > Nargiz > Glubochanka > Ulbinka-25 > Samal > Omskaya-18 > Kutulukskaya > Zaulbinka > Lyazzat > Erythrospermum-606 (Figure 1).

Regarding zinc, the results are the following: by the growth of surface organs at a high zinc concentration, genotypes can be arranged as follows: Zaulbinka > Altai > Ulbinka-25 > Lyazzat > Glubochanka > Kutulukskaya > Nargiz > Omskaya-18 > Samal > Erythrospermum-606 (Figure 2).

Based on the received data, we see that in both cases, by the growth of surface sprouts, Altai is the wheat variety least exposed to effect of zinc and cadmium ions, while surface sprouts of variety Erythrospermum-606 acutely react on the presence of heavy metal ions.

Based on the received data, we see that in both cases, by the growth of surface sprouts, Altai is the wheat variety least exposed to effect of zinc and cadmium ions, while surface sprouts of variety Erythrospermum-606 acutely react on the presence of heavy metal ions. Samal, Omskaya-18, Kutulukskaya and Zaulbinka wheat varieties showed average resistance to the cadmium effect according to the growth parameters of surface organs. When introducing zinc to the cultivation medium, growth of surface organs of varieties Lyazzat, Glubochanka, Kutulukskaya, Nargiz, Omskaya-18 is inhibited at the average degree, comparing to other varieties, which is the indicator of average metal endurance. By the root growth, at introducing cadmium to the nutrition medium, genotypes can be arranged the following way: Nargiz > Ulbinka-25 > Altai > Glubochanka > Omskaya-18 > Zaulbinka > Lyazzat > Kutulukskaya > Erythrospermum-606 > Samal (Figure 3).

Altai, Ulbinka-25 and Nargiz have the most resistant to cadmium root system. Root growth of varieties Nargiz, Ulbinka-25 and Altai is inhibited in much less degree comparing to other genotypes at a high cadmium concentration in cultivation medium. Varieties Glubochanka, Omskaya-18, Zaulbinka and Lyazzat have an average root resistance level. Plant roots of varieties Erythrospermum-606 and Samal turned to be the most non-resistant to the adverse cadmium effect (Figure 3). When studying the impact of zinc on growth parameters of varieties from the Eastern Kazakhstan Agricultural Scientific Research Institute collection, it was shown that by the root growth at a high zinc concentration genotypes can be arranged as follows: Altai > Ulbinka-25 > Zaulbinka > Lyazzat > Glubochanka > Nargiz > Omskaya-18 > Samal > Erythrospermum-606 > Kutulukskaya (Figure 4).

Varieties Altai, Ulbinka-25 and Zaulbinka have the most zinc resistant root system. Lyazzat, Glubochanka and Nargiz have an average roots resistance level. Altai, Ulbinka-25 and Zaulbinka varieties' root growth is inhibited less comparing to other genotypes that were introduced to zinc growth medium.

Varieties Omskaya-18, Samal and Erythrospermum-606 are less resistant to the adverse effect of zinc.

The most non-resistant to the adverse effect of zinc are plant roots of variety Kutulukskaya.

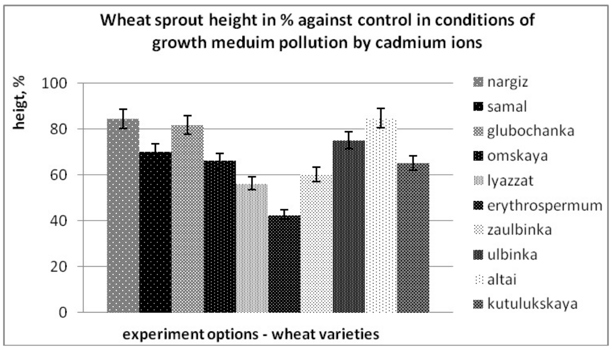

Figure 1 – Influence of cadmium ions presence in growth medium on sprout growth of different wheat genotypes.

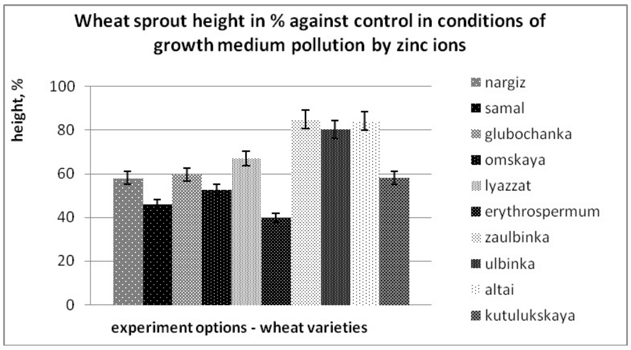

Figure 2 – Influence of zinc ions presence in growth medium on sprout growth of different wheat genotypes

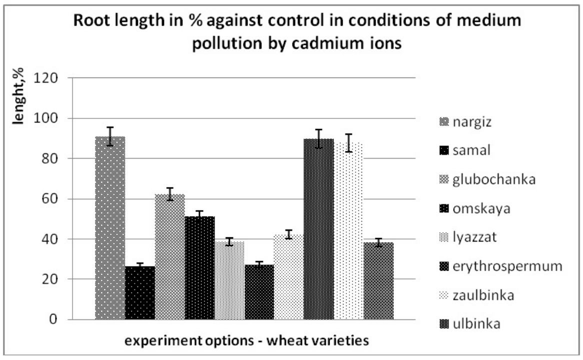

Figure 3 – Influence of cadmium ions presence in growth medium on root growth of different wheat genotypes

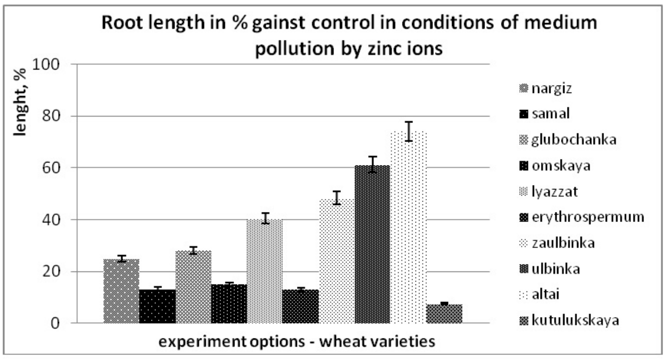

Figure 4 – Influence of zinc ions presence in growth medium on root growth of different wheat genotypes.

Varieties Nargiz, Ulbinka-25 and Altai have the highest, Glubochanka, Omskaya-18, Zaulbinka, Lyazzat Zaulbinka and Kutulukskaya have an average, and Erythrospermum-606 and Samal have the lowest Wilkins coefficient or index of tolerance at a high concentration of cadmium (Table 1).

Based on the results of the study of root growth in polluted by cadmium ions medium and on index of tolerance varieties Nargiz, Ulbinka-25 and Altai can be identified as genotypes with the most resistant towards the adverse effect of cadmium root system.

Altai variety has the highest, varieties Ulbinka-25 and Zaulbinka have an average, and Kutulukskaya and Erythrospermum-606 varieties have the lowest Wilkins coefficient at a high zinc concentration (Table 2). Based on the results of the study of root growth in polluted by zinc ions medium and on index of tolerance genotypes Altai and Ulbinka-25 can be identified as the most resistant toward the adverse effect of zinc root system.

The regularities of accumulation and distribution of Zn and Cd on the bodies of spring wheat depending on the genotypic differences of samples in a model experiment conditions have been studied.

Investigation of zinc content in aerial parts and roots of seedling of spring wheat of different genotypes showed that the smallest accumulation of zinc is in winter wheat aerial organs, when its content in the medium is 400 mg/l, observed in seedlings of varieties Samal and Lazzat. The highest zinc content in the aboveground organs observed in plants of variety Ulbinka.

Table 1 – Wilkins coefficient or tolerance index of wheat sprout roots in polluted by cadmium ions cultivation environment

Wheat varieties	Nargiz	Samal	Glubochanka	Omskaya-18	Lyaz-zat	Erythrospermum-606	Zaulbinka	Ulbinka-25	Altai	Kutu lukskaya
I_{me}	12.08	8.36	15.17	12.63	10.33	4.6	6.9	8.8	11.15	9.6
I_c	13.29	31.46	24.36	24.60	26.70	16.9	16.3	9.8	12.7	25
I_t	0.91	0.27	0.62	0.51	0.39	0.27	0.42	0.90	0.88	0.38

Table 2 – Wilkins coefficient or tolerance index of wheat sprout roots in polluted by zinc ions cultivation environment

Wheat varieties	Nargiz	Samal	Glubochanka	Omskaya-18	Lyaz-zat	Erythrospermum-606	Zaulbinka	Ulbinka-25	Altai	Kutulukskaya
I_{me}	3.32	4.17	6.87	3.72	10.79	2.2	7.9	6	9.4	1.9
I_c	13.29	31.46	24.36	24.60	26.70	16.9	16.3	9.8	12.7	25
I_t	0.25	0.13	0.28	0.15	0.40	0.13	0.48	0.61	0.74	0.08

Study of zinc accumulation in plant roots, at its content in the medium of 400 mg/l, showed that its least amount is contained in the seedling roots of varieties Altai, Lyazzat, Glubochanka and Samal. The highest zinc content in the roots was observed for plant varieties Ulbinka 25.

According to the study of the effect of zinc on the growth parameters of 10-day-old wheat seedlings as well as according to the study of the accumulation of zinc in the roots and aerial parts of plants of different winter wheat genotypes, variety Altai can be identified as a genotype with the highest root resistance to zinc action and variety Lyazzat and Samal as a resistant to zinc translocation into above-ground organs.

Study of zinc accumulation in roots and aerial parts, as well as growth parameters of wheat seedlings of different genotypes allowed to reveal the most sensitive and resistant genotypes. The most resistant to zinc genotypes are varieties of spring wheat Altai, Lyazzat and Samal, and most sensitive – spring wheat variety Ulbinka 25.

Study of the content of cadmium in the aerial parts and roots of seedlings of different genotypes of spring wheat showed that at a dose of cadmium in the environment of 40 mg/L, the lowest number in its roots accumulate sprouts of spring wheat varieties Kutulukskaya, Nargiz, 25 Ulbinka and Samal. The greatest amount of cadmium in the roots of plants accumulates variety of spring wheat Erythrospermum 606. The remaining varieties occupy an intermediate position between them. Thus, the smallest accumulation of cadmium is observed in the roots of spring wheat varieties Kutulukskaya, Nargiz, Ulbinka 25 and Samal.

Cadmium content in aboveground organs of studied seedlings of spring wheat genotypes showed that at a dose of cadmium in the environment of 40 mg/l, in above-ground organs least amount accumulate sprouts of spring wheat varieties Lyazzat, Omskaya 18 and Altai.

In these experimental conditions the greatest amount of cadmium in the aerial parts accumulate seedlings of spring wheat variety Erythrospermum 606.

The remaining varieties occupies an intermediate position between them. Thus, the greatest resistance to the inflow of cadmium in the aerial organs of winter wheat varieties showed Lyazzat, Omskaya 18 and Altai.

According to the study of the accumulation of cadmium in the roots and aerial parts of plants of different spring wheat genotypes can be identified varieties Kutulukskaya, Nargis, Ulbinka 25 and

Samal as the genotypes with the greatest root resistance to cadmium and varieties Lyazzat, Omskaya 18 and Altai as the genotypes resistant to the translocation of cadmium in above-ground organs. The study of cadmium accumulation in the roots and aerial parts, as well as growth parameters of wheat seedlings of different varieties allowed to reveal the most sensitive and resistant genotypes. The most resistant to cadmium were spring wheat genotypes Nargiz, Ulbinka 25 and the Altai, and the most sensitive – variety of spring wheat Erythrospermum 606.

During the research testing of spring wheat contrasting by resistance genotypes we tested on permeability of cell membranes. Membrane permeability change is one of the symptoms of plants reaction to the external influence. It is known, that the exit of electrolytes is the membrane permeability function, which in its turn describes the damage extent [7]. Therefore, genotypes resistant to adverse environmental factors must have different physicochemical membrane characteristics.

Study on the cadmium ions influence on cell membrane permeability of surface organs of resistant Altai and sensitive Erythrospermum-606 wheat genotypes, showed that the permeability of cell membranes for electrolytes increase at a high cadmium (40 mg /l) concentration (Table 3).

The leaf tissues cell membranes permeability of winter wheat variety Altai increased for 23% under the cadmium influence regarding control.

Leaf cell membrane permeability of spring wheat variety Erythrospermum-606 increased for 75% under the cadmium influence (Table 3). It should be mentioned that cell membrane permeability for electrolytes of more resistant wheat variety Altai's changed less comparing to more sensitive genotype Erythrospermum-606.

Table 3 – Influence of cadmium ions on the electrolyte output from leaf tissue of 10-day-old wheat seedlings

Variants	% output of electrolytes	% to control
Variety Altai		
Control	2.52±	100
Cd 40 mg/l	3.17±	123
Variety Erythrospermum		
Control	3.15±	100
Cd 40 mg/l	5.52±	175

Study on the influence of zinc ions on the cell membrane permeability of surface organs of resistant Altai and sensitive Erythrospermum-606 wheat genotypes, showed that cell membrane permeability for electrolytes increases at a high concentration of zinc (400 mg/L) (Table 4). Cell membranes permeability of leaf tissues of spring wheat variety Altai increased for 13% under the zinc influence regarding control.

Leaf cell membrane permeability of winter wheat variety Erythrospermum-606 increased by 45% under the zinc influence relatively to control (Table 4).

Table 4 – Influence of zinc ions on electrolytes output from leaf tissue of 10-day old wheat seedlings

Variants	% output of electrolytes	% to control
Variety Altai		
Control	2.52±	100
Zn 400 mg/L	2.84±	113
Variety Erythrospermum		
Control	3.15±	100
Zn 400 mg/L	4.63±	145

It should be mentioned that cell membrane permeability for electrolytes of resistant wheat variety Altai changed less comparing to more sensitive Erythrospermum-606, both under the cadmium and under zinc effect. The reason for strong inhibition of spring wheat Erythrospermum's-606 growth processes, as well as the relative stability of Altai varieties to the heavy metals toxic effect can be cell membrane's different ability to resist the stress, particularly the strong effect of heavy metals.

Membrane permeability change under stressful conditions proves structural restructure of membranes, which defines plants' potentially possible mechanisms to resist adverse environmental factors [8]. As a result of change of cell membrane permeability under influence of various factors, the intensive output of electrolytes from cells is happening. Intensity of secretion of electrolytes depends on varietal features.

Conclusion

As a result of a comprehensive study of zinc and cadmium effect on the growth of surface organs and sprout roots of different wheat genotypes, resistant and sensitive by growth parameters winter wheat genotypes were identified in the laboratory conditions.

According to the study, as the effect of zinc on the growth parameters of 10-day-old wheat seedlings and the accumulation of zinc in the roots and aerial parts of plants of winter wheat different genotypes can be identified variety Altai as a genotype with the highest root resistance to zinc action and variety Lyazzat and Samal as a resistant to zinc translocation into above-ground organs.

According to the study the accumulation of cadmium in the roots and aerial parts of plants of different spring wheat genotypes can be identified varieties Kutulukskaya, Nargis, Ulbinka 25 and Samal as the genotypes with the greatest root resistance to cadmium and varieties Lyazzat, Omskaya 18 and Altai as the genotypes resistant to the translocation of cadmium in above-ground organs.

Membrane permeability of more resistant wheat variety was changing in less degree comparing to more sensitive wheat genotype, both under the cadmium and the zinc effect. This fact indicates that the overall resistance of plants may be due to their cell membrane resistance towards the stressor effect.

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Distant and intraspecific hybridization, induced mutagenesis in soft bread wheat

Abstract: Increase wheat yields by improving its genotype – one of the most urgent problems of the rural economy. At present, using traditional methods of selection and genetic studies, such as backcross selection, distant hybridization, experimental mutagenesis, increased efficiency of obtaining genetically modified and improved forms of wheat. The high content of mono phosphorus (H_3PO_4, 5%) leads to a weak mutagenic effect, and its 0.1% concentration promotes the development of plants biomass. Fertility rates of soft wheat hybrids crosses with wild species (T. timopheevii, T. dicoccum and T. kiharae) depend on the direction of crossing and genotypes of initial varieties. Wild species as the parent component positively affect the high percentage of grain fertility. Genome of Nadezhda variety is compatible with genomes of wild species [1, 2].

Key words: breeding, chemical mutagenesis, isogenic lines, replacement of chromosomes, distant hybridization, variety, wheat chromosomes.

Introduction

By chemical mutagenesis new forms are obtained qualitatively, such as, dwarf mutants in wheat and barley, ultra-fast mutants in wheat and barley resistant to fungal diseases of plants form, high-lysine and highly productive mutants [3]. These facts indicate that mutants obtained by using chemical mutagens can successfully serve as progenitors of new high-yielding varieties. However, obtaining mutants and their study – is only the first stage of selection work. It is possible to use hybridization in selection of mutations. More important is the use of mutants in hybridization to obtain positive transgressions [4-8]. Preparation of mutants and their use for hybridization requires the study of genetic nature of emerging changes, which is crucial for the selection of effective mutagens with specific action, and to broaden and deepen understanding of the nature of wheat evolution. Mutants having complex morphological, physiological and biochemical changes affecting economically valuable properties can be further used to locate genes that determine the trait followed Intervarietal replacement of chromosomes. Isogenic lines are convenient objects for many biological and agricultural experiments. Main advantage of these lines is high genetic similarity among themselves and with the control line, which allows estimating the contribution to the formation of crop marking characteristics and applying them as effective donor marker signs [1, 2]. One of the possibilities to create new varieties with economically valuable traits, and primarily in the direction of selection on productivity and disease resistance to wheat rust, and their improvement in economically valuable attributes is the method of hybridization. Interspecific hybridization in wheat breeding to leaf rust resistance requires use of *T. timopheevii* [3]. In order to overcome hybrids sterility methods facilitating the gene transfer from distant wheat species have been recently developed. Some of them are based on the methods of chromosome engineering, others on methods of genetic control of meiotic recombination, third on the methods of genetic engineering. The aim of the current research is the obtainment of mutants with agriculturally valuable traits, distant and interspecific wheat hybrids and their breeding analysis.

Materials and methods

Spring soft wheat Kazakhstanskaya 126 variety (*Triticum aestivum* L. *var. ferrugineum* Al.), a series of its monosomic lines and such varieties as Nadezhda, Kazakhstanskaya 4 and Shagala served the objects of the current research. Kazakhstanskaya 126 variety was developed at Kazakh SRI of Agriculture and crop production by crossing soft wheat Lutescence 47 with the local variety Kozhebiday and subsequent two-time selection. Isogenic lines of Avocet variety by *Yr* genes, *T. timopheevii* species. Wheat grains of Nadezhda and Kazakhstanskaya 126 varieties were processed by phosphoric acid (H_3PO_4) in 5-10% aqueous solutions. For that different concentrations of phosphoric acid: 0.01; 0.1 and 0.5% were tested. Wheat grains were then incubated in a solution of appropriate concentration.

Results and their discussion

Comparative study of the effect of different concentrations of phosphoric acid has shown that 5% is set as the optimum concentration of the substance to study the ontogeny and cell division activity of root meristem of wheat germ. Effect of chemical compounds has been considered previously in studies of different directions. However, the genetic basis of variations in plant reaction to the action of these compounds has not been studied. Below is the data for the study of reactions of treated grains under laboratory conditions (Table 1).

Table 1 – Study of cell division and aberrations in anaphase of mitosis

Mutagen and its concentration,%	Total number of analyzed cells	Aberrations	The average percentage of affected cells
Kazakhstanskaya 126			
Control	750	5	0.66±0.01
H_3PO_4 0.1%	750	8	1.00±0.01
H_3PO_4 0.01%	750	11	1.40±0.01
H_3PO_4 0.5%	750	29	3.80±0.40
Nadezhda			
Control	750	3	0.40±0.01
H_3PO_4 0.1%	750	14	1.86±0.02
H_3PO_4 0.01%	750	17	2.26±0.01
H_3PO_4 0.5%	750	37	4.9±0.04

The treated grains were sown in test sites. Phenological observations showed that high level of mono phosphorus (5%) leads to a weak mutagenic effect, while its 0.1% concentration contributes to the development of the biomass. Weak mutagenic effect of 5% concentration is apparently linked to a strong acidification of pH. This is proven by some aberrations of chromosomes in mitosis and meiosis disturbances in plants treated with H_3PO_4 (Figure 1). Mitosis in mutant plants was accompanied by a massive sticking of chromosomes (pyknosis) and offset spindle of metaphase plate (Figure 2).

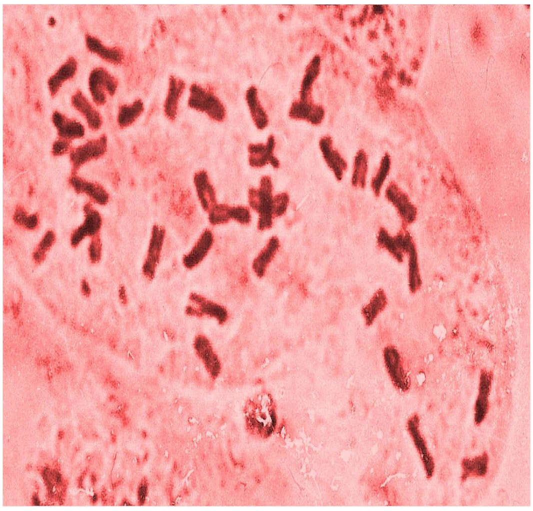

Figure 1 – Mitosis of mutant plants, ditelocentrics are indicated by arrow

Figure 2 – Sticking of chromosomes in plants, treated with phosphoric acid

Mutagenic effect and its importance in the breeding are determined by the results of the mitotic activity and the nature of the aberrations in cell division. They allow us to determine the degree of variability in plants obtained by the action of chemical and physical factors.

The inducing activity of a specific phosphoric acid concentration (5%) on grain germination, cell division and aberrations in mitosis of meristematic cells of test options compared with control obtained by the action of H_3PO_4 was studied. The action of the different concentrations of the chemical compound (H_3PO_4) was observed within the plant ontogeny. Thus, 0.1% H_3PO_4 concentration has a minor deviation (1.00 ± 0.01) on the normal course of mitosis compared to control (0.66 ± 0.01).

Structural analysis of elements of productivity isogenic lines revealed three lines – IL-Hg, IL-BgHg and IL-Pc, differing significantly by increase of productivity indicators of the spike and 1000 grain weight in comparison with control (Table 2). Isogenic line IL-Hg with hairy spike can be morphologically well-tested during the heading stage and has more saturated color of glume compared to the control. Indicators of spike productivity and weight of 1000 grains of the line IL-Hg was significantly higher than such in control (Table 2).

The length of the spike averaged 13.0 ± 0.2 cm. with the number of spikelets counting 20.0 ± 0.4. The number of grains in the main spike counts 63.2 ± 1.0 with a weight of 2.9 ± 0.1 g. Grain is medium size, oval with shallow groove. The average value of the weight of 1000 grains was 48.1 ± 1.4 g, in comparison with control – 44.7 ± 0.7 g.

Isogenic line IL-BgHg has a hairy, black ear. The median length of the spike in IL-BgHg line was 13.1 ± 0.1 cm. The number of spikelets on average counts 20.0 ± 0.1. the number of grains 65.4 ± 0.2, which was significantly higher than control. Grain size is medium, the groove is not deep. The weight of 1000 grains in line IL-BgHg significantly exceeds such in control counting 49.7 ± 0.3 g ($P<0.001$). Observed increase in spike productivity indices in lines IL-BgHg and IL-Hg can possibly be associated with the presence of a dominant allele of glume pubescence Hg in these lines. Isogenic line IL-Pc is characterized by the purple color of straw. The length of the spike in line IL-Pc in average is 12.5 ± 0.5 cm. Number of spikelets 19.6 ± 0.2, number of grains 63.2 ± 1.0. Weight of grain from the main spike in average is 2.7 ± 0.1. Major grain has articulate groove. The weight of 1000 grains in line IL-Pc is 48.4 ± 0.6 g, deviation from control is significant under at $P<0.001$. The increase in the average weight of grain from isogenic line IL-Pc is confirmed by the improved grain filling. This can possibly be associated with the increase in productivity of photosynthesis, due to the intensification of this process in anthocyanin containing plants.

Table 2 – Elements of productivity of spike of morphologically marked isogenic lines

Variety/line	Productivity of the main spike				
	Length of spike, cm	Number of spikelets, pc.	Number of grains, pc.	Weight of grain, g	Weight of 1000 grains, g
K. 126	12.2±0.1	19.0±0.3	51.7±1.6	2.4±0.1	44.7±0.7
IL-Hg	13.0±0.2***	20.0±0.4**	63.2±1.0***	2.9±0.1***	48.1±1.4**
IL-Pc	12.5±0.5	19.6±0.2	63.2±1.0***	2.7±0.1*	48.4±0.6***
IL-BgHg	13.1±0.1***	20.0±0.1***	65.4±0.2***	3.0±0.1***	49.7±0.3***
Deviation from control is significant under * P<0.05 ** P<0.01 *** P<0.001					

Earlier upon action with 0.01% aqueous solution of cadmium chloride as a mutagen on the variety Kazakhstanskaya 3 and Shagala mutant lines: L1, L2 and L3 were selected. Mutant forms have long spikes, elongated glumes, glassy large grain, anthocyanin coloration of the stem and leaf axils eyelets, as well as the high weight of 1000 grains. Several lines were higher and thicker culm, thickening and lengthening of the stem nodes, increased productive tillering. These selection and mutant forms of the important features, firmly inherited from generation to generation (M1-M6). In this regard, one of the objectives of this study is to localize genes responsible for economic-valuable signs of mutant forms. It is known that the elongation glumes spike was positively correlated with elongated grains. This fact is a direct proof of the high productivity of the ear mutant form A1. Under natural conditions, when intraspecific hybridization to obtain such form is rarely possible. Therefore, before using a mutant in order to hybridization it was necessary to genetically examine this property of wheat using the method of chromosome engineering. To carry out this work obtained F_1 hybrids seeds using a variety of monosomic lines Kazakhstanskaya 126 in the amount of 22 crossing combinations. Comparative monosomic analysis of the hybrids on the basis of extension glume will be held study of F_1 offspring based on harvest of 2013. Identification of mono- and disomics of Kazakhstanskaya 126 variety served as prerequisite for this work.

Interspecific hybridization. Tetraploid endemic species Triticum timopheevii Zhuk. (gentic formula AtAtGG) is characterized by a unique gene pool controlling resistance to many diseases of wheat. Creating and intensive involvement in the selection process of wheat donors with effective Lr-resistance genes transmitted from wild relatives could significantly expand its genetic basis for one or the other economically valuable traits. However, despite the difficulties (the sterility of hybrids and cytological instability), the literature contains information about the migration of a number of genes resistant to brown stem rust, powdery mildew by T. timopheevii to soft wheat. The published information about the compatibility of species T. aestivum and T. timopheevii is contradictory due to specific ecological zones [3, 4]. Using Twell method contributed to a sharp increase in the volume and improve the pollination quality, but in general – hybridization. High performance pollinating (up to 90 spikes per hour) by native pollen permitted analysis of the actual compatibility of the initial parental forms. Table 3 shows the results of cross-species hybridization.

Table 3 – Fertility of reciprocal hybrids of distant hybridization

№	Combination of breeding	Number of		Percentage of grain folding,%
		pollinated flowers	folded grains	
	Soft wheat x T.timopheevi			
1	F_0(*T.timopheevi* x Nadezhda)	190	119	62.63
2	F_0(Nadezhda x *T.timopheevi*)	72	11	15.28
3	F_0(*T.timopheevi* x к-2780)	150	61	40.67
4	F_0(к-2780 x *T.timopheevi*)	56	6	10
5	F_0(32 shortst. x *T.timopheevi*)	56	0	0
	Soft wheat x T.dicoccum			
1	F_0(*T. dicoccum* x Nadezhda)	282	181	64.18
2	F_0(Nadezhda x T.*dicoccum*)	156	41	26.28
3	F_0 (T.*dicoccum* x к-2780)	150	71	47.33
4	F_0(к-2780 x *t.dicoccum*)	130	14	10.77
5	F_0(*T.dicoccum* x 32 shortst.)	32	17	53.12
6	F_0(32 shortst. x *T.kiharae*)	33	0	0
	Soft wheat x T.kiharae			
1	F_0 (*T.kiharae* x Immune1498)	84	34	40.47
2	F_0 (Immune1498 x *T.kiharae*)	108	12	11.11
3	F_0 (*T.kiharae* x к-2780)	32	17	53.12
4	F_0(к-2780 x *T.kiharae*)	102	17	16.66
5	F_0(*T.kiharae* x 15/20977)	18	8	44.44
6	F_0(15/20977 x *T.kihara*)	118	14	11.86
7	F_0 (T.*kiharae* x Nadezhda)	50	29	58
8	F_0 (Nadezhda x *T.kiharae*)	52	10	19.23
9	F_0(USA18 x *T.kiharae*)	48	4	8.33
10	F_0 (*T.kiharae* x USA18)	22	12	54.54
11	F_0 (*T.kiharae* x USA19)	24	12	50
12	F_0 (USA19 x *T.kiharae*)	82	23	28.05

Hybrids with T. timopheevii. Experimental data shown in Table 3 suggests that the hybridization with different wild cultures of wheat species is successful. However, tying of grains in various combinations ranges from 0 to 64.18%. Apparently, the percentage depends on the genotype of variety from which samples were taken for cross-breeding, as well as from the crossing direction. Thus, the percentage of successful crosses of T. timopheevii with soft wheat is relatively high in the case, when the wild form is taken as the parent form. Depending on the number of successfully pollinated spikes the number of hybrid progeny grains varies. Compatibility level of T. timopheevii with soft wheat variety Nadezhda is relatively high, and the average is about 62.63%, k-2780 – 40.67%, and the percentage of backcrossing luck in hybrid progeny plummets 15.28% and 10%, respectively. F_1 hybrids (T. timopheevii to x-2780) of 150 – 61%, and 56 from the reciprocal mating pollinated flowers ensued only 10% of the grain.

Hybrids with T. dicoccum. F_1 hybrid offspring with wild species T. dicoccum with soft wheat was similar to the results of previous combinations produced with T. timopheevii. It is interesting to note that in this case the percentage of luck is much higher than, those combinations where grade Nadezhda served as a father. Thus, from 282 pollinated flowers luck percentage was 64.18%, and in the reciprocal crosses from 156 pollinated flowers tie a percentage of grains appeared 26.28%. Percentage of luck in direct (T. dicoccum x k-2780) crossed with the sample to 2780, amounted to 47.33% and -10.77% in reverse.

Hybrids with T. kiharae. Hybrids of wheat with T. kiharae, less productive than the hybrids with the previous combinations. However, in this case, there is a sharp drop in interest luck compared with those combinations where the parent form is taken T. kiharae. For example, the percentage of good luck in the forward mating ranged from 54.54% to 40.47% and 28.05% from reverse to 8.33%. This variety of indicators can be explained by the genotype – by environmental conditions for growing plants.

Thus, the study of reciprocal hybrids F_1, obtained by crossing wheat with wild species – T. timopheevi, T. dicoccum, revealed clear differences in the percentage of zavyazyvaemost grains. In plants, a hybrid combination with T. timopheevi, T. dicoccum and T. kiharae cytoplasm wheat, ie, under which the mother plants as soft wheat has been used, the percentage of luck somewhat lower compared to the hybrids, which served as the parent form of wild species. Hence, one can adopt clearly that the use of the wild-type form as maternal genomes increases compatibility than in the opposite mating. For hybrids derived from crosses with T. timopheevi soft wheat characteristic heteroplasmic condition: simultaneously present copies of the wild (the parent) and wheat (paternal) types.

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Frequency of vernalization requirement associated dominant *VRN-A1* gene and earliness related *Esp-A1* candidate genes in advanced wheat mutant lines and effect of allele on flowering time

Abstract: Seeds of the spring bread wheat variety Zhenis, registered in Kazakhstan, were irradiated with 100 and 200 Gray doses from a [^{60}Co] γ-ray radiation source. Promising advanced M_5 mutant lines were obtained and evaluated for mapping density of vernalization requirement associated dominant VRN-A1 gene and earliness related Esp-A1 candidate genes in 30 spring wheat mutant lines along with parent. Differences among generated mutant lines and parent were observed on frequency of distribution of VRN-A1 gene and Eps-A1 candidate gene alleles. Allelic variation at the VRN-A1 loci was observed in the 8 mutant lines (26.6%), mostly in 100 Gy mutant lines. A dominant (VRN) allele at its loci, which was present and indicated in these lines is sufficient to confer a spring growth habit. As for the candidate gene Esp-A1, eleven 100 Gy M_5 mutant and six 200 Gy M_5 lines, had Esp-A1 candidate gene allele. The two lines 24(2) and 43(4) carrying dominant allele of VRN-A1 had higher thousand kernel weight. Mutant lines carrying allele of Eps-A1 candidate gene found significantly differed from the parent especially in 100 Gy lines (73.3%) in flowering time.

Key words. spring wheat, mutation breeding, M_5 mutant lines, VRN-A1 allelic variation, Eps-A^m1 candidate gene allele.

