

Analytical Methods

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3 **Selective extraction of progesterone hormone from environmental and**
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6 **biological samples using poly pyrrole molecularly imprinted polymer and**
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10 **determination by gas chromatography**
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Abstract

A Molecularly Imprinted Polymer (MIP) was chemically prepared for the selective batch extraction of progesterone (PGN) hormone by chemical oxidation of pyrrole using FeCl_3 . A non-imprinted polymer (NIP) was also synthesized in the absence of PGN. The developed sorbents were characterized by FT-IR and SEM techniques. Gas Chromatography coupled with Flame Ionization Detector (GC-FID) analysis was applied for quantitative determination. The parameters affecting the efficiency of this method were thoroughly investigated. The imprinted polymer showed specificity towards template PGN. Investigation of the selectivity characteristics revealed that, compared to similar compounds, the developed polymer showed selectivity towards PGN. Some parameters like desorption solvent, amount of sorbent, pH, and ionic strength were optimized and the measurements were all conducted under optimal conditions. The limit of detection (LOD) and limit of quantification (LOQ) of the method were obtained as 0.625 and 1.875 ng/mL, respectively. Application of MIP for separation and pre-concentration of PGN in environmental and biological samples (urine, blood, hospital waste water and tap water) was examined.

Keywords: Molecularly Imprinted Polymer, poly pyrrole, Environmental and Biological Samples, Progesterone, GC-FID.

Introduction

Progesterone (PGN) is a naturally occurring estronic compound and a product of cholesterol long biosynthetic pathway. This steroid is responsible for reproductive-related activities such as breast glandular development, the endometrial aspects of the menstrual cycle, and the establishment and maintenance of pregnancy.^{1,2} In humans, although PGN produced is naturally produced in both sexes, women have much higher concentration.³ Progesterone is required for normal functioning and native circulation in the human body; however, carcinogenic effects are possible at enhanced levels.⁴ Progesterone is confirmed as animal carcinogens with neoplastigenic, tumorigenic, and teratogenic effects⁵⁻⁸ Progesterone is a poison by intravenous and intraperitoneal routes. It causes developmental abnormalities in the urogenital system. The effects of progesterone on men include changes in spermatogenesis, prostate, seminal vesicle, Cowper's gland, and accessory glands along with impotence and breast enlargement. The effects of progesterone on women include changes in the menstrual cycle, uterus, cervix, and vagina.⁹ The analysis of organic contaminants in complex matrices at low level of concentration requires a procedure of pretreatment in order to extract and preconcentrate the analytes. For many years, the environmental determination of steroid sex hormones has been dominated by the use of techniques, such as High-performance liquid chromatography (HPLC-UV)¹⁰⁻¹², (HPLC-DAD)¹³,¹⁴, immunoassays and gas chromatography/ mass spectrometry (GC/MS)¹⁵⁻¹⁹ Individual reference ranges are required for each immunoassay, since the hormone concentrations measured in the same sample may vary considerably depending on the kit used. In each case, extensive evaluation is required. Patient follow-up over time or between laboratories, as well as longitudinal studies are extremely difficult. However, GC-MS requires a time consuming workup and derivatization of the samples, and the GC step prior to MS prolongs the analytical run time considerably.²⁰ Liquid chromatography coupled with mass spectrometry (LC-MS) and LC-MS/MS can be used to directly analyze estrogens without prior derivatization of the sample, but the instrument used is very expensive.²¹ Consequently, for obtaining accurate results in analyzing trace amounts of the PGN hormone without applying typical expensive methods, pre-concentration and sample preparation are required. Molecularly imprinted polymers are new selective sorbents with molecular recognition sites designed for a particular analyte.²²⁻²⁹ Compared to other recognition systems, molecular imprinted polymers possess many promising characteristics and properties such as low cost and easy synthesis, high stability to harsh chemical and physical conditions, and excellent

