COPPER(II) DETERMINATION BY IMMobilized 
UREaSE INHIBITION IN A SPECTROMETRIC FLOW-
INJECTION SYSTEM

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ABSTRACT
A flow-injection system for analysis of urea and copper(II) ions based on the immobilized urease onto seven modified acrylonitrile copolymer membranes was studied. The most suitable ones for the both flow-injection analysis were selected. The urease systems with membranes modified with NaOH+2-dimethylaminoethyl methacrylate (DMAEM), NaOH+DMAEM+ethylene diamine (EDA) provided higher urea sensitivity and precision of the measurements (R=0.99 and RSD about 3%). The calibration curves for copper (II) ions show that the systems with urease immobilized on membrane modified with NaOH+EDA and hydrazinium sulfate (NH₂NH₂.H₂SO₄) had the highest sensitivity to Cu(II). The detection limit of Cu(II) for these systems was 0.1 mg l⁻¹. The correlation coefficient of the linear intervals (R=0.99) and standard deviation (about 4.5 %), which guarantees good reproducibility of the copper analysis. The operational stability of the flow system with membrane modified with NaOH+EDA for analysis of Cu(II) was studied. The investigated immobilized system reached 50% inhibition after 25 days. The stability of immobilized urease membrane during storage was determined. The FIA sensitivity was found to remain almost the same for 40 days. The inhibition effect caused by a mixture of Cu(II), Zn(II) and Ni(II) ions in model solution and real water was also determined.

Introduction
Heavy metals are widely spread in nature and contaminated environments. Due to their less biosusceptibility and toxic effect on organisms, the potential risk of heavy metals to human health and ecology has received much attention.

A number of biosensors for heavy metal trace measurement have been previously investigated (1-3). Some are based on urease because of its stability, very high sensitivity to inhibitors and low cost (4, 5). The immobilization of enzymes plays an important role in the development of enzyme-based biosensors due to its sensitivity, dynamic range, response time, and storage period of the devices. There are a number of publications describing the use of immobilized urease on membranes for heavy metal determination (6-8). The application of immobilized urease has several benefits in comparison with free enzyme: (1) the consumption of the enzyme is 10³ times lower; (2) the differential mode of operation enables a non-specific output signal to be suppressed; (3) preincubation takes place in the real sample solution while the measurements can be carried out in the buffer solution.

* Abbreviations: acrylonitrile = AN; 2-dimethylaminoethyl methacrylate (DMAEM); ethylene diamine = EDA; flow injection analysis = FIA; glutaraldehyde = GA; hydroxylammonium sulfate = NH₂OH.H₂SO₄; hydrazinium sulfate = NH₂NH₂.H₂SO₄; polyacrylonitrile = PAN
For further development of urease-based biosensors for heavy metal ions the determination of increase in sensitivity is very important. It has been shown (9) that a properly chosen support for immobilization considerably improves enzyme inhibitor sensitivity compared to that of free enzyme. Volotovsky et al. (10) immobilized urease in a negatively charged polymer (Naftion) in order to accumulate heavy metal cations in the biomatrix, in such a way as to improve sensor sensitivity to heavy metal ions.

The aim of the present work was to design a flow-injection system for analysis of urea and copper(II) ions based on the immobilized urease onto seven modified acrylonitrile copolymer membranes. The most suitable one for the two flow-injection analysis were selected.

**Materials and Methods**

Ultrafiltration membranes of AN copolymer (acrylonitrile – 91.3%; methylmethacrylate – 7.3%, sodium vinylsulfonate – 1.4%) supplied by Spartak Co., Bourgas, Bulgaria with cut-off 10 000 Da were used. The specific area of the membranes was 10 m²g⁻¹ and average pore size 0.02 µm. The membranes were modified with the following agents: sodium hydroxide, hydrochloric acid, nitric acid, hydrogen peroxide, methanol, ferrous ammonium sulfate, hydroxylammonium sulfate (NH₂OH.H₂SO₄), ethylene diamine (EDA), glutaraldehyde (GA), hydrazinium sulfate (NH₂NH₂.H₂SO₄), dimethyl sulfoxide, 2-dimethylaminoethyl methacrylate (DMAEM), produced by Fluka, Switzerland.