Introduction

Bread wheat (*Triticum aestivum* L.) is one of the staple crops with global economic importance and it plays an imperative role in food security. Wheat is successfully grown in different geographical regions of the world from 67°N to 45°S [1]. Optimum growth temperature for wheat is 25°C, but satisfactory yields have been achieved in temperatures ranging from 3 to 32°C [2]. The cultivation of hexaploid wheat in a wide range of environments has become possible mainly due to selection of optimum flowering time [3]. Wheat yield has been increased globally through modification of its developmental pattern that best suit particular growing conditions.

The optimization of flowering time in wheat is critical for plant growth and development in seed crops, to maximize grain yields. Flowering time is a complex character, which exhibits a continuous variation. Pre-anthesis developmental phases in wheat are mainly controlled by vernalization (*Vrn*) and photoperiod (*Ppd*) response, and earliness per se genes [4]. Therefore, adaptability and yield potential of wheat in different agroclimates are mainly determined by these three genes systems and their interactions with growing temperatures [3].

Varietal differences in flowering time other than those due to photoperiod and vernalization response have been observed in wheat [15]. Such genetic differences are termed ‘‘earliness per se’’, ‘narrow-sense earliness’ or ‘intrinsic earliness’. Earliness per se is the difference in flowering times of varieties whose requirements of vernalization and photoperiod have been fulfilled. Photoperiod and vernalization response genes control flowering time of wheat in response to specific day length and temperature, whereas, earliness per se genes affect flowering time independent of environmental stimuli. Major vernalization and photoperiod genes may be regarded as ‘modifiers of earliness’ because they influence flowering only in response to certain environmental conditions, but earliness is determined by a minimum vegetative growth that can initiate floral primordia independent of external stimuli.

The genes responsible for variation for ear emergence have been categorized as earliness per se (eps) [5]. The *Eps* genes are thought to be involved in different developmental phases including the transition from vegetative to reproductive growth, early and late spike development, stem elongation and heading, which determine grain yield components [5, 7]. They are also considered to be involved in the fine tuning of wheat flowering time within mega-environments [6] and are responsible for wide adaptation of wheat to different environments and regulation of flowering independent of both vernalization and photoperiod. Flowering time QTLs genes are found on almost all the wheat chromosomes [7] and eps generally causes differences of a few days (1–5) in flowering time [6]. However, knowledge of their identities, mechanism and the physiological and agronomic implications of different alleles/allelic combinations are poorly understood.

Over the years, wheat breeding has reduced genetic diversity with the replacement of traditional cultivars by modern high yielding varieties (FAO Document Repository). Genetic variation is prerequisite for crop improvement. Effective breeding programs for many agronomical traits require genetic variation, which has to be separable from non-genetic effects. It is widely believed that the genetic diversity of major crops, have suffered an overall reduction over time, primarily as a consequence of breeding processes and more recently due to the recurrent use of adapted germplasm and the adoption of breeding schemes that do not favour wide spread genetic recombination [8, 9].

Induced mutagenesis is a powerful tool to generate genetic variation from which desired mutants can be selected [11]. Over the past 80 years, it has been successfully applied to produce new mutant varieties in both seed and vegetatively-propagated crops [10,11, 12,13]. According to the FAO/IAEA Mutant Variety Database there are 3220 worldwide mutant plant varieties of 214 plant species as of 2014 (http://mvgs.iaea.org/). The use of mutagenesis in breeding involves the selection of individual mutants with improved traits and their incorporation into breeding programmes [10]. The release of mutant varieties has an enormous economic impact on agriculture and food production that is currently valued at billions of dollars from millions of cultivated hectares. Exploiting natural or induced genetic variability is a proven method for improving crops, and the use of mutagenesis to create novel variation is particularly valuable in major food crops that have restricted genetic variability [10]. Mutant lines developed in wheat and other crops have great potential for direct release or in cross-breeding programmes that develop new varieties [11,12]. Nuclear techniques contribute significantly in developing superior crop varieties in seed and vegetatively-propagated crops [12, 13].

The objective of this study was to develop new M_5 advanced wheat mutant lines and evaluate them along with parent for mapping density of dominant *VRN-A1* gene and *Esp-A1* candidate gene*,* and to determine effect of allele of *Eps-A1* candidate gene on flowering time in mutant germplasm and parent.

Materials and methods

Plant material and application of induced mutation

Seeds of uniform size of the spring bread wheat variety Zhenis (*Triticum aestivum L.)* were irradiated with 100 and 200 Gy radiation doses from a [^{60}Co] γ-ray radiation source at the Kazakh Nuclear Centre. Seeds were planted immediately after irradiation in order to raise M_1 plants. The M_1 generation was grown in the experimental field of the Kazakh Institute of Agricultural and Farming in Almaty. Single spikes were harvested from each plant to raise the M_2 generation, and the selection of the best lines from M_1 to M_5 was carried out based on individual plants. The M_3 and M_4 generation were grown with a randomized block design with three repetitions. The best lines were tested with their parent to select the best mutants. Seeds of best mutants were selected individually in each generation. It should be noted that standard wheat agriculture practices were followed. After harvesting M_5 plants, 15 advanced wheat M_5 lines in each type of mutant germplasm, 100 Gy radiation dose lines: 5(4), 6(4), 6(5), 6(13), 13(3), 18(5), (1), 24(2), 25(2), 26(6), 26(7), 26(9), 26(10), 30(1), 36(1), and 200 Gy radiation dose lines: 43(1), 43(3), 43(4), 45(1), 45(2), 45(3), 48(3), 49(2), 49(4), 49(6), 50(7), 51(1), 51(2), 51(8) and 53(2) were selected and used for evaluation of distribution frequency of *VRN-A1-F and Eps-A1* candidate genes.

Sampling and measurements

To measure productivity component, as the thousand grain weight, from the total number of grains (threshed 10 spikes), the weight in grams were measured in 2 samples, each 100 seeds, and thousand grain weight were calculated accordingly per line.

DNA extraction and SSR primer sources

Genomic DNA was isolated from leaves of 10-day-old seedlings of following CTAB extraction methods with our modifications [14]. DNA concentration was determined with the Eppendorf BioPhotometer plus.

Allelic variants at the *VRN* locus were detected using two gene-specific primers in a multiplex PCR assay, in which the primer pair:*VRN-A1-F*(5'TGAAAGGAAAAATTCTGCTCGT-3') *VRN-A1-R* (5'GGCAAACGGAATTACCAAATTGGTAGATTCCGTTTGCC-3') are produced by a 798 bp fragment in the genotype with *VRN* alleles.

PCR was performed in a C1000 thermal cycler with a dual 48/48 fast-reaction module (Bio-Rad corporation, USA) and with 25 µl volume of 10xPCR buffer with 25 mM of $MgCl_2$, 200 µM of the dNTPs, 5 pmol of the primer *VRN-A1-F*, 2.5 pmol *VRN-1R* and *VRN-1 F*, one unit of Taq DNA polymerase, and 50–70 ng of template DNA. Thermocycling conditions were as follows: one cycle of 60 s at 95°C, 39 cycles of 20 s at 94°C, 30 s at 58°C, 30 s at 72°C, and then a final extension step at 72°C for 5 min. Amplified PCR products were separated on 1% agarose gel at 100 V stained with ethidium bromide and then visualized with UV light (Bio-Imaging System, Mini Bis Pro).

Allelic variants at the candidate gene *Eps-A^{m}1* locus were detected using two candidate gene-specific primer pairs in a multiplex PCR assay, in which the primer pair *Eps-A^{m}1-F*–(5'–CACATATCTGGCACCCACA–3') and *Eps-A^{m}1-R*-(3'-TGCTCATGAGTTTTCC-TCTGAA-3') are produced by a 798 bp fragment in the genotype with *Eps-A^{m}1*-allele [7]. PCR reaction mixture (total 10 µl) consisted of 10xPCR buffer with 25 mM of $MgCl_2$, 10 mM of the dNTPs, 5 pmol of the primer *Eps-A^{m}1-F* and 2.5 pmol *Eps-A^{m}1-R*, 2U/µl of Taq DNA polymerase, and 100 ng of template DNA. Thermocycling conditions were as follows: one cycle of 30 s at 94^{o}C, 30 s at 57°C, 30 s at 72°C, total 37 cycles. Amplification was carried out in the Multigene^{TM} OptiMax Thermal Cycler with a dual 48/48 fast-reaction module (Labnet International, Inc). Amplified PCR products were separated on 1% agarose gel at 100 V stained with ethidium bromide and then visualized with UV light (the ENDURO™ GDS Gel Documentation System, Labnet International, Inc).

Recording of flowering time observations

Pot experiments were conducted in a greenhouse at Institute for Cereal Crops Improvement, Department of Molecular Biology and Ecology of Plants, Tel Aviv University to record flowering time observations. The experiment was set up in a factorial design with parent cv and mutant lines and 3 replications. Seeds were germinated in germination paper and seedlings were planted 4 days later. Thirty seeds were planted in each pot. Plants were grown in 2 cm deep of plastic pots (top diameter was 16 cm, a bottom diameter is of 12 cm, and a height of 18 cm) containing 2 kg of soil at constant temperature (23^{o}C) and a long-day photoperiod (16 h) in a naturally lit greenhouse. The plants were watered once a week at 70% field capacity. Flowering time was estimated as number of days from the day of sowing to the day of spike emergence. To record the flowering date, wheat plots were inspected once in two days. It counts as the number of start flowering spikes. At this time, the number of leaves formed on the tiller was also recorded.

Statistical Analysis

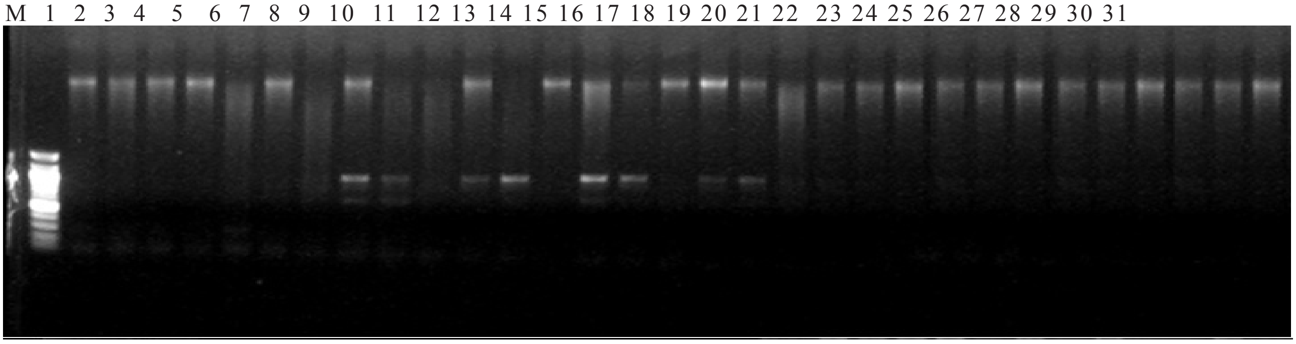
All data were evaluated in R 3.0.2 (R Core Development Team 2013). The simultaneous tests of general linear hypotheses, Dunnett Contrasts, were used for multiple comparisons of the means. Summary data are reported as mean values ± standard deviations. A p-value of less than 0.05 was considered statistically significant.

Results and discussion

One of the most important components of adaptation is flowering time, which is essentially determined by gene groups that regulate the vernalization requirement, i.e. the cold period to induce the transition from the vegetative to the generative phase (*VRN* genes) and the photoperiod sensitivity (*PPD* genes) and poorly defined 'earliness per se' (eps) or 'narrow sense' earliness genes [15].

In our study, SSR markers were used to detect the frequency of the dominant *VRN-A1* allele in M_5 mutant lines developed by using 100 and 200 Gy doses on genetic background of spring wheat cv. Zhenis along with parent. We revealed that allelic variation was observed at the dominant *VRN-A1* loci in the six 100 Gy lines, 24(2), 25(2), 26(7), 26(9), 30(1), 36(1), (40.0%) and two 200 Gy lines, 43(4), 43(3), (13.3%), all in all, eight M_5 mutant are different from the parent and other lines showing the presence of the dominant *VRN-A1* allele (Figure 1). Based on the frequency of the dominant *VRN-A1* allele in mutant lines, it is possible identify them as spring wheat (dominant). Thus, spring type allele of *VRN-A1* was present in 26.6% mutant lines, mostly in 100 Gy mutant lines. This finding may indicate the possible potential of induced mutagenesis for changing the development of the spring growth habit.

We observed that the two lines, 24(2) and 43(4) carrying dominant allele of *VRN-A1* had significantly higher thousand kernel weight (51.69±1.58 and 74.65±1.62 g, $p < 0.005$, respectively) than parent (38.08±3.67 g).



M, 1-cv.Zhenis, 1-5(4), 2-6(4),3-6(5), 4-6(13),5-13(3), 6-18(5), 7-(24(1), 8-24(2), 9-25(2), 10-26(6), 11-26(7), 12-26(9), 13-26(10), 14-30(1), 15- 36(1), 16-43(1), 17-43(3), 18-43(4), 19-45(1), 20-45(2), 21-45(3), 22-48(3), 23-49(2), 25-49(4), 26-49(6), 27-50(7), 28-51(1), 29-51(2), 30-51(8), 31-53(2).

Figure 1 – Amplification products obtained from M$_5$ mutant lines generated by treating 100 and 200 Gy radiation doses on cv. Zhenis of spring wheat genetic background and the parent in PCR, with the primer pair of the dominant *VRN-A1* gene.

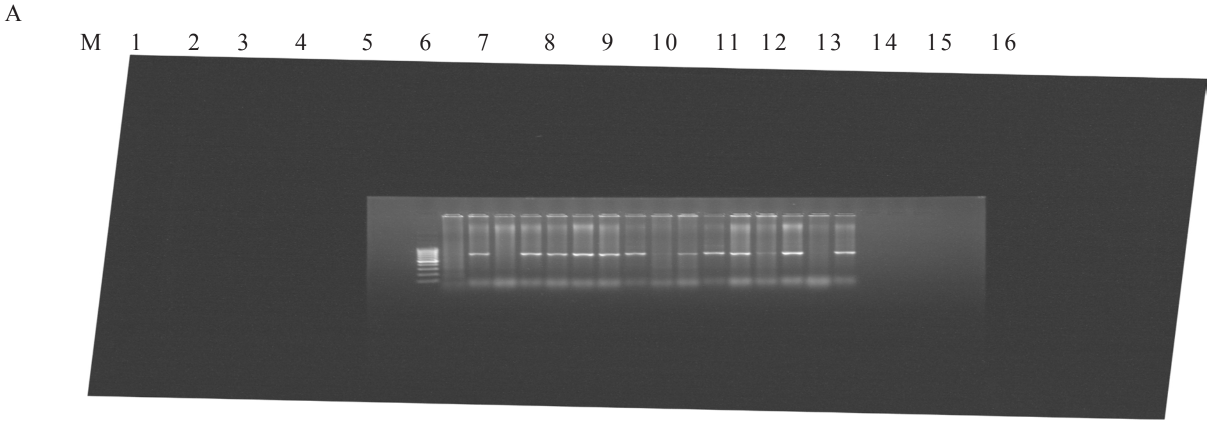
It was reported, in wheat, the *VRN-A1*, *VRN-B1*, and *VRN-D1* genes have the greatest effect on the vernalization response, which are located on the long arm of the homologous chromosomes 5A, 5B, and 5D. Depending on the ratio of dominant and recessive alleles in the *VRN* genes in the three genomes of hexaploid wheat, cultivars could be distinguished as winter (recessive) or spring (dominant) varieties, while genotypes with the facultative habit have various combinations of dominant and recessive alleles. The dominant *VRN-A1a* allele has the most pronounced genetic effect on the development of the spring growth habit analysed the *VRN-A1* locus in 200 lines of hexaploid wheat with different growth habits (68 winter and 132 spring) and confirmed the presence of the recessive *VRN-A1* allele in winter wheat. In spring wheat, 55% carried the *VRN-A1a* allele and only 6% had the *VRN-A1b* allele. For the remaining lines, they confirmed the occurrence of the recessive *VRN-A1* allele characterized the *VRN-1* locus of 117 spring wheat cultivars from Argentina and California. The dominant *VRN-A1* allele was identified in 56.5% and *VRN-D1* in around 42% of cultivars, regardless of region of origin. Molecular characterization of vernalization response genes in 40 spring wheat cultivars from Western Canada showed that 34 carried the dominant *VRN-A1a* allele. Based on272 wheat cultivars from different geographical regions, it was concluded that difference in VRN genotypes are connected to their origin. In European wheat cultivars, the most frequent allele is *VRN-A1*, the dominant *VRN-B1* allele is of moderate frequency, and the dominant *VRN-D1* allele is very rare.

Genetic variation in emergence and maturation of wheat ears is the consequence of allelic variation at loci controlling the vegetative to floral transition, inflorescence development and stem extension. This variation has major implications for yield potential, abiotic and biotic stress tolerance/avoidance, interactions with agronomic interventions, and our ability for predictive breeding of germplasm adapted to specific environments [15].

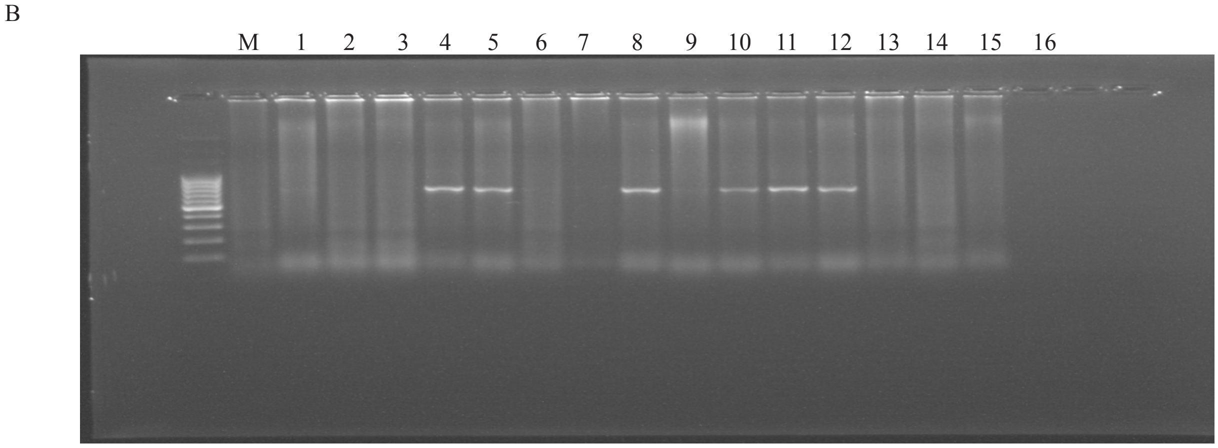
We investigated genetic density for $Eps\text{-}A^{m}1$ candidate gene in developed using 100 and 200 Gy radiation doses and genetic background of spring wheat cv. Zhenis by characterisation of allelic variation with PCR markers its flanking (Figure 2). If compare to the parent variety, we revealed that eleven 100 Gy M$_5$ mutant, 5(4), 6(5), 6(13), 13(3), 18(5), 24(1), 25(2), 26(6), 26(7), 26(10) and 36(1) (73.3%) had candidate gene $Eps\text{-}A^{m}1$ allele (Figure 2A).

In the case of 200 Gy mutant germplasm, six M$_5$ lines, 45(1), 45(2), 49(2), 49(6), 50(7) and 51(1), (40.0%) showed amplification of this candidate gene the fragment (Figure 2B). This finding may indicate that in 200 Gy lines, the presence of candidate gene $Esp\text{-}A^{m}1$ allele in numerical terms, as in the case of the VRN-A1 genes is less than those in 100 Gy radiation dose mutant germplasm.

Comparison of the mutant lines with respect to density mapping in terms of allelic variants at the *VRN-A1* locus and candidate gene $Eps\text{-}A^{m}1$ showed that among the M$_5$ lines identified as the genotypes are characterized by their difference in allele frequency from other lines and parent only one line 36(1) combined the two alleles, *VRN-A1* and candidate gene $Eps\text{-}A^{m}1$. This finding may suggest that induced mutagenesis may be effective tool to change allelic variation at the $Eps\text{-}A^{m}1$ locus candidate gene.



M, 1-cv.Zhenis, 2-5(4),3-6(4), 4-6(5), 5-6(13), 6-13(3), 7-18(5), 8-24(1), 9-24(2), 10-25(2), 11-26(6), 12-26(7), 13-26(9), 14-26(10), 15-30(1), 16-36(1).



M, 1-cv.Zhenis, 2-43(1), 3-43(3), 4-43(4), 5-45(1), 6-45(2), 7-45(3), 8-48(3), 9-49(2), 10-49(4), 11-49(6), 12-50(7), 13-51(1), 14-51(2), 15-51(8), 16-53(2).

Figure 2 – Amplification products obtained from M_5 mutant lines of spring wheat developed with 100 Gy (A) and 200 Gy (B) and cv. Zhenis in PCR, with the primer pair of the candidate gene *Eps-A^{m}1*.

Genetic variation in emergence and maturation of wheat ears is the consequence of allelic variation at loci controlling the vegetative to floral transition, inflorescence development and stem extension. This variation has major implications for yield potential, abiotic and biotic stress tolerance/avoidance, interactions with agronomic interventions, and our ability for predictive breeding of germplasm adapted to specific environments. The timing of ear emergence is fundamental to plant survival in that it allows plant species to flower at the most suitable period which will allow pollination, seed set and dispersal [15].

Effect of the candidate gene *Eps-A^{m}1* allele was determined on the flowering time of wheat developmental phases. Mutant populations differed in their flowering times, having less means, particularly 100 Gy lines (87±12.86 days) than parent (115±18.23) (Table 1). Such observed differences among genotypes could be attributed to effect of high mapping density of candidate gene *Eps-A^{m}1* alleles.

This finding may indicate the potential of induced mutagenesis for changing the flowering time, independently of vernalization and photoperiod, and are important for the fine tuning of flowering time and for the wide adaptation of wheat to different environments. Generated M_5 mutant lines of spring wheat on cv. Zhenis parent grown in Kazakhstan as genetic background can be classified as new sources having genetic potential to synchronize their flowering time with favourable environmental conditions.

Varietal differences in flowering time other than those due to photoperiod and vernalization response have been observed in wheat [15]. Such genetic differences are termed ‘‘earliness per se’’ , ‘narrow-sense earliness’ or ‘intrinsic earliness’.

Table 1 – Descriptive statistics of flowering time among spring wheat mutant lines and cv. Zhenis parent

Variable	cv. Zhenis (parent)	100 Gy M_5 lines	200 Gy M_5 lines
Lines numbers	30	30	30
Flowering days (from sowing to flowering days)	115±18.23	87±12.86	103±15.45
Number of main spike at the beginning of flowering	94±52	120±52	97±36

Earliness per se is the difference in flowering times of varieties whose requirements of vernalization and photoperiod have been fulfilled. Photoperiod and vernalization response genes control flowering time of wheat in response to specific day length and temperature, whereas, earliness per se genes affect flowering time independent of environmental stimuli. Major vernalization and photoperiod genes may be regarded as 'modifiers of earliness' because they influence flowering only in response to certain environmental conditions, but earliness is determined by a minimum vegetative growth that can initiate floral primordia independent of external stimuli.

Earliness per se is highly heritable and can, therefore, be effectively utilized in breeding programs to shorten wheat's life cycle independent of other environmental factors known to modify flowering time.

This study provides effective approach of induced mutagenesis with gamma rays treatment for generation of considerable genetic variability between and within mutant populations of spring common wheat variety Zhenis. It allows to identify of new M_5 mutant lines with allelic variation of the dominant *VRN-A1* and earliness related *Esp-A1* candidate genes in advanced wheat mutant lines and effect of allele on flowering time. Developed mutant wheat germplasm offers great possibilities for exploitation of physiological and biochemical aspects of the three genetic constituents of flowering and maturity time in wheat.

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Nitrate reductase in detached embryos may serve as a marker of the preharvest tolerance of wheat seeds

Abstract: We have observed an easily determinable parameter indicative of genetic pre-harvest sprouting (PHS) tolerance – the presence of an endosperm factor, presumably ABA, capable of inhibiting nitrate reductase (NR) induction in the embryo in the presence of NO_3. This finding has importance not only for the early rapid screening of PHS tolerant of cereal cultivars but may also be an important tool to determine the mechanism of NR inhibition either by genetic repression or by post-translational down regulation of NR activity. In this work we sought a simple relationship between ABA content and NR activity level that we assumed to be closely related to PHS susceptibility. Studied the relationship between ABA content, dry weight and ability to germinate in seeds of examined wheat varieties resistant to preharvest sprouting Lutescens 70, and unstable – Novosibirskaya 67. The data obtained are shown that the level of ABA reaching its maximum to 40-45 days . The maximum content of ABA for both varieties accounted for 40 days after flowering, when the grains reach maximum wet weight in the future, as the ripening hormone levels quickly decreased with the decrease in fresh weight of grain . The level of ABA in embryos unstable to pre-harvest sprouting wheat Novosibirskaya 67 ranged from 20 to 40% below the level of the hormone in the grains resistant Lutescens 70 wheat. The results showed a significant increase in the ability to germinate, since it is a phase 40-45 DAP. Reduction of endogenous ABA content in grains at the final stage of maturation, with a relatively high percentage of germination, can be explained by the need and ensuring to the start of the germination process of the seed.

Key words: Pre-harvest sprouting (PHS), nitrate reductase (NR), abscisic acid (ABA), wheat seeds, PHS tolerant cultivar of wheat.

Introduction

Pre-harvest germination (PHS) is the widespread phenomenon resulting in inferiority of grain, to decrease in its consumer qualities and respectively the prices. The phenomenon of pre-harvest germination depends on many reasons and, first of all, from genetic signs of wheat, from a phytohormone of abscisic acid (ABA) and the gibberellic acid (GA), germinations regulating process dormancy of seeds, and also from climatic factors such as humidity, low temperatures, during the period maturing grains, and from existence in the soil of enough of Mo for growth and viability of plants. Precocious germination of wheat grain is a serious problem in wheat production. The phenomenon of germination of physiologically mature cereal grains in the ear or panicle, usually under wet conditions shortly before harvest, is termed as pre-harvest sprouting (PHS) or vivipary. PHS occurs in many cereal crops such as wheat, barley, maize, and rice in most region of the world. PHS not only causes reduction of grain yield, but also affects the quality of grains, resulting into significant economic losses. Therefore the physiological, genetic, and environmental basis of PHS susceptibility in wheat have been a subject of intensive research during the past three decades [1].

ABA, a plant growth regulator, plays a pivotal role in the establishment of primary dormancy. ABA in mature grain has been shown to prolong dormancy in wheat by blocking germination of both the whole caryopses and the embryos. ABA is not only confined to mature grain, and it has been shown by Derera [2], that ABA levels in rise grain increase during their development. Therefore, ABA acts prior to maturity to inhibit germination. The transient rise in ABA content at approximately mid-development coincides with the establishment of dormancy. However, it also coincides with several other major events in seed development, such as dehydration, solidification of endosperm, testa formation and seed abscission [3, 4]. A major step forward in seed dormancy research was

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made with the introduction of ABA-deficient mutants of Arabidopsis, maize, pea and tomato. Many of these mutants have in common lack of dormancy Gibberellic acid (GA) induces seed germination. GA increases activity of enzymes such as α-amylase, protease, hemicellulase and more. ABA blocks GA induced gene transcription and enzyme production, while at the same time it stimulates the production of specific proteins, such as LEAs and the accumulation of anthocyanins and transcripts encoding storage proteins [4].

In wheat, barley, and sorghum cultivars that are susceptible to pre-harvest sprouting, there is a relative sensitivity of the embryo to ABA as compared to resistant cultivars, generally in the absence of any substantial differences in ABA content, or a higher ABA content in the susceptible cultivars [5, 6]. Interestingly, the expression pattern of the sorghum VP1 gene is different in two cultivars genotypes exhibiting differential resistance to preharvest sprouting at physiological maturity. More specifically, in the embryos of the susceptible cultivar, transcripts encoding VP1 peak at a relatively early stage of grain development (20 days after pollination), whereas the peak in the resistant cultivar occurs at much later developmental stages, when seed maturation is almost complete [5]. Tanner showed that PHS in corn was linked with deficiency of molybdenum [6]. Premature germination in maize was eliminated by Mo applications to the soil or the leaves. Foliar application of Mo to wheat growing in sand led to increased seed dormancy [7, 8] and the concomitant increase of ABA content in newly produced seeds.

In higher plants, ABA is derived from an epoxycarotenoid precursor that is oxidatively cleaved to produce xanthoxin [9, 10]. Following the cleavage, xanthoxin is converted to ABA by a series of ring modifications to yield abscisic aldehyde, which is oxidized to ABA by AO (EC 1.2.3.14), a molybdenum-containing enzyme [11, 12]. In addition to AO, plant MoCo-containing enzymes include nitrate reductase (NR; EC 1.7.1.1) and XDH (EC 1.17.1.4) [13]. XDH and AO have been characterized from various organisms and shown to be homodimers of 150-kDa subunits that have a high similarity in their amino acid sequence and motifs, among which are the binding sites for two iron-sulfur centers and a MoCo binding region [14, 15, 16]. Whereas, NR requires a dioxo-molybdenum, center XDH and AO contain mono-oxoMoCo in which the second oxygen was replaced by a sulfur ligand.

It has been shown that mutations of genes in synthesis of the carotenoid precursors of ABA resulted in the pre-harvest sprouting, which is consequence of ABA deficiency**.** The phenomenon is due to the lack of ABA in the young seeds and/or their insensitivity to the dormancy-inducing hormone [17].

Materials and methods

Experiments to determine the activities of Mo-enzymes as early markers of PHS were carried out in PHS tolerant (Latescence 70) and sensitive (Novosibirskaya 67) wheat varieties during seed germination. Wheat seeds of these varieties were obtained from the N. Baraev Cereal Research Institute at Shortandy (Kazakhstan). Preparation of seed tissue extracts and the activities of molybdoenzymes, aldehyde oxidase (AO), xanthine dehydrogenase (XDH) and nitrate reductase (NR) in the aleurone layers, endosperms and embryos were determined according to [18, 19].

NR activity was determined *in vitro* according to Savidov et al. [18] **.**The reaction medium contained 50 mM Na-phosphate buffer (pH = 7.7), 5 mM KNO_3 and 10 μM FAD. The reaction was started by the addition of the enzyme (i.e., seed part extract). After 10 min of incubation at 28^{o}C, the reaction was terminated by adding 50 μl of a mixture of 0.3 mM phenazinemethosulfate (PMS) and 1 M Zn acetate (1:1) to remove residual NADPH from the assay medium. This step was followed by the addition of 0.1 ml of a mixture of 0.02% (NED) and 1% (SA) (1:1) for nitrite determination. Reaction mixtures were centrifuged at 10000 rpm for 5 min, and their absorbance was read at 540 nm after 20 min.

AO activity was detected in 7.5% polyacrylamide gels after protein fractionation by native gel electrophoresis using indole-3-aldehyde as substrates for AO according to [19]. The samples, which contained approximately 20-25 µg of soluble protein, were loaded onto wells and carry out electrophoresis at 4^{o}C for 1.5-2 hours. Then, the gels were equilibrated and washed in 100 mM sodium phosphate buffer (pH=7.5) by gentle shaking for 10 min, which was followed by incubation in a reaction mixture containing 0.1 mM PMS, 1 mM 3(4,5-dimethylthiazolyl-2)2,5-diphenyltetrazolium-bromide (MTT) and 1 mM specific XDH/AO substrate. The activities of XDH and AO were estimated based on the amount of MTT reduction that resulted in the development of specific formazan bands. The intensity of formazan bands was directly proportional to enzyme activity in the presence of excess substrate and tetrazolium salt. Quantitative analyses were made by scanning the formazan bands in the gel with a computing laser densitometer (Molecular Dynamics) using the software provided (Image Quant version 3.19.4).

Results and their discussion

Mo-enzyme activities in the parts of dry wheat seeds. The activities of NR and AO were determined in different parts of dry dormant seeds – the embryo, endosperm and aleurone layer. The embryo and aleurone layer extracts showed AO activity while these activities were not detected in the endosperm

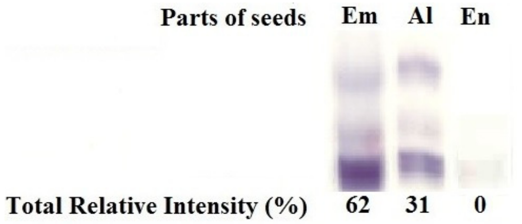

Figure 1 – The activity of AO in different parts of dormant seeds from the wheat cultivar Lutescens 70 (Em – embryo, Al – aleurone layer, En – endosperm)

Dry seeds were incubated in distilled for 24 h, after which only the embryo showed no NR activity. NR activity developed in seed embryos only after incubation in the presence of KNO_3 and its levels in embryos of both wheat varieties were nearly the same (not shown). Early results showed that NR-antibodies did not cross-react with protein of extracts of wheat seed embryos, endosperm and aleurone layer [20].

Mo-enzymes in developing and mature seeds. The activities of NR, XDH and AO in developing seeds were determined at ten days intervals starting from 10th day after pollination (DPA) (Table 2). Water content of the seed attained a peak at 30 DPA and then started declining to its lowest level a maturity. A linear increase in fresh as well as dry matter of the seed was observed between 10 and 30 DPA (not shown). XDH activity in the embryo and aleurone layer in maturing seeds of both varieties remained at a steady level. A slow increasing activity of AO reached a maximum at 40 DPA (Table 2).