1
2 reusability.³⁰ The molecular imprinting technique, first proposed by Wulff and Sarhan in 1972,³¹ is one of the promising and
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4 facile methods to impart molecular recognition sites in synthetic polymers.
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6 Direct detection and determination of target PGN in complex matrices such as waste water and physiological fluids can be a
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8 difficult task for which sample clean-up and applying preconcentration methods are needed. Using the MIP, an imprinted
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10 polymer should be able to selectively recognize the main analytical target by making use of several noncovalent interactions.
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12 Polypyrrole(PPy) is an important conducting polymer with high electrical conductivity which owing to its easy synthesis and
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14 good environmental stability is one of the most extensively studied materials.³⁰ Although various studies have discussed the
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16 electropolymerization of PPy films³², there is still little information about using PPy for chemical synthesis of MIPs^{33,34}. PPy can
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18 be formed chemically or electrochemically through oxidative polymerization of pyrrole monomer readily in the presence of
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20 different oxidants, such as FeCl₃²⁶ and K₂S₂O₈.³⁵ The physical form of polypyrrole is usually an intractable powder resulting
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22 from chemical polymerization and an insoluble film which is produced as a result of electropolymerization. At room
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24 temperature chemically formed polypyrrole has a larger surface area than that formed by electrochemical methods and is a
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26 good absorbent³⁶.
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28 The aim of the present study was to study the affinity-based separation and pre-concentration of PGN in biological and
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30 environmental samples using chemically synthesized PPy-MIP and determination with the detection of GC-FID that is a simple,
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32 an accessible and a low-cost method. An additional advantage of this method is that it does not use the derivatization process.
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34 Few studies have been carried out without the application of a derivatization step.³⁷
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46 Experimental

47 Chemicals and Reagents

48 All solvents used were HPLC grade and all reagents were of analytical grade supplied by Merck (Darmstadt, Germany).
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50 Pyrrole and FeCl₃ were analytical grade supplied by Merck as well (Darmstadt, Germany).
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52 PGN was purchased from Iran hormone Institute (Tehran, Iran). Ultra-pure water was produced by a Milli-Q system
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54 (Bedford, MA, USA). Stock solution of PGN (100 mg/L) was prepared by dissolving calculated amounts in methanol.
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2 The fresh working solutions were prepared daily by diluting the stock solution in de-ionized water. All experiments
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4 were carried out at room temperature, 25 ± 0.5 °C.

6 **Apparatus**

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8 The samples were analysed by (GC-FID) (VARIAN CP - 3800) using 30 m long columns, CP-Sil 8 CB with I.D. 0.25mm and
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10 1 μ m film thickness. H₂ was used as carrier gas with 1 mL/min constant flow compensation, split ratio was 5:1,
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12 injection temperature of 305°C, oven temperature 300 °C and FID temperature 300 °C. Total run time was 20 min.

13 Ultrasonic bath (Elmasonic D-7822, Germany) was used for sonication during preparation of functionalized
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15 nanomagnetites. The prepared MIP was analysed using a FT-IR instrument (M-500 Fast-Scan IR Spectrometer Buck
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17 Scientific, East Norwalk, CT 06855, United States) and microstructure of samples was investigated by scanning
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19 electron microscopy (SEM) (KYKY EM3200 scanning electron microscope). Stirring of the solutions was carried out by a
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21 Heidolph MR3001 magnetic stirrer (Schwabach, Germany) and a 8 mm \times 1.5 mm magnetic stirring bar.

22 **Preparation of the MIP and NIP with Bulk Polymerization**

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24 For polymer preparation, 1 mmol (300 mg) of template and 4 mmol of pyrrole were dissolved in 40 mL of methanol:
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26 water (3:2 v/v) with an oxidizing solution of FeCl₃ (9.2 mmol). The synthesis was allowed to proceed at 5–7 °C^{26, 38, 39}

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28 The syntheses were performed without agitation and under argon gas atmosphere. The precipitate MIP was collected
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30 by filtration after 24 h, rinsed with distilled water and dried at 25-35 °C. As a reference, a non-imprinted polymer (NIP)
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32 was simultaneously prepared in the same way but without the addition of the template.

