ISSN 0233–7657. Biopolymers and Cell. 2014. Vol. 30. N 3. P. 209–215 doi: http://dx.doi.org/10.7124/bc.000898

UDC 577.1 + 573.6 + 543.393 + 543.556 + 004.942

Colorimetric biomimetic sensor systems based on molecularly imprinted polymer membranes for highly-selective detection of phenol in environmental samples

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Aim. *Development of an easy-to-use colorimetric sensor system for fast and accurate detection of phenol in environmental samples.* ***Methods***. *Technique of molecular imprinting, method of in situ polymerization of molecularly imprinted polymer membranes.* ***Results***. *The proposed sensor is based on free-standing molecularly imprinted polymer (MIP) membranes, synthesized by in situ polymerization, and having in their structure artificial binding sites capable of selective phenol recognition. The quantitative detection of phenol, selectively adsorbed by the MIP membranes, is based on its reaction with 4-aminoantipyrine, which gives a pink-colored product. The in- tensity of staining of the MIP membrane is proportional to phenol concentration in the analyzed sample. Phenol can be detected within the range 50 nM–10 mM with limit of detection 50 nM, which corresponds to the concentrations that have to be detected in natural and waste waters in accordance with environmental protection standards. Stability of the MIP-membrane-based sensors was assessed during 12 months storage at room temperature.* ***Conclusions***. *The sensor system provides highly-selective and sensitive detection of phenol in both model and real (drinking, natural, and waste) water samples. As compared to traditional methods of phenol detection, the proposed system is characterized by simplicity of operation and can be used in non-laboratory conditions.*

Keywords: phenol, molecularly imprinted polymer membranes, sensors, test-systems, colorimetry.

Introduction. Contamination of environment, including natural waters, foodstuffs and drinking water is one of the worldwide problems. Population upsurge, urbanization, as well as intensification of agricultural and industrial development resulted in a three-fold increase in water consumption. At the same time, these factors caused a significant deterioration of water quality. Phenols are widespread water pollutants. These compounds are widely used as antiseptics inhibiting bacterial and fungal growth in industrial water supply systems, in production of paper, some medical preparations, phenol-formaldehyde resins, synthetic fibers, and plastics [1].

Phenols present in environment influence animals and humans health. They can be adsorbed through skin, gastrointestinal tract, respiratory system and cause burns, edemas, and intoxication. Phenols cause acute lesions of central nervous system, liver, kidney, myocardium, blood, and other tissues. Moreover, phenol is an

endocrine disrupting compound, causing malfunction of endocrine system at very low concentrations [2]. Therefore, monitoring phenol content in water as well as development of easy-to-use and convenient methods for its rapid and accurate detetion is of great importance for analytical biotechnology. There are a number of traditional analytical methods of phenol detection, including HPLC [3], GC [4], these methods in combination with mass-spectrometry [5, 6], and spectrophotometric methods [7]. A number of biosensors were also proposed for phenol detection in aqueous samples [8, 9].

However, traditional instrumental methods don't provide a possibility of fast and effective in-field analysis, they are time-consuming and normally need complicated procedure of the sample pre-treatment, *i. e.* pre-concentration. Biosensors are recognized to be the most effective tools of modern analytical biotechnology. However, instability of selective elements based on natural receptors, antibodies and enzymes is a significant drawback, which limits their wide practical application. At the same time, biosensors and sensor systems based on molecularly imprinted polymers (MIPs) mimicking active sites of biological molecules can provide a promising alternative [10, 11]. For instance, MIP membranes-based sensors provide high selectivity and sensitivity of the assays as well as rapid and accurate analysis in non-laboratory conditions due to their extraordinary stability in extreme environments [12–14]. We have shown that MIP membranes are capable of selective recognition of target analytes and generation of the sensor response, which can be easily registered [15, 16].

The present research is aimed at synthesis of phenol-selective binding sites in the structure of free-standing MIP membranes and development of colorimetric sensor systems for phenol detection in drinking and environmental water samples.