Immobilization of urease (Merck, Germany, 270 U mg⁻¹) onto the membranes of polyacrylonitrile (PAN) was carried out with glutaraldehyde (Fluka, Switzerland).

All other reagents were analytical grade. All the solutions used (without solutions used for modification) were prepared with bidistilled water.

**Chemical modifications of PAN membranes**

1. Modification with NaOH+EDA

Membrane with 100 cm² surface area was immersed in 100 ml 15% solution of sodium hydroxide (NaOH) for 60 min at 50°C. The membrane was then washed with distilled water and placed in diluted solution of hydrochloric acid for 120 min. After that, the membrane was immersed in 100 ml 10% solution of EDA for 60 min at room temperature. This modification was selected to obtain primary amino and carboxylic groups (11).

2. Modification with NaOH+GA+EDA

Amine enriched PAN membrane modified according to 1 was immersed in 12.5% solution of GA in borate buffer with pH 8 for 20 min at room temperature. The membrane was then washed with 100 mM phosphate buffer (pH 7.5) and immersed in 30 ml 500 mM aqueous solution of EDA at 4°C for 24 h. This modification produced also primary amino and carboxylic groups (12).

3. Modification with NH₂OH.H₂SO₄

Membrane with 100 cm² surface area was immersed in 100 ml 5% solution of dimethylformamidine for 30 min at room temperature. The swollen membrane was then immersed in 100 ml 10% solution of NH₂OH.H₂SO₄ at 40°C for 120 min. With this modification we introduced primary amino and amidoxyne groups (11).

4. Modification with H₂O₂

Membrane with 100 cm² surface area was immersed in a solution containing: 11.5 ml CH₃OH; 0.5 ml H₂O, 0.24 ml dimethyl sulfoxide, 2-dimethylaminoethyl methacrylate (DMAEM), produced by Fluka, Switzerland.

Immobilization of urease (Merck, Germany, 270 U mg⁻¹) onto the membranes of polyacrylonitrile (PAN) was carried out with glutaraldehyde (Fluka, Switzerland).

All other reagents were analytical grade. All the solutions used (without solutions used for modification) were prepared with bidistilled water.
according to 1 was immersed in 10 mM aqueous solution of ferrous ammonium sulfate (pH 5.5) for 10 min. It was then washed with distilled water and immersed for 15 min in 10% solution of 2-dimethylaminoethyl methacrylate (DMAEM) preliminarily neutralized with acid. Then 1 ml 0.3% hydrogen peroxide was added to obtain a mixture with pH 4. The modification was carried out at 40°C for 4 h and produced tertiary amino and carboxylic groups (11).

7. Modification with NaOH+DMAEM+EDA

PAN membrane modified according to 1 was treated with 10% solution of DMAEM as described in 6. Then the membrane was immersed in 100 ml 10% solution of EDA for 60 min at room temperature. This modification introduced primary amino, tertiary amino and carboxylic groups (12).

Finally, all the modified membranes were washed thoroughly with distilled water.

The amounts of functional basic and acid groups in the modified membranes were measured by residual titration in heterogeneous medium (14).

**Immobilization of urease on the modified PAN membranes**

The immobilization of urease on the modified membranes was made under preliminary determined optimal conditions. Each of the modified membranes (10 cm²) was immersed in 12% solution of glutaraldehyde for 1 h at 4°C. Then the membranes were washed with bidistilled water and after that were immersed in 0.1% solution of urease in sodiumphosphate buffer with pH 5.8. The membranes were kept in enzyme solution for 24 h at 4°C. Finally, the membranes with the immobilized enzyme were washed with bidistilled water and 60 mM solution of phosphate buffer.

The amount of protein bound to the modified membranes was determined by the method of Lowry. The method is based on spectrophotometric measurement of the blue colour resulting from the cupric ions with peptide bonds in alkali medium and from the reaction of the aminoacidous residues with Folin reagent (15).

