Molecularly Imprinted Membranes for Selective Separations

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Knowing that copolymers of acrylonitrile (AN) with acrylic acid (AA) can be used for the molecular imprinting, three AN: AA copolymers were synthesized by radical copolymerization in emulsion without emulsifier. The copolymer and diosgenin were dissolved in dimethylformamide (DMF) in order to obtain casting solutions with various polymer and template concentrations. The influence of the copolymer and template on the rheological behavior of the solutions was studied in order to establish the best parameters for the casting solutions preparation. The polymer solutions were transformed in membranes by phase inversion in water at room temperature (20°C). By casting the polymer solutions on a glass surface and drawing down with a scraper, a 200 μm thickness film resulted. The diosgenin was extracted from the membrane structure with ethanol, methanol or chloroform in an ultrasonic bath. The molecular imprinting was proved by HPLC on liquid (the extracting solvent after the membrane extraction) and solid phases (after dissolution of membrane parts in DMF). Comparing the sorption capacities of the imprinted materials, for two steroids (diosgenin and stigmasterol), the membrane selectivity towards the template was proved.

Keywords: imprinted polymer, molecular recognition, solid phase extraction, bioactive substances, phase inversion

The discovery of new phenomena and processes at nanometric scale provides science with a large area of instruments, materials, devices and systems with unique features.

A molecularly imprinted polymer is a polymer that formed in the presence of a molecule (called template) that is extracted afterwards, thus leaving complementary molecular cavities behind with specific electronic surroundings. The affinity for the target molecule suggests that they can be used in applications of advanced separations, biosensors, the mechanism being similar to antibodies and enzymes.

Since being pioneered [1], molecular imprinting has become an effective way to prepare polymer materials that show a "memory effect" toward their templates and has been extensively studied by several groups [2-6]. Molecular imprinting can also be considered as the selective manipulation of the shape, size and chemical functionality of a polymer matrix by a template molecule. The synthesis and processing capability of the nanoparticles with tailored structure and improved properties ensures tremendous perspectives for biomedical, environment and analytical applications: mimics for biological receptors [7], recognition of elements in sensors [8], stationary phases for chromatography [9] and solid phase extraction [10], micro extraction fibers [11], catalysis [12], molecularly imprinted membranes (MIM) [4], porous and imprinted membrane for dialysis [13]. The general principles, areas of applications and limitations of imprinted polymers have been extensively reviewed in the last years [14, 15].

Imprinting may be achieved by 2 approaches: polymerization and phase inversion [16].

The polymerization is conducted in a solvent (porogen) which facilitate the formation of template - monomer complex by stabilization of interactions [17]. This complex is then fixed into a spatial arrangement by the inclusion of a high proportion of cross-linking monomer, which confers rigidity to the polymer network. Removal of the template species affords nano-cavites, which are complementary in size, shape and chemical functionality to the templated species. These cavities have the ability to selectively rebind the template [18].

In the phase inversion approach the template is incorporated into the polymer matrix by phase inversion [19 - 22]. Removal of the template affords a cavity, which is complementary in size, shape and functionality to the template molecule. The phase inversion method has the advantage that it starts from an already prepared polymer. The main problems that have to be solved in this case are finding a good solvent common for the matrix copolymer and for the imprint and finding an optimum composition for the coagulation bath, so that the imprint diffusion in the bath or the chemical alteration would not take place.

The most common method for preparing molecularly imprinted polymers suitable for molecularly imprinted solid phase extraction (MISPE) consists in bulk thermal- (or photo-) polymerization that produces a monolithic polymer that has to be crushed and sieved to obtain particles of the desired size distribution. This method, by far the most popular, presents several attractive properties. It is fast and simple in its practical execution, it does not require particular skills of the operator, it is widely reported in literature for many different templates and it does not require sophisticated or expensive instrumentation [23].

However, the procedure of grinding and sieving is difficult, and it causes a substantial loss of useful polymer. Most of the lost polymer is a very fine sub-micrometric powder, which could adhere to the bigger particles and cause excessively high backpressures in a SPE column during the extraction procedure, especially with on-line devices. Moreover, the bulk polymerization cannot be scaled-up.