Materials and methods. *Materials*. Acrylamide (AA), 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPSA), 4-aminoantipyrine, acetonitrile, ammonium hydroxide, N,N-dimethylformamide, itaconic acid (IA), ketal (2,2-dimethoxy-2-phenylacetone), *o*-cresol, *p*-cresol, N,N'-methylene-bisacrylamide (MBAA), methacrylic acid (MA), 2-nitrophenol, 3-nitrophenol, 4-nitrophenol, triethyleneglycoldimethacrylate (TEGDMA), polyethyleneglycol Mw 20 000 (PEG 20 000), pyrocatechol, potassium ferricyanide were purchased from («Sigma-Aldrich», USA). Oligourethaneacrylate (OUA) was synthesized according to [17] and kindly provided by Dr. Matyushov (Institute of Macromolecular Chemistry, Kyiv, Ukraine).

Synthesis of MIP membranes by in situ polymerization. MIP membranes capable of selective recognition of phenols were obtained through radical photo-initiated co-polymerization of a functional monomer (AA, AMPS, IA, MA), TEGDMA and OUA. Functional monomers with the highest binding to phenol were selected using computational modeling [16]. The ratio TEGDMA/OUA (85/15) in the monomer composition was optimized earlier [15]. Ketal (2,2-dimethoxy-2-phenylacetone) was used as an initiator of radical photopolymerization. To increase accessibility of phenol-selective binding sites in MIP membranes, they were formed according to the principle of semi-interpenetrating polymer network formation. A mixture of dimethylformamide (50 vol%) and PEG 20 000 (15 wt%) was used as a porogen. Molar ratios phenol/functional monomer in the initial mixture of monomers were 1:1, 1:2, 1:3, and 1:4. Typical mixture of monomers for the synthesis of phenol-selective MIP membranes contained 20 mg phenol, 55.3 mg IA (for the molar ratio 1:2), 293 mg TEGDMA, 51.7 mg OUA, 50 vol % DMF, and 0.5 wt% ketal. Monomer mixture was polymerized between two glass slides fixed at a distance 60 μm. Radical polymerization was initiated by UV-irradiation (λ = = 365 nm) and performed for 30 min. Blank membranes were synthesized from the same mixture of monomers, except for phenol. Template molecules and non-polymerized components were extracted from the fully-formed membranes with hot ethanol in Soxhlet apparatus for 8 h. Polymeric porogen (PEG 20 000) was removed by extraction in water for 8 h (until the constant weight of the samples was reached).

Calibration of the colorimetric sensor system for phenol detection. Samples of phenol-imprinted and blank membranes (1 × 1 cm) were used for the adsorption of phenol from 50 nM–10 mM standard phenol aqueous solutions. Phenol, which was selectively adsorbed by the binding sites in MIP membranes structure, was visualized after its interaction with 4-aminoantipyrine in alkaline media in the presence of potassium ferricyanide. The adsorption procedure was followed by wa-

shing with distilled water, containing 5 % acetonitrile. The membrane samples were wetted with the mixture of 2 % aqueous 4-aminoantipyrine and 10 % ammonium hydroxide (1/3). Then the samples were treated with 2 % aqueous $K_3[Fe(CN)_6]$, which resulted in immediate formation of a pink-colored staining with the intensity, proportional to phenol concentration in the analyzed solutions. Intensity of staining was estimated using «Scion Image J» software («Wayne Rasband, Inc.», USA).

Spectrophotometric detection of phenol. 180 μl of the 50 nM–10 mM standard phenol solution or analyzed aqueous sample, 60 μl of the mixture of 2 % aqueous 4-aminoantipyrine and 10 % NH_4OH (1:3) and 30 μl of 2 % aqueous $K_3[Fe(CN)_6]$ were mixed in the polystyrene microtiter plate wells. The absorbance values were measured at $\lambda = 450$ nm using microplate reader DYNEX Technologies (UK). All measurements were made in triplicate.