The activities of free and immobilized urease was determined by measuring the ammonium obtained by the incubation of urea with the enzyme. The amount of NH₃ released was determined spectrophotometrically by measuring the intensity of the coloured compound formed with the addition of Nessler’s reagent (16).

**Flow injection analysis (FIA) module**

The arrangements of the FIA module used in this study are shown in Fig. 1. A three-channel peristaltic pump (MCP-552) was used to deliver the solutions to the manifold at the flow rates (in ml min⁻¹). This module included a membrane reactor (2 mm height, 30 mm inner diameter, 1.4 cm³ working volume) with immobilized urease membrane. Standards of the inhibitor Cu(II) ions (0.1÷2 mg l⁻¹) were injected by means of an injection port. All connecting lines were of 0.5 mm and were kept as short as possible. The flow-through spectrophotometer (LKB, model 2051) was set at 460 nm.

**Analytical procedure for urea determination**

The operation of urea FIA system was tested by the injection of 100 µL urea solution with concentration from 0.01 to 10 mM into the carrier stream (sodium phosphate buffer, 60 mM, pH opt for each immobilized system, presented in Table 1) under pressure 1.10⁵ Pa. The amount of the released NH₃ was determined spectrophotometrically with the addition of Nessler’s reagent after the membrane bioreactor.

**Analytical procedure for inhibitive determination of Cu(II) ions**

The urease inhibition of Cu(II) ions results in a reduction of the spectrophotometrical signal. In the first step of the assay the maximum absorption signal was detected as function of urease activity by permeation of urea stream (10 mM urea in 60 mM sodium phosphate buffer with pH opt for each immobilized system, Table 1) through the enzyme membrane reactor under pres-
TABLE 1

<table>
<thead>
<tr>
<th>№</th>
<th>Modification</th>
<th>Type of functional groups</th>
<th>Basic groups (mgeq g⁻¹)</th>
<th>Acid groups (mgeq g⁻¹)</th>
<th>Bound protein (µg cm⁻²)</th>
<th>pH opt</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>NaOH+EDA</td>
<td>-NH₂; -COOH</td>
<td>0.25</td>
<td>0.14</td>
<td>27</td>
<td>6.8</td>
</tr>
<tr>
<td>2.</td>
<td>NaOH+GA+EDA</td>
<td>-NH₂; -COOH</td>
<td>0.33</td>
<td>0.04</td>
<td>41</td>
<td>6.6</td>
</tr>
<tr>
<td>3.</td>
<td>NH₂OH.H₂SO₄</td>
<td>=N-OH₂; -NH₂</td>
<td>0.22</td>
<td>0.08</td>
<td>25</td>
<td>7.0</td>
</tr>
<tr>
<td>4.</td>
<td>H₂O₂</td>
<td>-CONH₂</td>
<td>0.22</td>
<td>-</td>
<td>24</td>
<td>5.4</td>
</tr>
<tr>
<td>5.</td>
<td>NH₂NH₂.H₂SO₄</td>
<td>=NH₂; -NHNH₂</td>
<td>0.13</td>
<td>0.08</td>
<td>26</td>
<td>6.8</td>
</tr>
<tr>
<td>6.</td>
<td>NaOH+DMAEM</td>
<td>-NR₃; -NH₂; -COOH</td>
<td>0.56</td>
<td>0.10</td>
<td>47</td>
<td>6.2</td>
</tr>
<tr>
<td>7.</td>
<td>NaOH+DMAEM+EDA</td>
<td>-NR₃; -NH₂; -COOH</td>
<td>0.62</td>
<td>0.03</td>
<td>48</td>
<td>5.8</td>
</tr>
</tbody>
</table>

sure 1.10⁵ Pa (necessary time about 2 min). Then the standard of Cu(II) ion (100 µL) was injected in urea stream. The immobilized enzyme was preincubated in the test solution for a definite time (10 min). After the inactivation, urea stream with pH opt was directed through the enzyme reactor for about 2 min. The maximum absorption signal as function of urease activity was detected again. The difference between the two absorption values was used to calculate the degree of urease inhibition by the metal ion.