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Recently, the preparation, morphology and diffusive permeability of MIP membranes have aroused increasing attention [24, 25]. The ability of MIP membranes to change their diffusive permeability automatically by responding to the presence of template molecules is the most interesting phenomenon. MIP membranes may be applicable as novel separation devices, chemical sensors with high stability and selectivity, drug delivery systems (DDS) with molecular recognition and biomimetic membranes. However, it is desirable to increase the selectivity of these membranes to make them suitable for practical application. In order to achieve this aim it is necessary to improve the understanding of the basic nature and recognition mechanism of MIP membranes by preparing MIP membranes with the use of different functional monomers.

H-Indole-3-acetic acid (IAA) is a plant hormone that exists in most plants and regulates growth and development in plants. An IAA-imprinted polymer with *N*,*N*- dimethyl-aminoethyl methacrylate as the functional monomer in chloroform was prepared [26].

Also 9-Vinyladenine was synthesized as a novel functional monomer for molecular imprinting techniques [27].

Methacrylic acid (MAA) is the most widely used functional monomer in molecular imprinting. However, because of the weak hydrogen-bonding interactions between MAA and some template molecules in polar solvents, the MIPs made with the use of MAA in polar solvents exhibited only very weak recognition [26] and in some cases no recognition at all [5, 28].

The molecularly imprinted polymers recognize the template molecule by polar bonds, but it was proved that even the non-polar bonds can be used, although these are, in general, weaker than the polar ones. For the polar interactions the target molecule must possess functional groups, more often acid or base. Using the non-polar interactions, other molecules, like polyaromatic hydrocarbons, which are plane molecules, without functional groups [29] can be imprinted.

Apart from the more obvious recognition properties of molecularly imprinted polymers, their physical and chemical characteristics are highly appealing. These materials exhibit high physical and chemical resistance towards various external degrading factors. Thus, molecularly imprinted polymers are remarkably stable against mechanical stress, elevated temperatures and high pressures, resistant against treatment with acid, base or metal ions and stable in a wide range of solvents. The storage endurance of the polymers is also very high: storage for several years at ambient temperature leads to no apparent reduction in performance. Further, the polymers can be used repeatedly, in excess of 100 times during periods of years without loss of the "memory effect". In comparison with natural, biological recognition sites, which are often proteins, these properties are highly advantageous [30].

As economical, rapid and selective clean-up methods (relying on "intelligent" materials) are needed, solid phase extraction and clean-up methods based on molecularly imprinted polymers (MISPE) seem to represent natural candidates. Recent years have seen a significant increase of the MISPE technique in the food contaminant analysis [23]. In fact, this technique seems to be particularly suitable for extractive applications where analytic selectivity in the presence of very complex samples represents the main problem.

In this study, a procedure for diosgenin molecularly imprinted membranes obtaining was elaborated, using the phase inversion method [16, 19]. The chosen method for this type of MISPE was phase inversion, because of the above presented advantages over the polymerization method. The chosen substance to be imprinted in was diosgenin. Diosgenin, a steroid sapogenin, is the product of hydrolysis by acids, strong bases, or enzymes of saponins. It has an estrogenic bioactivity [31] and it can reduce the cholesterol from blood. It can be transformed in pregnelonone and progesterone. It is extracted especially from Dioscorea species. However the extracts are very complex mixtures, from which the separation by classic methods is very laborious. This is the reason for proposing the use of the molecularly imprinted polymers, known for their high selectivity.

Experimental part

In order to obtain molecularly imprinted polymers with diosgenin, by phase inversion, the next steps were followed [19].

The acrylic copolymer preparation

Knowing that copolymers of acrylonitrile (AN) with acrylic acid (AA) [20, 32] can be used for the molecular imprinting, three AN: AA copolymers were synthesized, by radical copolymerization of the acrylonitrile with acrylic acid, in aqueous environment, initiated with the redox system: potassium persulfate – sodium metabisulfite. In the case of other AN copolymers it was proved that this kind of reaction is a radical copolymerization in emulsion without emulsifier [6, 33, 34].

The synthesis recipe was (mass percent): monomers concentration: 15%, potassium persulfate (PK) concentration (calculated based on monomers): 0.5%, sodium metabisulfite (MS) concentration (relative to the monomers): 0.5%, H_2SO_4 concentration (relative to the monomers): 0.3%, polymerization temperature: 45°C, polymerization time: 90 min.