Results and discussion. Detection of phenol, which is selectively adsorbed by artificial receptor sites in the MIP membranes structure is based on its ability to form coloured complexes with 4-aminoantipyrine in alkaline media in the presence of potassium ferricyanide [18]. Intensity of the membrane staining is proportional to phenol concentration in the analyzed sample. To provide better accessibility of the receptor sites to phenol, MIP membranes were synthesized by *in situ* polymerization according to the principle of the semi-IPN formation [19]. Influence of the type of the functional monomer used for the membrane synthesis as well as molar ratio between the template and a functional monomer on analytical characteristics of corresponding sensor systems was investigated. General selectivity of the sensor systems and effectiveness of their application for phenol analysis in natural and waste waters was analysed.

It is widely recognized that binding energy between the template and functional monomers directly influence affinity and selectivity of artificial receptor sites in the resulting polymer. The method of computational modelling was demonstrated to be effective for the selection of the optimal functional monomers for both MIPs and MIP membranes synthesis [12, 16, 20]. According to our previous results [16], IA, AMPSA, AA, and MA, providing binding energies: –34.80 kcal/mol, –30.86 kcal/mol, –24.14 kcal/mol, and –23.17 kcal/mol, respectively are the best functional monomers for the

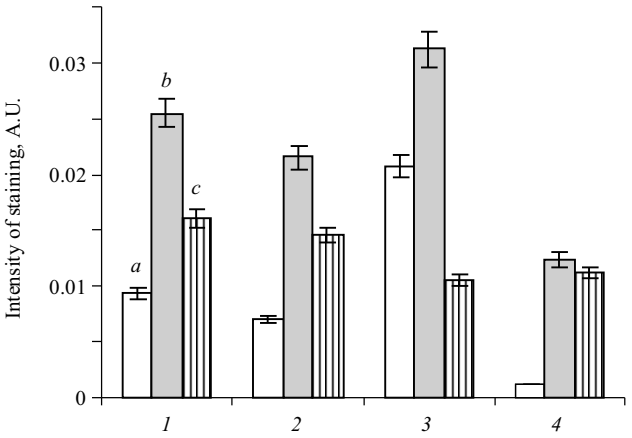

Fig. 1. Dependence of phenol selective adsorption on the type of a functional monomer used for membrane synthesis: *1* – acrylamide; *2* – methacrylic acid; *3* – itaconic acid; *4* – 2-acrylamido-2-methyl-1-propansulfonic acid (*a* – selective absorption; *b* – MIP; *c* – blank). Aqueous solution of phenol (500 μM) was used for the adsorption experiments

synthesis of the phenol-selective MIPs. These monomers were used in the present research for the MIP membranes synthesis.

It was shown that the MIP membranes formed using IA as a functional monomer were the most effective for the construction of the colorimetric sensor systems. These membranes revealed both the highest intensity of staining as compared to the MIP membranes synthesized with the other functional monomers as well as the highest levels of selective phenol adsorption (which were estimated as a difference in staining of MIP and corresponding blank membranes) (Fig. 1). Importantly, this result was in a good accordance with data of computational modelling [16]. According to these data, IA was shown to give the highest binding energy with phenol as compared to the other three functional monomers. At the same time the level of non-specific binding of phenol by the blank membranes was quite significant in all cases (Fig. 1).

First of all, it can be associated with the high levels of non-specific phenol adsorption by MIP and blank membranes caused by hydrophobic interactions. This also can be explained by the fact that the formation of selective binding sites requires multiple interactions between monomers and a template, which is difficult to achieve for monofuncitonal [21].