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34 The obtained MIP was crushed and ground. The template molecules were extracted with methanol via Soxhlet
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36 extraction until the template molecules were undetectable in the washing solution by GC-FID. Then, the particles
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38 dried to constant weight under vacuum at 70 °C.

39 **Extraction procedure**

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42 The extraction of all samples involved in this study was carried out in 500 ml flasks at room temperature. The PGN
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44 standard solution or the spiked sample solution (20 mL) was adopted throughout the study. The MIP or NIP/PPY was
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46 immersed into the sample followed by sonication for a fixed duration. The sorbent was then collected by filtering.

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48 Afterwards, methanol (0.5 mL) was added and sonicated for 35min. The methanol desorption solvent was then
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50 transferred out of the flask for GC-FID analysis. After desorption, MIP was pulled out and immersed into methanol.

51 **The real samples analysis**

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54 The serum and urine samples were kindly donated by healthy 6-9 month pregnant volunteers. The volunteers were informed
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56 about the main objective and use of the present study and their full consent was obtained. The blood sample was allowed to
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2 coagulate at room temperature or at 4 °C for 2 hours and then spin down the sample. The blood samples were centrifuged at
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4 4000×g for 10 min at 4 °C then resulted Serum samples were collected in siliconized plastic tubes without any additives and
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6 stored at -20 °C (Becton Dickinson, Cat. No. L10263-00). In general, about half of the total blood volume can be recovered as
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8 serum.⁴⁰ For urine samples, the pH was adjusted to 7.3±0.1 with 3M NaOH or 1M HCl and ascorbic acid was added (0.03%, w/v)
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10 to 1mL urine aliquots, which were also frozen at -20 °C until analysis without further preparation.⁴¹ Before applying the raw
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12 sewage samples and water samples, they were filtered through a cellulose membrane (millipore) of 0.45 μm pore size.⁴² The
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14 hospital wastewater samples were collected in polyethylene flasks out of the main sewer of the hospital using a pump collector.
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16 The samples were immediately transported to the laboratory, partitioned into aliquots, and either tested directly or
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18 refrigerated at -20 °C.
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23 Selection of porogen solvent

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26 Porogenic solvents play an important role in the formation of the porous structure of MIP, known as macro porous polymers.⁴³
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28 It is known that the nature and level of porogenic solvents determine the strength of non-covalent interactions and influence
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30 polymer morphology which obviously and directly affects the performance of MIP. Template molecule and initiator monomer
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32 have to be soluble in the porogenic solvents and the porogenic solvents should produce large pores, in order to assure good
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34 flow-through properties of the resulting polymer.⁴⁴ In this study, polymerization was carried out in the most frequently used
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36 porogens, i.e. water, chloroform, methanol and acetone in the presence of constant amounts of functional monomer (4 mmol)
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38 and template (1 mmol).
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44 The solvent should be able to solve template, monomer and oxidant. Although it was found that water had great ability in
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46 making the pyrrole MIP, PGN solubility in water was very low; therefore, methanol was used as a solvent. After obtaining better
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48 results through increasing the amount of water to methanol, the amount of methanol/water was optimized. Other solvents
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50 such as acetone and chloroform were used as solvents which were not acceptable due to the high volatility and the mixture of
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52 methanol and water (3/2) v/v was used as porogen donor solvent (Fig. 1).
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56 Insert Fig.1
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Optimization of template to functional monomer molar ratio

Formation of a complex between the template molecule and functional monomers by self-assembly process is the first step in the preparation of MIP. The monomer that can interact with the template most intensively will give the complex the highest stability.⁴⁵ The molar relationship between the functional monomer (M) and template (T) has been found to be important with respect to the number and quality of MIP recognition sites. Low M/T ratios afford less than optimal complexation on account of insufficient functional monomer. Too high an M/T ratio, the extreme case, being a non-imprinted polymer, yields non-selective binding.^{34, 46, 47} The molar ratio of the template to the functional monomer must be optimized. For this purpose, various template to functional monomer molar ratio were tasted. The results revealed that 1/4 mmol was the optimum amount of template to monomer molar ratio (Fig. 2).