Table 1 – Activities of AO, XDH and NR in different seed parts of PHS-tolerant cultivar Lutescence 70

Seed parts	AO[1]	XDH[2]	NR[3]
Embryo	75.7 ± 6.3	1.5 ± 0.2	0.0
Aleurone layer	55.3 ± 4.5	1.2 ± 0.3	0.0
Endosperm	0.0	0.0	0.0

[1]nmol phenantridone mg^{-1} protein min^{-1}; [2]µmol NADH mg^{-1} $hour^{-1}$; [3]µmol NO_{2-} mg^{-1} $hour^{-1}$

Low AO activity was detected in the embryo and aleurone layer of seeds until the age of 30 DPA. After this age the activity of the enzyme in the embryo increased steeply reaching a maximum at 40 DPA, the enzyme activity in the aleurone layer increased slightly. Thereafter, AO activity in the embryo fell significantly at full maturity of the seeds, while AO activity in the aleurone layer remained at a steady level.

Table 2 – Changes in AO, XDH and NR activities in seed embryo during seed development and maturation

DPA	10	20	30	40	50	60	FM
AO	10.2±1.7	11.8±2.1	18.3±2.4	67.4±8.7	65.7±9.3	66.4±7.4	66.8±7.4
XDH	16±0.2	1.6±0.2	1.7±0.3	1.8±0.2	1.9±0.3	1.9±0.2	1.9±0.2
NR	0.0	0.0	0.0	0.3±0.02	2.3±0.1	4.7±0.5	5.1±0.7

As can be seen from the Figure 2, the activity of AO is beginning to show up in the initial period of maturation (25DAP), and in all parts of the seed. 35-40 days in the endosperm, and particularly in the germinal part of the activity of the enzyme increases about 10 times compared with the early stages of ripening grains. In electrophoretic spectrum first appear cathodic components (AO1) involved in the biosynthesis of ABA (30 DAP).

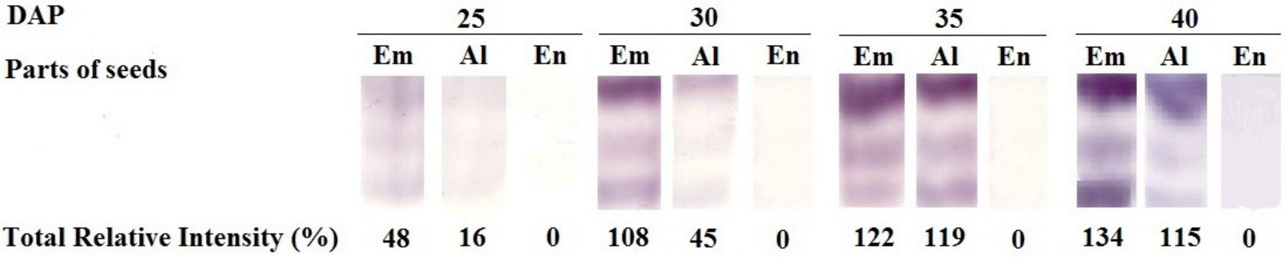

Figure 2 – The activity of AO in different parts of developing and maturing seeds of the wheat cultivar Lutescens 70 (Em – embryo, Al – aleurone layer, En – endosperm)

In subsequent phase of ripening the increase in the activity of AO is due to a cathodic activation of the enzyme and the appearance in the spectrum of the anode forms (AO1 and AO3).

Approximately the 40th DAP fresh weight of the grain reaches a maximum and then begins the stage of dehydration or drying and, at this time gradually decreases the level of the AO. It should be noted that AO1 throughout the ripening period is a major component in the germ.

Studied the relationship between ABA content, dry weight and ability to germinate in seeds of examined wheat varieties resistant to preharvest sprouting Lutescens 70, and unstable – Novosibirskaya 67.

The data obtained are shown in Table 3, from which it follows that the level of ABA after reaching its maximum of 40-45 days began quickly to decline towards the end of ripening process (55 DAP). Tabular data show a marked increase in ability to germinate, starting from the phase of 40-45 DAP.

Table 3 – Dynamics of changes in ABA and the ability to germinate wheat seeds at different stages of maturation

DAP	Lutescens 70			Novosibirskaya 67		
	ABA ng/mg of dried weight	% germination	Dried weight, %	ABA ng/mg of dried weight	% germination	Dried weight, %
20	0.70	0.0	35	0.45	0.0	37
25	0.65	3-5	50	0.45	6-8	40
30	0.75	7-10	65	0.45	10-13	56
35	0.80	11-12	75	0.55	13-16	75
40	0.95	10-11	89	0.60	15-19	86
45	0.85	14-16	92	0.60	22-30	88
50	0.60	20	95	0.40	32-45	92
55	0.60	22-25	98	0.35	40-60	95
Complete germination	0.50	25	100*	0.30	55-65	95

*100%- dry weight of 50 seeds

Such temporal coincidence, apparently, is not accidental, given the fact that ABA is an important endogenous regulator (inhibitor) of germination. May decrease endogenous ABA content in the grains at the final stage of ripening, with relatively high germination percentage can be explained by the need and ensuring to the start of the germination process of the seed.

There was no NR activity detectable *in vivo* and *in vitro* in the embryo, endosperm and aleurone layer of developing seeds. In contrast, when seeds were removed from the spikes that were at different stages of maturation and incubated in 50 mM KNO_3 solution for 30 hours, NR activity was detected in embryos starting at 40 DAP (Table 2).

The maximum level of NR activity was observed in the embryo portion of mature seeds.

NR activity in germinating seeds of PHS-tolerant and sensitive wheat cultivars. The following experiments were carried out to study the activities of NR in pre-harvest sprouting tolerant and sensitive wheat varieties during their germination. The seeds were separated into aleurone layer, endosperm and embryo. These parts of dry seeds showed no detectable NR activity. Seeds were incubated in distilled water for 24-30 h, then separated and into their parts to determine NR activity in each of the tissues. NR activity appeared in seed embryo only after incubating them in the presence of KNO_3 and its levels varied among wheat varieties (Table 3).

Seeds were incubated in nitrate and at appropriate times embryos were removed from the seeds to estimate the enzyme activity. In the present set of experiments embryos were first excised and then incubated in nitrate. Study of NR induction in the excised embryos showed a significant level of the NR activity as early as 6 h of germination.

Table 4 – NR activity of attached and detached seed embryos of PHS-tolerant Lutescence 70 and PHS-sensitive Novosibirskaya 67 wheat varieties

Wheat varieties	Type of embryo	Incubation time, h					
		0	6	12	18	24	30
PHS-tolerant	Attached	0.0	0.0	0.0	0.0	0.0	0.0
	Detached	0.0	2.7±0.2	4.3±0.7	3.2±0.5	2.5±0.4	1.8±0.2
PHS-sensitive	Attached	0.0	0.5±0.1	1.2±0.1	2.4±0.3	1.9±0.3	1.3±0.2
	Detached	0.0	3.2±0.4	3.2±0.4	4.8±0.6	4.2±0.5	3.6±0.6

After this, the enzyme activity increased and showed a peak at 12 h of incubation followed by a decrease in the level of the enzyme activity which came to the minimum activity after 30 h of incubation (Table 4). It is interesting to note that the embryos of ungerminated wheat seeds of both varieties could not synthesize NR in the presence of 60 mM KNO_3 after 12 h induction. Since wheat seeds germinate in the absence of nitrate and without NR synthesis, seed germination is not depended on nitrate assimilation in the embryo.

Since in PHS-tolerant seeds the presence of the endosperm delayed NR induction, attempts were made to study effects of cross-combinations of PHS-tolerant and PHS-sensitive endosperm extracts on the induction of NR in their detached embryos.

Results of these experiments are shown in Table 5.

Table 5 – Effects of cross-combinations of fresh endosperm extracts of PHS-tolerant and sensitive on NR-induction in their detached embryos in after 12 h induction presence of 60 mM

Combinations of endosperm extracts and detached embryos in KNO_3	NR activity in detached embryos
Detached embryo of PHS-tolerant seed in	4.6 ± 0.9
Endosperm of PHS-tolerant seed + its detached embryo	0.5 ± 0.1
Detached embryo of PHS-sensitive seed	5.5 ± 0.7
Endosperm of PHS-sensitive seed + its detached embryo	2.9 ± 0.6
Endosperm of PHS-tolerant seed + detached embryo of PHS-sensitive seed	1.9 ± 0.3
Endosperm of PHS-sensitive seed + detached embryo of PHS- tolerant seed	0.8 ± 0.1

The endosperm of PHS-tolerant wheat seeds contains a factor inhibiting NR induction by nitrate in embryo, while this factor is not present in PHS-sensitive cultivars.

This inhibitory factor present in the endosperm gets gradually destroyed with time (not shown).

Seed sensitivity of different wheat varieties to PHS depends on the levels of ABA in their endosperm and embryo, i.e. PHS-tolerant wheat seeds contain higher concentrations of ABA [21]. On the basis of these facts we proposed that ABA regulates the activity or synthesis of NR in wheat embryo. Detached embryos of PHS-tolerant seeds were incubated for 12 h in the presence of different ABA concentrations plus 60 mM KNO_3.

Increasing concentrations of ABA increase the inhibition of NR activity in detached wheat embryo (Table 5).

Table 6 – Time course decrease of NR activity in PHS-tolerant embryo by increasing ABA concentrations (NR activity in nmoles of nitrite/embryo/h)

ABA concentrations	Control	10 nM	100 nM	1.0 µM
NR activity	4.8 ± 0.8	4.3 ±0.8	3.5 ± 0.3	1.3 ± 0.2

Since ABA content in both seed types is nearly the same the endosperm of PHS-sensitive seeds may contain higher concentrations of ABA inactivating enzymes than the PHS-tolerant seeds, and this is one of reasons of PHS-sensitivity of wheat seeds. These observations are significant in view of the fact that NR is induced in wheat seed embryo linked to the seed tolerance to PHS. The inhibition of NR in detached embryos following 4-6 h of imbibition by endosperm extract may serve as an early marker of the tolerance of wheat seeds to PHS.

In the embryos present in the intact seed there was no NR activity until 24 h of incubation. It appears that the presence of endosperm in the intact seed inhibited the induction of NR in the embryos during the (first 24 h) germination of the PHS-tolerant wheat seeds but not in the embryos of the PHS-sensitive wheat seeds. Thus, only the endosperm of PHS-tolerant seed appeared to inhibit NR induction.

Other enzymes of nitrate assimilation pathway, such as nitrite reductase, glutamine synthase and glutamate dehydrogenase were also found to be present in *in situ* embryos during the initial stages of germination in wheat (not shown). Thus, it appears that among the key enzymes of nitrate assimilation only NR shows a long lag period in its development that seems to be exerted by the endosperm of PHS-sensitive seeds for NR.

The level of the inhibitory endosperm factor of PHS-sensitive wheat seeds appears to be lower in PHS-tolerant seeds. The inhibitory factor gradually disappears with seed aging, similarly to the fate of the dormancy controlling ABA, the level of which determines the PHS sensitivity of the seeds, suggesting that ABA may regulate the synthesis of NR in wheat embryos and perhaps in other plant tissues [22]. These observations are significant in view of the fact that NR induction in wheat seed embryos correlates with their level of PHS tolerance. Thus, the 4-6 h inhibition by the endosperm extract of NR in detached embryos may serve as a marker of the PHS tolerance of wheat seeds, and may be used as an early parameter for fast genetic screening.

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Phenolic profiles and brine shrimp cytotoxicity of the ethanolic extract from the aerial part of *Crocus alatavicus* L.

Abstract: The aim of this study is to investigate the phenolic profiles and evaluate the brine shrimp cytotoxic activity of the ethanolic extract from the aerial part of *C. alatavicus*, an endemic species of Kazakhstan flora. Nine phenolic compounds were identified and quantified in the extract by high-performance liquid chromatography method. Preliminary cytotoxicity of the extract was determined by the brine shrimp (*Artemiasalina*) assay. The results reveal that the ethanolic extract from the aerial part of *Crocus alatavicus* exhibit a high cytotoxicity with LC_{50} 15.71 µg/mL.

Key words: *Crocusalatavicus*, phenolic compound, cytotoxicity, brine shrimp, extract.

Introduction

Phenolic compounds are the substances containing benzoic nucleus with one or several hydroxylic groups and their derivatives. Higher plants synthesize several thousand known different phenolic compounds. These compounds are the products of secondary metabolism. Phenolics are produced in plant organism for protection against environmental stress, such as high light, low temperatures, UV radiation, various pathogens and nutrient deficiency. Phenolics play important roles in plant development, provide structural integrity and scaffolding support to plants [1].

Crocus alatavicus is an early spring ephemeral and geophytic-geocarpic species of the Iridaceae family. It is an endemic of Kazakhstan flora belonging to the group of bulbous and tuberous plant. Wild habitat of this species is the Kora river middle flow, Kopal district, Taldykurgan region and South-East Kazakhstan [2]. The species is interested as a source of naturally active substances that have many useful biological properties. Accumulation of the bioactive compounds is associated with the geophytic life-form of this plant and its ephemeral development cycle.It was usedas spasmolytic, anti-inflammatory, bactericidal and antiviral agents in traditional medicine [3]. The flowers were usedas diuretic, for treatment of abdominal illness and to improve hormonal regulation of women [4]. Dried stigmas are appliedfor coloring food products and impart flavor for them [5].

Phytochemical constituents and biological activities of this species insufficiently explored. It is revealedthe predominant content of secondary metabolites in the aerial part of this plant [6]. The leaves contain ascorbic acid and the stigmas of flowers contain yellow pigment available for food colorant [7]. Anthocyanins such as delphinidin 3-*O*-β-rutinoside and petunidin 3-*O*-β-rutinoside and flavonoids such as myricetin, quercetin, kaempferol have been investigated in flowers [8].Total antioxidant, radical scavenging and antibacterial activities of the various extracts from different part of *C. alatavicus*were reported [6].

Investigation of the phytochemical constituents and potential biological properties of plants is importance for their use in different industries. In recent years, the medicinal properties of plants are investigated due to their potent pharmacological activities. Among the metabolites of plants, phenolicshave been examined for their biological activities including antioxidant, radical scavenging, antibacterial and cytotoxic activities.

The purpose of this study is to investigate the phenolic profiles and evaluate the cytotoxic effect of the ethanolic extract from the aerial part of *C. alatavicus*.

Materials and methods

Plant material and preparation of the extract

The aerial part of *C. alatavicus* was collected in Almaty region ($43^0 22'14'$) at the flowering phase in March, 2015. Collected materials were cleaned, air dried in the shade at room temperature.

Powdered samples (10 g) were three times extracted with 100 ml of 96% ethanol at 60 °C for 6 hours in a water bath shaker. After the filtration with Watman No: 1 filter paper the solvent was concentrated under vacuum (48-49 °C) by evaporating to dryness. The extract were stored at -20 ºC until use.

HPLC analysis was performed according to the method of Caponio [9]. The extract sample was prepared by dissolving the 0.1 g of the dried extract in 1 ml methanol followed by filtration over 0.45 µM Nylon Syringe Filter. 9 standard phenolic compounds (gallic acid, 3.4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, chlorogenic acid, vanillic acid, caffeic acid, p-coumaric acid, ferulic acid, cinnamic acid) have been analyzed.

The operating mode of HPLC:
Chromatograph: Shimadzu Prominence
Column: Agilent Eclipse XDB-C18 (250 mm x 4.6 mm, 5 µm particle size)
Mobile Phase: A: 3% formic acid; B: methanol
Injection Volume and Flow Rate: 20 µl, 1 ml/min
Detector: DAD (SPD-M20A) (λ=280 nm)
Column Oven and Temperature: CTO-10 ASvp, 30°C
Pump: LC-20 AT
Auto Sampler: SIL-20ACHT
Computer Program: LC Solution
Brine Shrimp Test

The test was performed as described in Meyer [10] and McLaughlin [11]. The extract was dissolved in artificial seawater and was tested at the concentrations of 10, 50, 100, 500 and 1000 µg/ml. Brine shrimp eggs (*Artemia salina* Leach, USA) were hatched in artificial sea water at room temperature.

After 48 h, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. Ten nauplii were placed in each vial containing 4.5 ml of brine solution and 0.5 ml of the plant extractand maintained at room temperature for 24 h under the light. After 24 h, the number of surviving shrimps at each concentration of the extract were counted and LC50 values were calculated using EPA probit analyzer version 1.5. The test was performed in triplicate.

Results and their discussion

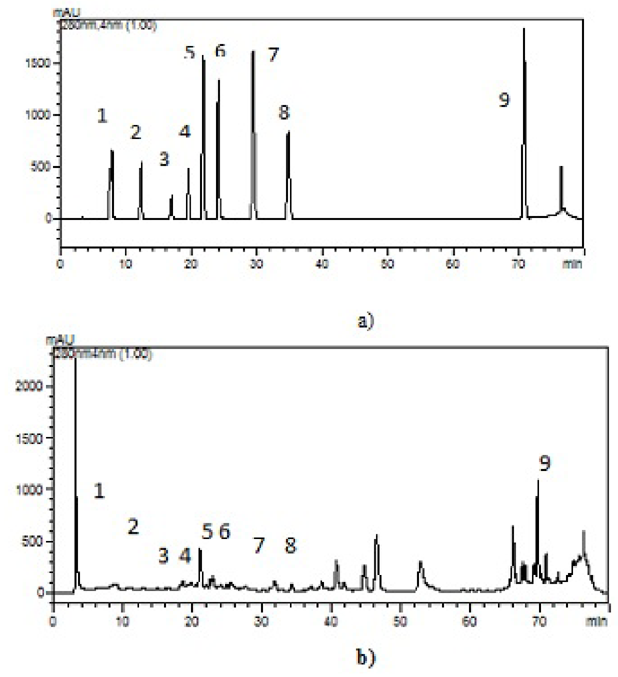
According to the previous study [6] among the various extracts from *C. alatavicus* the highest total phenolic content (72.29 mgGAE/g) was observed in the ethanolic extract from the aerial part. It is the reason to investigate some phenolic compounds in this extract.

In this study, 9 standard phenolic compounds (gallic acid, 3.4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, chlorogenic acid, vanillic acid, caffeic acid, p-coumaric acid, ferulic acid, cinnamic acid) have been analyzed.

The phenolic compounds were identified based on their retention times (RT) and quantified according to the respective standard calibration curves. Each of the phenolic compound was expressed as µg/g of extract.

The chromatograms of the standards and the ethanolic extract from the aerial part of *C. alatavicus*are presented in Figure 1.

Quantitative results (Table 1) show that 4-hydroxybenzoic acid (1491.1 ± 0.13 µg/g) has the highest concentration value over other benzoic acid derivatives: gallic acid = 289.8 ± 1.03µg/g, vanillic acid = 156.7 ± 0.03 µg/g and 3.4-dihydroxybenzoic acid = 59.6 ± 0.82 µg/g. Hydroxicinnamic acid derivatives as caffeic acid (468.7 ± 0.44 µg/g), ferulic acid (397.3 ± 0.02 µg/g), chlorogenic acid (77.5 ± 0.09 µg/g) and p-coumaric acid (20.2 ± 0.28 µg/g) also were identified and quantified in the extract. Unsaturated carboxilic acid namely cinnamic acid was determined in quantities of 238.3 ± 0.61 µg/g extract.



a)

b)

Figure 1 – HPLC profiles: a) chromatogram of the standards; b) chromatogram of the phenolic compounds in ethanol extract from the aerial part of *C. alatavicus:*
1 – gallic acid; 2 – 3.4-dihydroxybenzoic acid;
3 – 4-hydroxybenzoic acid; 4 – chlorogenic acid;
5 – vanillic acid; 6 – coffeic acid; 7 – p-coumaric acid; 8 – ferulic acid; 9 – cinnamic acid.

Table 1 – RTs (min) and values of regression (r^2) for standard phenolic compounds and their content (µg/g) in the ethanolic extract from the aerial part of *C. alatavicus*

Peak no	Phenolic compound	RT ± SD	r^2	Content in the extract: mean ± SD (µg/g)
1	Gallic acid	7.8 ± 0.05	>0.9999	289.8 ± 1.03
2	3.4-Dihydroxybenzoic acid	12.2 ± 0.03	>0.9998	59.6 ± 0.82
3	4-Hydroxybenzoic acid	16.9 ± 0.05	>0.9999	1491.1 ± 0.13
4	Chlorogenic acid	19.4 ± 0.04	>0.9994	77.5 ± 0.09
5	Vanillic acid	21.7 ± 0.06	>0.9991	156.7 ± 0.03
6	Coffeic acid	24.6 ± 0.08	>0.9997	468.7 ± 0.44
7	p-Coumaric acid	29.3 ± 0.05	>0.9998	20.2 ± 0.28
8	Ferulic acid	34.7 ± 0.08	>0.9998	397.3 ± 0.02
9	Cinnamic acid	70.7 ± 0.07	>0.9998	238.3 ± 0.61

The same content of p-coumaric acid (25.36 µg/g) was detected by HPLC in the methanolic extract of *C. baytopiorum*[12]. Cumaric, chlorogenic and gallic acid was identified in the methanolic extracts of *C. sativus* stigmas [13]. Karimi et al. [14] have the concentration of gallic acid found in *C. sativus* stigmas to be 1.82 mg/g dry sample. It is markedly higher compared to the gallic acid content (289.8 µg/g) in ethanolic extract from aerial part of *C. alatavicus*.

Brine shrimp bioassay is one of the most useful and rapid tool for the screening of biochemical activity and it is used to determine the toxicity of a wide variety of products. This method is considered as a broad measure of antitumor activity [15]. It is the first study on cytotoxicity of *C. alatavicus*.

The results (Table 2) reveal that the extract from the aerial part of *C. alatavicus* exhibit LC_{50} with 15.71 µg/mL. According to Meyer [10], extracts obtained from natural products which have $LC_{50} \leq 1.0$ mg/mL are known to possess toxic effects.

The ethanolic extract from the aerial part of *C. alatavicus* possesses high brine shrimp cytotoxic activity and it probably due to the presence of phenolic hydroxyl group in the extract.

Table 2 – Brine shrimp cytotoxicity of *C. alatavicus*

Part of plant	Concentration, µg/ml	Number of shrimps surviving after 24 h			Total number of surviving shrimps	Lethality,%	LC_{50}, µg/ml
		T_1*	T_2	T_3			
Aerial part	10	3	2	2	7	76.6	15.71±0.5
	50	2	2	1	5	83.3	
	100	0	2	1	3	90.0	
	500	0	1	0	1	96.6	
	1000	0	1	0	1	96.6	
T*- parallels of the experience							

In many studies it is reported the high cytotoxicity of *C. sativus*. Safronal andcrocin isolated from *C. sativus* shows cytotoxicity against *Artemiasalina* with LC_{50} 14.3 µg/mL and 147.036 µg/mL, respectively.

High inhibitory effects of the main activity substances of saffron were found in suspension of *Agrobacterium tumefaciens* (LC_{50} 0.31 and 2.34 µg/mL) [16].

Anticancer, anti-tumor and cytotoxic effects of saffron and its constituents have been studied on another test-systems [17].

Conclusions

This is the first report that shows the phenolic compound analysis and brine shrimp cytotoxicity of *C. alatavicus* extract from aerial part. Some phenolic compounds were identified and quantified by HPLC. Analized extract showed a high brine shrimp cytotoxicity and can be considered as a promising candidate for a plant derived antitumor agent. Future studies should be carried out to determine the cytotoxicity of *C. alatavicus* on cancer cell lines *in vitro*.

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Influence of heavy metals on fluorescence activity of perspective strains of microalgae and cyanobacteria

Abstract: The article presents the results of a study of the effect of heavy metals on fluorescence activity of microalgae and cyanobacteria. Based on the results of the study determined that the collection of strains of microalgae and cyanobacteria 5 crops resistant to heavy metals, 4 cultures are more sensitive to the studied concentrations of heavy metals. Some strains under the influence of the zinc and copper was observed phenomenon of plasmolysis and deformation of the cells. Influence of heavy metals on cells of microalgae and cyanobacteria have identified a number of surveyed metals toxicity, which is as follows: $Cu^2 + > Zn^2 + > Co^2 + > Ni^{2+}$. From cultures of microalgae and cyanobacteria the following types of selected for further study, as promising strains for bioremediation of contaminated aquatic ecosystems, various heavy metals: *Phormidium autumnale* I-5, *Anabaena variabilis* RI-5, *Synechococcus elongatus* I-4, *Chlorella vulgaris* sp BB-2, *Chlamydomonas reinhardtii* B -4.

Key words: microalgae, cyanobacteria, heavy metals.

Introduction

Environmental pollution by heavy metals is a worldwide phenomenon. Anthropogenic activity is one of the major culprits to the release of heavy metals to the environment. To date, many approaches, e.g. analytical chemistry approaches and biological approaches have been developed to detect the presence of heavy metals. In biological approaches, many cells, e.g. bacteria, algae, cyanobacteria, have been used as the bioindicators or biological reporter groups in biosensors [1]. These cells reflect the real physiochemical toxicity of pollutants to the living organisms. As one of the smallest living entities, these cells can produce distinct responses towards certain analytes, with high sensitivity and rapid responses [2].

From a biological point of view, heavy metals can be divided into two categories: essential and non-essential [3]. However, essential heavy metals have even been reported to be toxic at high concentrations. For example, some heavy metals including copper, zinc, nickel and chromium, are essential for growth at very low concentrations but toxic at slightly levels [4]. The concentration 5 x 10-6 – 10-5 $mol.L^{-1}$ Co^{2+} exerted maximal stimulatory effect on *Chlorella pyrenoidosa* cells at the exponential growth phase in terms of fresh weight (150-160% increase), dry weight (50-60% increase), chlorophylls a and b (45-65% increase), total carotenoids (55-65% increase), water-soluble proteins (19-20% increase) and monosaccharides content (55-60% increase), when compared to the control culture. The effect of Co^{2+} on *Chlamydomonas reinhardtii* observed reduction of growth at 10 ppm Co^{2+} and without change in the morphology of the cells or pH. At 20 ppm Co^{2+}, on the other hand, growth was considerably reduced compared to the control and the colour of the organism became paler and the cells clumped. In addition, the pH value was lower compared to the pH of the control at the end of experimental period. Lu et al. demonstrated that chlorophyll fluorescence analysis could be a useful physiological tool to assess early stages of change in photosynthetic performance of algae in response to heavy metal pollution [5].

In this study, the fluorescence response of microalgae and cyanobacteria to the exposure of heavy metals was determined.

Materials and methods

Isolation and purification of the algae and cyanobacteria: *Chlorella vulgaris sp BB-2* and *Ankistrodesmus sp BI-1* were isolated from the lake Balkhash, *Scenedesmus quadricauda B-1* and *Chlamydomonas reinhardtii B -4* were isolated from the lake Bilikol. Cyanobacteria *Oscillatoria tenuis* RI-4, *Nostoc calcicola* RI-3 and *Anabaena variabilis* RI-5 were isolated from the river Irtysh, *Phormidium autumnale*

I-5 and *Synechococcus elongatus* I- 4 were isolated from the river Ilek. One single cell from each colony was isolated, transferred to fresh solid medium and subjected to repeated subculturing on fresh solid media before transfer to sterilized liquid nutrient media. Cultures were checked regularly microscopically. These cultures were deemed axenic [6].

Nutrient solution and Culture technique: Tamia medium was used for cultivation of microalgae. Medium BG-1 was used for the cultivation of cyanobacteria. The culture illumination was provided by fluorescent tube lamps giving a light intensity of 120 watts[6].

For water pollution modelling *medium* TM used aqueous solutions of copper sulfate ($CuSO_4 5H_2O \times$), cobalt sulfate ($CoSO_4 \times 7H_2O$), zinc sulfate ($ZnSO_4 \times 7H_2O$), nickel sulfate ($NiSO_4 \times 7H_2O$). The solutions prepared in distilled water, creating concentrations of metal ions: 0.001, 0.01, 0.1 and 1 mg/L.

Fluorescence intensity measurement carried out on spectrofluorimeter Fluorolog -02-panorama (λvozb = 400 nm, λreg = 685 nm) at the end of the experiment (72 hours), the expected relative changes in option (I) in% (Formula 1).

$$I = \frac{I_o}{I_k} \cdot 100\% \qquad (1)$$

where the I_o is the average value of the intensity of fluorescence in the experience, I_k-average value of the intensity of fluorescence in the control [7].

Results and their discussion

Culture exposed to heavy metals, Co2 +, Ni2 +, Zn2 +, Cu2 + at concentrations of 0.001, 0.01, 0.1 and 1 mg/l.,0 the choice of metals due to their the wide spread in natural waters. According to the data content of metals in surface waters far exceeded MAC [8].

Now in ecophysiological studies are widely used indicator of chlorophyll fluorescence. Growth and photosynthetic activity of algae and cyanobacteria that determine the intensity of fluorescence of chlorophyll, are markers of toxicity and the extent of tolerance organisms to this medium. We examined the effect of HM on selected productive strains of 4 cultures of microalgae and cyanobacteria cultures 5. Relative values of chlorophyll fluorescence intensity allocated microalgae with various concentrations of HM are represented on Figure 1. For 100% accepted by the intensity of fluorescence of cells in medium without HM.

The study of the greatest toxicity to selected algae possess the copper ions: fluorescence intensity of all cultures in the presence of copper in the investigated range of concentrations of 0.001-1.0 mg/L decreased to 50-88%. The smallest impact provided nickel and cobalt ions: fluorescence intensity if available microalgae in medium in the same concentrations decreased at 20-60%. Most stable to HM are *Chlorella vulgaris sp* BB-2*, Chlamydomonas reinhardtii* B -4. Most sensitive to the toxic effects of salts of heavy metals are strains of *Scenedesmus quadricauda* B-1 and *Ankistrodesmus sp.* BI-1. Heavy metals in the cells of these strains caused strong plasmolysis, an increase in cell size. This process is clearly observed in the concentration of 1.0 mg/L, Cu^{2+} (Figure 1).

Sensitivity strains to heavy metals may be perspective in bioassay to assess the degree of contamination of water bodies with salts of heavy metals. According to the literature value of fluorescence intensity is closely linked to the absolute number of living cells in culture, then there are HM inhibit microalgae photosynthesis process that affects the viability of cells and their size [9].

Based on the results of the study the effects of these heavy metals on cells of cyanobacteria, it was found that for selected from various sources of cyanobacteria of the four heavy metals studied most toxic are copper and zinc. Least toxic metal turned out to be a nickel. In the initial period of cultivation of cyanobacteria with the introduction of heavy metals resulted in lengthening the lag-phase in all cultures. But in the presence of heavy metals nickel, cobalt linear growth of cyanobacteria quickly restored, and fluorescence intensity crops accounted for 45-55% lower than controls. When making copper and zinc in concentrations of 0.001 mg/L 0.01 mg/L 0.1 mg/L fluorescence intensity declined to 60-80% (Figure 2).

The intensity of the fluorescence *Oscillatoria RI-4* and *Nostoc calcicola RI-3* in all concentrations of copper and zinc declined, and the concentration of 1.0 mg/l fully inhibited the process of photosynthesis. Cultures of cyanobacteria *Oscillatoria RI-4* and *Nostoc calcicola RI-3* proved to be more sensitive to copper and zinc ions. The remaining strains of cyanobacteria extracted from various sources, namely, *Phormidium autumnale I-5, Anabaena variabilis RI-5, Synechococcus elongatus I-4* were found to be more tolerant to the effects of heavy metals.

Based on the results of the study determined that the collection of strains of microalgae and cyanobacteria 5 crops resistant to heavy metals, 4 cultures are more sensitive to the studied concentrations of heavy metals.