Since the best recognition properties were demonstrated for MIP membranes synthesized using IA, this

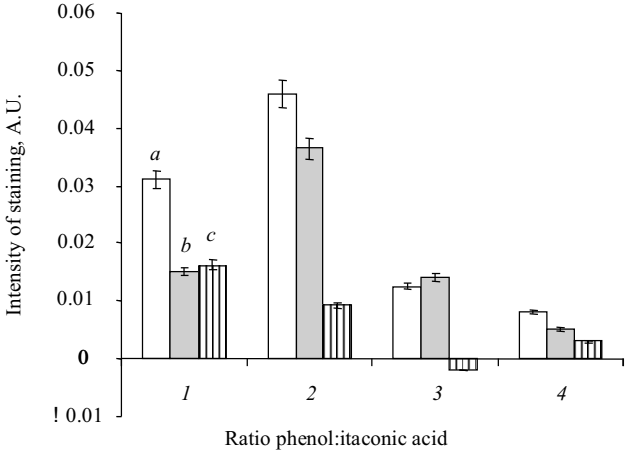

Fig. 2. Dependence of intensity of staining of MIP and blank membranes synthesized with itaconic acid as a functional monomer on the ratio of phenol:functional monomer in the monomer mixture: *1* – 1:1; *2* – 1:2; *3* – 1:3; *4* – 1:4 (*a* – MIP; *b* – blank; *c* – selective adsorption). Aqueous solution of phenol (500 μM) was used for the adsorption experiments

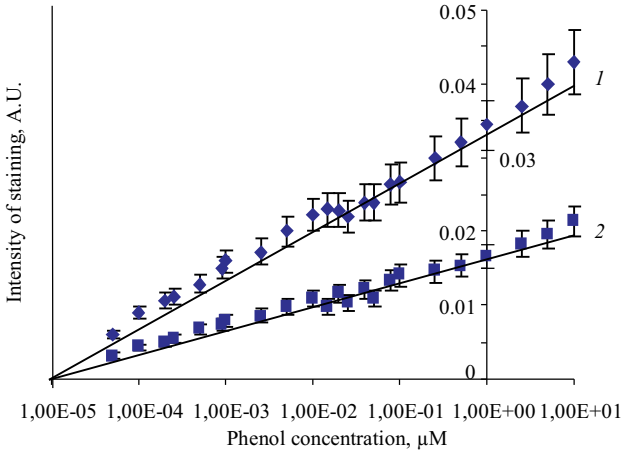

Fig. 3. Calibration plot of the colorimetric sensor system for phenol detection in aqueous samples: *1* – MIP membrane; *2* – blank membrane