**Results and Discussion**

**Urea determination**

The aim of the first investigation is to design a flow-injection system for analysis of urea using the seven immobilized systems. It was important to find which immobilized system would be the most suitable one for this analysis. Before carrying out the experiments with all the immobilization systems, the optimal flow rate for the analysis had to be found. For these experiments, urease immobilized onto the membrane modified with NH₂OH.H₂SO₄ was used. The substrate flow rate was varied from 0.6 to 1.4 ml min⁻¹. The analysis was carried out with 10 mM urea solution at the optimal temperature (30°C) and optimal pH (7.0) for the urease bound to the studied carrier. The results are shown in Fig. 2.

The highest absorption signal was measured at flow rate of 1 ml min⁻¹. It should be noted that this value significantly differs from the other values of flow rates studied. The volume of the injected urea sample was varied from 100 to 500 µL. The best FIA peak was observed with a sample volume of 100 µL. Similar effects of sample volumes were reported by other authors (17, 18). After that the experiments were carried out with all the immobilized systems studied (urease bound to various modi-
fied membranes) mounted consecutively in the flow apparatus and 100 µL urea solutions with concentrations from 0.01 to 10 mM were injected into the flow-injection system. The results are shown in Fig. 3.

For most of the systems studied, the calibration curves were linear in the concentration range from 0.01 mM to 1.25 mM. It should be noted that the slope of calibration curves for some of the urease bound carriers were higher, i.e. the system for urease analysis based on them would be more sensitive. These were the membranes modified with NaOH+DMAEM, NaOH+DMAEM+EDA and NaOH+GA+EDA. The higher sensitivity of these immobilized systems was supposed to be due to the higher content of bound protein (Table 1). The lowest sensitivity was observed for the membrane modified with NH₂NH₂.H₂SO₄. Hence, any of these three membrane systems should be used for analysis of urea because they would provide higher sensitivity and precision of the measurements. As can be seen from the Table 2, the correlation coefficient of linear intervals are R=0.99 and standard deviation about 3%, which guarantees good reproducibility of the urea analysis.

**Inhibitive determination of Cu(II) ions**

Further, experiments with the same immobilized systems were carried out under the same conditions as discussed above (flow cell, flow rate, temperature, pH, etc.) but Cu(II) ions were injected in the urea stream (10 mM urea in 60 mM sodium phosphate buffer with pH opt for each immobilized system: from 5.4 to 7). This particular urea concentration was used since, as it has been mentioned above, the absorption signal was highest and no substrate inhibition was observed. There was no precipitation of copper phosphate at pH values below 7. The experiments were carried out by analytical the procedure described above.

The calibration curves for determination of Cu(II) ions with urease immobilized on all types of modified membranes are presented in Fig 4. The calibration curves show that the systems with urease immobilized on the membranes modified with NaOH+EDA and NH₂NH₂.H₂SO₄ had the highest sensitivity to Cu(II). The slope of the calibration curves of the systems: urease-modified membranes with H₂O₂ was the highest, but the linearity was within narrow interval. The lowest detection limit of Cu(II) for urease immobilized on the membranes modified with NaOH+EDA, NaOH+GA+EDA, NH₂OH.H₂SO₄ and NH₂NH₂.H₂SO₄ was 0.1 mg l⁻¹ (Fig. 4, Table 3). It should be noted that these values are lower than these reported by some authors (7, 10) but higher than the values reported by others (5, 19). It was also found that the Cu(II) ion limit detection was higher for systems with modified membranes containing higher amount of amino groups and bound enzyme (modification with DMAEM and DMAEM+EDA, Table 1). Bryce et al. (17) noted that the sensitivity of heavy metal analysis should increase with the decrease of the amount of active enzyme, which followed from the inhibition kinetics with no respect of enzyme and inhibitor used. This fact was observed in our experiment. Other authors (9) reported that a properly chosen carrier type could considerably improve enzyme inhibitor sensitivity.