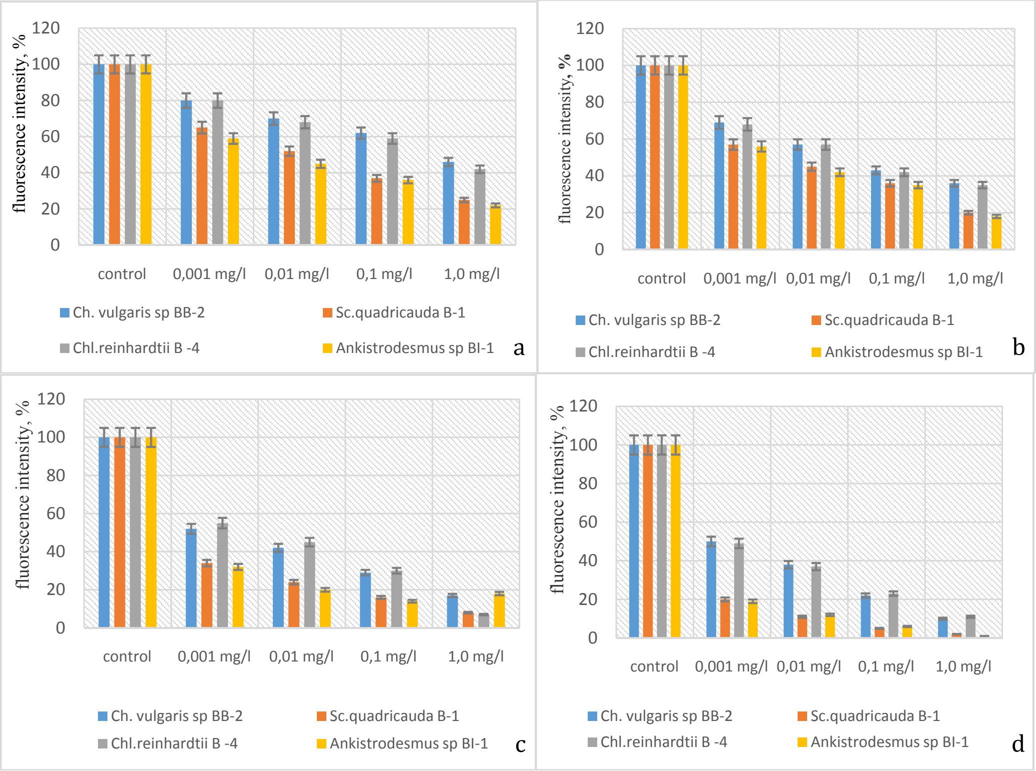
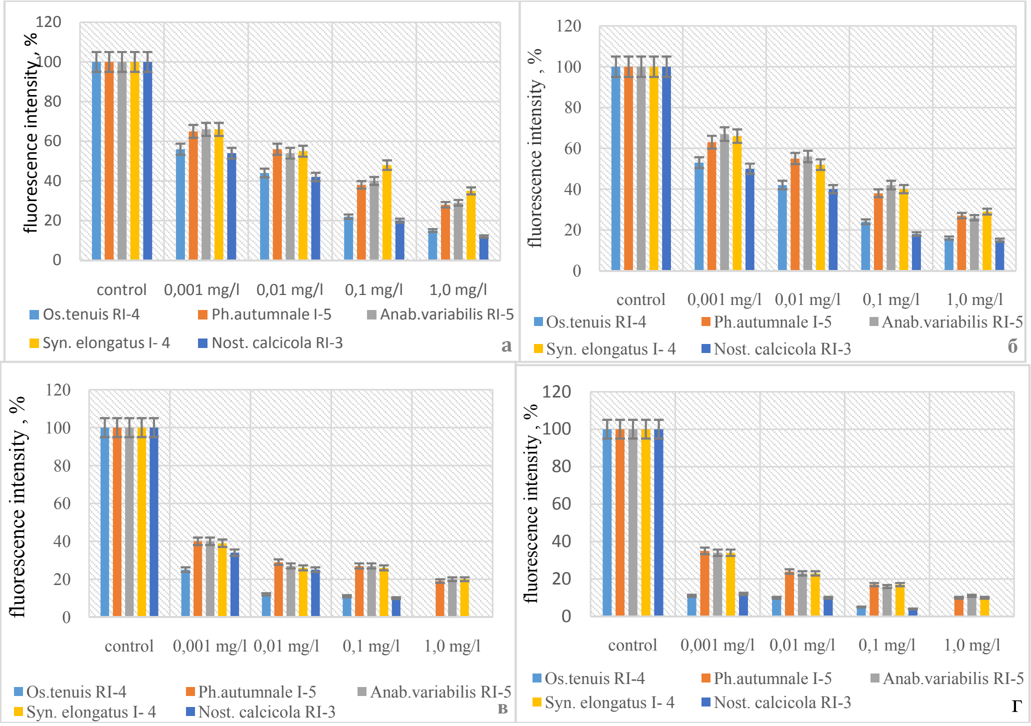


Figure 1 – Relative intensity values of chlorophyll fluorescence of microalgae, subjected to the effects of heavy metals in different concentrations, a – Co^{2+}, b – Ni^{2+}, c – Zn^{2+}, d – Cu^{2+}



а

Figure 2 – Relative intensity values of chlorophyll fluorescence of cyanobacteria, subjected to the effects of heavy metals in different concentrations, а – Co^{2+}, б – Ni^{2+}, в – Zn^{2+}, г – Cu^{2+}

Some strains under the influence of the zinc and copper was observed phenomenon of plasmolysis and deformation of the cells. Influence of heavy metals on cells of microalgae and cyanobacteria have identified a number of surveyed metals toxicity, which is as follows: $Cu_2 + > Zn_2 + > Co_2 + > Ni_2 +$. From cultures of microalgae and cyanobacteria the following types of selected for further study, as promising strains for bioremediation of contaminated aquatic ecosystems, various heavy metals: *Phormidium autumnale I-5, Anabaena variabilis RI-5, Synechococcus elongatus I-4, Chlorella vulgaris sp BB-2, Chlamydomonas reinhardtii B -4.*

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Identification of flavonoids in plant samples by gas chromatography-mass spectrometry with pre-derivatization

Abstract: Plants of Artemisia L. genus are important source of flavonoids – a group of biologically active substances, which are widely used in the perfume, alcoholic beverage, pharmaceutical industry and medicine. Flavonoids have antiseptic, anti-tumor, anti-inflammatory, antibacterial, antituberculosis actions. Therefore, testing and introduction of many types of Artemisia to the culture, and their selection in Kazakhstan is up-and-coming. Qualitative composition of wormwood Artemisia scopariaWaldst. et Kit flavonoids collected in Almaty and Ile-Balkhash region in 2013 year was studied by gas chromatography-mass spectrometry method. Samples were prepared by extraction three times with 40% ethanol solution. During the identification of flavonoids by gas chromatography-mass spectrometry pre-derivatization of the compounds was carried out by N,O-Bis(trimethylsilyl) trifluoroacetamide, which is the most reactive derivatizing agent to increase the volatility and thermal stabilityofstudied substances. Kaempferol and quercetin were found as a result of analysis.

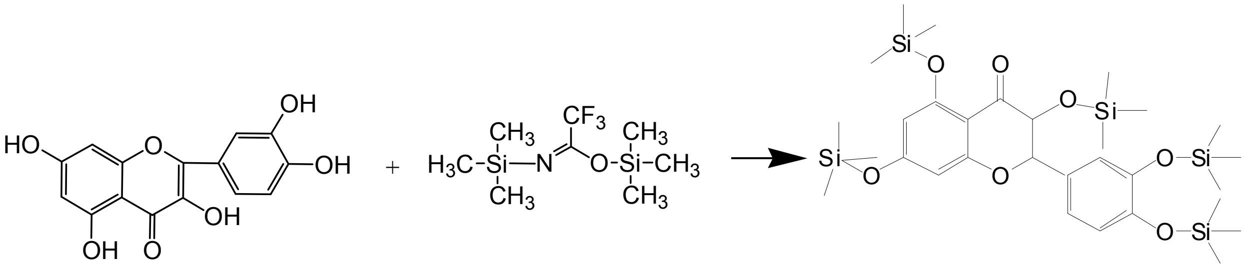
Key words: analysis; Artemisia; derivatization; flavonoids; gas chromatography; kaempferol; mass spectrometry; quercetin.

Introduction

According to scientists [1], the linearity ranges of the measured concentrations of phenolic compounds in the analysis by GC-FID is considerably wider than when analyzing by GC-MS, but the limits of detection using mass spectrometric detector is 2-3 times lower. Scientists [2] used the technique of GC-FID described in work [3], and it was possible to identify a number of flavonol glycosides in bearberry extracts. Researchers [4] used capillary gas chromatography with flame ionization detector to assess the content of flavones, flavonones, flavonoles and isoflavones in legumes. According to the method [5] the content of isoflavones in soybean was determined, the authors were able to identify three aglyconesin plant sample:quercetin, genistein anddaidzein.

Gas chromatography-mass spectrometry is used to analyze the different objects. American scientists [6] had developed a technique of GC-MS with electron impact ionization for the analysis of biological fluids (human plasma, urine) on the content of flavonoids and phenolic acids after consumption of cranberry juice. In research [7] a method of determining the cinnamic acid, catechins and flavonols in red wines proposed.

A method for analyzing of olive oil on the content of phenolic compounds described in [8]. Detection of phenolic compounds was performedusing time-of-flight mass spectrometer with chemical ionization at atmospheric pressure. Detection limit for luteolin was 1.1 μg/g, for apigenin - 0.6 μg/g, for ferulic and coumaric acids - 0.2 μg/g. The authors of [9] carried out the analyses of the content of more than 20 different phenolic compounds and accompanying substances in aloe extract by GC-MS. Application of GC-MS in the analysis of mosses, herbs, fruits and vegetables is described in [10-12]. Most known methods of derivatization are alkylation and silylation. The mechanism of derivatization includes replacing the acidic hydrogen atom in the molecule of phenolic compound (for example, -OH and -COOH groups) by the alkyl or silyl group [13]. Various silylating agents: trimethylchlorosilane (TMCS), bis-trimethylsilylacetamide (BSA) dimethylsilylacetate (DMTSA) et al are used for derivatization. [7]. Among all the listed reagents N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) has the highest silylating ability which also modifies the surface of the sorbent column, which greatly improves the resolution of peaks. Pyridine can be added to speed up derivarization, because BSTFA slowly reactes with tertiary or quaternary hydroxyl groups [13]. In [7.10] pyridine is initially added to the solution of the phenolic compounds, followed by addition of the excess of derivatizing agent and holded at a temperature of 50-80°C. Scientists [12] studied and optimized derivatization by variating temperature from 50 to 80°C and time from 10 min to 24 hrs. It was found that, mixture should be holded at 70°C for 2 hours for the completion of the reaction. Derivatization reactions on the example of interaction quercetin with BSTFA:



Determination of flavonoids may be carried out with using the gas chromatography method, which is one of the most sensitive methods of analysis. For identification of flavonoids with gas chromatography using flame ionization and mass spectrometric detectors.

Thus, gas chromatography with mass spectrometric detector becamewidespread receives for determination and identification phenolic compounds in plant samples, because it ensures component separation of complex natural matrices. In this article we solved the problem of flavonoids identification in plant samples by gas chromatography-mass spectrometry with pre derivatization. Analysis by GC- MS was carried out on a chromatograph Agilent 7890A / 5975C (USA). For automation of selection, sample preparation and sample introduction, gas chromatography-mass spectrometer equipped with an autosampler CTC-Combi-PAL (CTC Analytics AG, Switzerland). To control the whole gas chromatographic system, recording and processing of chromatographic data using software Agilent MSD ChemStation (version 1701EA). Processing included the determination of the retention times, peak heights and areas as well as processing of resulting in a mass spectrometric detector spectral information. To decrypt the obtained results was used the mass spectra library Wiley 7th edition and NIST'02 (the total number of spectra in the library - more than 550 thousand.). Chromatographic parameters are presented in Table 1.

Materials and methods

Auxiliary materials for GC -MS

Helium grade "A" (99.995%) (Orenburg, Russia);

Column: DB-5MS, length 60 m, internal diameter 0.25 mm, film thickness 0.25 µm (Agilent, USA);

Microsyringe for Combi-PAL autosampler, volume of 10µL (Hamilton, Switzerland);

Conical vials 1.5 mL with teflon caps and ultrapure silicone gaskets (CTC Analytics AG);

For derivatization, 50µL of individual flavonoids were dried and placed vials. 50 µLof N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 50 µL of pyridine were added and the mixture was kept for 2 hours on water bath at 70°C and then chromatographed.

Analysis by GC- MS was carried out using Agilent 7890A/5975C (USA). For automation of sample preparation and introduction gas chromatography-mass spectrometer is equipped with CTC-Combi-PAL (CTC Analytics AG, Switzerland). Agilent MSD ChemStation (version 1701EA) software was used to control the whole gas chromatographic system, to recording and to process chromatographic data. Processing included determination of retention times, peak heights and areas as well as processing of resulting mass spectral information. Wiley 7th edition and NIST'02mass spectra library (the total number of spectra in the library - more than 550 thousand.) was used to process the obtained results was used Chromatographic parameters are presented in Table 1.

Results and discussion

Objects of study are eight species of Artemisia wormwood:

- Artemisia juncea Kar.et Kir;
- Artemisia terrae-albaeKrasch;
- Artemisia scopariaWaldst. et Kit;
- Artemisia dracunculus L;
- Artemisia sieversianaWilld;
- Artemisia nitrosaWeb.exStrechm;
- Artemisia vulgaris L;
- Artemisia Absinthium L.

Flavonoids are used as standard samples:

Quercetin 95%; Myricetin 96.0%; Kaempferol 97.0%; Naringenin 95%; Hesperetin 95%; Apigenin 97%; Catechin 99.0%; Isorhamnetin95.0%; Luteolin97.0% (Sigma Aldrich) and Ruthin 96.18% (Russian Federation).

Solvents:

- Distilled water;
- Concentrated hydrochloric acid (Russian Federation);
- Methanol (AppliChem);
- Chloroform 99.8% (Sigma Aldrich).

Eluents:

- Acetonitrile 99.8%, (Sigma Aldrich);
- Analytical grade phosphoric acid (Reahim, Russia).

For determining flavonoids, plant samples were kept and preparated according to the scheme shown on Figure 1.

Table 1 – Chromatographic parameters of flavonoids by GC-MS

Parameter	Value
Inlet temperature	240°C
Flow rate carrier gas (helium)	1 mL / min (constant flow)
Oven temperatureprogramme	40 ° C (holding 10 min), heating to 300°C at the rate of 10°C for min (20 min)
MSD interface temperature	240°C
Detection mode	m/z 34-1000 SCPN
Analysis time, min	56

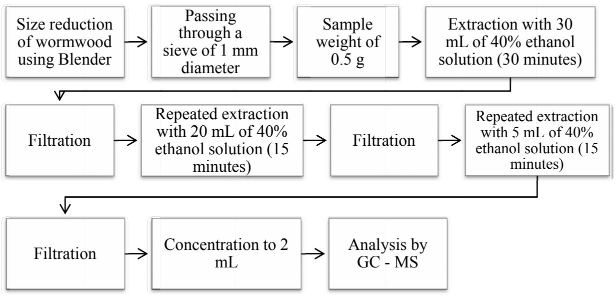
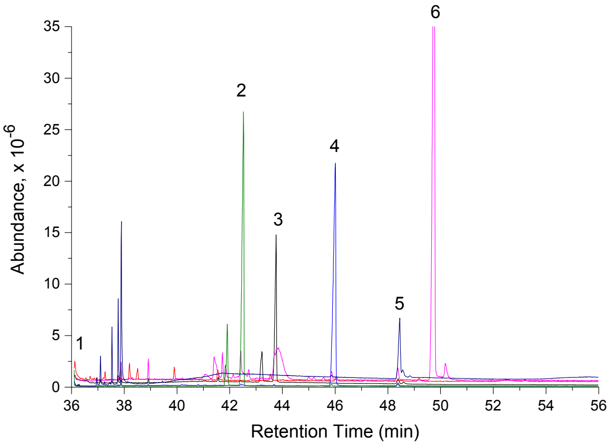


Figure 1 – Scheme of sample preparation of plants to analyze



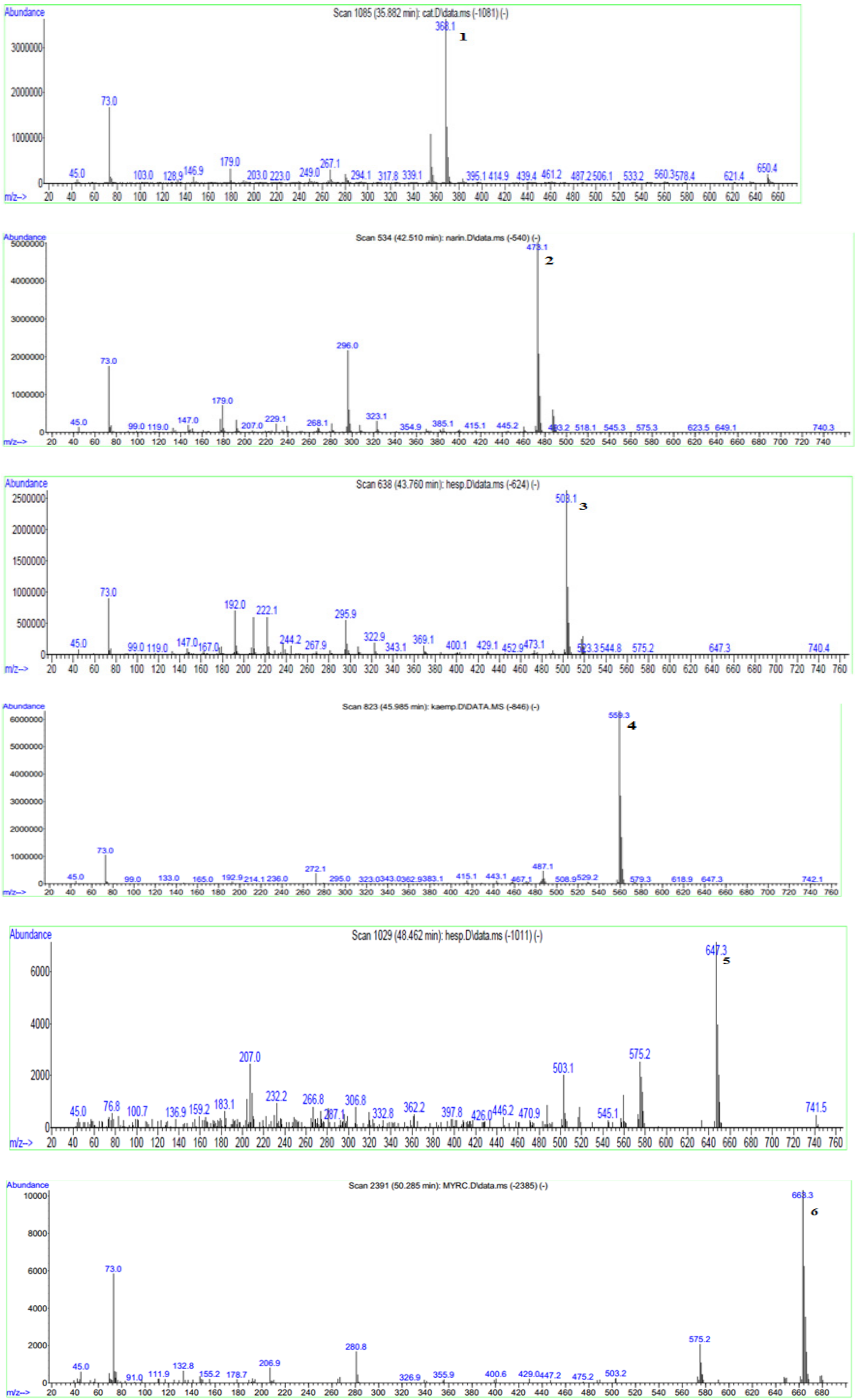
1 – catechin; 2 – naringenin; 3 – hesperidin; 4 – kaempferol; 5 – quercetin; 6 – myricetin.

Figure 2 – Chromatogram of the individual flavonoids

As a result of carried research separation of mixed flavonoids is achieved. Chromatogram of separation six standard flavonoids is presented below on Figure 2.

On the Figure 3 trimethylsilyl (TMS) derivates of six flavonoids are shown according to their eluation order.

For flavonoids identification parameters of retention and retention time of specific ions and their TMS-derivates are set (Table 2). According to the mass spectra of TMS derivatives various fragment ions compoundsare formed in the result of ionization/


Scan 1085 (35.882 min): cat.D\data.ms (-1081) (-)

Scan 534 (42.510 min): narin.D\data.ms (-540) (-)

Scan 638 (43.760 min): hesp.D\data.ms (-624) (-)

Scan 823 (45.985 min): kaemp.D\DATA.MS (-846) (-)

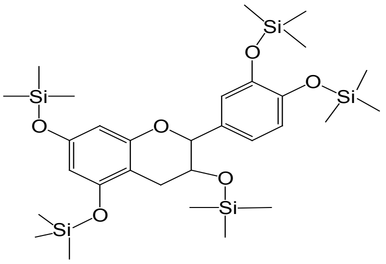
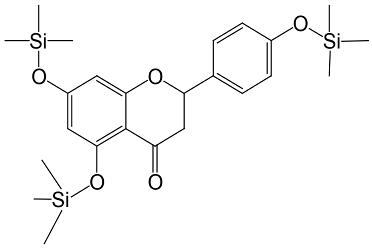
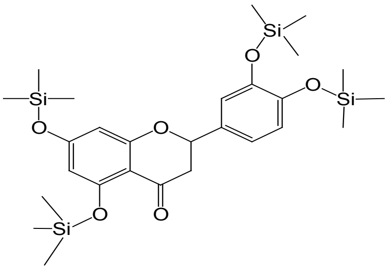
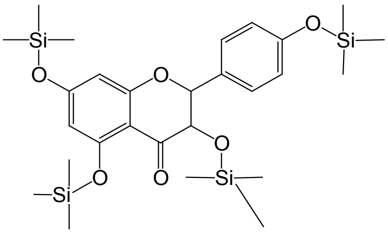
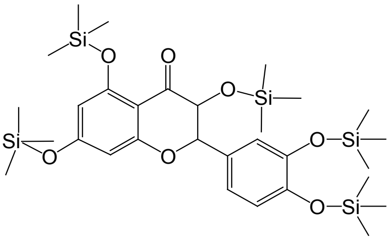
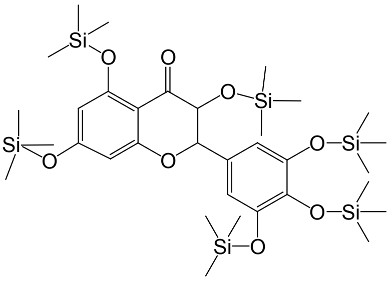
Scan 1029 (48.462 min): hesp.D\data.ms (-1011) (-)

Scan 2391 (50.285 min): MYRC.D\data.ms (-2385) (-)

1 – catechin; 2 – naringenin; 3 – hesperidin; 4 – kaempferol; 5 – quercetin; 6 – myricetin.

Figure 3 – Mass spectra of individual flavonoids

Table 2 - Parameters of identification of flavonoids

Compound	Formula of TMS derivatives	MM	Number of OH groups	Retention time, min	m / z
Catechin		650	5	35.8	368 (999) 369 (338) 355 (295) 370 (157)
Naringenin		488	3	42.5	473 (847) 296 (458) 474 (334) 179 (234) 475 (154) 297 (125) 177 (123)
Hesperidin		519	3	43.7	503 (999) 504 (421) 192 (295) 222 (217) 209 (215)
Kaempferol		577	4	45.9	559 (999) 560 (489) 487 (65)
Quercetin		663	5	48.4	647 (999) 648 (567) 649 (330) 559 (169) 650 (112)
Myricetin		751	6	50.2	735 (452) 736 (285) 737 (180) 647 (143)

Identification of flavonoids was performed by retention parameters and mass spectra of substances. From all the samples selected in 2013, flavonoids were found only in sample №3, such as kaempferol and quercetin. The chromatogram of the wormwood extracts №3 - Artemisia scopariaWaldst. et Kit is presented in Figure 4.

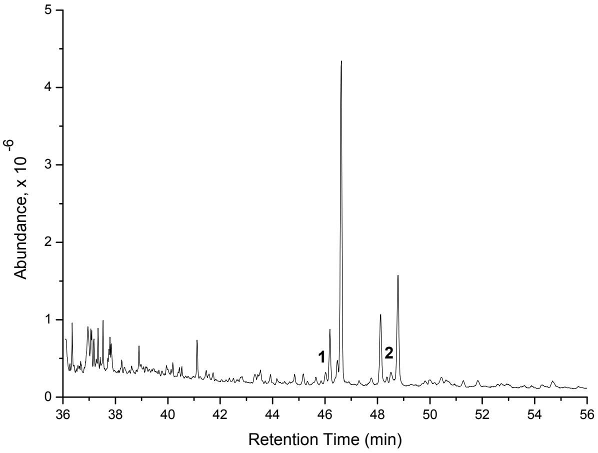


Figure 4 – The chromatogram of the wormwood extracts №3 – Artemisia scopariaWaldst. et Kit (1 kaempferol – 45.9 min 2 – quercetin – 48.4 min)

Conclusion

According to the analysis of samples of wormwood were summed the following conclusions:

1. Before testing on GC- MS is necessary to remove of flavonoids aglycones and polar solvents in the sample because the sugar overload operation of the mass spectrometer;
2. Application derivatizing agent contributes to the volatility of investigated substances, thermal stability and better separation;
3. In the sample of wormwood Artemisia scopariaWaldst. et Kit were found kaempferol and quercetin, which are then assumed to allocate from this type of plant.

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Synthesis and mucoadhesive study of thiolated hydrogels based on 2-hydroxyethylacrylate and 2-hydroxyethylmethylacrylate copolymers

Abstract: Hydrogels and copolymers of 2-hydroxyethylmethacrylate and 2-hydroxyethylacrylate structured with 3-mercaptopropyltrimethoxysilane nanoparticles of different composition and nanoparticles concentration have been synthesized and characterized using IR-spectroscopy, SEM, small angle X-ray scattering, compression mechanical test, thermogravimetric analysis, iodometry. Mucoadhesive properties of obtained hydrogels were investigated. Study revealed that increase in concentration of hydrophilic component, nanoparticles content and crosslinking agent concentration reflects an increase of adhesion time of obtained hydrogel materials. Drug loading and drug release studies of HEA-HEMA-MPTS samples with metronidazole were conducted. It was observed that thiolated samples absorb more drug amount than non-thiolated ones. As nanoparticles concentration increases metronidazole content rises respectively. Drug release studies revealed that the higher concentration of 3-MPTS nanoparticles in hydrogels results in prolongation of drug release but concentration of discharged metronidazole decreases.

Key words: organosilane thiol nanoparticles, hydrogels, mucoadhesion, 3-mercaptopropyltrimethoxysilane

Introduction

Thiolated polymers represent new class of mucoadhesive drug delivery systems. The attachment of non-thiolated mucoadhesive polymers to the mucus layer has been achieved only by non-covalent bonds such as hydrogen bonds and ionic interactions. Accordingly, they provide only a weak adhesion, which in many cases insufficient to guarantee the localization of a drug delivery system at a given target site. Thiolated novel polymers are capable of forming covalent bonds with mucus through disulphide bridges formation. Mechanism of thiolated polymers attachment mimics the mechanism occurring in biological systems. Besides, nonionic polymers show weak mucoadhesion comparing to ionic. In this regard, thiolation of nonionic polymers will allow significantly improve their ability to retain on mucous surfaces [1]. Synthesis of such polymers will provide hydrogels with better mucoadhesive characteristics suitable for using them as buccal drug delivery systems. Drug delivery to target organ through classic routes of administration is usually complicated, however buccal drug delivery offers the possibility of circumventing the hepatic ‘first-pass’ elimination that follows gastrointestinal absorption, degradation in the gastrointestinal tract is also avoided [2]. Therefore, creation of buccal drug forms appears to be a very promising direction of investigation.

Particularly in this work we synthesized homopolymers and copolymers of 2-hydroxyethylmetacrylate (HEMA) and 2-hydroxyethylacrylate (HEA) structured with 3-mercaptopropyltrimethoxysilane (MPTS) nanoparticles of different composition and nanoparticles concentration. The thiolation was confirmed by different physical-chemical methods. Mucoadhesive properties of synthesized hydrogels were investigated. Drug loading and drug release studies of thiolated HEA-HEMA samples with metronidazole were conducted.

Materials and methods

Materials. 3-Mercaptopropyltrimethoxysilane (95%) (supplied by Sigma-Aldrich, Inc., dimethylsulfoxide «Chemical Pure» grade (supplied by AppliChem), 2-Hydroxyethylmetacrylate (96%) (supplied by Acros Organics), N,N’-methylene-bis-acrylamide (supplied by AppliChem), ammonia persulfate (supplied by Sigma-Aldrich, Inc.), 2-Hydroxyethylacrylate (96%) (supplied by Sigma-Aldrich, Inc.), starch were used without preliminary treatment.

$Na_2HPO_4*10H_2O$, $NaHCO_3$, $CaCl_2$ (supplied by Laborfarma) were used as purchased.

HCl, NaOH, C_2H_5OH were used without preliminary treatment.

Dialysis membrane MWCO 12-14 kDa (Medicell International Ltd, UK).

Synthesis of 3-MPTS nanoparticles and hydrogels based on HEMA, HEA and 3-MPTS nanoparticles. Preparation of 3-MPTS nanoparticles was conducted as described in [3].Synthesis of hydrogels made by free radical polymerization was conducted in the presence of ammonium persulfate as the initiator and N,N'-methylene-bis-acrylamide as crosslinking agent, at a temperature of 40°C. The initial monomer mixture was purged with argon. Hydrogels and copolymers with different concentrations of monomers and nanoparticles in the initial monomer mixture have been synthesized. Also hydrogels without 3-MPTS nanoparticles were synthesized.

Methods of characterization. Chemical structure of obtained copolymers was studied by IR-spectroscopy, on Perkin Elmer FTIR-Spectrum 400 (USA).

Morphology and structure of obtained copolymers were characterized by SEM using FESEM Hitachi SU8220 (Japan).

In order to verify presence of nanoparticles in structure of obtained hydrogels and determination of their size and placement small angle x-ray scattering method was used. It was performed on the combined system of small and wide angle X-ray scattering (Hecus, Austria).

Compression test of obtained copolymers was performed by TA.XT. Texture Analyser (England). Elastic modulus was calculated by the following formula:

$$E=\mathrm{tg}\ \alpha$$

Every copolymer composition was tested three times and average values were calculated.

Thermogravimetric analysis was conducted on Netzsch STA 449 F3 Jupiter (Germany) with heating rate 10°C/min in nitrogen atmosphere.

Iodometry. The amount of thiol groups on the hydrogel HEA-HEMA-MPTS was determined using iodometric titration method proposed by Bernkop-Schnürch [4]. First, 30.00 mg of each polymer were hydrated in 9.0 ml of deionized water. The pH-value was then adjusted to 2–3 by adding 1 M HCl. After the addition of 300 μL of aqueous starch solution (1%), samples were titrated with an aqueous iod solution (1.00 mM) until a permanent light blue colour became visible.

Preparation of artificial saliva solution. Artificial saliva solution was prepared by method proposed in [5]. Na_2HPO_4 (0,671 g), $NaHCO_3$ (1,05 g), $CaCl_2$ (0,092 g) were dissolved in distilled water (499,5 ml) with 1M HCl (1,56 ml) addition.

Mucoadhesion studies. In order to evaluate the retention time of hydrogels on mucosa surface the "falling liquid method" was used. It is represented at Figure 1. Preliminarily dried polymer samples were attached to freshly excised buccal porcine mucosa, which has been sticked to a glass pad 2. Glass funnel 1 with artificial saliva solution was placed over the mucosal surface with polymer sample. Flow was regulated by faucet up to 20 drops in a minute (imitating the saliva release). Forementioned glass pad was disposed in a transparent beaker 3 to collect the washings. Received washing were continuously returned back to the funnel. The detachment, disintegration of test hydrogel samples was observed within a time period of 4 h.

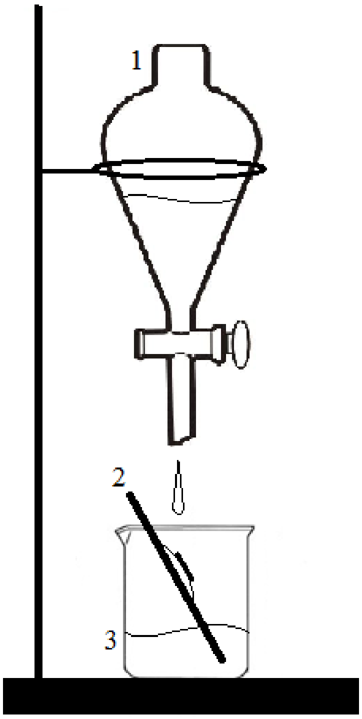


Figure 1 – Schematic illustration of "falling liquid method"

Drug loading. 0,8 % Metronidazole solution was prepared by dissolving metronidazole powder in distilled water. Then preliminarily dried samples were immersed in this solution for 48 hours until equilibrium swelling. Concentration of metronidazole loaded in hydrogels was determined by measuring its residual concentration in solution by UV-spectroscopy (absorbance of metronidazole at 320 nm). For these purposes UV-spectrophotometer Shimadzu UV/VIS-2401 PC was used. Before that, calibration curve of metronidazole in water solutions was plotted.

Drug release studies. The measurement of metronidazole release was conducted using Franz cell chamber. The cellulose membrane separated the donor compartment containing the sample being analyzed from the receptor compartment filled with collection medium. Artificial saliva solution was used as the collection medium. Diffusion of the drug from polymer sample across the membrane was monitored by taking sample of the receptor medium. At predetermined time points, an aliquot of medium was removed from the receptor compartment and analyzed by UV-spectrophotometer Shimadzu UV/VIS-2401 PC. The aliquot was measured at 320 nm. Then it was returned back to receptor compartment. Determination of drug concentration was carried out using calibration curve of metronidazole in artificial saliva solution.

Results and their discussion

The characterization of synthesized hydrogels was carried out using different physical-chemical methods: FTIR spectroscopy, scanning electron microscopy, thermal analysis, small angle X-ray scattering, mechanical test, “falling liquid method”, Franz cell method.

FTIR-spectroscopy. The investigation of obtained composite materials by FTIR-spectroscopy confirmed the presence for C-S (691 cm^{-1}) and S-S at 491 cm^{-1} bonds formation (Figure 2) [6]. However, it’s difficult to provide good evidence for –SH groups presence, because that groups give very weak signal in IR-spectra.