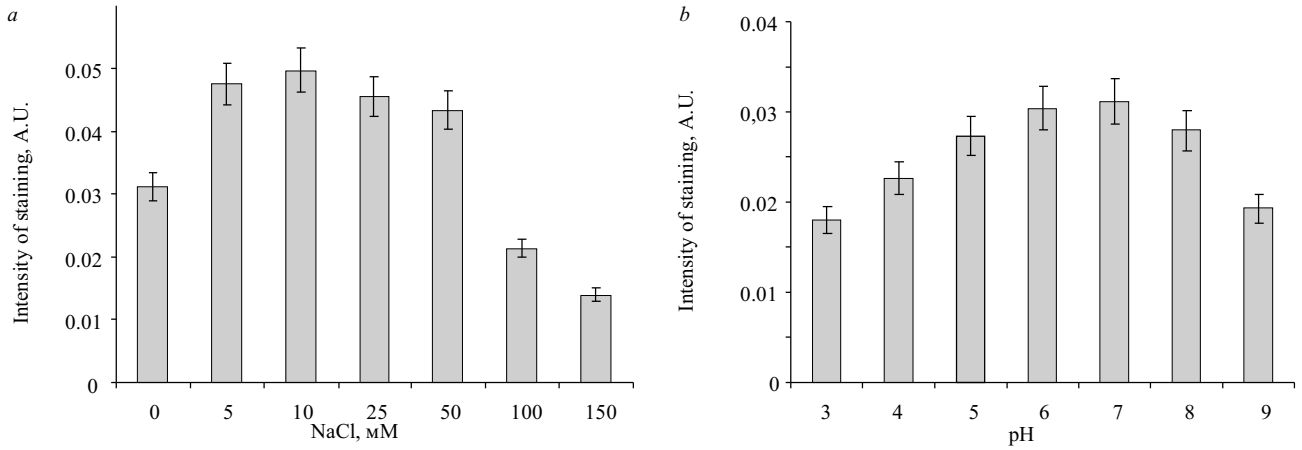

Fig. 4. Dependence of intensity of staining of phenol-selective MIP membranes synthesized using itaconic acid as a functional monomer on NaCl concentration (*a*) and on pH (*b*) of the analyzed sample. Aqueous 500 μM phenol solution was used for adsorption experiments

monomer was chosen for the further investigation. Theoretically, not all molecules of a functional monomer present in a monomer mixture are included in the specific binding sites. There is a balance between high concentrations of the monomers required to shift equilibrium in monomer mixture toward formation of monomer-template complex, and between impact of «free» monomers on the high level of non-specific binding in the resulting polymer. To optimize polymer specificity, a set of MIP and corresponding blank membranes was synthesized from the monomer mixtures with the different molar ratio phenol-IA (1:1, 1:2, 1:3, and 1:4). The ability of these membranes to adsorb phenol was analyzed by monitoring formation of the colored complexes on their surface. The optimal recognition properties were observed for the MIP membranes synthesized using 1:1 ratio phenol-IA (Fig. 2). Apparently, in the case of higher content of the functional monomer in the initial mixture, random distribution of the excess of functional groups on the membrane surface results in high levels of non-specific binding, which are not associated with the effect of imprinting.

Typical calibration curve of the developed colorimetric sensor system is shown in Fig. 3. It was demonst-

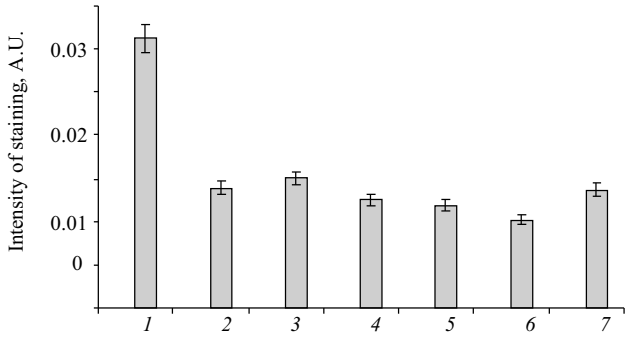

Fig. 5. Cross-reactivity of the colorimetric sensor system based on MIP membranes. Aqueous solutions (500 μM) of phenol and its analogues were used for the adsorption experiments: *1* – phenol; *2* – 2; *3* – 3-nitrophenol; *4* – 4-nitrophenol; *5* – *p*-cresol; *6* – resorcinol; *7* – pyrocatechol

fect the adsorption capability of the MIP membranes (Fig. 4 *a*).

However, the further increase in salt concentration up to 150 mM caused a significant decrease in sensor response values.

The influence of pH of the analyzed sample on value of the sensor response was also studied. Since pH of natural waters varies from acidic (pH = 3) to alkaline (pH 9), influence of the sample pH on sensor responses was investigated in the pH range from 3 to 9. It was shown that the most effective phenol binding was achieved at pH 6– 8, which corresponds to pH of river and lake water (Fig. 4 *b*).