Insert Fig.2

Optimization of template removal conditions

All MIP absorbents need the extracted analyte desorption using some specified methods.^{46, 48} In this study, desorption of the analyte was carried out by solvent washing assisted by applying soxhlet extraction. Thus, methanol, acetonitrile, chloroform, dichloromethane, and acetone, as removing solvents, were studied. As the results show, methanol was the best extractor solvent (Fig. 3).

Insert Fig.3

The minimum time and peak area are desirable for complete removing of template. So, Time periods from 8 to 48 hr were investigated using a Soxhlet extractor. According to the results, 24 hr was selected (Fig. 4).

Insert Fig.4

Live subject statement

All experiments were performed in compliance with the relevant laws and institutional guidelines and also state the institutional committee(s) that have approved the experiments. The authors also confirm informed consent was obtained for any experimentation with human subjects.

Results and Discussion

Characterization of MIP-Poly pyrrole (PPy)

Fourier Transform Infrared Spectroscopy analysis in FTIR spectrum of MIP-PPy, The bands at 1553 and 1456 cm^{-1} may be assigned to typical C=C stretching of pyrrole ring. The bands at 1384, 1048 cm^{-1} may correspond to C–N stretching vibration in the ring and =C–H band in-plane deformation in pyrrole unites. The broad band at 1179 cm^{-1} may be assigned for N–C stretching band. The broad peak at 3428 cm^{-1} corresponds to N-H stretch in pyrroles. The IR peak observed at 616, 926 cm^{-1} may be assigned to the =C–H out of plane vibration indicating polymerization of pyrrole.^{26, 49-51}

Scanning electron microscopy (SEM) (Fig. 5a and b) shows the surface structure and pore structure of MIP-PPY and NIP-PPY, respectively. The MIP showed high porosity. This characteristic is important in extraction procedures because it favors interactions between the analytes and the absorbent. The NIP appeared to consist of larger cluster units and less pores with small cavities and surface area than those of the MIPs, indicating that increasing the surface area of MIPs was because of imprinting. The possible reaction between polymer and template could be hydrogen bonding (Fig.6).

Insert Fig.5

Insert Fig.6

Experimental optimization for the MIP extraction conditions

In order to obtain high preconcentration and extraction efficiency of the analytes, the main parameters were optimized as indicated below. Optimization of PGN extraction conditions by MIP and NIP were performed simultaneously.

Effect of the donor phase volume

The effect of sample volume on the extraction efficiency of PGN was studied. In the present study, the phase ratio of donor and acceptor solutions was optimized by changing the volume of the donor phase between 5 and 30mL, while the volume of acceptor phase was kept constant at 0.5mL. The MIP and NIP (each 100.0 mg) were prepared with the above requirements and

1
2 used in contact with volumes of 3 ppm PGN for 30 min in the ultrasonic bath. The desorption was performed by 0.5 mL of
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4 methanol in an ultrasonic bath for 30 min and the temperature was 30[^]C. Then, one micro liter of methanol extraction solution
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6 was injected into the GC-FID system. The preconcentration factor can be improved by increasing the volume ratio of donor and
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8 acceptor phases.⁵²⁻⁵⁴ According to the results, the optimum donor phase volume was obtained 20ml for preconcentration of
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10 PGN (Fig.7).
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14 Insert Fig.7
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16 **Effect of sorbent weight**

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18 For effective imprinted materials, the specific cavities that match the size, shape and functionality of the template molecule
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20 should be formed. Thus, the capability of special adsorption for template molecule is a vital property to evaluate the imprinted
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22 materials.¹² The effect of dosage of the MIP on the extraction efficiency of the PGN from spiked sample was studied.
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24 15.0-125.0 mg MIP and NIP prepared with the above requirements were used in contact with 20 ml of 3 ppm PGN at pH 7 for
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26 30 min in the ultrasonic bath. The desorption was performed by 0.5 mL of methanol in an ultrasonic bath for 30 min and the
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28 temperature was 30[^]C. Then, one micro liter of extracted solution was injected into the GC-FID system. Fig.8 represents that
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30 the best adsorption was obtained by 100 mg of sorbent.
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32 Insert Fig.8
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34 **Effect of pH on the extraction**