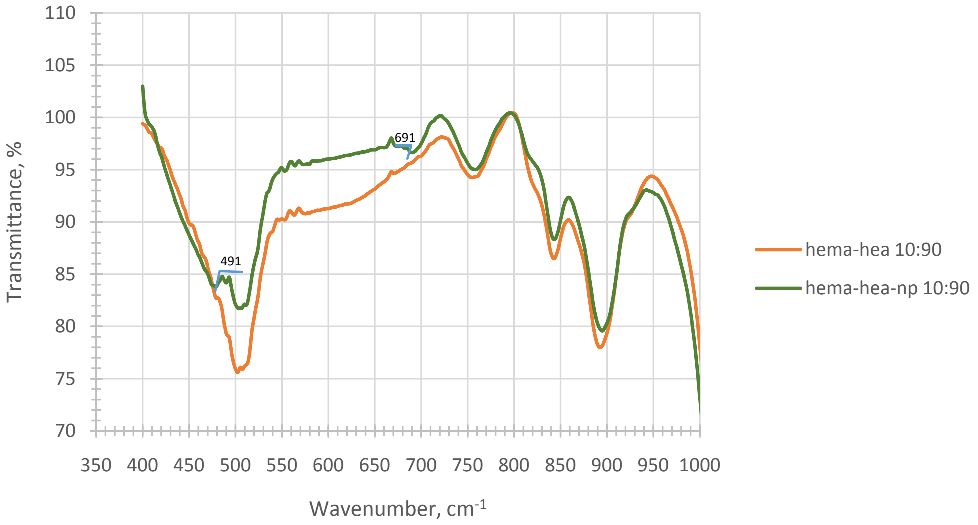


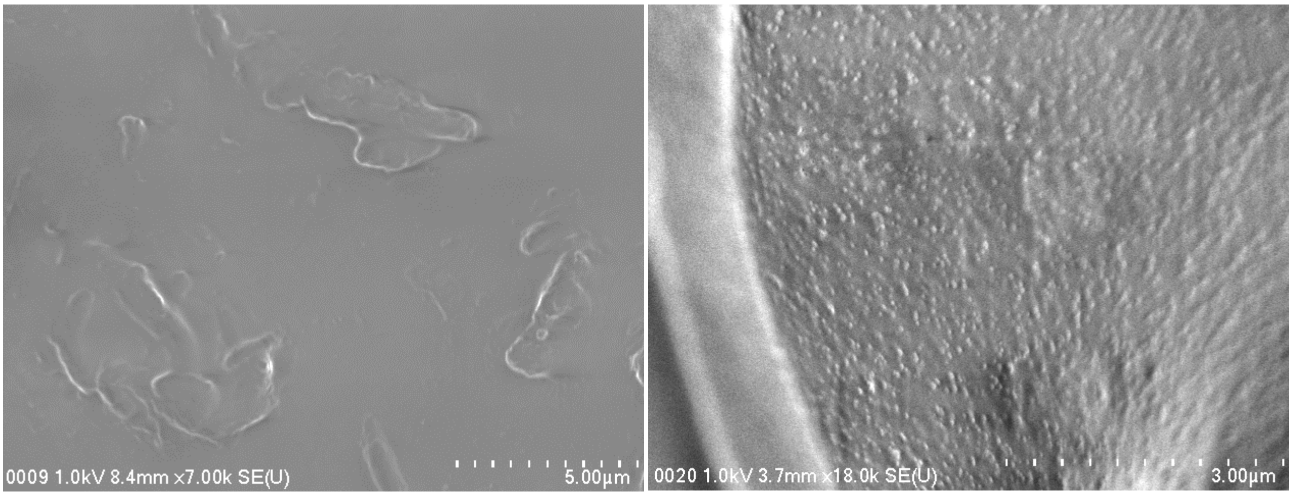
Figure 2 – IR-spectra of thiolated and non-thiolated HEA-HEMA copolymer

Scanning electron microscopy (SEM). In present work SEM was used and obtained microphotographs are presented on Figure 3. Comparing the images of thiolated (b) and non-thiolated (a) hydrogel samples revealed that small nanoparticles are present in gel structure with satisfying homogeneous distribution. Also the size of 3-MPTS nanoparticles can be determined and is around 60 nm.

Small angle X-ray Scattering (SAXS). Nanoparticles presence and their size distribution in polymer matrix were investigated by small angle X-ray scattering spectroscopy (Figure 4). Average size of nanoparticles incorporated in polymer network is about 60-70 nm which is in good correlation with results obtained by SEM.

Thermogravimetric analysis. Thermogravimetric analysis was conducted on Netzsch STA 449 F3 Jupiter (Germany) with heating rate 10^{o}C/min in nitrogen atmosphere. Samples were heated under temperature range of 30-600^{o}C.

From thermogravimetric curves it was possible to determine the final residues % and then calculate the mass of 3-MPTS nanoparticles in hydrogel samples. Obtained data are presented in Table 1. It was observed that with increase of nanoparticles concentration in initial monomer mixture the percentage of final residues of nanoparticles incorporated into HEA-MPTS and HEA-HEMA-MPTS hydrogel samples and their weight also increased.



a *b*

Figure 3 – Microphotographs of HEA-HEMA copolymers without (a) and with 3-MPTS nanoparticles (b)

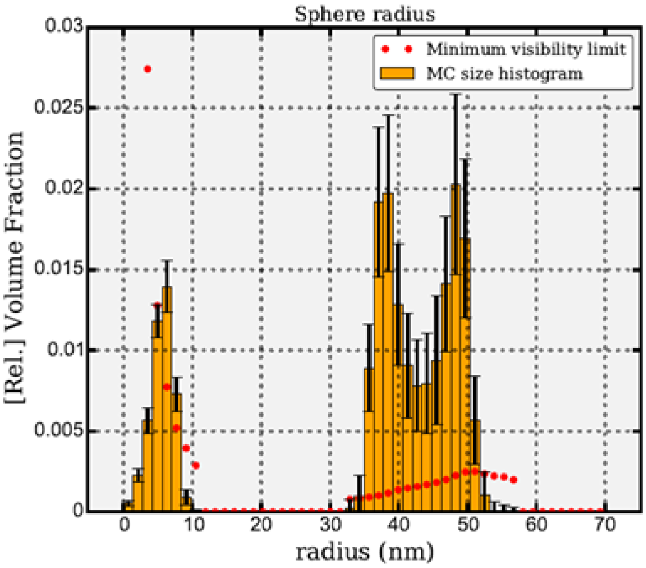


Figure 4 – Small Angle X-ray Scattering of thiolated hydrogel

Table 1 – TGA final residues analysis and calculation of 3-MPTS nanoparticles concentration in hydrogels.

Sample	C_{NP} in IMM, weight %	Final residues of hydrogels, weight %	Weight of sample, mg	C_{NP} in HEA-HEMA hydrogels, weight %	Weight of 3-MPTS nanoparticles in hydrogel sample, mg
HEA	-	10		-	-
HEA-MPTS	0,26	12	18,090	2	0,362
	0,36	13	11,458	3	0,344
	0,40	14	7,490	4	0,300
HEA-HEMA	-	8	22,728	-	-
HEA-HEMA-MPTS	0,26	10	15,402	2	0,308
	0,36	13	13,255	5	0,663

Compression test. Mechanical properties of synthesized materials were investigated by mechanical test in compression mode. Compression test showed that hydrogels based on HEA, HEMA and 3-MPTS nanoparticles are sufficiently elastically strong and flexible. This is due to the fact that glass transition temperature of HEA lies in the negative temperatures, which results in demonstration of highly elastic properties at room temperatures. Thus, while the compression analysis of hydrogel samples containing HEA was performed, their destruction was not observed, and when the load was removed, the hydrogel shape returned to its original state.

The elastic modulus E for analysed hydrogels were calculated from stress-strain curves. Results are shown in the Tables 2 and 3. The elastic modulus was determined as the slope of the initial straight-line section of the deformation curve. The growth of the concentration of the crosslinking agent and nanoparticles in the gel promoted increase of elastic modulus values by 2-3 times for certain samples.

Table 2 – Values of elastic modulus E for HEA-HEMA-MPTS hydrogels of different HEA-HEMA ratios and concentrations of nanoparticles

Composition of initial monomer mixture		Concentration of nanoparticles, mg/ml	Concentration of crosslinking agent, mol %	Elastic modulus E, Pa
[HEMA]		-	0,125	48 700,55
		2,45		121 780,60
[HEA-HEMA]	70:30	-	0,125	7 281,75
		2,45		9 021,91
	80:20	-	0,125	1 685,90
		2,45		8 530,34
		3,43		9 701,46
		3,92		15 720,57
	90:10	-	0,125	1 312,77
		2,45		5 053,85
		3,43		12 492,13
		3,92		17 408,85
[HEA]		-	0,100	5 126,72
		-	0,125	13 194,04
		2,45	0,100	1 768,32
		3,43	0,100	2 713,48
		3,92	0,100	5 925,39
		4,41	0,100	8 406,60

It is clear that the presence of nanoparticles in a gel contributes to a slight decrease in the elasticity of the polymer network, thus increasing its rigidity. This is probably due to the higher number of crosslinks in the network, which reduce flexibility and mobility of macromolecules.

When studying the effect of the monomer ratio in the initial monomer blend on the mechanical properties of copolymers, it was observed that reduction of the HEA content in the copolymer hydrogel the rigidity of the network increased.

When studying the effect of the concentration of crosslinking agent on the physico-mechanical properties, it was found that the capacity for high elastic deformation is the lowest for hydrogel samples with maximum concentration of methylene bis-acrylamide.

Thiol groups content determination. Many methods have been developed for the quantification of thiol groups; electromagnetic resonance spectroscopy, high-performance liquid chromatography with electrochemical detection, capillary electrophoresis, liquid chromatography with mass spectrometry, enzymatic methods and, the traditional analytical approaches based on the thiol derivation procedure, obtaining compounds detectable by UV–visible spectroscopy or fluorimetric. However, all these methods require elaborate equipment and are very expensive. Therefore, in this work thiol content determination was carried out by simple iodometric titration. Although, it's less sensitive and precise, this method is highly appreciated for its easiness, rapidity and no necessity in aggressive procedures.

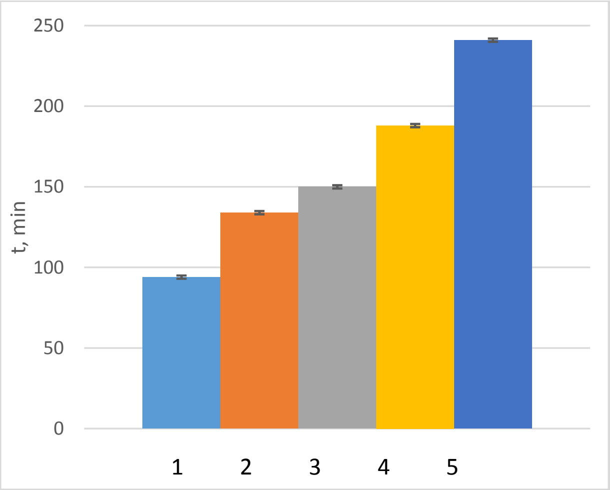
Table 3 – Impact of crosslinking agent concentration on mechanical properties of HEA-HEMA-MPTS hydrogels

Composition of initial monomer mixture		Concentration of nanoparticles, mg/ml	Concentration of crosslinking agent, mol %	Elastic modulus E, Pa
[HEA-HEMA]	80:20	2,45	0,075	6 215,07
		2,45	0,125	8 530,34
		2,45	0,175	15 404,79
		2,45	0,225	34 366,51

Results obtained by iodometric titration are presented at Table 4. First of all, it's vital to say that thiol group content increase with the concentration of nanoparticles in initial blend. For example for thiolated HEA-HEMA copolymers with monomer ratio 80:20 thiol content appears to be 32,9; 36,2 and 45,6 µmol per g polymer for nanoparticle concentration 2,45; 3,43 and 3,92 mg/ml respectively. Another point is that increase in hydrophilicity of copolymer composition results in worse thiolation which is due to hydrophobic nature of nanoparticles.

Table 4 – Dependence of thiol groups content of HEA-HEMA-MPTS hydrogels from nanoparticles concentration

Composition of initial monomer mixture		Concentration of nanoparticles, mg/ml	Concentration of crosslinking agent, mol %	Thiol content, µmol per g polymer
[HEMA]		2,45	0,125	27,8
[HEA-HEMA]	70:30	2,45	0,125	22,7
	80:20	2,45	0,125	32,9
		3,43		36,2
		3,92		45,6
	90:10	2,45	0,125	23,9
		3,43		34,4
		3,92		40,0
[HEA]		2,45	0,100	13,3
		3,43		20
		3,92		23,3
		4,41		26,7



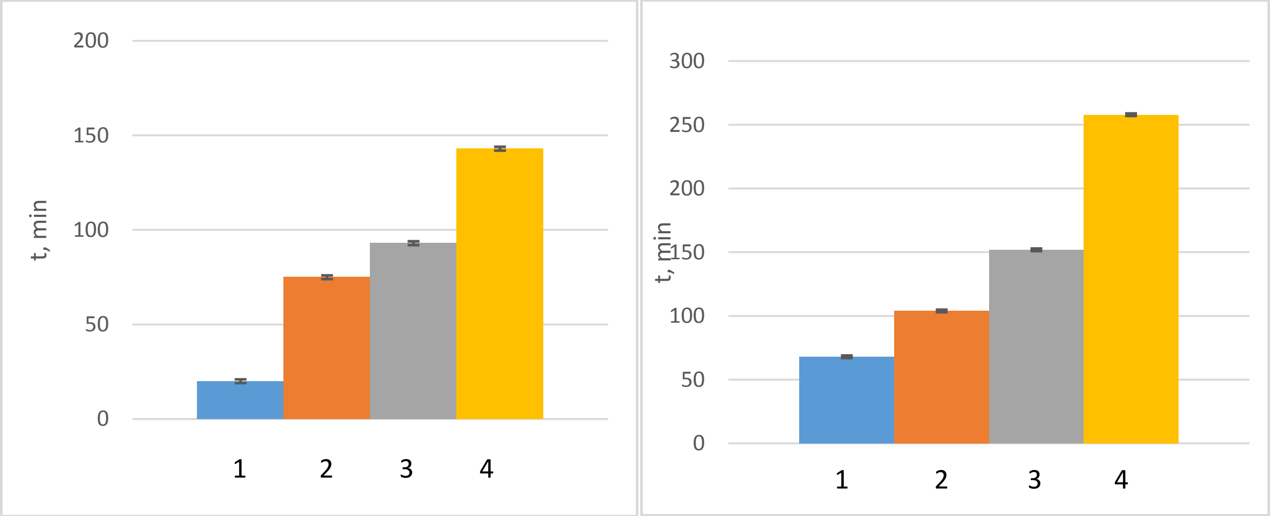
C_{NP} = 0 mg/ml (1); 2,45 mg/ml (2);
3,43 mg/ml (3); 3,92 mg/ml (4); 4,41 mg/ml (5)

Figure 5 – Impact of 3-MPTS nanoparticles concentration on retention time of HEA-MPTS hydrogels

Mucoadhesive studies. Retention time of homopolymer and copolymer hydrogels on mucosal surfaces was evaluated by "falling liquid method".

From Figures 5–6 it's seen that increase of 3-MPTS nanoparticle concentration results in longer retention time for thiolated homopolymer and copolymer hydrogels. Such effect was observed for HEA-HEMA-MPTS hydrogels of different copolymer ratio. Consequently, it should be noted that increase of thiol moieties in hydrogel composition in fact promotes better mucoadhesion.

Influence of IMM ration on mucoadhesive properties was studied. Figure 7 represents dependence of retention time for HEA-HEMA-MPTS hydrogels from copolymer content. The data obtained on this dependence appears to suggest that increase of HEA concentration as hydrophilic component in hydrogel composition results in improvement of mucoadhesion.



[HEA:HEMA], mol % = [80:20]

[HEA:HEMA], mol % = [90:10]

C_{NP} = 0 mg/ml (1); 2,45 mg/ml (2); 3,43 mg/ml (3); 3,92 mg/ml (4)

Figure 6 – Impact of 3-MPTS nanoparticles concentration on retention time of HEA-HEMA-MPTS hydrogels

Drug loading. In present work as a model drug for buccal drug delivery forms the metronidazole was chosen. Metronidazole is an antibiotic used to medicate infections of oral cavity [7], so our choice was determined upon this fact. Metronidazole was loaded into obtained thiolated hydrogels by simple absorption method.

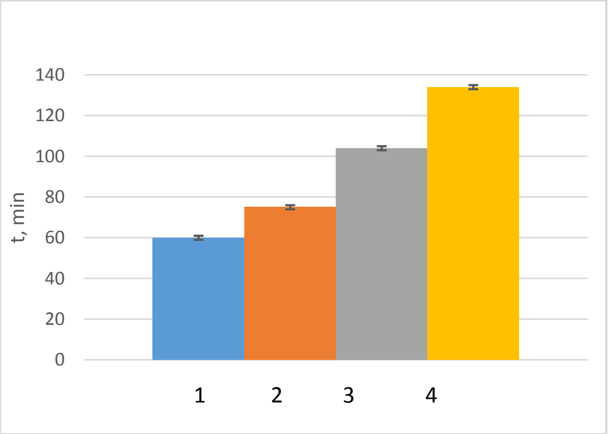
Content of metronidazole loaded into polymer samples is presented in Table 6.

It should be noted that thiolated samples absorb more drug than non-thiolated ones. As nanoparticles concentration increases metronidazole content rises respectively. For example for HEA-HEMA-MPTS sample with copolymer ratio 90:10 and nanoparticles concentration 2,45; 3,43 and 3,92 mg/ml masses of loaded metronidazole were 0,0112; 0,0123 and 0,0132 g respectively.

Another pattern that could be derived is that with increase of HEA content in copolymer samples concentration of loaded drug decreases. For instance, samples with nanoparticles concentration of 2,45 ml/ml and copolymer ratio 70:30, 80:20, 90:10 showed drug concentration of 0,0126; 0,0098. Hypothetically, it's caused by hydrophobic character of metronidazole, so that metronidazole showed affinity to hydrophobic nature of HEMA.

Drug release studies. The measurement of metronidazole release was conducted using Franz cell chamber. The Franz Cell chamber is an in vitro skin permeation test method frequently used in formulation development. In this work permeation of drug was observed through cellulose membrane. Artificial saliva solution was used as collection medium.

From Figure 8 it's seen that increase of nanoparticles concentration promotes more prolonged release of metronidazole. As concentration of nanoparticles becomes higher metronidazole concentrations decrease.



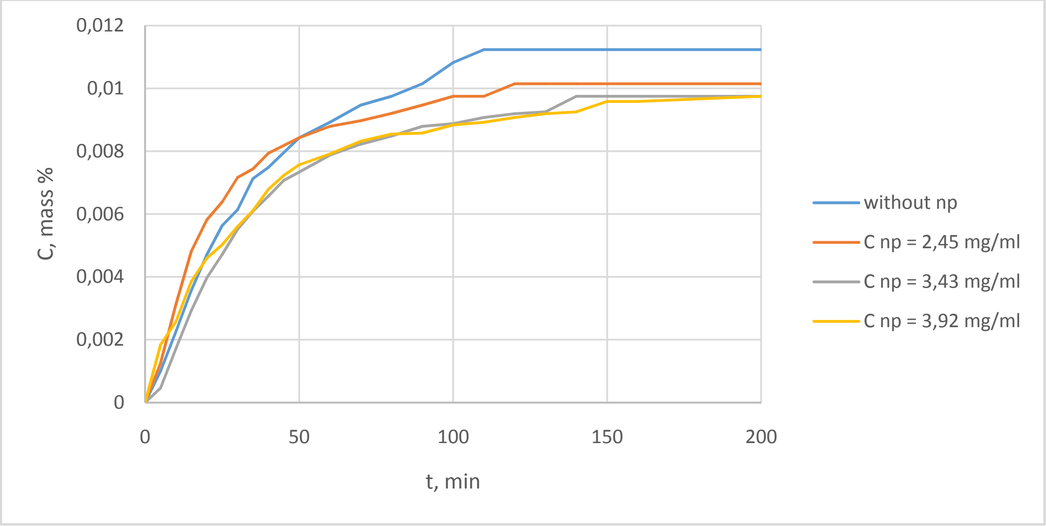
[HEA:HEMA], mol % = 70:30 (1); 80:20 (2); 90:10 (3); [HEA] (4)

C_{NP} = 2,45 mg/ml

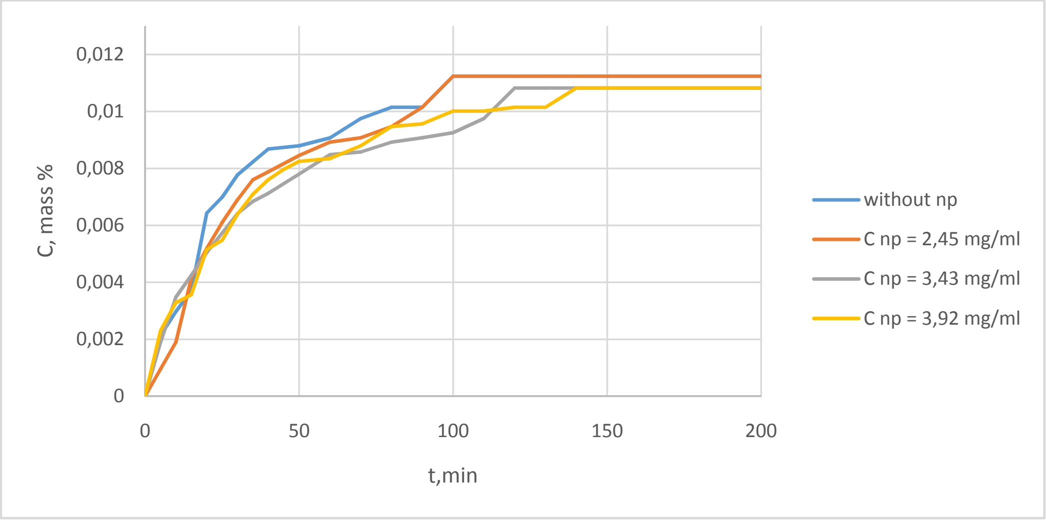
Figure 7 – Impact of copolymer IMM ration on retention time of HEA-HEMA-MPTS hydrogels

Table 6 – Metronidazole concentrations loaded in non-thiolated and thiolated HEA-HEMA hydrogels

Composition of initial monomer mixture		Concentration of nanoparticles, mg/ml	Concentration of crosslinking agent, mol %	Mass of loaded metronidazole, g	Concentration of loaded metronidazole, g/g of polymer
[HEMA]		-	0,125	0,0125	0,2147
		2,45		0,0135	0,1777
[HEA-HEMA]	70:30	-	0,125	0,0120	0,1921
		2,45		0,0126	0,2603
	80:20	-	0,125	0,0084	0,1132
		2,45		0,0098	0,2357
		3,43		0,0108	0,1735
		3,92		0,0192	0,3751
	90:10	-	0,125	0,0075	0,1157
		2,45		0,0112	0,1752
		3,43		0,0123	0,1636
		3,92		0,0132	0,2052
[HEA]		-	0,125	0,0112	0,2463



a. [HEA:HEMA], mol % = [80:20]



b. [HEA:HEMA], mol % = [90:10]

Figure 8 – Impact of nanoparticles concentration on kinetics of metronidazole release.

Figure 9 shows that with increase of hydrophilic component in copolymer ratio the concentration of released metronidazole grows. Also drug release duration become shorter as time of plateau appearance moves to smaller values. Consequently samples with copolymer ratio 90:10 release drug more quickly.

Conclusion

Hydrogels based on HEMA and HEA copolymers structured with 3-MPTS nanoparticles of different concentration have been synthesized and characterized using various physical-chemical methods.

Small angle X-ray scattering and scanning electron microscopy confirmed presence of incorporated nanoparticles. These methods allowed determination of nanoparticles size and their distribution in polymer matrix. Average size of nanoparticles incorporated in polymer network was about 60-70 nm.

Thiolation of copolymers was confirmed by FT-IR-spectroscopy and iodometry as well. Iodometric quantification showed that concentration of thiolated samples lays in concentration range 12-46 µmole per g of hydrogel.

Thermogravimetric analysis displayed that with increase of nanoparticles concentration in initial monomer mixture the mass fraction of residues of HEA-MPTS and HEA-HEMA-MPTS hydrogel samples also increased.

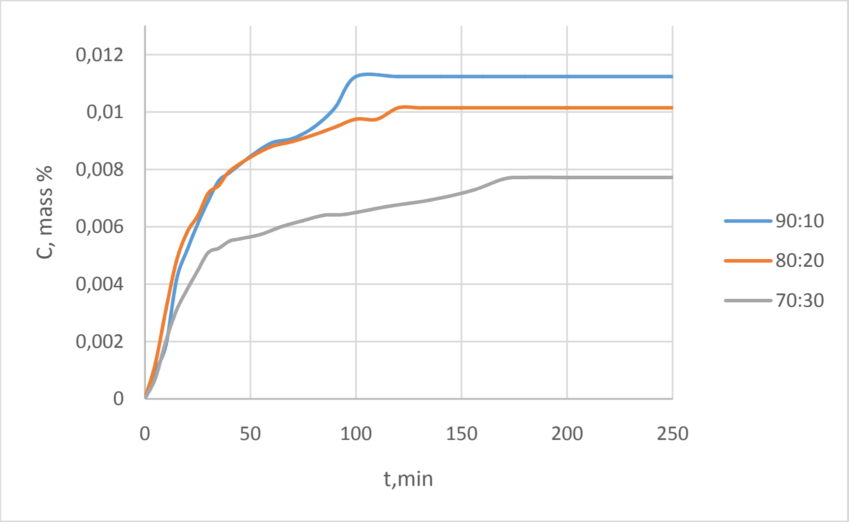


Figure 9 – Impact of HEA-HEMA copolymers ratio on kinetics of metronidazole release.

Compression test showed that the presence of nanoparticles in a gel contributes to a slight decrease in the elasticity of the polymer network increasing its strength. The growth of the concentration of the crosslinking agent and nanoparticles in the gel promoted increase of elastic modulus values by 2-3 times for certain samples.

Mucoadhesion studies showed that increase in concentration of hydrophilic component HEA in hydrogel, nanoparticles content and crosslinking agent concentration reflects an increase of adhesion time of obtained hydrogel materials.

Drug loading and drug release studies of HEA-HEMA-MPTS samples with metronidazole were conducted. It was observed that thiolated samples absorb more drug than non-thiolated ones. As nanoparticles concentration increases metronidazole content rises respectively. With increase of HEA content in copolymer samples concentration of loaded drug decreases.

Drug release studies revealed that the higher concentration of 3-MPTS nanoparticles in hydrogels results in prolongation of drug release but concentration of discharged metronidazole decreases. When hydrophilic component in copolymer content increases the concentration and rate of released metronidazole also growing.

Modifications of hydrogel materials by thiolation lead to broadening of their application possibilities. As a result of this research new thiolated polymers with improved mucoadhesive properties were obtained. And mucoadhesive studies of their application as a buccal dosage forms were performed as well.

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Mucoadhesive properties study of films based on N-vinylpirrolidone-2-hydroxymethylacrylate and acrylic acid copolymers

Abstract: The films based on N-vinylpirrolidone and 2-hydroxymethylacrylate (NVP-HEMA) copolymers and N-vinylpirrolidone-2-hydroxymethylacrylate and acrylic acid (NVP-HEMA-AA) triple copolymers were synthesized by free radical copolymerization. Complex formation of linear NVP-HEMA and NVP-HEMA-AA with mucin was studied. It has been found that the complex formation is a result of specific hydrogen bonds and van der Waals interactions. Also the pH of complexation for binary and triple copolymers system is around 5.5-6.5. The investigation of mucoadhesive properties of films based on linear copolymers was carried out and the impact of their composition on retention times of films on mucosa surface of pork buccal was istablished. The presence of HEMA and AA in copolymers composition increases the adhesive properties of films. Thermal treatment of polymeric films based on the NVP, HEMA and AA in order to get crosslinked structure resulted in lowering their mucoadhesive properties. Thus, it is found that the retention time of the films on mucosal surfaces of the pork oral cavity primarily depends on the composition of copolymers and a the way of films preparation.

Key words: mucoadhesion, hydroxymethylacrylate, N-vinylpirrolidone, acrylic acid.

Introduction

At the beginning of the XXI century there was a popular trend toward scientists developing a new mucoadhesive dosage forms. The main positive property of these delivery systems is consolidating on the mucous membranes, which is used as a way of solving the problem of low bioavailability of conventional formulations used in the oral cavity, on the surface of the eye or other parts of the body. In these parts of the body prolonged retention of drugs are prevented by movement of tissue and formation of different secrets. Application of mucoadhesive ointments based on polyacrylic acid and polymethyl methacrylate was a first theme of early investigations.

At the present time various types of synthetic and natural polymers get popular for the development of new pharmaceutical drug delivery systems. Examples of such polymers are derivatives of cellulose such as hydroxypropylcellulose (HPC), hydroxypropylmethylcellulose (HPMC), hydroxyethylcellulose (HEC), sodium carboxymethylcellulose (KMC), polyacrylic acid derivatives, polyacrylates, polycarbophyl, a copolymer of methyl vinyl ether and methacrylic acid, poly-2-hydroxyethylmethacrylate, polyvinylpyrrolidone (PVP), polyvinyl alcohol (PVA), chitosan, xanthan, pectin and alginate.

When polymers are moistered, they have viscous forms, which increase their residence time on the mucosal surface and result in adhesive interactions [1]. Adhesive interactions include hydrogenous bonds, hydrophobic interactions, van-der-Waals interactions, electrostatic coupling, covalent bonds [2,3]. For these reasons, high-molecular compounds with a high content of polar groups such as COOH and OH, characterized by a stronger mucoadhesion with minimal exposure of toxins [2].

Mucoadhesive properties depend on molecular weight of polymers, their flexibility, degree of hydration, hydrophilic-hydrophobic balance and the presence of crosslinking units in the macromolecules inspite of influence various functional groups in a polymer chain [4-7]. At present paper the polymeric films were formed from new potentially mucoadhesive copolymers based on NVP, HEMA and AA and their ability to retain on mucosa surface was studied.

Materials and methods

Materials

N-vinylpirrolidone, 2-hydroxymethylacrylate and mucin from porcine stomach type II were purchased from Sigma Aldrich (Great Britain) and used without purification. Acrilic acid was purchased from Russia with mark «clean». Azobisisobutyronitrile was purchased from Acros (USA).

Artificial saliva solution was prepared according to the method described in paper [8]. The chemical

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composition of the artificial saliva: disodium hydrogenorthophosphate (Na_2HPO_4), sodium hydrogencarbonate ($NaHCO_3$), calcium chloride ($CaCl_2$), distilled water (H_2O) and a solution of 1M hydrochloric acid (HCl).

Synthesis of copolymers based on NVP, HEMA and AA.

The copolymers of different composition were synthesized by free radical copolymerization method. Synthesis was carried out in glass ampoules with alcohol as a solvent and azobisisobutyronitrile as an initiator at temperature of 60°C (333 K). The ratio monomer mixture: solvent was 30:70 vol. %. For oxygen expulsion the reaction mixture was blown by argon during 15 minutes. Synthesised copolymers were purified from unreacted monomers by multi-reprecipitation from an ethanol solution to ethyl acetate. After that copolymers were dried in vacuum oven for a few days till constant weight.

Films preparation.

The aqueous copolymer solutionts with concentrations of 0.1, 0.3 and 0.5 M were prepared and cast onto plastic molds. After that the samples were dried for 24 hours in the open air at room temperature to constant weight.

The film thickness was measured in five different places (center and four corners) by digital micrometer.

Crosslinked films preparation and characterisation.

Crosslinking of polymers was performed by their thermal treatment. Previously prepared films by casting to plastic molds were placed into drying oven at the three temperature regimes, such as 90°C, 110°C and 130°C for 5, 7 and 9 hours.

The swelling degree of thermo-crosslinked films was calculated using the following equation:

$$\alpha = \frac{m_{swel} - m_{dry}}{m_{dry}}$$

где, m_{swel} – weight of equilibrially swelled film; $m_{сух}$ – weight of dry film.

Interpolymer complex solutions were prepared by mixing the polymers at certain ratio for 5 minutes to reach equilibrium and measured.

Methods of analysis

IR spectra of copolymers were registered using IR spectrophotometer «Satellite FTIR Mattson» (USA) in the mid-IR area. For analysis the tablets were prepared from the powders of copolymers and calcined potassium bromide.

Turbidimetric analysis of interactions between mucin and water solutions of copolymers was carried out using UV-spectrophotometer «Shimadzu UV/VIS-2401 PC» under the following conditions: T= 25^{0}C (298K) and λ=400 nm. To maintain the appropriate temperature the cuvettes with thermostatic regulated cell were used.

Rheological method of copolymers mucoadhesive properties evaluation was carried out using microviscometer «Lovis 2000 M/ME AntonPaar» (Austria).

Sizes of copolymers and mucin-copolymer complexes was determined at 25°C by dynamic light scattering on «Malvern Zetasizer Nano-S» (Malvern Instruments, Great Britain).

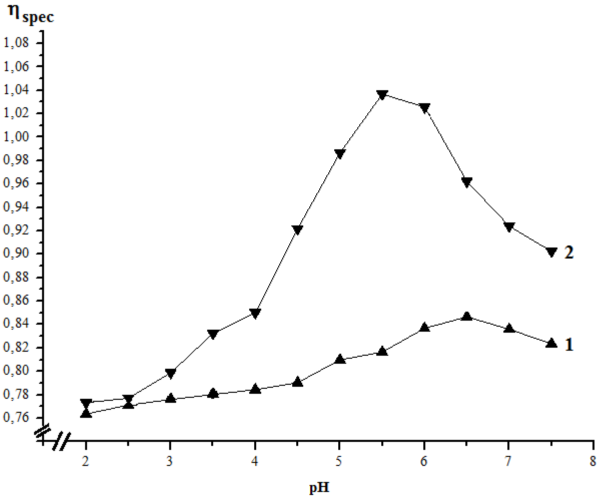
Mucoadhesive properties of films was studied by fixing their sample to a freshly prepared mucosa of pork cheek and set on a rotating disk (rotation speed 59 rpm). After that the disc was placed in a solution of artificial saliva (pH 6,5-6,8, and T=36,5^{0}C). The time required for complete separation or dissolution of the film was determined visually.