The weight ratio between monomers was 70:30, 80:20 or 90:10. The addition order of the chemicals in the reactor was: water, sulfuric acid, AN, AA and then MS. Nitrogen was sparged into solution for 10 – 15 min to remove oxygen, which is an AN polymerization inhibitor. Then PK solution was added, the flask was introduced in the thermostated bath and mixing started. After 90 min, the reaction was stopped.

The obtained AN: AA copolymers were analyzed for chemical composition (by Kjeldal method) and relative viscosity (0,3% copolymer in DMF at 25°C).

Imprinting the membranes

The copolymers prepared above were used to cast DMF solutions, having 8, 10 and 12% concentration (copolymer in DMF) and 4 and 5% diosgenin concentration (based polymer). The dissolution of the copolymer lasted 45 min while that of diosgenin 15 min, both at 70 – 75°C. The casting solutions were rheologically characterized on a Rheotest 2 apparatus (Germany) having coaxial cylinders.

Preparation of membranes

The polymer solutions were casted at room temperature onto a glass plate (70 x 100 x 4 mm) and a draw-down technique was used to produce films of 200μm wet thickness. This film was quickly immersed in a water coagulation bath.The phase inversion occurred at room temperature.

Extracting the template

In order to extract diosgenin (the template molecule), the membranes were introduced in a beaker placed in an ultrasonic bath and left 60 min for extraction with methanol, ethanol or chloroform at a solid: liquid ratio 1: 100. After

extraction the membranes were maintained in distilled water until using.

In order to analyze the solid phase, the polymer membranes were dried under air after sorption, and then were dissolved in DMF, to obtain a 1% solution.

HPLC analyses for the liquid phase were performed on a Varian Pro Star instrument, with Inertsil ODS 3 C18 25 cm x 4.6 cm column, UV-Vis detector at λ = 193 nm, mobile phase acetonitryl 90% / water 10%, flow 1 mL/min and sample volume 25µL.

HPLC analyses for the solid phase performed on an Agilent Technologies 1200 series instrument with PLGel MiXED C column, refractive index detector (RID), using DMF as mobile phase and a 1 mL/min flow.

In order to compare the sorption capacities of the two steroids (diosgenin and stigmasterol) upon the imprinted materials, the retention time and the corresponding area for a 0.1% steroid solution in DMF were determined.

Results and discussions

The obtained AN: AA copolymers presented the chemical composition and relative viscosities presented in table 1.

In order to analyze and compare the obtained solutions, the study of their rheological behaviour was used as investigating method. Preliminary rheological tests revealed that the copolymer prepared from the monomers mixture AN:AA 90:10 does not have the desired rheological behaviour. So that only two types of copolymers were included in the study (70:30 and 80:20 AN:AA). Three different concentrations of copolymer in DMF (8, 10 and 12%) and two different concentration of diosgenin were tested (4 and 5%). The influence of diosgenin concentration at 25°C, on the rheological behaviour of a 10% DMF solution, for the 80:20 AN: AA copolymer is shown in figure 1. One can observe that by adding diosgenin in copolymer solution, the average dynamic viscosity is rising and the dilatant flow character is being amplified in the small share rates range. At big shear rate range the solution behaviour is light pseudo plastic, almost newtonian.

The obtained membranes have a porous surface (fig. 2) and an asymmetric structure.

The presence of diosgenin in the membrane after phase inversion was proved by analyzing the liquid phase by HPLC with UV-Vis detector. The liquid phase means the solvent after the extraction. The extraction of diosgenin can be performed with ethanol, methanol and chloroform. Firstly the retention time of diosgenin was determined by HPLC of ethanol p.a. and of a 0.05% diosgenin in ethanol p.a. (fig. 3). The retention time for diosgenin is almost the same in methanol, chloroform and ethanol (fig. 4).

From figure *5* it seems that is sufficient only one operation of extraction to remove the most important quantity of diosgenin from the membrane.

The demonstration for the membrane's molecular imprinting with diosgenin was performed also by analyzing the solid phase (parts of the copolymer membrane). For this aim, parts of the membranes at different stages of the process were dissolved in DMF and were analyzed by HPLC method. Firstly it was determined the chromatogram for a copolymer – DMF solution (fig.6). The copolymer has a retention time of 5.5 ±0.2 min.