The linear range for an enzyme system with the membrane modified with NaOH+
EDA is from 0.1 to 1.0 mg l\(^{-1}\) Cu(II) (Table 3). Other authors, too, have reported the same range for analysis of heavy metal ions using flow injection analysis with immobilized urease (20). For the rest of the systems, linearity was within narrower interval, except for the system immobilized onto the membrane modified with NH\(_2\)OH.H\(_2\)SO\(_4\). In the latter case, the detection limit of Cu(II) was low as well – 0.1 mg l\(^{-1}\), but the calibration curve had smaller slope. Nevertheless, this system could be preferred for its wider linear range. It should be noted here that the influence of the matrix is different

**Table 2**

<table>
<thead>
<tr>
<th>№</th>
<th>Modification</th>
<th>Equation</th>
<th>(R^2)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>NaOH+EDA</td>
<td>(\text{A, } \text{AU} = 0.245 \text{ [urea]} + 0.0089)</td>
<td>0.9947</td>
<td>2.10</td>
</tr>
<tr>
<td>2.</td>
<td>NaOH+GA+EDA</td>
<td>(\text{A, } \text{AU} = 0.346 \text{ [urea]} + 0.0583)</td>
<td>0.9933</td>
<td>2.50</td>
</tr>
<tr>
<td>3.</td>
<td>NH(_2)OH.H(_2)SO(_4)</td>
<td>(\text{A, } \text{AU} = 0.3007 \text{ [urea]} + 0.0467)</td>
<td>0.9973</td>
<td>2.45</td>
</tr>
<tr>
<td>4.</td>
<td>H(_2)O(_2)</td>
<td>(\text{A, } \text{AU} = 0.255 \text{ [urea]} - 0.0067)</td>
<td>0.999</td>
<td>2.15</td>
</tr>
<tr>
<td>5.</td>
<td>NH(_2)NH(_2)H(_2)SO(_4)</td>
<td>(\text{A, } \text{AU} = 0.1867 \text{ [urea]} - 0.048)</td>
<td>0.9948</td>
<td>1.75</td>
</tr>
<tr>
<td>6.</td>
<td>NaOH+DMAEM</td>
<td>(\text{A, } \text{AU} = 0.3814 \text{ [urea]} + 0.0905)</td>
<td>0.9831</td>
<td>3.10</td>
</tr>
<tr>
<td>7.</td>
<td>NaOH+DMAEM+EDA</td>
<td>(\text{Abs} = 0.3415 \text{ [urea]} + 0.0704)</td>
<td>0.9956</td>
<td>2.90</td>
</tr>
</tbody>
</table>

**Fig. 3.** The calibration curves for urea determination by urease immobilized onto PAN membranes modified with: A) NaOH+EDA (□), NaOH+GA+EDA (△), hydroxylammonium sulfate (◇) and B) H\(_2\)O\(_2\) (●), hydrazinium sulfate (♦), NaOH+DMAEM (♦), NaOH+DMAEM+EDA (x)

**Fig. 4.** The calibration curves for Cu(II) determination by free (○) and urease immobilized onto PAN membranes modified with: A) NaOH+EDA (□), NaOH+GA+EDA (△), hydroxylammonium sulfate (◇) and B) H\(_2\)O\(_2\) (●), hydrazinium sulfate (♦), NaOH+DMAEM (♦), NaOH+DMAEM+EDA (x)
 TABLE 3