Results and their discussion

Copolymers of different composition were synthesized with the following ratio of monomers in the initial monomer mixture (IMM) [NVP]:[HEMA] = 90:10, 80:20 and 70:30 mol% and [NVP]:[HEMA]-[AK] = 90:10-5, 80:20-5 and 70:30-5 mol%.

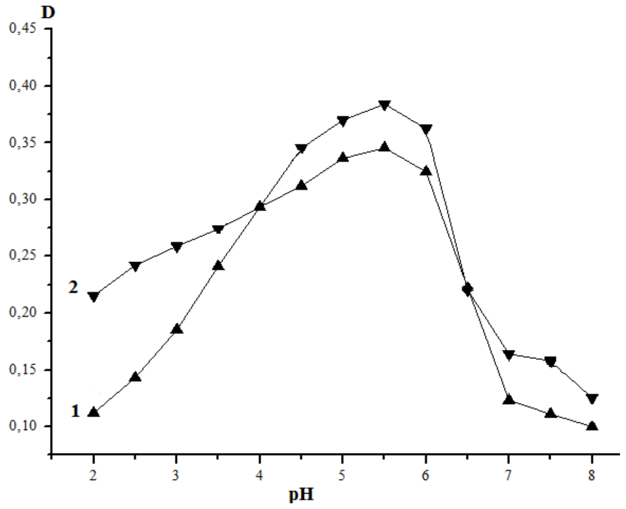
The impact of solution pH on complex formation between mucin and synthesized copolymers was studied using viscosimetric and turbidimetric titration. Graphical results of complexation study are depicted at figures 1 and 2. The critical pH value of complexation was found to be between pH 5.5-6.5. Moreover, pH of complexation for terpolymers system lies in the region closer to 5,5. The observed shift of pH complexation for tercopolymers in acidic region is due to suppression of acrylic acid carboxyl groups dissociation and electrostatic repulsion between the charged groups of mucin and copolymers.

Influence of copolymers solution concentration on their interaction with mucin molecules was studied (figure 3). Conjugated proteins of mucin have a negative charge. As a result of interaction between mucin and copolymer solutions, particles of copolymers adsorbed onto surface of conjugated proteins. And if concentration of copolymers in solution increases, there will be aggregation with mucin particles. pH of complexation descends if concentraion of polymers increases, because of growth of copolymer particles participated in complexation with mucin (figure 3).



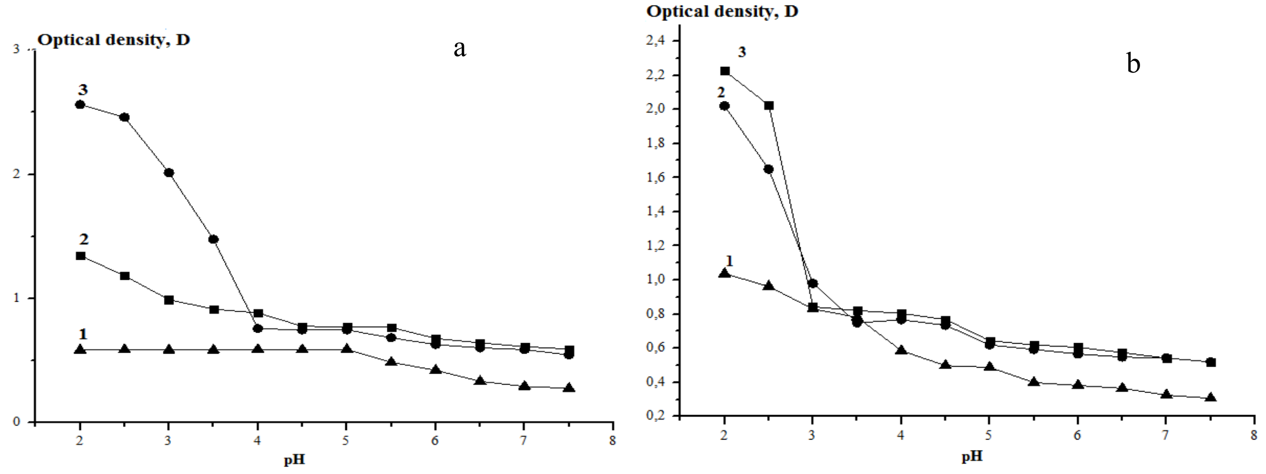
Concentration of mucin and copolymer solutions is 1 mg/mL
[NVP:HEMA] =[80:20] (1);
[NVP:HEMA]-[AA] =[80:20]-[5] mol% (2).

Figure 1 – Viscometric titration of copolymer solutions with mucin



[Concentration of mucin and copolymer solutions is 1 mg/mL
[NVP:HEMA] =[80:20] (1);
[NVP:HEMA]-[AA] =[80:20]-[5] mol% (2).

Figure 2 – Turbidimetric titration of copolymer solutions with mucin



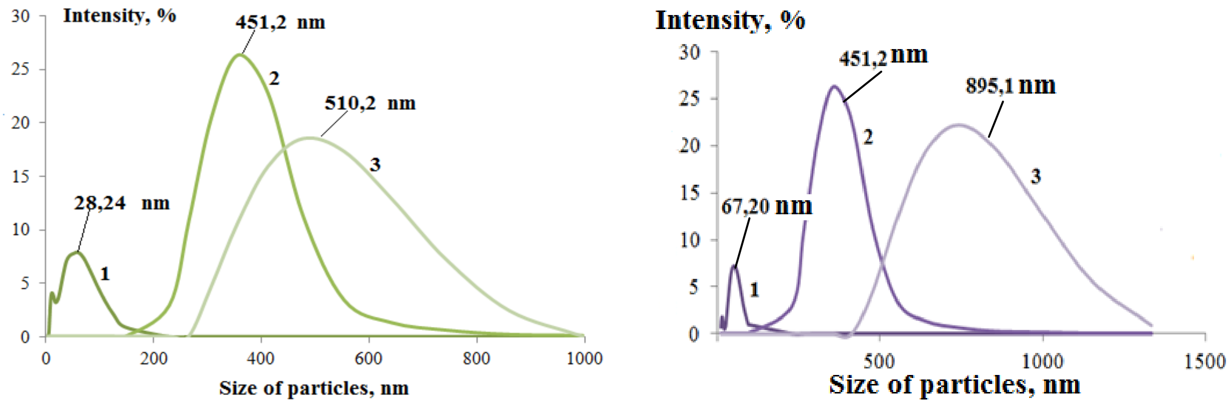
C_{cpl} 0,003 M (1); 0,01 M (2); 0,05 M (3)
[NVP:HEMA] =[80:20] (a); [NVP:HEMA]-[AA] =[80:20]-[5] mol% (b)

Figure 3 – Turbidimetric study of copolymer solutions concentration impact on complex formation with mucin molecules

Dynamic light scattering was used also to study the complex formation between macromolecules of copolymers and mucin. In order to confirm interaction the particle sizes of copolymers, mucine and their mixtures were determined. Results of analysis are presented in figures 4 and 5. It can be seen from the figures, that after mixing the copolymers solution with mucin the intermolecular complex formation occur which is resulting in increasing the particle size of macromolecular agglomerations.

Linear and thermally crosslinked polymeric films based on NVP-HEMA and AA copolymers were casted and swelling degree of crosslinked samples was studied. Kinetics of films swelling was analysed to determine the possibility of their using as mucoadhesive materials. For this purpose a films were thermally treated at T = 90, 110 and 130 °C for 5, 7 and 9 hours. Usually, thermal treatment is performed to improve the mechanical properties of the products, reduce the residual tension, which accumulate in the products during molding and to reduce the content of volatiles in the material.

The swelling kinetics and degree of different films was estimated in distilled water, ethanol-water solution (50:50 vol.%) and a solution of artificial saliva. Results of analysis are presented in figure 6. The content of acrylic acid chains in copolymers improves swelling ability of films in all kind of surrounding mediums. This is due to the fact that carboxylic groups of acrylic acid enhance the hydrophylic properties.

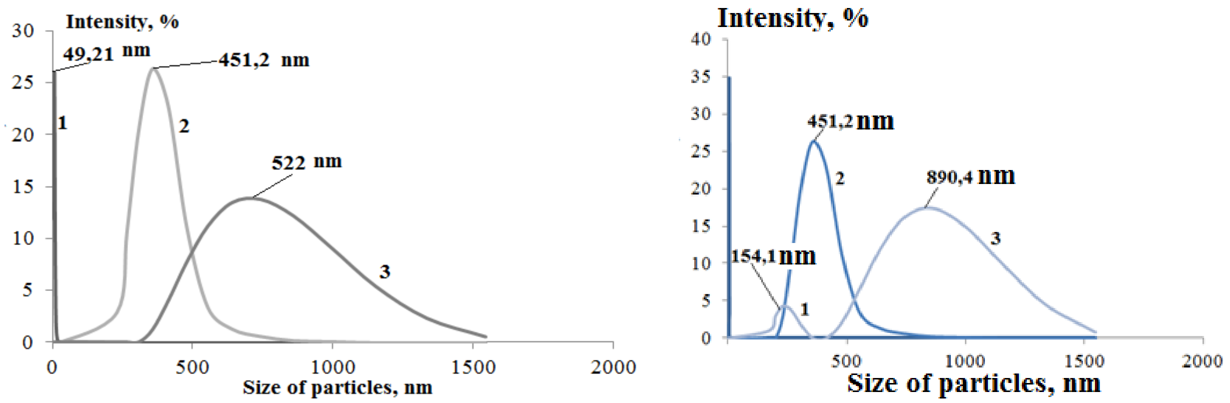


1 – [NVP:HEMA] [90:10] mol%; 2 – mucin;
3 – mixture of mucin with NVP:HEMA copolymer

1 – [NVP:HEMA-AA] [90:10]-(5) mol%; 2 – mucine;
3 – mixture of mucin with NVP:HEMA-AA copolymer

[mucin]:[copolymer] = [1:1] by volume, T = 25°C, pH = 6.5~6.8.

Figure 4 – Graphs of particles size distribution of individual copolymer, mucin and their mixtures



1 – [NVP:HEMA] [80:20] mol%; 2 – mucin;
3 – mixture of mucin with NVP:HEMA copolymer

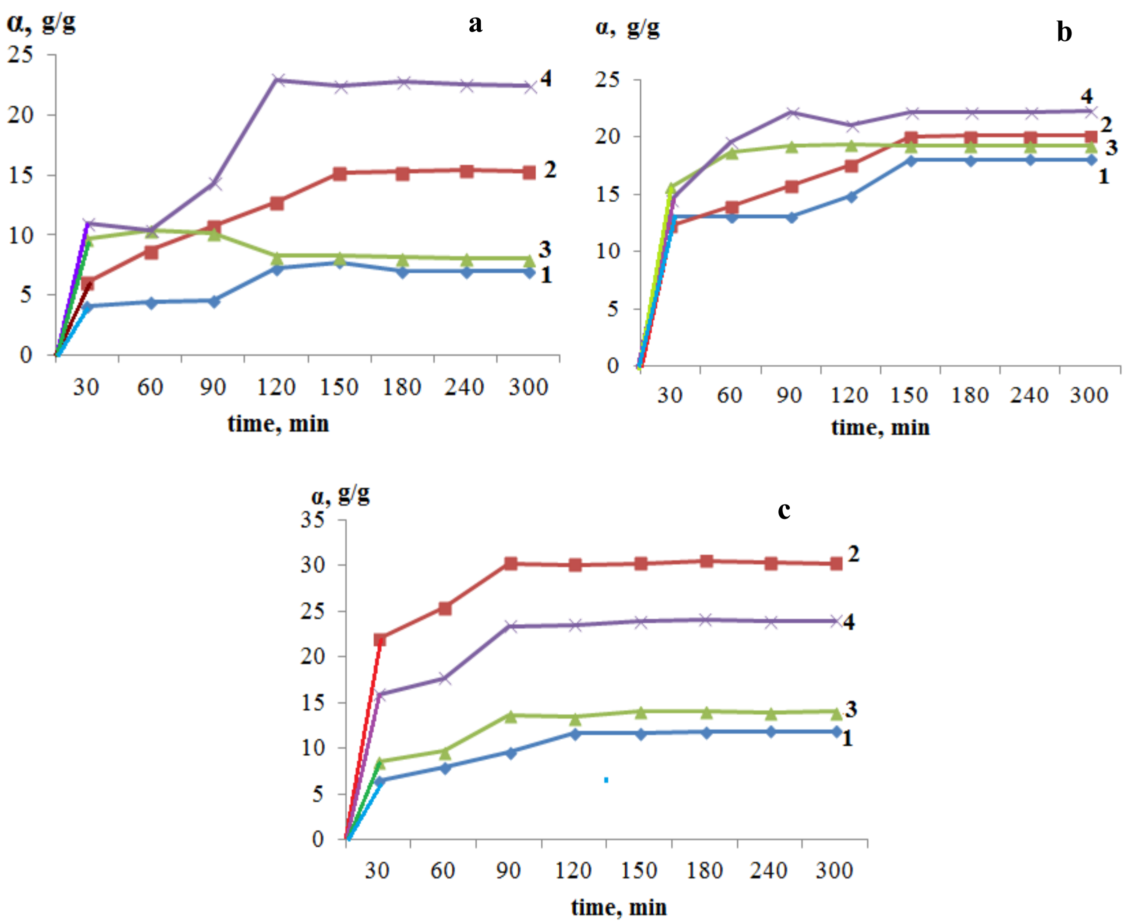
1 – [NVP:HEMA-AA] [80:20-(5%)]; 2 – mucine;
3 – mixture of mucin with NVP:HEMA-AA copolymer

[mucin]:[copolymer] = [1:1] by volume, T = 25°C, pH = 6.5~6.8.

Figure 5 – Graphs of particles size distribution of individual copolymer, mucin and their mixtures

Mucoadhesive properties of crosslinked and non-crosslinked films based on NVP, HEMA and AA copolymers were studied. Thus, a sample (film) is brought into contact with fresh pork buccal mucosa and fixed on rotating disk. The system was immersed in a solution simulating the environment of the oral cavity (pH = 6.5~6.8) at T = 35-36 °C. The retention time or dissolution time of films were determined visually. Results are represented in a table 1 and 2. For the films based on linear copolymers NVP-HEMA and NVP-HEMA-AA the presence of AA in IMM results in enhancing the mucoadhesive properties and increasing the retention time up to 5 minutes. In case of thermally crosslinked films the samples containing

AA posses lower ability to adhere to mucus surface after heating them probably because the crosslinking process involves the carboxylic groups of AA and acrylate groups of HEMA which responsible to mucoadhesion. Also increasing the heating temperature for copolymers crosslinking results in worsening the mucoadhesive properties of polymeric films.



[NVP:HEMA] =[90:10] (1); [NVP:HEMA]-[AA] =[90:10]-[5] (2);
[NVP:HEMA] =[80:20] (3); [NVP:HEMA]-[AA] =[80:20]-[5] (4) mol%
Thermal crosslinking conditions: 110 °C and 5 hours

Figure 6 – Swelling kinetics of films based of NVP-HEMA and NVP-HEMA-AA copolymers in water (a), ethanol-water solution (b) and solution of artificial saliva (c)

Table 1 – Mucoadhesive properties of non-crosslinked films based on copolymers of NVP, HEMA and AA.

IMM content of copolymers, mol%	Retention/dissolution time, min	Observation
[NVP:HEMA] =[90:10]	25	Detachment
[NVP:HEMA]-[AK] =[90:10]-[5]	30	Dissolution
[NVP:HEMA] =[80:20]	26	Detachment
[NVP:HEMA]-[AK] =[80:20]-[5]	32	Dissolution

Table 2 – Influence of crosslinking temperature on retention time of thermo-cross linked films based on copolymers of NVP, HEMA and AA

IMM content of copolymers, mol%	Crosslinking temperature		
	90°C	110°C	130°C
[NVP:HEMA] =[90:10]	30 min	10 min	5-10 min
[NVP:HEMA]-[AA] =[90:10]-[5]	20 min	5 min	5 min

Conclusion

As a result of investigation the complex formation between mucin and copolymers based on N-vinylpirrolidone, 2-hydroxymethilacrylate and acrilic acid. Impact of pH on interaction of copolymers with mucine molecules was observed. The maximum complexation was observed at interval of pH 5.5-6.5. Also the presence of interaction of copolymers with mucin was proved using DLS analysis which shows significant growth of particle size of macromolecules after mixing them. The films with linear and crosslinked structure based on copolymers were obtained and their mucoadhesive properties were studied. Retention time of films on pork bucal mucosal surface was found to be depended on composition of copolymers and temperature of thermal crosslinking of copolymers. Presence of AA increases the retention time of films but thermal treating reduces the mucoadhesive properties of the films, which is apparently due to the fact that thermal crosslinking proceeds by functional groups of AA and HEMA, which are responsible for the mucoadhesive properties of the films.

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The effect of TDI, PTMG and DMPA on the physico-mechanical properties of polyurethane dispersion containing aromatic isocyanate

Abstract: The present paper relates to environment-friendly polyurethane dispersion having a high solids content of polyurethane polymer based on aromatic isocyanate. A series of water dispersion polyurethanes (PUDs) were prepared by polyaddition reaction using toluene diisocyanate (TDI), poly(oxytetramethylene) glycol (PTMG), and dimethylol propionic acid (DMPA). Physico-mechanical properties of PUDs were studied by average particle size, viscosity, contact angle, tensile strength and elongation. IR spectroscopy was used to check the end of polymerization reaction and characterization of polymer. Results revealed that tensile strength, tear strength, hardness, contact angle and viscosity increase with increase of the amount of TDI, PTMG and DMPA in the PUDs. Elongation at break and average particle size decrease with increase of TDI, PTMG and DMPA. The increase of physico-mechanical properties are attributable to the increase of free NCO content and hard segment in the prepolymer of high content of TDI, PTMG and DMPA.

Key words: polyurethanes; dispersions; mechanical properties; physical properties; TDI.

Introduction

Polyurethane dispersions (PUDs] are well known and used in the production of different of useful polyurethane products, such as; adhesive, coatings, sealants, elastomers, fibers and foams [1-5]. PUDs are binary colloidal systems having polyurethane particle dispersed in aqueous phase together with an active hydrogen-containing chain extender, such as diamine [6].Researchers are imperative develop PUD mainly due to the evolution of legislation towards reducing the VOC (volatile organic concentration) and the creation of environmentally friendly products.Wang et al [7] developed PUD by using trimethylolpropane, which was modified by 3- aminopropyltriethoxysilane to form highly crosslinked PUD. The results showed that, as the mass fraction of 3- aminopropyltriethoxysilane increases from 0% to 10%, water and ethanol absorption decreased.Vanesa at al [8] provided some useful indications on how to optimize the properties of polyurethane dispersions prepared by the prepolymer mixing technique without the use of any organic solvent. They used aliphatic and aromatic diisocyanates with the same polyols and chain extenders. In particular, it showed that aliphatic diisocyanates like [α, α, α\, α\-tetramethyl-1,3-xylylene diisocyanate (TMXDI) and isophorone diisocyanate (IPDI)] are much more appropriate than aromatic structures or symmetric aliphatic ones and that the [NCO]/[OH] ratio plays a fundamental role and gives the most promising products in terms of small particle size in the dispersions and physical properties of the final polymer films.Lee et al [9] modified polyurethane dispersion by enhancing the molecular weight in the preparation of the aqueous polyurethane dispersions derived from various polycarbonatediols, di (4-isocyanatocyclohexyl)methane (HMDI), and various carboxylic diols, including dimethylol propionic acid (DMPA), dimethylol butyric acid (DMBA), and a carboxylic polycaprolactonediol. The films of the polyurethane dispersions derived from the carboxylic polycaprolactonediol were significantly softer, and had lower tensile strength and moduli, and higher elongation than those derived from DMPA and DMBA. The dynamic mechanical property data indicate that the HMDI based polyurethanes dispersion films derived from DMBA and DMPA can exhibit higher temperature resistance than the isophorone diisocyanate (IPDI) based ones. Negim et al [10] studied the effect of NCO/OH ratio on the physico-mechanical properties of PUD. PUD were prepared by polyaddition reaction using isophorone diisocyanate (IPDI), toluene diisocyanate (TDI), poly(oxytetramethylene) glycol (PTMG), dimethylol propionic acid (DMPA) and poly(oxypropylene) poly(oxyethylene) triol (FA-703). Results revealed that molecular weight, T_g, tensile strength, tear strength, hardness, contact angle and average particle size increase with increase of NCO/OH molar ratios. Elongation at break and viscosity decrease with increase of NCO/OH molar ratios. The increase of molecular weight, T_g, tensile strength, tear strength, hardness, contact angle and average particle size are attributable to the increase of free NCO content and hard segment in the prepolymer of high NCO/OH molar ratio.

The present study deals with the preparation of PUDs from poly (oxytetramethylene) glycol (PTMG), dimethylol propionic acid (DMPA), isophorone diisocyanate (IPDI) The influence of variable TDI, TMPG and DMPA amounts on the physical and mechanical properties of emulsion-cast films was studied.

Materials and methods

Materials

Poly(oxytetramethylene) glycol (PTMG, M_w = 2000, OH number = 55 mg/g, Korea PTG, Korea) was dried and degassed at 80 ^{o}C, 1 – 2 mm Hg for 2 h before use. Dimethylol propionic acid (DMPA, M_w =134.13, Aldrich) was dried at 50 ^{o}C for 48 h, while toluene diisocyanate (TDI, M_w = 174.16, Merck) was used as received. Triethylamine (TEA, M_w = 101.19, Merck) was dried over molecular sieves (Å), ethylenediamine (EDA, M_w =60.1, Merck), N-methyl-2-pyrrolidinone (NMP, Fluka) and deionized (DI) water was used throughout the reaction.

Preparation of pre-polymer

The PUDs were prepared as previously described in the literature [11-18]. Polymerization was performed in a 500 mL round-bottom, four-necked separable flask with a mechanical stirrer, thermometer, and condenser with drying tube. The reaction was carried out in an N_2 atmosphere in a constant-temperature oil bath. TDI and polyol were charged into the reactor and the mixture was heated at 100 ^{o}C for 1 h. After that, DMPA and NMP were added to the mixture and the reaction proceeded at the same temperature until the theoretical NCO value was reached, as determined by the di-n-butylamine titration method [19, 20].Samples were prepared by different TDI, PTMG and DMPA, which are shown in Table 1.

Table 1 – Feed compositions of PUD synthesized with variable contents of TDI, PTMG and DMPA.

Samples	A1	A2	A3
Materials	Wt., gm		
PTMG	361	237.3	159.6
TDI	62.87176	41.328168	27.795936
DMPA	10.7988063	7.2	4.8166083
TEA	8.1468069	5.431804927	3.6337329
Water	568.526	372.819	252.4
EDA	10.84805	7.130865	4.79598
OH	0.1805	0.11865	0.0798
NCO	0.361	0.2373	0.1596
NCO/OH	2	2	2

Neutralization and dispersion of the prepolymer

After the prepolymer temperature dropped to 40 ^{o}C, the carboxylic acid groups were neutralized by the addition of triethylamine (TEA) and the degree of neutralization is 100%. The mixture was stirred for further 20 min to ensure the reaction was completed. Then, the prepolymers were dispersed by adding distilled water to the prepolymer solution which was stirred vigorously. The addition rate was controlled carefully using a tubing pump with a calibrated flow rate [21]. Finally 10 wt% ethylenediamine aqueous solution was added to extend the chain at room temperature**.** The emulsion was stable for more than 6 months after preparation at room temperature.

Film preparation

Films were prepared by casting the aqueous dispersions on surfaces and allowing them to dry at room temperature for 7 days, and then at 60 °C, for 12 hours [22-23].The films were stored in a desiccator at room temperature for further characterization and measurements.

Measurements

Particle size and distribution were measured by laser light scattering (Sema Tech, SEM-633, He-Ne laser). The samples were diluted to the required concentration with distilled water before the measurement. FTIR spectra were recorded on a Bruker Tensor 37 FTIR spectrometer. The viscosity (η) of the dispersions was measured using a Brookfield viscometer (Model LVTDV-II) at a shear rate of 100 S-1 at 25°C. The contact angle formed between the water drops and the surface of the sample was measured using contact angle measuring system CAHN DCA-322 analyzer operated at 25°C with water drop, and a velocity of 100 μm/s. The drop of water was mounted on the surface to be tested with a micro-syringe and contact angle was measured from the view of water drops as observed on monitor. Results are the mean value of three measurements on different parts of the film. The tensile properties of the emulsion cast films were measured by using MTS 10/M tensile testing machine at a crosshead speed of 50 mm/min. An average of at least 4 measurements was taken and the 1-kN load cell was used. Shore A hardness was measured using an indentation hardness tester according to ASTM D2240-75.

Results and their discussion

FT-IR analysis

IR spectrum obtained from the cast film is shown in Figure 1. This analysis was used to check the end of polymerization reaction, verifying the disappearance of the NCO at 2265 $cm^{-1}$and the appearance of N-H at 3291 cm^{-1}. The presence of expected peaks implies that the reaction was completed and the predesigned PU was formed. IR spectra also contains all the related information on the primarily structure of the final polymer. An absorption band of the N-H stretching mode at 3291 $cm^{-1}$was observed. Aliphatic C-H stretching mode of 2795– 2938 $cm^{-1}$and carbonyl (C=O) stretching absorption band at 1730 cm^{-1}were observed. N-H bending vibrations at 1550 cm^{-1}, C-O-C stretching absorption band corresponding to the ether oxygen of the soft segment at 1000–1150 cm^{-1}were also observed. The bands at 1507 cm-1 was also observed for aromatic ring stretch. These vibrations are strong evidence for the formation of PU. The N-H group in polyurethane could form hard segment H-bonding with the carbonyl oxygen and hard–soft H-bonding with the ether oxygen. The stronger hard–hard segment H-bonding acts as physical crosslinks leading to difficult segmental motion of the polymer chain, which results in a more significant phase separation between the hard and soft segments.

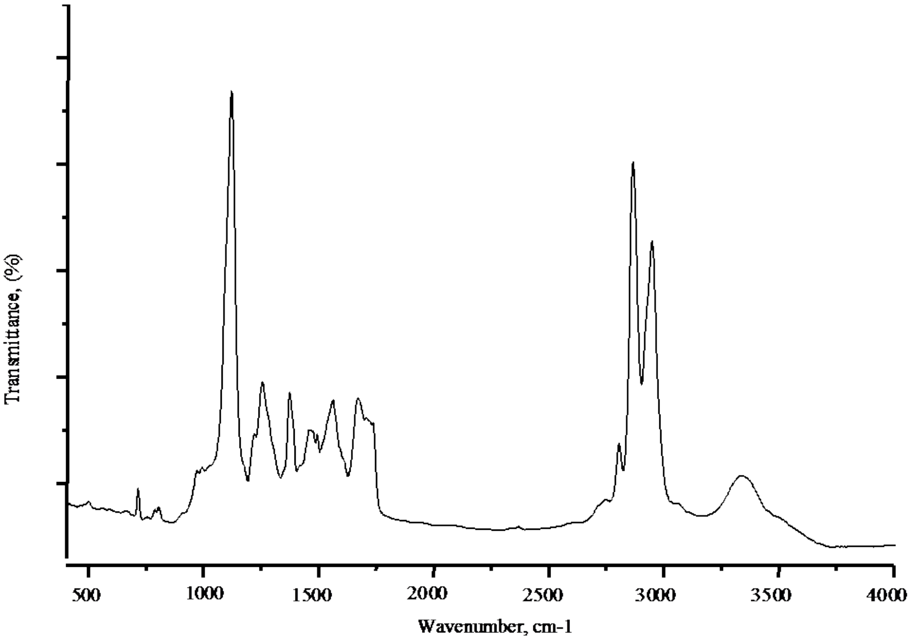

Figure 1 – FTIR of polyurethane dispersion.

Physical properties:

In PUD, the particle size could be controlled to some extent by emulsification conditions such as stirring speed or dispersing temperature which have an effect on the viscosity of prepolymer but it is mostly governed by the concentration of hydrophilic groups, i.e. carboxylic acids (24, 25). The particle size decreased as the DMPA content increased. The decreases of the particle size with increasing of DMPA content could be due to the stabilizing mechanism of the ionomer as shown in Table 2. However, particle size is important factor in PUD coatings. For example, in many surface coatings, relatively large particles are preferred to facilitate fast drying and relatively small particles are preferable when deep penetration of the dispersion into the substrate is an essential requirement.

Table 2 – Physical Properties of the PUD Cast Films

Samples	A1	A2	A3
Particle size, nm	50.7	56.8	67.8
Viscosity, cps	60	38	25
Contact angle, deg	30	45	48

Table 2 shows the increasing in PUD viscosity with the increasing amount of PTMG, MPA and TDI, which also led to the increase in the total ionic content. The ionic content affects the dispersion viscosity in two ways. Due to the higher ionic content, the particle size of the dispersion decreases, and hence, dispersion viscosity increases due to the increase in the effective volume of the dispersed particle. When the ionic content is high, the repulsions between the ionic groups increases, leading to higher viscosity. The water contact angle decreased as the amount of PTMG, MPA and TDI were increased as shown in Table 2. The increased contact angles using water means the films showed poorer wetting with decreasing PTMG, MPA and TDI content.

Mechanical properties:

The mechanical properties of the PUD films with respect to the amount of PTMG, DMPA and TDI are shown in Table 3. It can be seen that tensile, tear strength and elongation increased with increasing amounts of PTMG, DMPA and TDI. The increase of tensile strength and tear strength are associated with increasing TDI, while increasing of elongation is associated with increasing PTMG and DMPA.Authors concluded that the hard segments act as crosslinks, inhibiting stress-relaxation and inducing stress-crystallization of the soft segments that results in higher tensile strength [26, 27].

Table 3 – Mechanical properties of the PUD Cast Films

Samples	A1	A2	A3
Tensile strength, Kgf/cm^2	97.92	68.87	53.67
Tear strength, Kgf/cm^2	24.196	21.92	15.7
Elongation, %	751.33	272.5	225.87
Hardness, shore A	95	82	73

It can be seen that hardness increased with increasing amounts of TDI, PTMG and DMPA in the prepolymer as shown in Table 3. A1 showed the largest hardness compared with A3 and A2. This is presumably due to the increased hard segment contents and hydrogen bonding density of the PUDs.

Conclusion

Aqueous polyurethane dispersions were synthesized with different content of TDI, PTMG, and DMPA. The effect of TDI, PTMG, and DMPA on the particle size distribution, contact angle, viscosity and mechanical properties are studied. Average particle size of the prepared polyurethane emulsions decreases, and the viscosity increases with the increasing amount of TDI, PTMG, and DMPA. Tensile strength, tear strength, elongation at break, and contact angle increase with the increasing of the amount of TDI, PTMG, and DMPA. The increase in tensile properties is interpreted in terms of increasing hard segments, chain flexibility and their phase separation in high content of TDI, PTMG, and DMPA -based polyurethane.

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Development of polyurethane dispersion based on aromatic isocyanat: influence of NCO/OH ratio on physico-mechanical properties of PUD

Abstract: Aqueous polyurethane dispersions (PUDs) were prepared by the prepolymer mixing technique solvent-free using aromatic isocyanat[toluene diisocyanate (TDI)], poly(oxytetramethylene) glycol (PTMG), dimethylol propionic acid (DMPA). The properties of both the dispersion's and casting polymer films were characterized byparticle size analyzer, viscosity and tensile tester. IR spectroscopy was used to check the end of polymerization reaction and characterization of polymer. The effect of NCO/OH molar ratio on the physico-mechanical properties of the polyurethanes was studied. Results showed that tensile strength, contact angle and average particle size increase with increase of NCO/OH molar ratios. Elongation at break and viscosity decrease with increase of NCO/OH molar ratios.