Secondly, the diosgenin signal was found at 10.5 ±0.2 minutes (fig.7 and 8) and for stigmasterol at 11.5 ±0.2 minutes (fig. 8*).* Stigmasterol is a bioactive compound from the same class as diosgenin (steroids), chosen in order to test the solid phase extraction (SPE) selectivity of the imprinted cavities for the template molecule.

After extracting the membrane with ethanol, the diosgenin signal is decreasing, but does not disappear

Table 1
COMPOSITIONS AND RELATIVE VISCOSITIES FOR AN: AA COPOLYMERS

No.	Compositions of the AN:AA monomers mixture, % (mass)	Compositions of the AN:AA copolymer, % (mass)	Relative viscosities
1	70: 30	71.5: 28.5	1.93
2	80: 20	79: 21	1.84
3	90: 10	86: 14	1.68

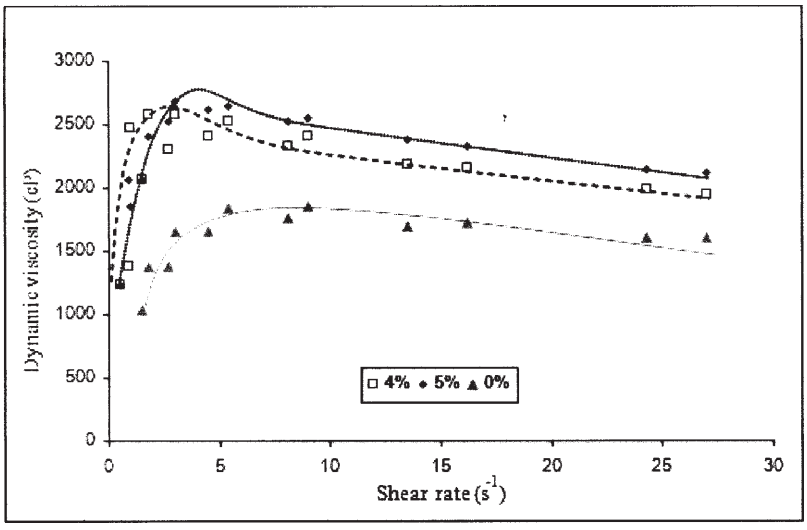

Fig. 1. Rheological behavior at 25°C of the copolymer solutions (80AN: 20AA), with 10% polymer concentration in DMF and different quantities of diosgenin (0, 4 and 5%- calculated to polymer)

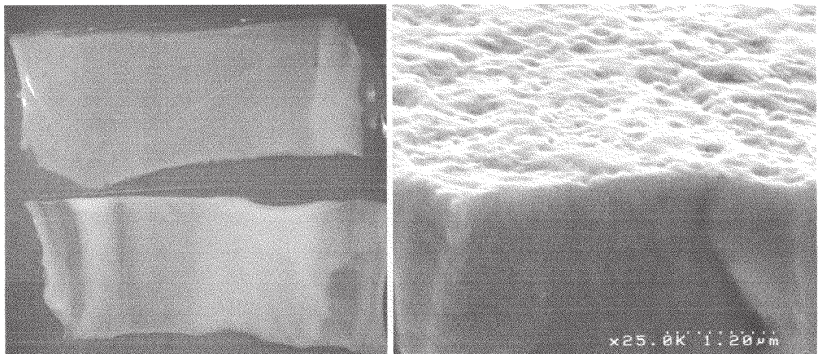

Fig. 2. Membranes obtained from copolymer solution (80AN: 20AA) in DMF with 10% polymer concentration and 4% diosgenin and a SEM photography