<table>
<thead>
<tr>
<th>№</th>
<th>Modification</th>
<th>Linear range (mg l⁻¹)</th>
<th>Equation of linear range</th>
<th>R²</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>NaOH+EDA</td>
<td>0.1÷1.0</td>
<td>I, % = 103.4 [Cu(II)] - 6.6</td>
<td>0.9976</td>
<td>2.15</td>
</tr>
<tr>
<td>2.</td>
<td>NaOH+GA+EDA</td>
<td>0.1÷0.75</td>
<td>I, % = 74.8 [Cu(II)] - 5.6</td>
<td>0.9996</td>
<td>2.05</td>
</tr>
<tr>
<td>3.</td>
<td>NH₂OH.H₂SO₄</td>
<td>0.1÷2.0</td>
<td>I, % = 39.2 [Cu(II)] - 0.4</td>
<td>0.9903</td>
<td>1.90</td>
</tr>
<tr>
<td>4.</td>
<td>H₂O₂</td>
<td>0.5÷0.75</td>
<td>I, % = 200 [Cu(II)] - 102</td>
<td>0.9999</td>
<td>4.55</td>
</tr>
<tr>
<td>5.</td>
<td>NH₂NH₂.H₂SO₄</td>
<td>0.1÷0.75</td>
<td>I, % = 102.3 [Cu(II)] - 6.7</td>
<td>0.9994</td>
<td>2.20</td>
</tr>
<tr>
<td>6.</td>
<td>NaOH+DMAEM</td>
<td>0.5÷1.5</td>
<td>I, % = 81.2 [Cu(II)] - 32.9</td>
<td>0.9985</td>
<td>3.50</td>
</tr>
<tr>
<td>7.</td>
<td>NaOH+DMAEM+EDA</td>
<td>0.5÷1.0</td>
<td>I, % = 78.2 [Cu(II)] - 35.9</td>
<td>0.9980</td>
<td>3.75</td>
</tr>
</tbody>
</table>

when analyzing urea and cupric ions. This confirms once again the effect of the carrier charge, which is due to its different interaction with urea and cupric ions when using the same type of immobilized system.

The calibration curve for the analysis of Cu(II) ion concentration obtained for free urease is presented in Fig. 4. The measurement conditions were optimal for free enzyme (60 mM sodium phosphate buffer, pH 5.8 (12), 28 ºC). The concentration of free urease (60 µg ml⁻¹, Table 1) was the average of all concentration values of the immobilized enzyme on all types of modified membranes. The results of the experiment unambiguously demonstrated the increase of the enzyme stability on immobilization against the metal ion inactivation. There are at least two causes for this: (1) the decrease of the thiol groups reaction ability (which are necessary for catalysis) after immobilization; (2) the reduction of the effective inhibitor concentrations due to binding of certain number of metal ions with the charge groups in the membrane.

The effect of ionic strength of solution over the inhibition of immobilized urease by heavy metal ions was investigated. It was found that the addition of 100 mM of NaCl into inhibitor solution did not lead to any notable changes in the sensor sensitivity.

Table 3 shows the equation describing the linear range of the calibration curve derived by the least squares method, the regression coefficient, linear range and precision expressed as standard deviation (%) for each immobilized system. The method reproducibility was determined from the standard deviation of 6 samples. The relative standard deviation of the biosensor did not exceed 4.5% for 6 continuous measurements.

An important issue is the possibility to restore the enzyme effectiveness after each analysis. The enzyme membrane was incubated in 80 mM EDTA for 5 min and detection response observed was found to be 100% of the initial one. The optimal incubation time of the metal ions sample with immobilized urease was studied preliminarily. For this purpose, the time was varied from 5 to 30 min. Loading times in excess of 10 min showed no increase in the levels of inhibition, and so a time of 10 min was chosen for determination of heavy metals concentration. The same results were obtained by other authors (10). In case of 40% inhibition of Cu(II) and soaking the biosensor in a 100mM EDTA solution for 5 min, 100% of initial sensitivity restored but in case of 80% inhibition-only 75%.

The operational stability of the flow enzyme system with the membrane modified with NaOH+EDA for analysis of Cu(II) was studied. The membrane with immobilized urease was kept nightly in contact with 1mM EDTA in 60 mM phosphate buffer solution (pH 6.8, 25 ºC). Four analyses were performed daily. The enzyme activity was preserved for 15 days. The investigated immobilized system reached 50% inhibition after 25 days.
### TABLE 4