Key words: polyurethanes; NCO/OH ratio; mechanical properties; physical properties; TDI

Introduction

Polyurethanes dispersions (PUDs) are a two-phase system comprised of polyurethane polymer particles suspended in an aqueous continue phase [1-3]. PUDs have been synthesized by a number of different process including, the acetone process, the melt dispersion process, and the prepolymer mixing process [4]. In the acetone process, urethane prepolymer is dissolved in an organic solvent, as acetone or methylethylketone. After polyaddition, water is added to the mixture until the system spontaneously under goes a phase inversion[5]. The acetone process yields is a high quality reproducible product, but the disadvantages of this process are organic solvents that required in the original polymer formation. In the melt dispersion process, the chain extension is initiated either before or during the dispersion with water, but the disadvantage of this process is the high viscosity in the final products that need heating eliminates or large amounts of organic solvent to reduce the viscosity [5-7]. In the prepolymer mixing process, surfactants are added before dispersion with water. Surfactant is a surface-active agent that containing carboxyl group typically dimethylolpropionic acid (DMPA). Advantages of this process is that polymer synthesis is carried out in the aqueous two phases system with little or no addition of organic solvent and ability to control the strength of cross-linking in the final polymer particle[8]. A number of researcher developed PUDs by different technique including, polyols, isosyanat, chain extension, neutralization step and NCO/OH ratio [9-14]. The NCO/OH molar ratio determines the molecular weight of the prepolymer in the first stage of the synthesis and consequently the relative proportion of the urethane and urea groups in the final polymer chain. As an ultimate result, this ratio will therefore affect both the properties of the dispersions, such as particle size, and the dry polymer thermal and mechanical properties [13]. As expected, and in agreement with previously reported results by other authors [15], physico-mechanical properties of polyurethane dispersion based on TDL and IDI increases with increasing NCO/OH ratio [2].In particular, it showed that aliphatic diisocyanates like TMXDI and IPDI are much more appropriate than aromatic structures or symmetric aliphatic ones and that the [NCO]/[OH] ratio plays a fundamental role and gives the most promising products in terms of small particle size in the dispersions and physical properties of the final polymer films, when it approaches a value of 2 [13].Negim et al [16] studied the effect of NCO/OH molar ratio on the physico-mechanical properties of the polyurethanes. Polyurethane dispersion were based on isophorone diisocyanate (IPDI), toluene diisocyanate (TDI), poly(oxytetramethylene) glycol (PTMG), dimethylol propionic acid (DMPA)

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and poly(oxypropylene) poly(oxyethylene) triol (FA-703). Results revealed that molecular weight, Tg, tensile strength, tear strength, hardness, contact angle and average particle size increase with increase of NCO/OH molar ratios. Elongation at break and viscosity decrease with increase of NCO/OH molar ratios. The increase of molecular weight, Tg, tensile strength, tear strength, hardness, contact angle and average particle size are attributable to the increase of free NCO content and hard segment in the prepolymer of high NCO/OH molar ratio.

The present article deals with a series of PUD formulations, used with variable NCO/OH ratios and involving aromatic diisocynates (TDI), poly(oxytetramethylene) glycol (PTMG), dimethylol propionic acid (DMPA). The characterization of the aqueous dispersions and cast films were studied.

Materials and methods

Materials

Poly(oxytetramethylene) glycol (PTMG, M_w = 2000, OH number = 55 mg/g, Korea PTG, Korea) was dried and degassed at 80 °C, 1 – 2 mm Hg for 2 h before use. Dimethylol propionic acid (DMPA, M_w =134.13, Aldrich) was dried at 50 °C for 48 h, while toluene diisocyanate (TDI, M_w = 174.16, Merck) was used as received. Triethylamine (TEA, M_w = 101.19, Merck) was dried over molecular sieves (Å), ethylenediamine (EDA, M_w =60.1, Merck), N-methyl-2-pyrrolidinone (NMP, Fluka) and deionized (DI) water was used throughout the reaction.

Preparation of pre-polymer

The PUDs were prepared as previously described in the literature [17-22]. Polymerization was performed in a 500 mL round-bottom, four-necked separable flask with a mechanical stirrer, thermometer, and condenser with drying tube. The reaction was carried out in an N_2 atmosphere in a constant-temperature oil bath. TDI and polyol were charged into the reactor and the mixture was heated at 100 °C for 1 h. After that, DMPA and NMP were added to the mixture and the reaction proceeded at the same temperature until the theoretical NCO value was reached, as determined by the di-n-butylamine titration method [19, 20].**T**he reaction scheme for the prepolymer preparation and the processes of dispersion and chain extension are shown in scheme 1. Samples were prepared by different NCO/OH molar ratios are shown in Table 1.

Table 1 – Feed compositions of PUD synthesized with variable NCO/OH ratio

Samples	A1	A2	A3
Materials	Wt. (gm)		
PTMG	140.00	140.00	140.00
DMPA	7.00	7.00	7.00
TDI	31.92	42.56	53.21
TEA	5.28	5.28	5.28
Water	351.30	378.95	404.47
EDA	3.67	7.34	11.02
NCO	0.1833	0.2444	0.3055
OH	0.1222	0.1222	0.1222
NCO/OH	1.50	2.00	2.50

Neutralization and dispersion of the prepolymer

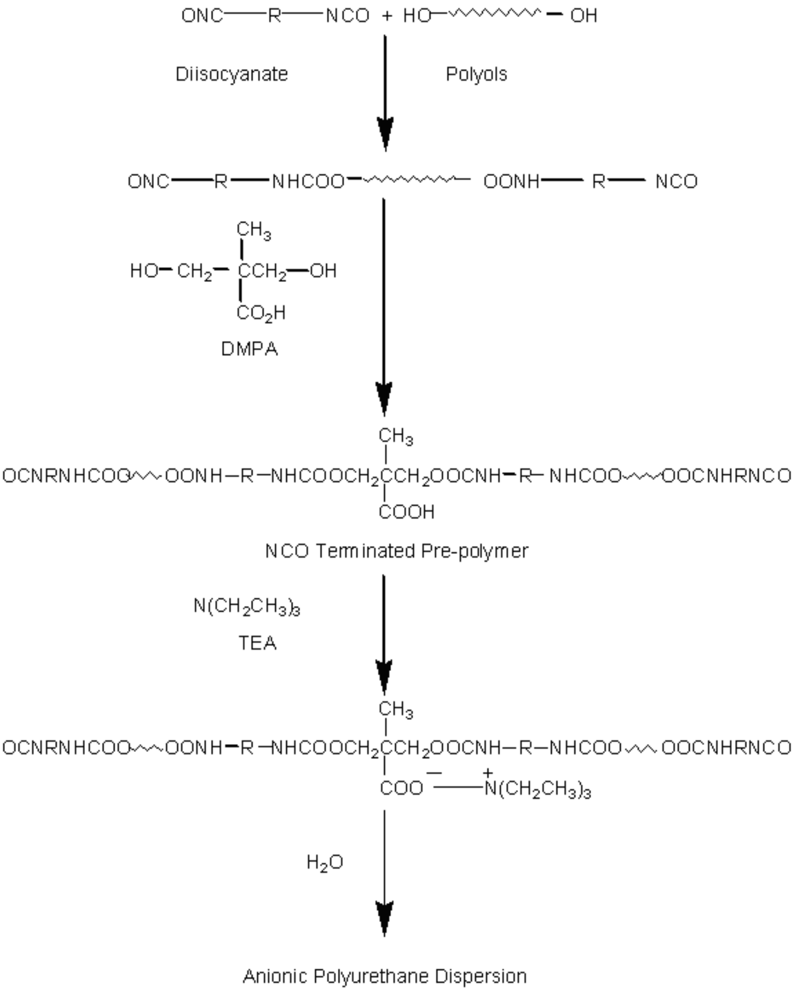
After the prepolymer temperature dropped to 40°C, the carboxylic acid groups were neutralized by the addition of triethylamine (TEA) and the degree of neutralization is 100%. The mixture was stirred for further 20 min to ensure the reaction was completed. Then, the prepolymers were dispersed by adding distilled water to the prepolymer solution which was stirred vigorously. The addition rate was controlled carefully using a tubing pump with a calibrated flow rate [21]. Finally, 10 wt% ethylenediamine aqueous solution was added to extend the chain at room temperature**.** The emulsion was stable for more than 6 months after preparation at room temperature.

Film preparation

Films were prepared by casting the aqueous dispersions on surfaces and allowing them to dry at room temperature for 7 days, and then at 60 °C, for 12 hours [22-23].The films were stored in a desiccator at room temperature for further characterization and measurements.

Measurements

Particle size and distribution were measured by laser light scattering (Sema Tech, SEM-633, He-Ne laser). The samples were diluted to the required concentration with distilled water before the measurement. FTIR spectra were recorded on a Bruker Tensor 37 FTIR spectrometer. The viscosity (η) of the dispersions was measured using a Brookfield viscometer (Model LVTDV-II) at a shear rate of 100 S^{-1} at 25°C.



Scheme 1 – Formation of PU dispersion having anionic centre.

The contact angle formed between the water drops and the surface of the sample was measured using contact angle measuring system CAHN DCA-322 analyzer operated at 25oC with water drop, and a velocity of 100 μm/s. The drop of water was mounted on the surface to be tested with a micro-syringe and contact angle was measured from the view of water drops as observed on monitor. Results are the mean value of three measurements on different parts of the film. The tensile properties of the emulsion cast films were measured by using MTS 10/M tensile testing machine at a crosshead speed of 50 mm/min. An average of at least 4 measurements was taken and the 1-kN load cell was used.

Results and discussion

FT-IR analysis

IR spectrum obtained from the cast film is shown in Figure 1. This analysis was used to check the end of polymerization reaction, verifying the disappearance of the NCO at 2265 cm^{-1}and the appearance of N-H at 3291 cm^{-1}. The presence of expected peaks implies that the reaction was completed and the pre-designed PU was formed. IR spectra also contains all the related information on the primarily structure of the final polymer. An absorption band of the N-H stretching mode at 3291 cm^{-1} was observed. Aliphatic C-H stretching mode of 2795– 2938 cm^{-1} and carbonyl (C=O) stretching absorption band at 1730 cm^{-1} were observed. N-H bending vibrations at 1550 cm^{-1}, C-O-C stretching absorption band corresponding to the ether oxygen of the soft segment at 1000–1150 cm^{-1} were also observed. The band at 1507 cm^{-1} was also observed for aromatic ring stretch. These vibrations are strong evidence for the formation of PU. The N-H group in polyurethane could form hard segment H-bonding with the carbonyl oxygen and hard–soft H-bonding with the ether oxygen. The stronger hard–hard segment H-bonding acts as physical crosslinks leading to difficult segmental motion of the polymer chain, which results in a more significant phase separation between the hard and soft segments.

Physical and mechanical properties

The effect of NCO/OH on the particle size and viscosity of PUD are shown in Figure 2. The result showed that particle size increased with increasing NCO/OH molar ratio. With increasing NCO/OH molar ratio, the number of ionic groups in PUDs decreased. However, the particle size of NCO/OH (1.5) has smaller particle size (35 nm), while NCO/OH (2.5) has larger particle size (76.5 nm) as shown in Figure 2. In general, the particle size is very important parameter in paint industrial; dispersions with relatively large average particle size are generally unstable with respect to sedimentation. Dispersions with smaller particle size are more useful since such dispersions have high surface energy, which enhances film formation. Figure 2 shows the effect of NCO/OH molar ratio on the viscosity of PUD. It can be seen that the viscosity increased with decreasing NCO/OH molar ratio. The increase in viscosity of PUD due to the decreasing of particle size. It is important in paint application that the viscosity of the prepolymer, so NCO/OH (2.5) has standard viscosity (50 cps) for paint application. A suitable viscosity range is required to avoid sagging (in case of low viscosity) and practical difficulty in application (encountered with very high viscosity).

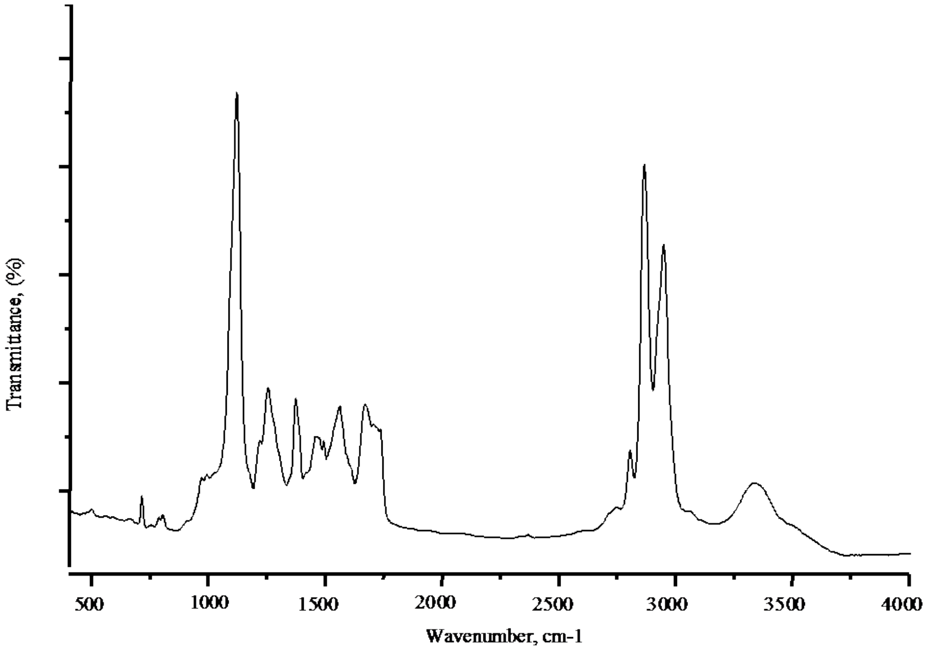


Figure 1 – FTIR of polyurethane dispersion.

Advancing and receding contact angle measurements of the films cast from PUDs could provide more information on the hydrophilicity of dried cast films. A better understanding of the hydrophobicity of the cast films could be obtained from dynamic contact angle studies rather than from swelling studies. The effect of NCO/OH molar ration on the contact angle of PUD films is shown in Figure 2. The results showed that contact angle decreased with increasing NCO/OH ratio. The results confirm that chain rigidity (NCO) is a more significant factor in controlling the contact angle, because chain rigidity does not allow the ionic groups to come near the particle surface (22, 23). From the contact angle test, gave information about the best molar ratio (NCO/OH) for painting application because the increased contact angles using water means the films showed poorer wetting, adhesion, and application. Generally, if the water contact angle is smaller than 90°, the solid surface is considered hydrophilic and if the water contact angle is larger than 90°, the solid surface is considered hydrophobic. Contact angle test showed that PUD films with different NCO/OH films are hydrophilic.

The NCO/OH molar ratio is important for the properties of PUDs. As shown in Figure 3, tensile strength increased and elongation at break decreased as the NCO/OH molar ratios increases. This may be due to the increase in hard segments (NCO) and hydrogen-bonding interactions between the chains in the PUDs. However, the mechanical properties of PUD prepared in second report are higher than that of prepared in the first report. NCO/OH.Also, it can be observed that the increase in NCO/OH molar ratio improved the mechanical properties because of increasing in the degree of inter-chain hydrogen bonding, which leads to the formation of more rigid film. The high quality industrial polymer should behaving adhesive and film forming (24).

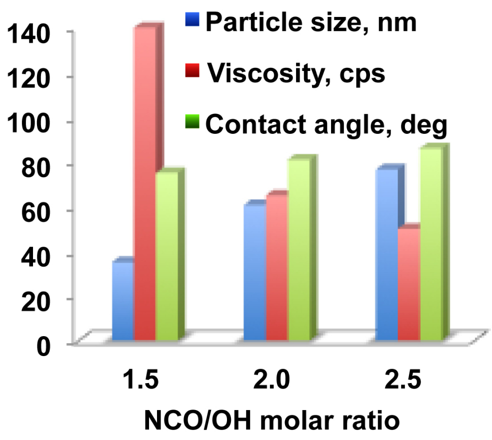


Figure 2 – The effect of NCO/OH ratio on particle size, viscosity and contact angle of PUD

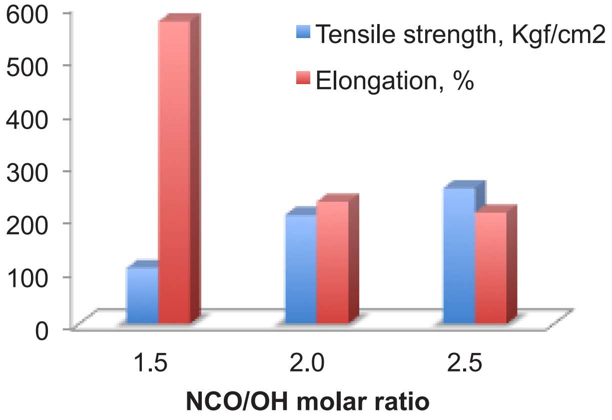


Figure 3 – The effect of NCO/OH molar ration on the tensile strength and elongation of PUD.

Conclusions

A series of PUDs were synthesized from TDI, PTMG and DMPA with different NCO/OH molar ratios according to the prepolymer mixing process. Average particle size of the prepared PUD emulsions increases and the viscosity decreases with the increasing NCO/OH molar ratio. Tensile strength and contact angle increase with the increasing of NCO/OH molar ratio. The increase in tensile properties is interpreted in terms of increasing hard segments in the prepolymer of high NCO/OH molar ratio.

Acknowledgements

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The possibilities of use of the polyelectrolyte solutions in membrane technology for desalination

Abstract: The possibilities of use polyacrylic acid (PAA) solutions in membrane technology for water desalination have been investigated. To control changes in salt concentration (K_3 [Fe $(CN)_6$]), in aqueous solution, separated using standard dialysis membranes by UV spectroscopy. Thus optical density was examined at different concentrations of the solutions of the polymer (PAA), red blood salt, effect of pH on the degree of desalination. It was determined that the optimal concentration of polymer upon purification of water is PAA 10%. But with reduction of concentration of salt from 0,1mol/l to 0,001 mol/l the degree of desalination is increased. Upon use of solutions with lower pH values such as pH=3 the degree of desalination is decreased.

Key words: dialysis membrane, polyacrylic acid, optical density, water purification, degree of desalination.

Introduction

Many human activities, such as drinking, agriculture, sanitation and electricity generation require particularly significant amount of water. Fortunately, in many cases, centers of population are located near sources of useable water. However, oceans, which cover more than 70% of the earth's surface and contain 97% of the earth's water, have salt water [1]. Since this salty water is unsuitable for many applications, it must be purified before it used.

As the population on earth continues grow, the existing water supply will be more and more lack. Hence, the more water is required for satisfaction of requirements of mankind,the desalination of sea water will become more and more important source of useful water [2].

Membrane systems are attractive since they provide an absolute barrier for pathogens and remove the turbidity,thus, increasing the palatability of the water. Some membrane systems rely on thepower of gravity as a driving force, thereby avoiding the use of pumps and electricity [3].

The membrane technology allows to receive a water of high degree of purification. The complex process of purification allows to carry out for one technological operation the removal of excess content of salts from water, almost completely to expel from the composition of water the microbiological and organic components [4].

Membrane installations allow to receive best quality drinking water, and also to receive the desalinated water for various industries. While unlike traditional methods of purification the use of reagents (coagulants, flocculant, acids, alkalis etc.) is sharply reduced. Meanwhile, compactness and power intensityof installations allows to saveindustrial sites and cost of maintenance [5].

As the world's population continues to grow, existing water supplies will become increasingly insufficient. As more and more water is required to meet mankind's needs, desalination of sea water will become an increasingly important source of useable water. Any comprehensive plan addressing mankind's energy usage or ecologic impact must account for the effect of desalination; responsible development requires attention to the most energy-efficient methods of purifying water [6].

Materials and methods

In this work, the possibilities of use of polyacrylic acid solutions with the concentration of 5 w%, 10 w%, 15 w%, ($K_3[Fe(CN)_6]$) salt with concentration of 0,001 mol/l, 0,01 mol/l, 0,1 mol/l in membrane technologies for water desalination have been investigated. Also for the study of pH effect the solutions of salt with pH = 3, 4, 5, 6 values were prepared. Measurements were carried out via the device shown below (Figure 1). The tube (1) filled with aqueous solution of polyacrylic acid (PAA), the lower part is closed by a standard dialysis membrane (3) that non-permeable for macromolecules, but easily permeable for molecules of low-molecular substances. Changes in the concentration of salts in the «external» and «internal» solutions was controled by UV spectroscopy at a wavelength λ = 400 (UV-spectrometer, Specord Plus200, Germany).

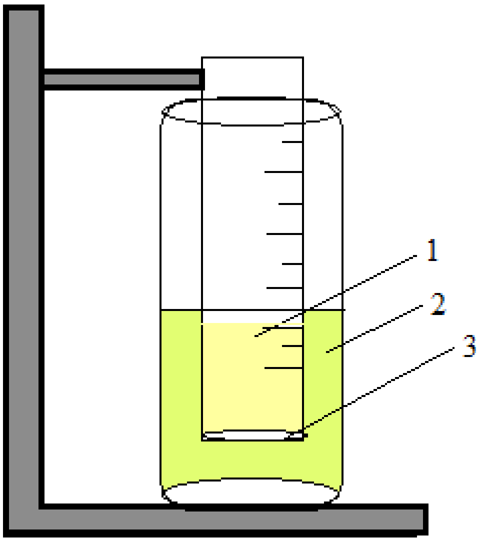

Figure 1 – The device for change of level of solution in internal and external solutions

1 – in an external vessel; 2 – in an internal tube: 3 – dialysis membrane

Result and their discussion

In the course of work, the effect of concentration of polymer on degree of water desalination (figure 2) was studied. It is necessary to emphasize that this experiment gives better result at high concentration ([PAA]=10%) of polymer comparable to concentration of macromolecules incrosslinked polymeric network of the same chemical nature.

It is quite explained as according to the classical theory of ideal solutions, the difference of osmotic pressure is defined by full number of the dissolved particles in each of unit ofthe system. The number of macromolecules having considerable molecular weight, remains small even at rather high mass concentrations of polymer. In case of suppression of ionization of carboxylic groups sharply decreases the quantity of osmotically active particles in solution.

However, at high concentrations ([PAA]=15%) of polymer considerable deviations from ideality as shown in the experiment are observed. At such concentrationsthe solution of polymer possesses the same degree of swelling, as the crosslinkedone. In a sense, the solution “swells” in the same way as the crosslinkednetwork (polymeric hydrogel), specifically,swells its constituent macromolecules, and partially permeable membrane plays the same role, as border of the sewed polymeric grid. In this regard, the optimal concentration of polymer upon water purification is PAA 10 w%.

This system has been used for the study of processes of swelling of PAA aqueous solution in solution ($K_3[Fe(CN)_6]$) salt. External solution contained 0,001 mol/l of salt in the initial state. “Internal solution” (i.e. the solution which contains in the internal tube, separated at the bottom from surrounding solution in a glass by the dialysis membrane) contains different concentration of PAA ([PAA]=5, 10, 15 w%). It should be noted that the system was set up in such a way that in the initial state the solution level in the tube (“internal solution”) was 20 mm below than the level of solution in the external glass. In the initial statethe external solution had the volume of 100 ml, “internal”– 19 ml. After 50 hours the volume of internal solution increased from 19 ml to 39 ml. Accordingly, the volume of external solution in 50 hours decreased to 20 ml.

It is seen that at initial stages small increase in optical density of internal solution because of salt penetration was observed.But at later stages clarification of internal solution owing to pure penetration into him from external solution is observed. While, as seen from data of figure 2, there is an increase of optical density of external solution owing to loss of clear water by him.

Therefore, presence in “internal” solution of ionized macromolecules of PAA substantially interferes with penetration through the membrane ofsalt ions from external solution in “internal” solution. As the result of this phenomenon through the membrane from external solution by power of osmotic pressure generally clean watergets into internal solution.

Also during the experiment the effect influence of concentration of salt ($K_3[Fe(CN)_6]$) in external solution on the degreeof water desalination. With the reduction of concentration of salt from 0,1 mol/l to 0,001 mol/l in external solution the effect of cleaning amplifies. Really, apparently from data of figure 3, in big concentration of salt the optical density of internal solution changes a little, and only the optical density of external solution as a result of pure transition from its to internal solution increases.

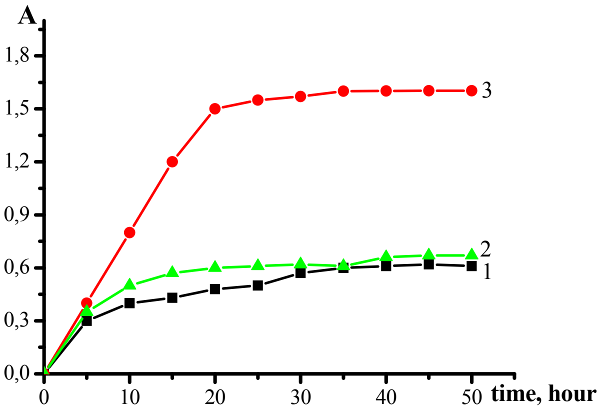

Figure 2 – The kinetics of change of optical density of the aqueous solution in the internal tube at different concentration of polymer

1 – C(PAA) = 5 w%; 2 – C(PAA)= 15 w%; 3 – C(PAA)=10 w%

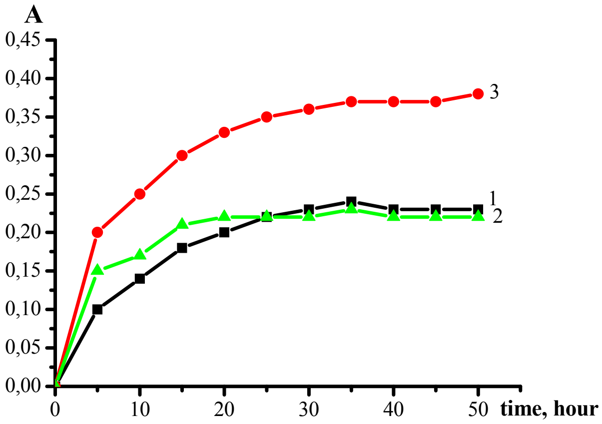

Figure 3 – The kinetics of change of optical density of the aqueous solution in the internal tube at different concentration of salt

1 – C($K_3[Fe(CN)_6]$)=0,1 mol/l; 2 –C($K_3[Fe(CN)_6]$)=0,01 mol/l; 3 –C($K_3[Fe(CN)_6]$)= 0,001 mol/l

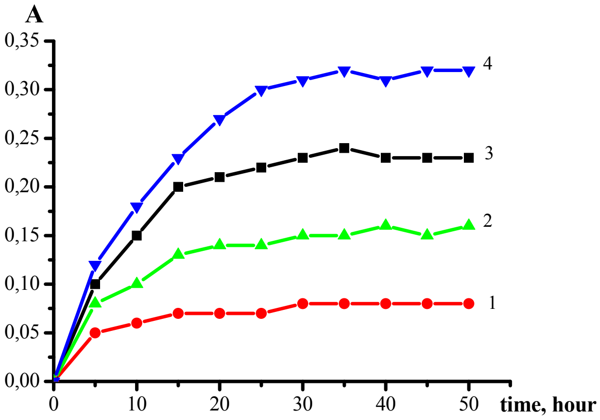

Figure 4 – The kinetics of change of optical density of the aqueous solution in the internal tube at different pH

1 –pH=3; 2 – pH=4; 3 – pH=5; 4 – pH=6

Besides, in this work the effect of pH on the degree of water desalination was investigated. It was observed that upon transition to solutions with lower pHthe degree of water desalination decreases. Optical density increases in internal solution, but remains during the long time (two days) is much lower than the optical density of external solution. Besides, decrease of pH the effect decreases owing to suppression of ionization of the PAA carboxyl groups and decrease of osmotic “activity” of PAA solution (Figure 4).

Conclusion

Thus, in this work the possibility of use the solutions of polyelectrolytes in membrane technology for desalinationhave been investigated. By UV-spectroscopy technique were studied absorbance of the solution at different concentrations of polymer [PAA]=5w%, 10 w%, 15w%. It was assumedthat existence into“internal’ solution of ionized macromolecules of PAA significantly interferes with the penetration through the membrane of ions of salt from external solution into “internal” solution. As the result of this phenomenon through the membrane from external solution bypower of osmotic pressuresolution generally clear water gets into internal.It wasshown that optimal concentration of polymer upon water purification is 10 % PAA. While with reduction of concentration of salt from 0,1 mol/l to 0,001 mol/l in the solution the degree of desalination amplifies. And upon transition to solutions with lower pH (pH=3) the effect of degree of desalination decreases.

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Nitrogen-containing inhibitors and their link to their protective mechanism with molecular structure

Abstract: A potentiometric method was employed to determine the protective effects of the investigated heterofunctional organic compounds, which are potential inhibitors of corrosion of steel, by hydrogen sulfide. According to the results of potentiometric measurements, the most effective of the studied substances can be classified as film-forming inhibitors of mixed effect with anodic predominance. The analysis of the change of the inhibitory properties of aminodiesters was carried out to explain the connection of the protective effect of the inhibitors with their molecular structure. It was based on the principle of linearity of free energy. It was found that the most effective aminodiesters are characterized by the high hydrophobicity and electron-deficient property of the substituents. The article presents an explanation of the difference of the protective effects of the studied compounds based on their molecular structure and the nature of the corrosive environment. The obtained results can be used when synthesizing organic compounds with high inhibitory efficiency.

Key words: Steel, Corrosion, Protective effect, Adsorption, Hydrogen sulfide inhibitor, Molecular structure, Hydrophobicity

Introduction

The material, economical and particularly losses from corrosion during extraction, storage, transportation and refining of oil. The equipment of oil wells and systems of oil and gas collection of numerous fields are intensely corroding as a result of rising from the layers of great amounts of corrosive and highly mineralized waters, which include in composition sodium, calcium, magnesium chlorides. Corrosive activity of layer waters is high due to the presence in them of carbonic acid, hydrogen sulfide, oxygen.

For the Republic of Kazakhstan, which is in possession of gigantic hydrocarbon resources the problem of anticorrosive defence of oil field industry is very topical. Great problems, posed before the workers of oil and gas industry for the further increase of volumes of production of oil and gas make it necessary the further increase of reliability and longevity of production equipment. Related to the development of oil fields of Western Kazakhstan containing in their products the high concentration of hydrogen sulfide, the corrosion of oilprocessing and mining equipment increases. Application of inhibitors during mining and transportation of oil and gas in the Republic of Kazakhstan is constrained by the deficit of domestic inhibitors of corrosion. Thus, the development of new highly effective inhibitors of hydrogen sulfide corrosion of steel is important problem of oil industry.

Dominant part of inhibitors of hydrogen sulfide corrosion, used for the protection of metallic equipment of oilgas complex represents the organic nitrogen containing compounds, in particular, amines and their derivatives. Their protective action is explained by the fact, that in the environment of hydrogen sulfide the adsorption of HS^{-1} ions is observed on the surface of the metal. These ions (HS^{-}) form the dipoles, negatively charged ends of which are directed towards the side of corrosive environment, which contributes to the adsorption of inhibitors of cationic type [1]. The strength of bond of inhibitor with the surface in the first approximation is determined by its adsorption properties (affinity of functional group twoards the surface, which being protected, and its surface activity). The necessary condition of the efficiency of inhibitors of hydrogen sulfide corrosion is their ability for donor-acceptor chemosorption, hydrophobicity and high surface activity.

The problem of relationships between the structure of inhibiting additives and their protective action have been discussed in literature for relatively long time. V.P. Grigoriev and V.V. Ekilik wrote remarkable monograph [2], where the link is considered between the chemical structure of organic surfactants

(SAS) with their inhibiting action on corrosion, hydrogenation of metals and other electrochemical processes. From the review of studies it is apparent, that there is no unique form of relationship. At present time in the question of link between the structure of SAS and their inhibiting action the dominating is the activation-blocking mechanism of action of inhibitors. Under this the main factors during selection of inhibitors are electronic density on adsorption center of molecule and its dipole moment. Structural factors causing the change of electron density on adsorption-active regions of molecule of organic compounds affect the protective properties. Introduction of electron-donor substituents (R) increases protective effect of aromatic and heterocyclic compounds. The presence of branched hydrocarbon chains near the functional group, center of adsorption decreases the efficiency of inhibitor. This is caused by the decrease of adhesion and difficulties in orientation of molecules during adsorption. Introduction into the molecule of inhibitor of aromatic cycles, as a rule, increases inhibiting effect, while benzene radicals, contributing to the formation of more dense protective layer, act more efficiently than phenolic. The substitution of simple bond by unsaturated, and also the increase of the number of last improves the adsorption of organic compounds on metals.

Triple bond from the viewpoint of adsorption is more effective than double bond, while the maximal effect is exhibited by the compounds with the end triple bond. High mobility of electronic cloud of multiple bonds makes them extremely reactive.

And, when in the result of occurrence of various chemical reactions with indicated compounds in the conditions of corrosion the products are formed contributing the main input into the inhibition of the process, the secondary inhibition takes place.

Great value is attributed to the interaction of inhibitors of corrosion due to P-electrons with the surface of metals. Among the compounds with double bonds the most effective molecules are with conjugated and aromatic systems. Such compounds are being held on the surface both due to the adsorption bond «nitrogen-metal», and P-electron interaction. Corrosion process is a very complex phenomenon, because the complication of molecules of inhibitor and the change of external condition may lead to observation of deviation from the regularities indicated above. Besides, during investigation of the effect of structure of the inhibitor on its protective properties the account of the charge of the metal is needed and its nature. Thus, for example, very often the strengthening of the adsorption bond «metal-inhibitor» with the formation of surface complexes contributes to the growth of the protection from the corrosion. But, if the strength of the bond «metal-inhibitor» approaches corresponding bond of individual compound, then the decomposition of the metal lattice occurs. As a result, inhibiting effect gets transformed to the stimulating effect.

It must be noted, that the account of simultaneous effect of various structural factors and electronic effects of the molecule on the dissolution of the metal is a very complex problem. Hence the degree of protection from corrosion is determined mainly by blocking and hydrophobisation of the metal surface by adsorbed molecules of inhibitor.

Some regularities of protective effect of inhibitors of corrosion may be explained by the presence of specific interaction «metal-inhibitor». During consideration of questions of inhibiting protection of metals it is usefull to apply the electronic conceptions of heterogeneour catalysis about chemosorptive ability of metals. According to these conceptions it is possible to expect the high protective effect, which is due to chemosorption for primarily transition metals. It is shown, that metals with completed d-levels hold on their surface the molecules of inhibitor primarily due to the forces of purely physical adsorption. Metals with incompleted d-level are characterized by the presence of notable specific adsorption. Formation of surface complexes has proven in the row of cases by spectral, crystallochemical and radiochemical measurements.