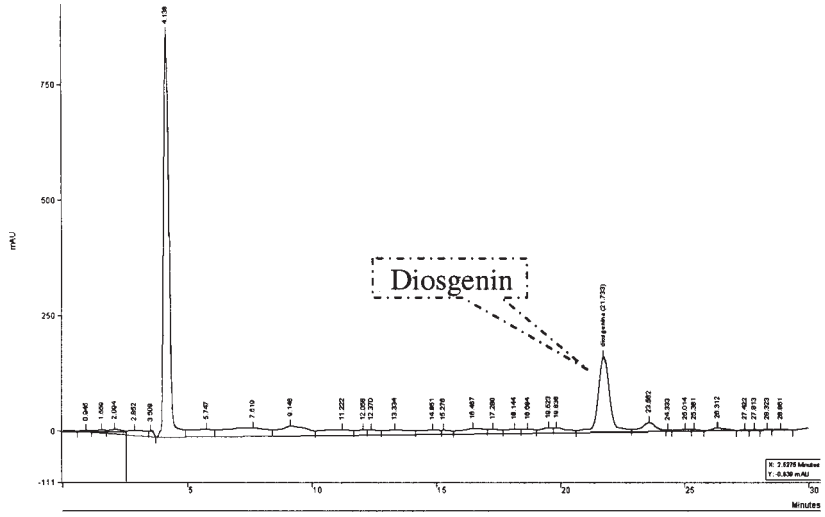

Fig. 3. HPLC chromatogram of the ethanol (white) and of a 0.05% diosgenin solution in ethanol (black)

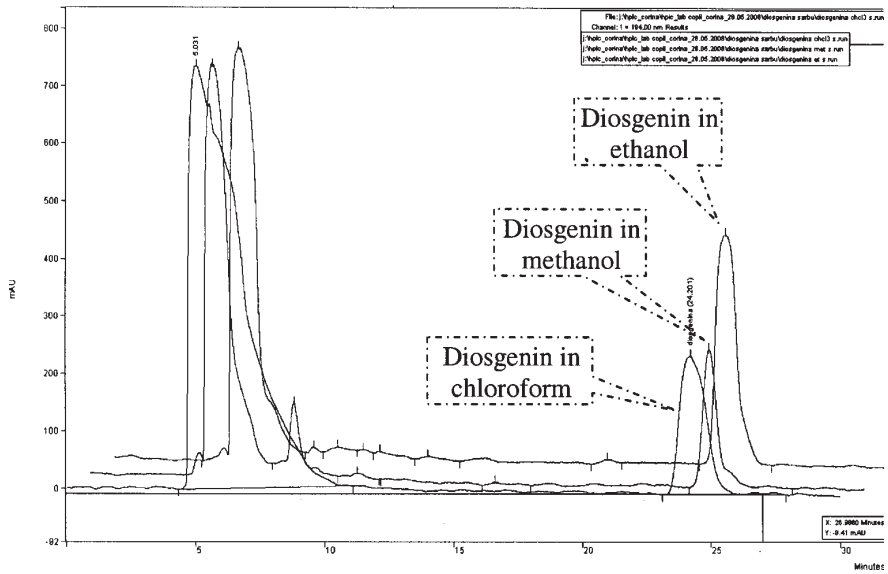

Fig. 4. HPLC chromatograms of the chloroform, methanol and ethanol solution after the extraction of polymer membranes obtained from 10% DMF solution of copolymer with 5% diosgenin