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37 The pH value of the sample solution plays an important role in the extraction procedure, since the pH of solution determines
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39 the existing states of the analytes and acidic or alkaline functional groups of MIP, the extraction efficiency of the targeted
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41 compounds can be significantly influenced by pH.^{14, 55} The effect of pH in the range from 2.0 to 12.0 was evaluated by adding
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43 the appropriate hydrochloric acid or sodium hydroxide solution to the aqueous donor phase. The results, as shown in Fig.9,
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45 demonstrate that the peak area generally increased with pH. For a pH value above 6.5, the extraction efficiency began to
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47 decrease. Therefore, a pH value of 6.5 was fixed. The reason of the above changes may be explained in this way that steroids
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49 are ionisable compounds the extraction yield of which might strongly be affected by changing the pH of the aqueous matrix.^{56,}
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54 Insert Fig.9
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56 **Effect of extraction time**

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2 As expected, the extracts obtained in this technique increased with the increase of extraction time. This is due to longer MIP-
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4 liquid contact time which lead to the extraction of more solute.⁴⁸ Comparing the area under the peaks obtained in the
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6 apparatus for 35 minutes, as the optimal amount of time, subsequent steps were performed (Fig.10).
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9 However, prolonged extraction time resulted in the reduction of extraction rate due to the reduction of mass transfer driving
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11 force.⁴⁸ There was no significant increase of extraction after 35 min of extraction time. Therefore, for further experiments, the
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13 same time span was applied.
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Insert Fig.10

Salt effect

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21 Addition of salt improves the extraction efficiency in many conventional extraction techniques.⁵⁸ This phenomenon is called
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23 “salting out effect”. NaCl is commonly added to analytical samples to improve their extraction efficiency. In the current study,
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25 the extraction was performed with 20 mL sample solution containing various concentrations of NaCl (0-25% w/v) to investigate
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27 the effects of salt on the extraction. As it is shown in fig. 11, with increasing the amount of NaCl to 10% (w/v), the peak area of
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29 PGN was increased about 2.5 times. Moreover, with increasing salt concentration more than 10.0% in the aqueous sample, the
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31 peak current decreased.

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33 An initial increase and then a decrease in extraction efficiency with an increase in the concentration of sodium chloride can be
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35 explained by two simultaneously occurring processes described by Lord and Pawliszyn.⁵⁹ Initially, extraction efficiency was
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37 increased due to salting out effect, i.e. water molecules form hydration spheres around the ionic salt molecules that reduce the
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39 concentration of water available to dissolve the analyte molecules, thereby driving the additional analytes into the MIP. In
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41 competition with this process, however, is the fact that polar molecules may participate in electrostatic interaction with the salt
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43 ions in solution, thereby reducing their ability to move into the extraction phase which creates a decrease in the extraction
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45 efficiency. Thus, initially, salting out effect is predominant and increases the extraction efficiency and then an electrostatic
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47 interaction that decreases the efficiency.
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51 The obtained results showed that the salt almost had a positive effect on the extraction efficiency of the PGN. The optimal
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53 concentration of NaCl was obtained at 10.0% (w/v) (Fig. 11).
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Insert Fig.11

The effect of acceptor phase volume

The choice of elution solvent is very important to guarantee the efficient elution of the analytes from the MIP. Among methanol, acetonitrile, acetone and dichloromethane, according to optimization of template removal conditions (Fig. 3), methanol was used as an eluent. Chloroform also had good results, but because of its high volatility, we preferred methanol as acceptor solvent.

The volume of methanol, as acceptor phase, was studied from 0.2 to 4.0 mL and increases in the analytical signal of PGN were observed when 0.5 mL of methanol was used. Then, 0.5 mL was selected for further experiments (Fig.12).