The main qualitative relationships between chemical structure and inhibiting effect of surface-active substances are presented above. The methods exist for quantitative account of the effect of chemical structure on inhibiting action of SAS. The attempts are presented in literature of utilization for these purposes of the ability of additives to change the value of certain factors influencing the occurrence of corrosive process.

Such factors were electric resistance on the boundary corroding metal — solution, the value of the potential of the corroding metal, the change of effective energy of activation of corrosive process. But the analysis of numerous experimental data shows, that there is no unique linked between these characteristics of corrosive process and inhibiting effect. Of significant interest is the comparison of data on adsorption of SAS and their effect on corrosion of metals. For the qualitative and quantitative assessment of adsorption of SAS on solid electrodes in comparison with their inhibiting action the great abundance was achieved by the method of measurement of differential capacity.

It must be noted that in contrast from the earlier works, when at the foundation of choice of inhibitors of corrosion was almost strictly empirical method, the number of research studies increases, where for the quantitative assessment of the effect of the structure of compounds on their inhibiting action the correlation analysis was used base on the principle of linearity of free energies (LFE principle) [3].

LFE principle of the reaction and activation found wide applications during interpretation of the effect of the nature of surface-active substances on the efficiency of their defensive action in acidic and neutral environments [4-6].

At present is may be considered proven the applicability of the principle of LFE for numerous electrochemical processes with participation of organic substances [7, 8].

During investigation of the mechanism of action of inhibitors of corrosion the link is important between electronic structure, physical-chemical properties and protective efficiency of compounds. This leads to possible interpretation and prognosis of anticorrosive properties based on molecular-mechanical and quantum-chemical investigations of the structure of molecules of inhibitors. For the solution of the problem “structure of inhibitor- anticorrosive efficiency”, along with the widely used principle of linearity of free energies, the principle of “rigid” and “soft” acids and based (RSKB) may be used [9-11]. In works [12, 13] for the description of the process of inhibition of corrosion of metals the scheme of quantitative assessment of parameters “rigidity” based on the energies of highest occupied molecular orbitals (HOMO) and lowest unoccupied (LUMO).

In work [14] the possibility of inhibition of hydrogen sulfide corrosion of steel by quaternary ammonium salts (QAS), synthesized from amines and aminoalcohols with various substituents R_i according to their reaction with benzene chloride. The main problem of this work was the investigation of the effect of chemical structure of QAS on their protective properties. It is noted, that efficiency of the protection of steels from corrosion with the help of QAS depends on their chemical structure, while hydrophobicity of inhibitor is the dominating factor. But the adequacy of description of protective properties increases under additional account of polar properties of substituents.

At present time there is no unique opinion related to the nature of the link of polar groups of organic inhibitors with protective metal. Definitely, the active participant in the process of adsorption is the polar end of molecule. It must be noted, that the formed surface compounds during chemosorption do not get released in the form of a new phase. The majority of organic inhibitors in acidic environments form with surface ions of the metal insoluble complex compounds. That is why the primary process during the action of numerous efficient inhibitors is the formation on the surface of the metal of protective film, representing the product of interaction between the metal, inhibitor and ions of corrosive active environment. The most important factor, determining the strong bond of inhibitor and the protected metal is the electronic density of the main metal of inhibitor, i.e. The number of free electrons, which it can spend on coordination bond. This condition is completely met of the nitrogen atom, which easily forms the coordination bond with metal.

Materials and methods

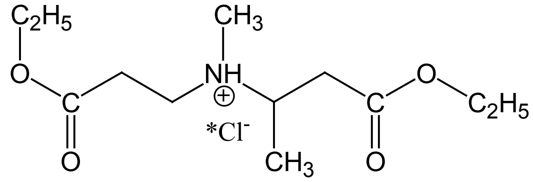
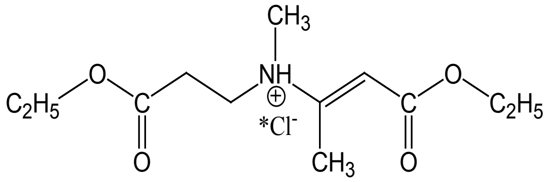
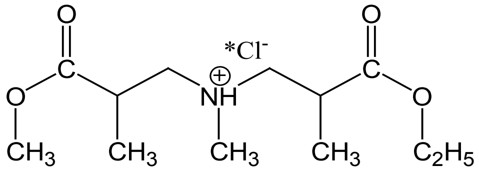
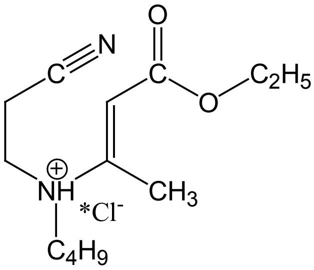
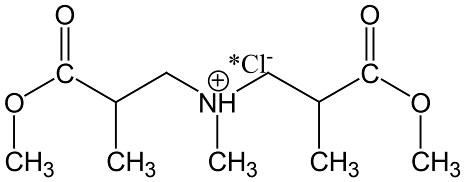
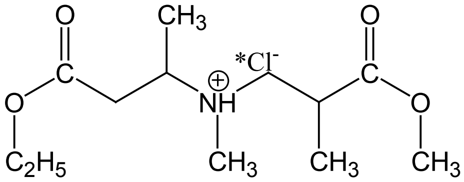
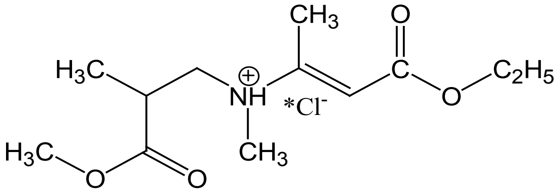
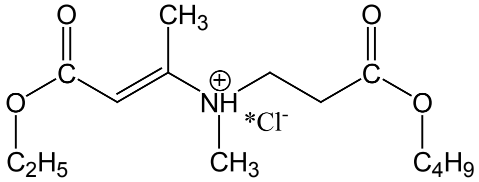
Objects of investigation in present work — nitrogen and oxygen containing organic compounds, synthesized on the basis of available raw materials and simple technology, which are the intermediate products of synthesis of new biologically active substances (table 1).

For the experimental determination of the rate of corrosion of steel electrode, the three-electrode cell was used with steel working electrode, platinum auxiliary electrode and silver-chloride comparative electrode. Polarization curves were images using potentiostat-galvanostat «PGSTAT30» (AUTOLAB, Netherlands, 2001).

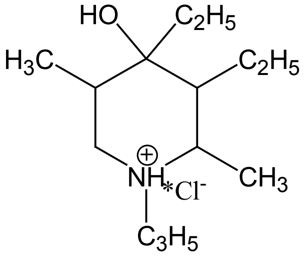
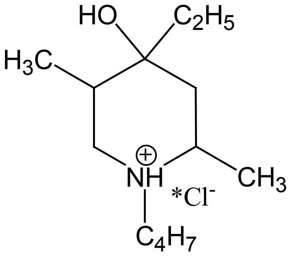
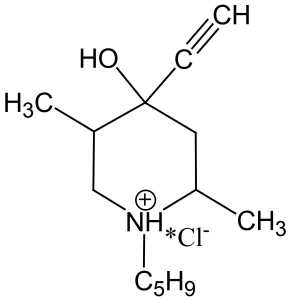
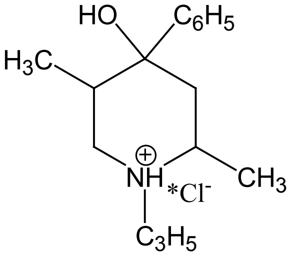
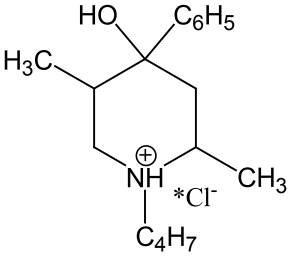
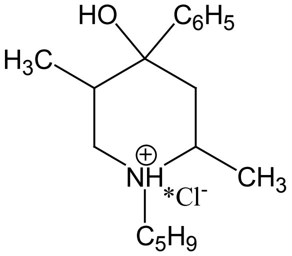
The working electrode was the rotating disk steel electrode. The background solution was the corrosive model environment (NACE), representing a 3% solution of sodium chloride, saturated H_2S (0,8g/l). For the saturation of the solutions of sodium sulfide was used. The concentration of hydrogen sulfide was controlled by iodometric method of back titration. The variation of concentrations of potential inhibitors of corrosion was carried out by the method of additives.

The preparation of the surface of working electrode before the imaging of polarization curves was carried out by mechanical technique. The surface of electrode was cleaned by micron abrasive material until the stage of smooth surface, was degreased by alcohol and washed by distilled water. The processing of surface of working electrode was carried out before every measurement.

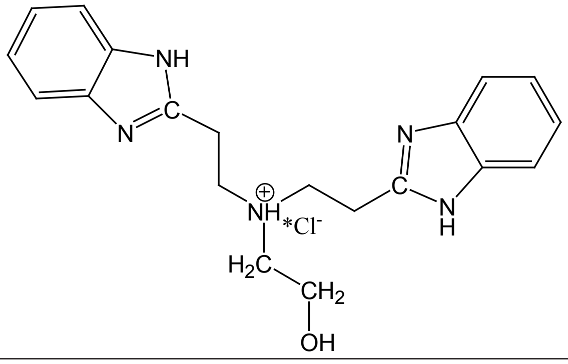
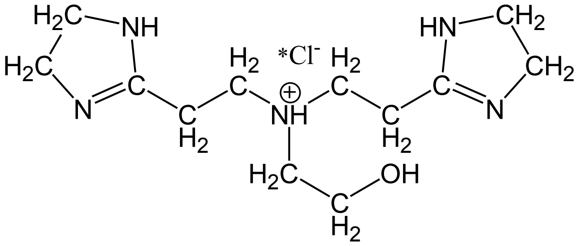
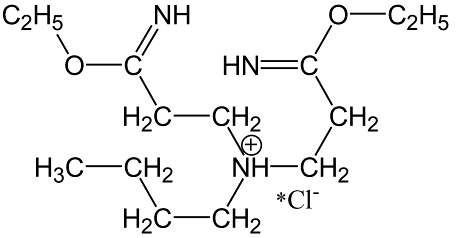
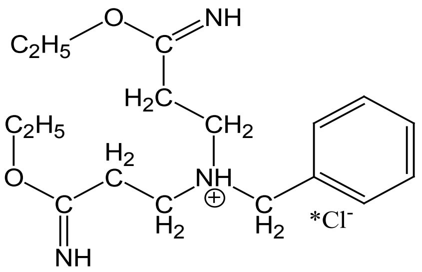
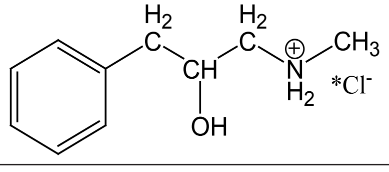
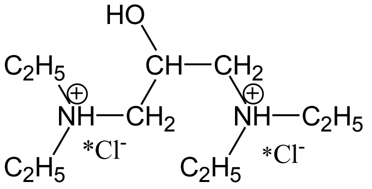
Table 1 – Structural formulae and names of investigated compounds

N	Structural formula	Title
1		N-(1-methyl-2-carboethoxyethyl)- N-(2-carboethoxyethyl)- methylamine hydrochloride
2		N-(1-methyl-2-carboethoxythene)- N-(2-carboethoxyethyl) methylamine hydrochloride
3		N-(2-methyl-2-carbomethylethoxyethyl)-(2-methyl-2-carboethoxyethyl)methylamine hydrochloride
4		N-(1-methyl-2-carboethoxyethene)- N-(2-cyanethyl)buthylamine hydrochloride
5		N,N -bis(2-methyl- 2-methoxyethyl)-methylamine hydrochloride
6		N -(2-methyl-2-carbomethoxyethyl)- N-(1-methyl-2-carboethoxy)-methylamine hydrochloride
7		N -methyl-(2-carbmethoxyethyl)-(1-methyl-2-carboethoxyethene) methylamine hydrochloride
8		N-(1-methyl-2-carboethoxyethene)- N-(2-carbobutoxyethyl)methylamine hydrochloride

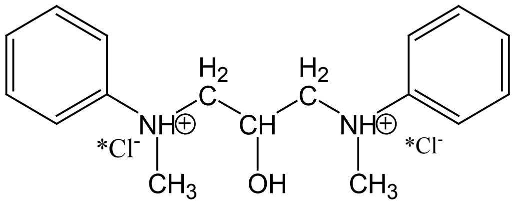
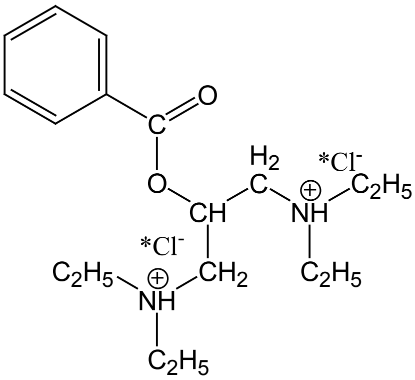
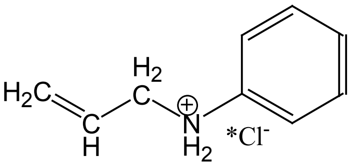
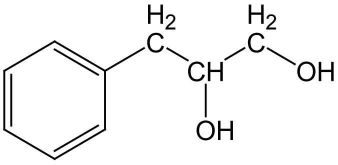
Continued table 1

N	Structural formula	Title
9		N- propenyl-2,5-dimethyl-4-ethylpiperidine-4-ol hydrochloride
10		N-(butene-2-yl)-2,5-dimethyl-4-ethylpiperiine-4-ol hydrochloride
11		N-(3-methylbutene-2-yl)-2,5-dimethyl-4-ethynylpiperidine-4-ol hydrochloride
12		N-allyl-2,5-dimethyl-4-phenylpiperidine-4-ol
13		N-butenyl-2,5-dimethyl-4-phenylpiperidine-4-ol hydrochloride
14		N-(3-methylbutene-2-yl)-2,5-dimethyl-4-phenyl-piperidine-4-ol hydrochloride

Continued table 1

N	Structural formula	Title
15		N, N-bis-[β, β`-ethyl-2-benzylimidazolyl]- 2-hydroxyethylamine hydrochloride
16		N, N-bis-[β, β`-imidazolylethyl]- 2-hydroxyethylamin hydrochloride
17		N, N-bis-(3-imino-3-ethoxypropyl)- N-butylamine hydrochloride
18		N, N-bis-(3-imino-3-ethoxypropyl)- N-benzylamine hydrochloride
19		1-phenyl-3-methylaminoisopropanole hydrochloride
20		1,3-bis-(diethylamino)isopropanol hydrochloride

Continued table 1

N	Structural formula	Title
21		1,3-bis-(N-methyl- N-phenyl)-isopropanol dihydrochloride
22		bensoate 1,3-bis-(diethylamino)-isopropanol dihydrochloride
23		N-allylaniline hydrochloride
24		3-phenyl-1,2-propandiole

Electrodes were placed into electrochemical cell with investigated medium and connected to the instrument. Polarization measurements were started after stabilization of stationary potential of corrosion. Obtained potentiodynamic curves were processed using Microcalc Origin program, which allowed determining the values of the rates of corrosion of steel electrode in various inhibited and non-inhibited environments [15].

Results and their discussion

Usually, the polarization curves that characterize the partial corrosion processes are presented in the form of semi-logarithmic dependence.

Polarization curves were obtained for all the above inhibitors at different concentrations in a corrosive environment. Figures 1-4 show the potentiodynamic curves for compounds 4, 8, 19, 21. Because of the large number of studied inhibitors, polarization curves are shown only for some of the substances. The selection of these inhibitors by the need to reflect the relationship of protective properties of the compounds with the values of corrosion currents observed at different concentrations of additives in a corrosive environment.

Based on potentiodynamic measurements the protective effects were computed of the investigated substances under corrosion of steel in the sodium chloride and chloride environment, saturated by hydrogen sulfide (tables 2 and 3). An important feature of the electrochemical reactions is the equivalence of their speed and the current density. This equivalence follows from Faraday's law. Protection efficiency is expressed by the degree of protection Z, which indicates how full to suppress the corrosion due to the use of a method:

$$Z = [(i_0 - i)/i_0] * 100\%$$

where i_0 – corrosion current density of metal in the solution without inhibitor; i – current density of corrosion in solution in the presence of the inhibitor.

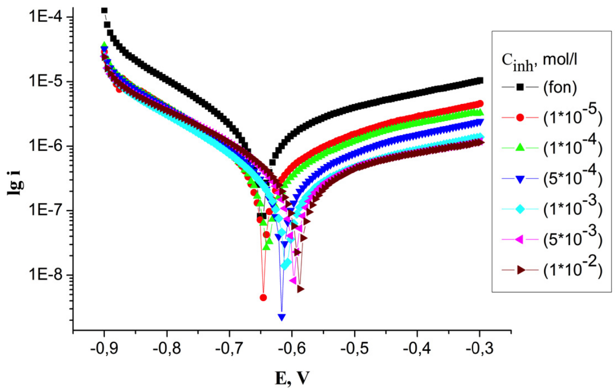


Figure 1 – Potentiodynamic curves of St3 in the solution of 3% NaCl + 0.8 g/l H_2S (pH = 5) with addition of inhibitor 8

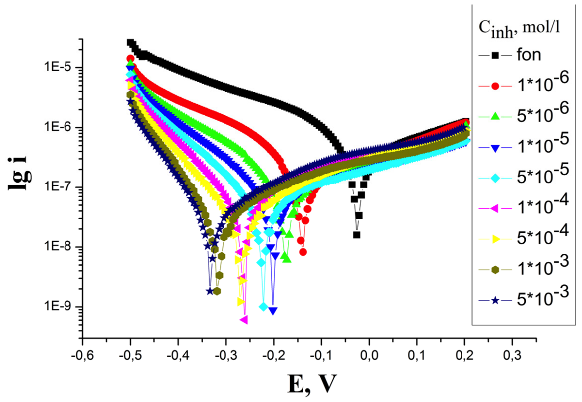


Figure 2 – Potentiodynamic curves of St3 in the solution of 3% NaCl with addition of inhibitor 4

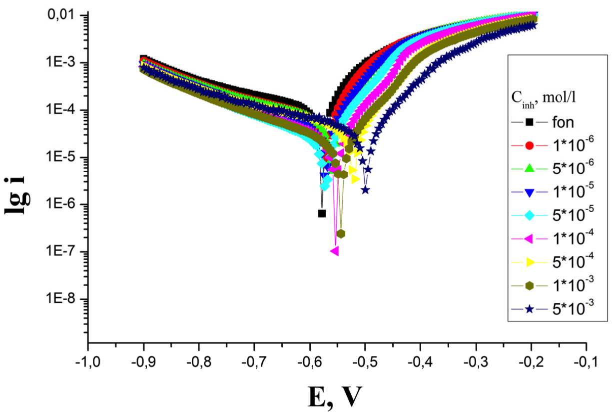


Figure 3 – Potentiodynamic curves of St3 in the solution of 3% NaCl + 0.8 g/l H_2S (pH = 5) with addition of inhibitor 19

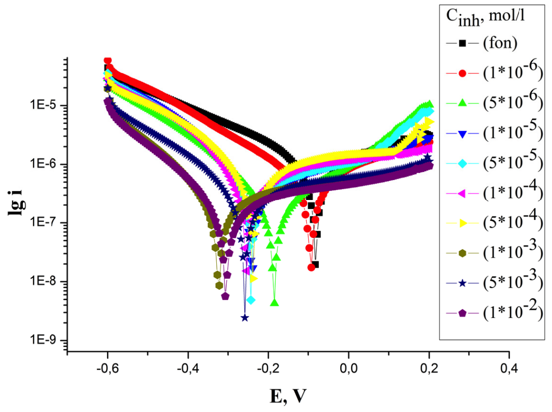


Figure 4 – Potentiodynamic curves of St3 in the solution of 3% NaCl with addition of inhibitor 21

Table 2 – Protective action of organic compounds (C=1*10^{-2} mol/l) in 3% solution NaCl

Inhibitor	Z_k ,%	Z_a, %	Inhibitor	Z_k ,%	Z_a, %	Inhibitor	Z_k ,%	Z_a, %
1	64.2	68.2	9	81.3	86.7	17	42.3	62.7
2	87.7	71.3	10	91.2	94.8	18	0	0
3	76.1	67.8	11	59.5	56.6	19	15.9	10.9
4	90.3	88.2	12	72.1	71.1	20	36.4	36.1
5	71.2	65.5	13	57.8	52.2	21	50.1	58.7
6	67.5	61.2	14	0	0	22	52.8	42.6
7	79.1	75.4	15	50.8	55.6	23	21.6	54.2
8	91.2	87.4	16	0	0	24	52.4	62.6

The presence of hydrogen sulfide in a corrosive environment increases the adsorption and, consequently, the protective ability of cationic inhibitors. Strengthening the protection of steel by organic cations is largely due to the fact that the specific adsorption of HS^- is recharge the surface facilitating the interaction with surface and cations, which creates an energy barrier (due to changes ψ_1-potential) and blocks the surface. In the sodium chloride medium, the additions of inhibitors cause displacement of the stationary potential of steel electrode in the cathode area and preferential inhibition of the cathodic process of discharge of hydrogen ion on the metal.

Accordingly, in a solution of sodium chloride cathodic protective effect of inhibitors are slightly higher. Apparently, at the cathodic areas of surface of the steel electrode take place the adsorption of cation-active inhibitors, which slow down the cathodic reaction. This is explained by a better adsorption of the tested inhibitors in comparison with the specific adsorption of chloride ions.

Table 3 – Protective action of organic substances (C=1*10^{-2} mol/l) in 3% solution NaCl + 0.8g/l H_2S

Inhibitor	Z_k ,%	Z_a, %	Inhibitor	Z_k ,%	Z_a, %	Inhibitor	Z_k ,%	Z_a, %
1	63.1	81.7	9	61.2	63.4	17	82.9	85.6
2	45.3	97.1	10	30.6	51.2	18	44.1	62.2
3	67.3	88.6	11	12.9	85.1	19	79.5	85.9
4	95.8	98.3	12	54.7	28.3	20	75.9	74.9
5	74.1	74.4	13	49.6	83.2	21	82.5	88.1
6	67.3	88.6	14	77.2	78.9	22	62.1	72.1
7	83.5	92.4	15	77.4	96.9	23	72.6	58.5
8	97.8	94.9	16	78.1	79.3	24	61.2	66.3

Most of the investigated compounds showing weaker protective properties in a sodium chloride medium. This is probably explained by lower adsorption of chloride ions in comparison with the hydrosulfide ions, i.e. “The effect of overcharges” in the hydrogen sulfide-containing medium is significantly higher than in the chloride medium.

According to the results of potentiodynamic measurements, the compounds investigated by us can be attributed to film-forming inhibitors. In contrast to the passive films, the defensive films are formed in the result of such interaction of inhibitor with metal or products of corrosion of metal, during which the material of the film contains either the molecules of inhibitor itself, or functional groups, cations or anions of inhibitor. Film-forming inhibitors exhibit the mixed character of protective action, i.e. form the films both on cathode and anode sections. Such inhibitors include the compounds investigated in our work. They may be attributed to the inhibitors with mixed anode-cathode control, but with domination of anodic. This is evidenced by the results of our potentiodynamic studies. In favor of the formation of protective films, say also a very small concentration of these compounds, leading to considerable lowering of the rate of corrosion.

There exist various approaches during consideration of the problem «structure – inhibiting action». The majority of experimental data on the link between the structure and inhibiting properties of SAS is possible based on assessment of electronic density on adsorption-active heteroatom. During this, the main criteria must be the electronic density and its dipole moment. However, in case of complex compounds, which are presented in this work, the account of simultaneous effect of various structural factors and electronic effects of molecule is very difficult.

Electronic density on adsorption center of molecule determines the donor function of molecule of inhibitor and, consequently, its surface activity. Introduction of substituents into the SAS molecule affects the electron density of adsorption center. To account the donor-acceptor properties of substituents σ^* – Taft constants are used, which reflect the inductive influence of substituent on reactive center of organic compound [16]. For R–CH_3 $\sigma^*=0$, i.e. methyl group — standard substituent. More electro-donor substituents, than metal are characterized by $\sigma^*<0$, and electro acceptor $\sigma^*>0$.

Molecules of inhibitor may be adsorbed on the surface of corroding metal, both by polar part, and lipophilic part, and also hydrophobic end. During description of physical-chemical interactions of inhibitor with metal hydrophobicity must be taken into account along with main parameters of molecules, polar and steric. Analysis of correlation «structure-property» for primary, secondary and tertiary amines evidences about the presence of quite clear dependence of protective effect on hydrophobicity coefficient. Hydrophobicity of organic compounds in various chemical and biochemical processes have been estimated for a long time by coefficient of their distribution (D) in the system composed of two immiscible liquids, for instance, octanol-water, or sum of constants of Rikker, computed using these coefficients of distribution. Constants of Rikker are known for the majority of fragments of molecules and reflect their relative hydrophobicity in comparison with hydrogen atom. Constant f is positive for hydrophobic and negative for hydrophilic substituent.

In our work the analysis is carried out of correlation of protective properties of the row of aminodiethers with their chemical structure based on the above described principle of linearity of free energies using f – constants of hydrophobicity and σ – Taft constants, taking into account the polar properties of substituents [17].

During analysis of presented in table 4 results with protective action of aminodiesters it can be seen, that the most effective are characterized by the highest hydrophobicity and more electron-acceptor properties of substituents, which is in accordance with peculiarities of hydrogen sulfide corrosion of steel.

Table 4 – Total hydrophobicity (∑f) and inductive effect (∑σ) of substituents in molecules of aminodiesters

Compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
∑σ	1.3	1.8	1.4	2.3	1.4	1.4	1.8	1.7
∑f	3.1	2.7	3.0	3.4	2.4	2.8	2.6	3.8

The investigated compounds may be separated into three groups. The first group (compounds 1-8) – these are the aminodiesters, the protective action of which is due to the presence of several reactive centers. Compounds 1, 3, 5, 6 have practically similar structure, composition and equal «concentration» of carbonyl and ether oxygen, which determines their similar efficiency. Compounds 2 and 7 are in possession of higher activity, which is due to the presence in their structure of C=C double bond, which leads to the increase of adsorption. Increase of adsorption during this leads to the increase of efficiency of inhibitor. Aminodiesters 4 and 8 are distinct from others relative by structure and composition compounds by the presence of cyanide group (inhibitor 4) and long hydrocarbon radical C_4H_9, which ensures high protective properties of these molecules.

Nitryl group possesses the high electron density of triple bond, which is characterized by a more strong electronegative inductive effect (Taft constant $\sigma = 3.38$). Besides, the long chain hydrophobic radical C_4H_9, oriented perpendicularly to the surface, creates the shielding protective layer and contributes to the strengthening of inhibiting activity of compounds 4 and 8. Increase of surface activity is related to the growth of hydrophobicity of molecules, more hydrophobic molecules are easily squeezed out from the volume of aqueous solution.

The second group – compounds 9-14 has similarities in chemical structure, namely, all of them contain heterocycle, which includes nitrogen heteroatom, existing in the state of sp^3 hybridization, due to which the nitrogen atom has unshared pair of ē able to enter the donor-acceptor interaction with hydrogen cations and metal surface. But the presence of hydrocarbon radical at nitrogen atom producing funnel-like rotational motion around the singular axes N-C, effectively shields the pair of electrons of nitrogen, which leads to the considerable decrease of adsorption properties of inhibitor molecule. In case of acidic environment, positively charged hydrogen — nitrogen center is also shielded by these radicals. Thus, the presence in molecules of heterocycles 9-14 of voluminous organic radical at nitrogen atom practically eliminates their ability to act in the quality of effective inhibitor of corrosion. It must be noted, that inhibitor 11, contains acetylenic bond, which is composed of hydrogen atom of end carbon atom, possessing acidic properties, i.e. having positive charge and allowing a nonsmall anodic protective effect of this compound. Inhibitors 12, 13, 14, are having in their composition phenyl group, possessing the high electronic density, exhibit particular activity. Electronic interaction due to the pi-electronic system of aromatic radical with incomplete d-levels of iron strengthen both adsorptive ability and input of shielding effect into the total protective effect.

The third group is the derivatives of aminodiesters and aminoalcohols. Compounds 17 and 18 contain in their composition -O-C=NH group. Nitrogen and oxygen of this group do not exhibit donor properties. Donor ability of unshared electronic pair of nitrogen atoms in these molecules is lowered. The reason is in the double bond C=N and oxygen atom, pulling the electronic density from nitrogen atom, which explains not considerably high protective properties. Active in all of these large molecules are only nitrogen atoms N*, possessing donor properties.

Relatively high protective properties are exhibited by compounds 21 and 22, which is due to the presence in the molecule of sp3-hybrid two atoms of nitrogen containing the unshared pairs of electrons and phenyl radicals. The presence of hydroxyl groups in compounds 19, 20 increases their hydrophilicity and lowers their inhibiting activity. Weak protective properties of substance 23 are due to the fact, that the pair of electrons of nitrogen to higher degree compared to other compounds exists in conjugation with benzene ring.

Inhibiting properties of compounds 15, 16 are not considerably high due to the weak donor activity of nitrogen atoms, entering the 5-member cycle, and also the presence of hydroxyl groups. In both inhibitors the actual active nitrogen atom is N*, entering the interaction with metal surface.

Inhibitor 24 does not possess protective properties, because it doesn't contain nitrogen atom, besides, the presence in its molecules of two hydroxyl groups having alcoholic character, makes them very hydrophilic. This compound exhibits non-high activity in the solution of sodium chloride. This, apparently, is related to the fact, that chloride ions possess the smallest adsorption ability, rather than hydrosulfide ions, which allows the presence of competing adsorption of organic molecules.

The major part of compounds of third group contains from 2 to more atoms of nitrogen. Usually the increase in the molecule of the number of nitrogen atoms, leads to the increase of efficiency of inhibition, because they bind the molecule with high number of surface atoms of iron. Besides, the molecules containing no less than 2 atoms of nitrogen, during interaction with charged surface of metal must exhibit the tendency towards the planar location. But this is true for flat configurations. In our compounds, having the complex structure the account of effect of various substituents on reactive centers of molecules is necessary, i.e. on electronic density of adsorption center, which determines the donor function of molecule of inhibitor and, consequently, its surface activity.

In inhibition of corrosion a substantial role is played by protonated and unprotonated molecules. The higher the negative charge on nitrogen atom of reactive center and higher the positive charge on hydrogens in protonated form, the better they will inhibit the corrosion due to the formation of strong coordination and hydrogen bonds with metallic surface.

In hydrogen sulfide containing environment the surface of the steel is charged negatively due to the specific adsorption of sulfide and hydrosulfide ions. Adsorption of HS- is proven by the method of radioactive measurements. Thus, electron acceptor properties of substituent groups increase the effective positive charge on reactive center – organic cation and increase it electrostatic interaction with the surface of steel, which is charged negatively due to the specific adsorption of sulfide- and hydrosulfide- ions.

Great part of studied substances exhibit more weaker protective properties in the sodium chloride medium. This, apparently, is explained by lower adsorption of chloride ions in comparison with hydrosulfide ions, i.e. «effect of recharge» in hydrogen sulfide environment, is considerably higher, then in chloride.

The link is established between protective effects of investigated compounds with their molecular structure. Higher inhibiting properties are possessed by compounds, having unsaturated bonds (compounds 2, 4, 7, 8, 11), phenyl radical (14, 21), ester group (1-8), hydrophilic radical C_4H_9 (4, 8). Compound 4, in molecule of which the triple end bond and double bond are present, may be attributed to the highly effective inhibitors of hydrogen sulfide corrosion. The increase in the molecule of inhibitor of the number of nitrogen atoms does not lead to the ordinary increase of protective action, which is related, apparently, with the complexity of the structure of investigated compounds and necessity of account of effect of various substituents on reactive centers of molecules, i.e. on electronic density of adsorption center. This says about the effect of spatial structure of molecules of inhibitor on its protective properties. Presence of nitrogen atom in compounds (9-14) leads to the decrease of their efficiency. The presence of hydroxylic groups in molecules of compounds (16, 19) increases hydrophilicity and lowers their inhibiting activity. Absence of nitrogen atom in molecule of compound 24 leads to a complete disappearance of protective ability in hydrogen sulfide environment.

Thus, the investigation of protective action of newly synthesized nitrogen-containing organic substances allowed to identify from them the most effective. Analysis of link of protective properties of investigated compounds with their chemical structure may form the foundation of aim-driven synthesis of organic substances with potentially high inhibiting efficiency.

Conclusions

1. Using the method of imaging of polarization curves the protective effects of a row of organic nitrogen-containing compounds were determined.

2. The link is established between inhibiting properties of investigated compounds and their molecular structure using the principle of linearity of free energies.

3. It is determined, that the effective inhibitors are characterized by the maximal hydrophobicity and higher electron acceptor properties of substituent groups. This is in accordance with the main peculiarities of corrosion of steel in hydrogen sulfide environments.

SAS– surface active substances

LFE principle – principle of linearity of free energies

RSKB – principle of "rigid" and "soft" acids and based

HOMO – highest occupied molecular orbitals

LUMO – lowest unoccupied molecular orbitals

QAS – quaternary ammonium salts

NACE – corrosive model environment

Z – degree of protection

i_0 – corrosion current density of metal in the solution without inhibitor;

i – current density of corrosion in solution in the presence of the inhibitor

σ* – Taft constants

f – constants of hydrophobicity

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