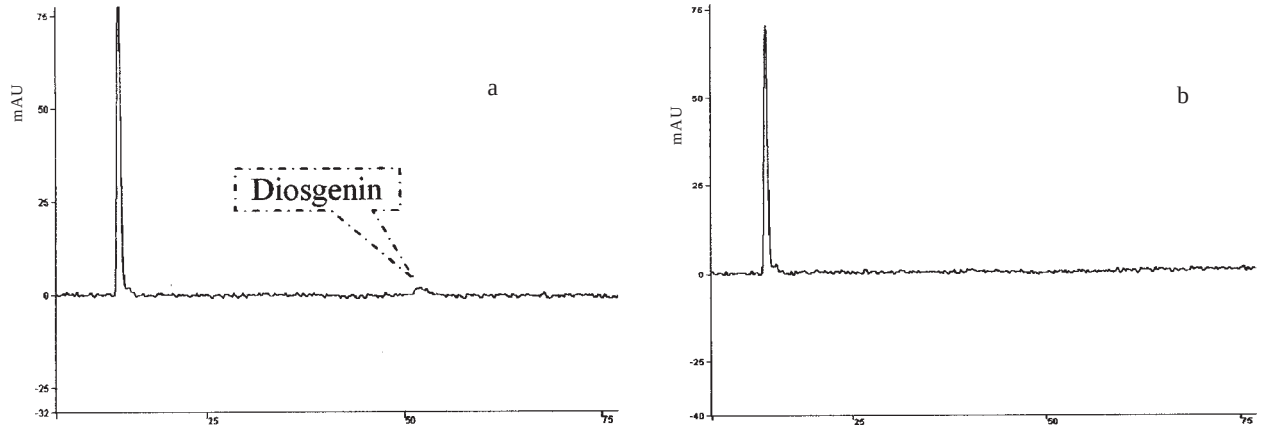

Fig. 5. Ethanol solution after the first (a) and second extraction (b) of the imprinted membrane

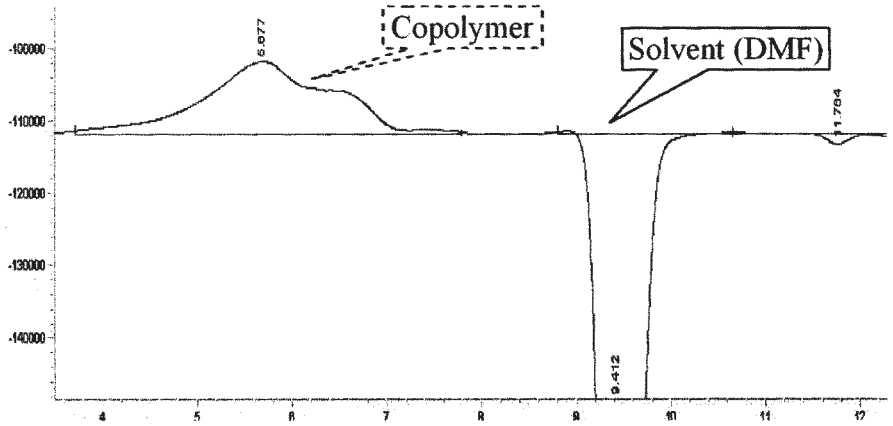

Fig. 6. HPLC chromatogram of a 1% solution AN:AA copolymer in DMF

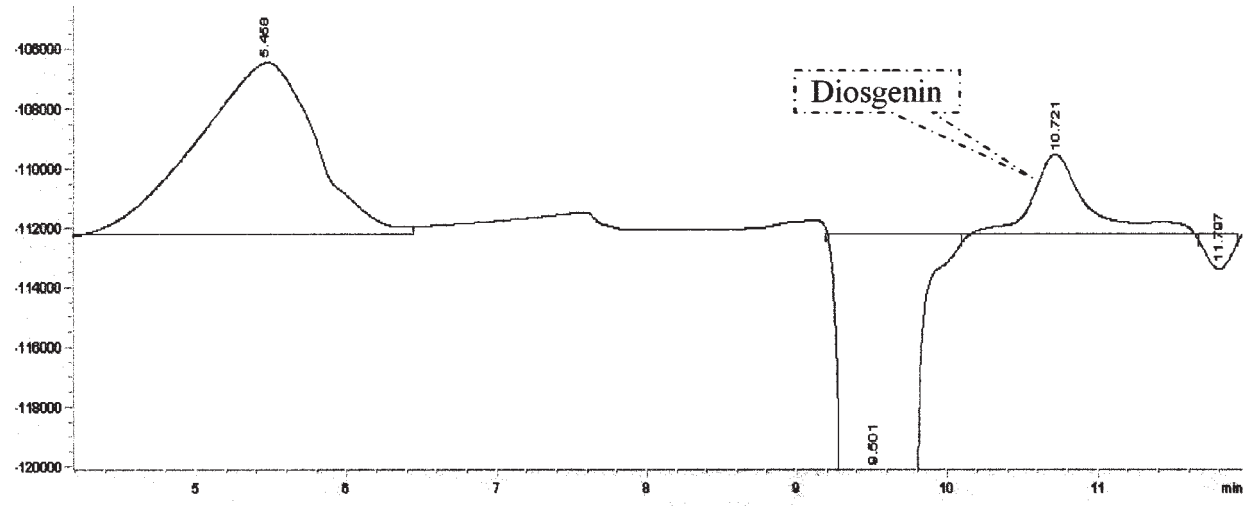

Fig. 7. HPLC chromatogram of a 1% solution polymer imprinted with diosgenin, before extraction