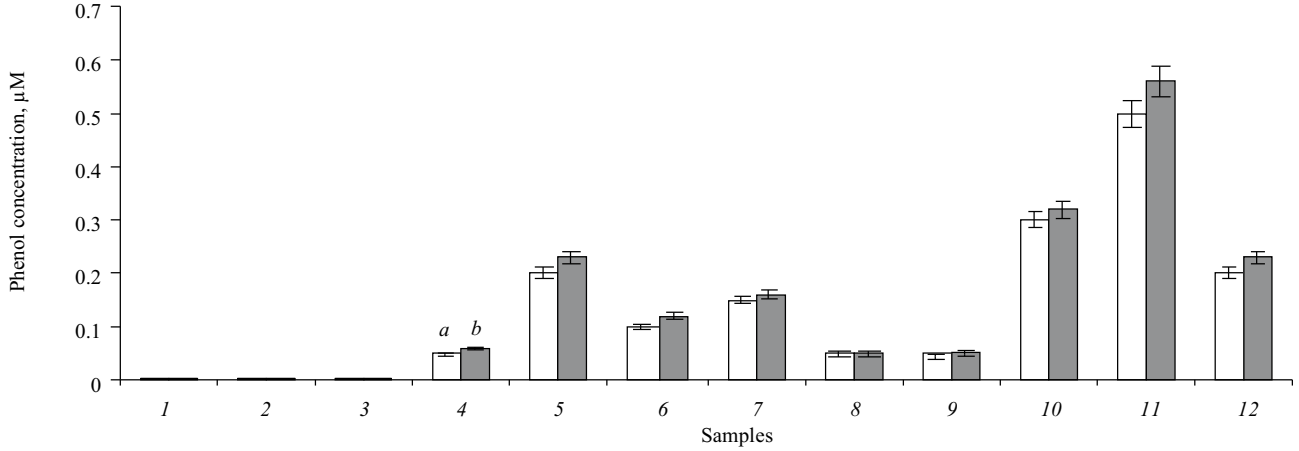

Fig. 6. Results of phenol detection obtained by the colorimetric sensor system based on MIP membranes (*a*) and traditional spectrophoto- metric method (*b*) in samples of tap, natural, and waste waters: *1* – borehole «Troyanda», Baryshivka, Kyiv region; *2* – tap water, Kyiv; *3* – source «Dubky», Kyiv; *4* – river water, river Syrets; *5* – milk plant «Ichnya», waste waters; *6* – pond «Ichnya»; *7* – Kyiv water channel, incoming water; *8* – Kyiv water channel, outcome water; *9* – Ukrainian Research Institute «UkrNIIPlastmash», waste waters; *10* – river Vita, v. Pyrogiv, Kyiv region; *11* – filtrate of the city dump (v. Pyrogiv, Kyiv region); *12* – river Stugna, Vasylkiv, Kyiv region

rated, that under optimized conditions, a significant difference between intensity of staining of MIP and blank membranes was observed. This indicates that phenol binding to MIP membrane is mainly determined by the presence of phenol-selective artificial receptor sites, confirming imprinting effect. The detection limit for phenol was estimated as 50 nM, while the detection range of the sensor system comprised 50 nM–10 mM.

Since the main working characteristics of biosensors are often significantly influenced by the composition of the analyzed sample, influence of ionic strength of the samples on capability of the biomimetic sensors to effective phenol binding was investigated. It has been shown that the increase in NaCl concentration in the analyzed sample up to 50 mM did not significantly af-

General selectivity of the colorimetric sensor systems based on MIP membranes was investigated using phenol structural analogues – 2-nitrophenol, 3-nitrophenol, 4-nitrophenol, *p*-cresol, resorcinol, and pyrocatechol. In all cases the developed sensor system possessed enhanced selectivity towards phenol (Fig. 5).

The developed colorimetric sensor systems were tested for phenol detection in both model and real environmental samples (drinking, natural and waste waters). It was demonstrated that the composition of the analyzed samples had insignificant influence on the accuracy of phenol detection using MIP membranes. The results of phenol detection using sensor method were in a good accordance with the results obtained using traditional spectrophotometric method (Fig. 6).

The stability of the MIP-membrane-based sensors stored at room temperature for 12 months was assessed, showing negligible changes in their performance during this period. As compared to the traditional instrumental analytical methods the developed system is highly-sensitive, easy-to-use, and can provide express-analysis of phenol content in water in real analytical applications. As compared to the existing biosensor methods of phenol detection, the proposed sensor system provides similar sensitivity and significantly higher storage stability.