<table>
<thead>
<tr>
<th>№</th>
<th>Linear range of Cu(II) (mg l⁻¹)</th>
<th>Detection</th>
<th>Inhib. time (min)</th>
<th>pH opt</th>
<th>References:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.13 ÷6.35</td>
<td>conductometric</td>
<td>15</td>
<td>7.4</td>
<td>(23)</td>
</tr>
<tr>
<td>2.</td>
<td>0.19 ÷0.64</td>
<td>pH metric</td>
<td>5</td>
<td>7.0</td>
<td>(10)</td>
</tr>
<tr>
<td>3.</td>
<td>0.006 ÷0.64</td>
<td>pH metric</td>
<td>30</td>
<td>7.0</td>
<td>(5)</td>
</tr>
<tr>
<td>4.</td>
<td>0.64 ÷6.4</td>
<td>Fluorometric</td>
<td>20</td>
<td>7.1</td>
<td>(7)</td>
</tr>
<tr>
<td>5.</td>
<td>0.1÷1.0</td>
<td>spectrophotometric</td>
<td>10</td>
<td>7.0</td>
<td>Modif. №1 in this paper</td>
</tr>
<tr>
<td>6.</td>
<td>0.1÷2.0</td>
<td>spectrophotometric</td>
<td>10</td>
<td>7.0</td>
<td>Modif. №3 in this paper</td>
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</table>

The stability of immobilized urease membrane (modified with NaOH+EDA) during storage was determined. The composition of the mixture used was 50% glycerol; 1 mM EDTA; 0.01% NaN₃ and 1 mM dithiothreitol in 60mM phosphate buffer, pH 6.8. Urea calibration curve was periodically registered. The FIA sensitivity was found to remain almost the same for 40 days. The basic characteristics of the flow-injection system for analysis of Cu(II) based on urease immobilized onto polyacrylonitrile membrane modified with NaOH+EDA (selection system) are shown in Table 4. Data from similar injections systems of other authors are also included for comparison. It was observed, that characteristics of our flow injection system were comparable with reported data.

**Preliminary application to water samples**

It was interesting to analyze the behavior of the flow system with immobilized urease on membrane modified with NaOH+EDA in samples containing a mixture of heavy metals and in real waters. First, experiments were carried out with model solution containing Cu(II), Zn(II) and Ni(II) in concentrations 3.53, 4.91 and 13.40 mg l⁻¹. These particular concentrations were selected because they correspond to the composition of water released from the galvanic facility of Metal-Varna Co. The results from the experiment are presented in Fig. 5. For comparison, samples containing the separate ion in the same concentrations were also analyzed. It should be noted that the registered inhibition affected by the model solution was slightly stronger than that of the sample containing Cu(II). It is known, that Zn(II) and Ni(II) were much weaker urease inhibitors than Cu(II), hence Cu(II) masked their inhibitory impact. It has been reported by some author (17, 21, 22). Other authors, who carried out experiments with mixtures of Hg (II), Cu(II) and Ni(II) ions observed that the degree of inhibition caused by the mixture was identical to that caused by the strongest inhibitor ion – Hg (II), (5). Therefore, this fact was confirmed in our case.

From practical point of view, the experiment carried out with real wastewaters from the galvanic facility of Metal-Varna Co was quite important. Using atomic absorption spectrophotometer Shimadzu AA-660, it was found that the sample contained 3.53 mg l⁻¹ Cu(II), 4.91 mg l⁻¹ Zn(II), 13.40 mg l⁻¹ Ni(II). The inhibition effect of the metals present in the real wastewater was identical to that of the metals in the model solution (Fig. 5). This result confirmed the potential capability of our flow injection system with immobilized urease on polymer membrane for determination of heavy metal ions.

**Conclusions**

In this study a FIA analysis of urea and copper(II) ions using the seven immobilized urease system was described. The most suitable carriers for creating of enzyme system, providing the highest sensitivity of FIA analysis of urea were the AN membrane modified with NaOH+DMAEM
and NaOH+DMAEM+EDA. It the same time the highest sensitivity of FIA analysis of copper(II) was observed with membranes modified with NaOH+EDA and NH$_2$NH$_2$•H$_2$SO$_4$. In these cases the lowest detection limit of Cu(II) was 0.1 mg l$^{-1}$. The correlation coefficient of linear intervals was R$=0.99$ and standard deviation about 4.5 %, which guaranteed good reproducibility of the copper(II) analysis. The inhibition effect caused by heavy metal ions in model solution and real waters, containing Cu(II), Zn(II), Ni(II) ions was also determined. It was found that the registered inhibition effect of the metals in the water was identical to that caused by the strongest inhibition ion – Cu(II) ions.

REFERENCES