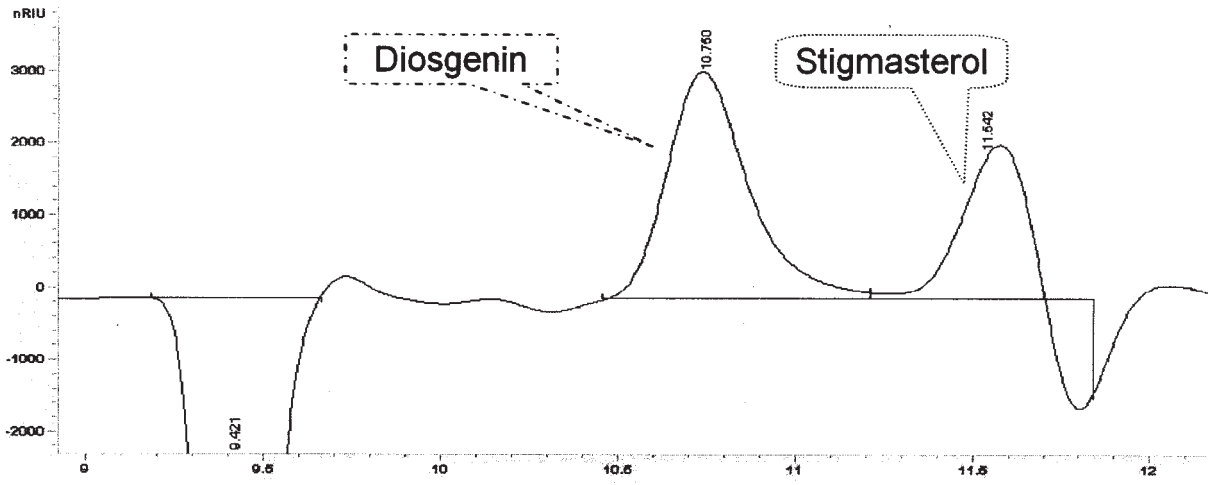

Fig. 8. HPLC chromatogram for a solution 0.5% diosgenin – 0.5% stigmasterol in DMF

suggesting that some diosgenin is retained into the internal pores (fig. 9).

The SPE capacity of the unimprinted membranes was also analyzed. The result was that the diosgenin is low extracted from a prepared solution (diosgenin in ethanol).

The selectivity of the imprinted cavities is revealed by the fact that, from a diosgenin – stigmasterol solution (fig. 8), only the diosgenin is well extracted (fig. 10).

Based on the peak areas coresponding to diosgenin from HPLC determinations, was evaluated that after phase inversion, about 72% from the diosgenin existing in the initial casting solution was found in the polymeric membranes. After the extraction with ethanol, about 25% diosgenin from the initial solution is still inside the membranes, meaning 35% from the diosgenin present in membrane at the imprinting step. As the HPLC studies with UV-VIS detector showed (fig. 5) that in the second extraction the diosgenin is not present anymore in the liquid phase, we presume that a part of the template is traped inside the closed pores and this is the reason why diosgenin can not be extracted anymore.