Conclusions. Free-standing MIP membranes capable of highly-selective phenol binding were synthesized by *in situ* polymerization and their composition optimized. The developed membranes were used in easy-to-use and inexpensive colorimetric sensor system for phenol detection in environmental samples. Their performance was characterized by low detection limit (50 nM), and wide detection range (50 nM–10 mM). The sensor system demonstrated high selectivity towards phenol and revealed relatively low binding of its structural analogues. The sensor system was shown to be effective for phenol detection in environmental samples (natural and waste waters), with the results of the detection in a good accordance with those obtained by the traditional spectrophotometric method.

Acknowledgement. Financial support from National Academy of Sciences of Ukraine (Programme «Sensors for medical-ecological and industrial purposes: metrological attestation and applications») is gratefully acknowledged.

Колориметричні сенсорні системи на основі полімерів-біоміметиків для високоселективного визначення фенолу у довкіллі

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Резюме

Мета. *Розробка простих у використанні колориметричних сенсорних систем для швидкого і точного визначення фенолу у зразках із довкілля.* ***Методи***. *Метод молекулярного імпринтингу, метод полімеризації in situ молекулярно імпринтованих полімерних (МІП) мембран.* ***Результати***. *Запропонований сенсор створено на основі МІП мембран, синтезованих методом полімеризації in situ, які мають у своїй структурі штучні рецепторні сайти зв'язування фенолу. Кількісне визначення фенолу, селективно адсорбованого МІП мембранами, грунтується на детекції забарвленого у малиновий колір продукту його реакції з 4-аміноантипірином. Інтенсивність забарвлення МІП мембран є пропорційною концентрації фенолу в аналізованому зразку. Фенол детектується у діапазоні 50 нМ–10 мМ, що відповідає концентраціям, які необхідно виявляти у природних і стічних водах. Стабільність сенсорних систем на основі МІП мембран становить12 місяців за кімнатної температури.* ***Висновки***. *Сенсорні системи забезпечують високоселективний і чутливий аналіз фенолу як у модельних, так і реальних зразках (питна, природна, стічна вода). Порівняно до традиційних методів визначення фенолу пропонована система є простою у використанні та може бути застосована за польових умов.*

Ключові слова: фенол, молекулярно імпринтовані полімерні мембрани, сенсори, тест-системи, колориметрія.

Колориметрические сенсорные системы на основе полимеров-биомиметиков для высокоселективного определения фенола в окружающей среде

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Резюме

Цель. *Разработка простых в использовании колориметрических сенсорных систем для быстрого и точного определения фенола в образцах из окружающей среды.* ***Методы***. *Метод молекулярного импринтинга, метод полимеризации in situ молекулярно импринтированных полимерных (МИП) мембран.* ***Результаты***. *Предложенный сенсор создан на основе МИП мембран, синтезированных методом полимеризации in situ, имеющих в своей структуре синтетические рецепторные сайты связывания фенола. Количественное определение фенола, селективно адсорбированного МИП мембранами, основано на детекции окрашенного в малиновый цвет продукта его реакции с 4-аминоантипирином. Интенсивность окрашивания МИП мембран пропорциональна концентрации фенола в анализируемом образце. Фенол можно детектировать в пределах 50 нМ–10 мМ, что соответствует концентрациям, которые необходимо выявлять в природных и сточных водах. Стабильность сенсорных систем на основе МИП мембран составляет 12 месяцев при комнатной температуре.* ***Выводы***. *Сенсорные системы обеспечивают высокоселективный и чувствительный анализ фенола как в модельных, так и реальных образцах (питьевая, природная и сточная вода). По сравнению с традиционными методами определения фенола предложенная система проста в использовании и может применяться в полевых условиях.*

Ключевые слова: фенол, молекулярно импринтированные полимерные мембраны, сенсоры, тест-системы, колориметрия.

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Received 15.03.14