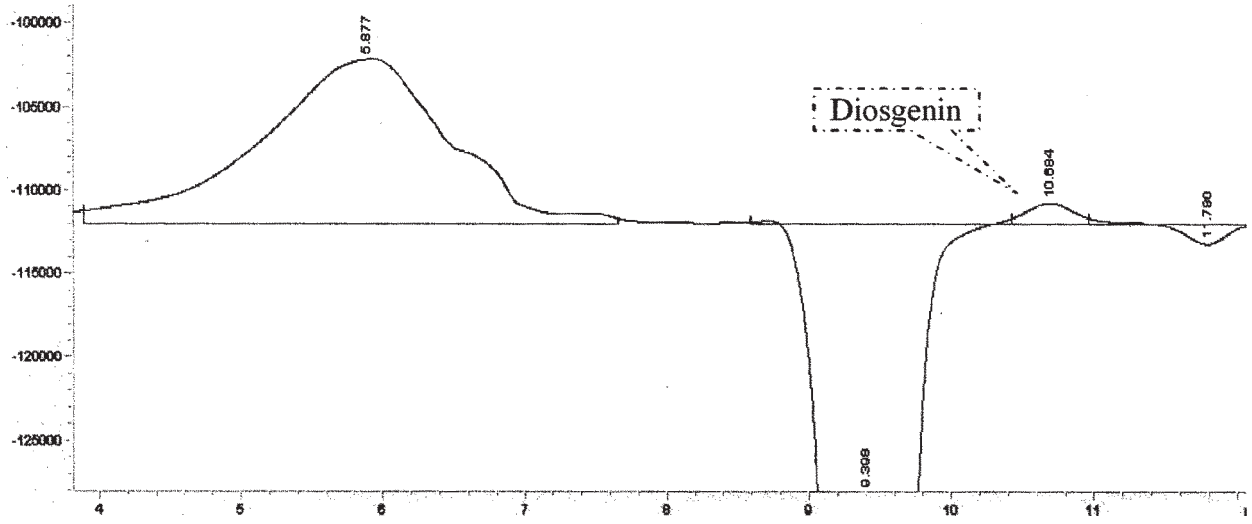

Fig. 9. HPLC chromatogram of a 1% solution polymer imprinted with diosgenin, after the extraction

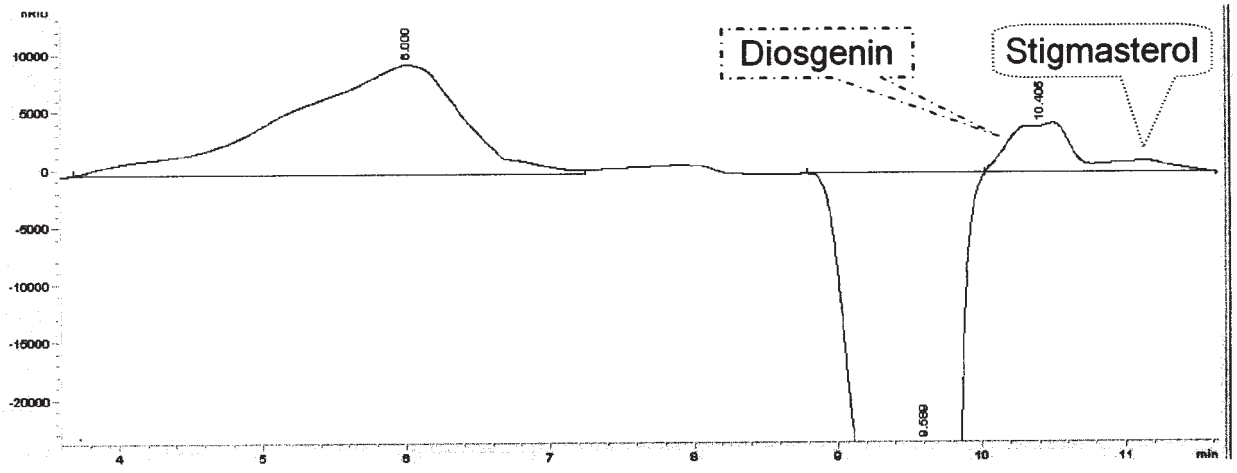

Fig. 10. HPLC chromatogram proving the selectivity of the molecular imprints towards the template molecule

Conclusions

When the phase inversion and extraction conditions are properly chosen, the obtaining of membranes, molecularly imprinted with diosgenin, is possible. The obtained membranes are porous and have an asymmetric structure.

The rheological behavior of casting polymer solution is dilatant at small shear rates and becomes pseudoplastic at higher shear rates.

The imprinting and the sorption capability were proved by HPLC with UV- VIS detector (for the liquid phase) and by HPLC with RID detector (for the solid phase).

The extracts obtained with three solvents contain diosgenin, proving that this steroid was introduced by phase inversion into the membrane and that the immobilized diosgenin can be extracted only from the external pores.

Analyzing the solid phase (parts of the membranes dissolved in DMF) the imprinting of the membranes with diosgenin was confirmed.

The competition for the imprinted cavities between two similar bioactive substances (diosgenin and stigmasterol) is unequal because the molecular places are selective to diosgenin and stigmasterol is in disadvantage.

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