Insert Fig.12

Effect of ultrasonic agitation desorption time

Minimum time with maximum peak area is desirable. The times from 10 to 50 min for desorption was investigated in ultrasonic bath. According to the results, 40 min was selected as desorption time. As can be seen after this time there were no changes in the peak area (Fig. 13).

Insert Fig.13

All optimized conditions, including PPy-MIP and extraction procedure are summarized in Table. 1.

Insert Table.1

Method performance

Validation procedure was performed using calibration solutions prepared in deionized water. The method was evaluated for linear range, limit of detection (LOD), limit of quantification (LOQ), correlation coefficient (R) and linear dynamic range (LDR) under the optimal conditions. The limit of detection was calculated experimentally as the minimum concentration providing chromatographic signals three times higher than background noise ($S/N = 3$). The calibration curve was plotted against the concentration levels of the PGN. For each level, five replicate extractions were performed. The calibration solutions were extracted with the mentioned PPy/MIP of PGN subsequently analyzed with the GC system.

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2 The LOD and LOQ were obtained 0.625 ng/mL and 1.875 ng/mL, respectively. Linearity was observed over a wide range of 1.25–
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4 5000 ng/mL through correlation coefficient of 0.9996 with the equation $Y=132351X-2582$.
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7 **Real samples analysis**

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10 The developed PPy/MIP was also used for the determination of the PGN in environmental and biological samples. The NIP/PPy
11 also used for comparison with MIP/PPy. Spiked samples at 0.1ng/mL level were analyzed using batch extraction method
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13 under the abovementioned optimized conditions. MIP batch extraction of all the samples involved in this study was carried out
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15 in 50 mL flasks at room temperature. 100mg of the MIP or NIP was added into the 20 mL of feed sample followed by sonication
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17 for 35min. The analyte-enriched sorbent, was then collected by filtration and vacuum was applied to drain the extract
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19 completely. Methanol (0.5 mL) was then added to the enriched sorbent. The methanol suspension of MIP or NIP was sonicated
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21 for 40min and filtered and 1 μ L of resultant elution solvent (methanol) was then transferred out of the flask via microsyringe
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23 and subjected for GC-FID analysis. The analytical results of waste water, tap water, urine and blood serum matrix are given in
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25 Table. 2 The relative recoveries of MIP in real samples were quite good; however, the NIP extraction capability was much lower
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27 than that of the MIP for PGN. During imprinting process, the binding sites of the functional monomer and PGN undergo some
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29 rearrangement for optimum configuration and this is sculptured during polymerization. Thus these sites retain memory of the
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31 shape and geometry of the PGN and resulted in the specific binding.⁶⁰ Fig 14-16 show the chromatogram of blood serum, urine
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33 and waste water samples respectively after a: MIP and b: NIP extraction. These samples were spiked with 0.1ppm PGN.
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42 Comparison of chromatograms validated the separation and enrichment effect of PPy-MIP material in real samples.
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45 Insert Fig.14

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2 Fig.17 shows the chromatogram of a: blood serum and b: urine samples related to pregnant women after MIP extraction. As
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4 can be seen PPy-MIP simply extracted and separated trace amount of PGN in these matrices. (Blood samples were a mixture of
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6 multiple samples)
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16 In comparison with other extraction methods (Table 3), by coupling this MIP batch extraction process with GC-FID
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18 quantification, acceptable level of detection sensitivity with conventional extraction time and sample volume was achieved for
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20 the determination of progesterone hormone in biological and environmental matrices.
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24 Insert Table.3
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31 **Selectivity of synthesized MIP**

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33 To compare the efficiency of the PGN imprinted polymer, specific binding studies were performed and the obtained results
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35 were compared with structurally similar compounds such as estradiol, testosterone and cholesterol hormones. Their chemical
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37 structures are shown in Fig. 18. To avoid the competitive adsorption, PGN and structural analogue solutions were prepared
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39 individually with the concentration of 3.00 mg/lit. It is clear that the extraction amounts of PGN, cholesterol, testosterone and
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41 estradiol with the MIP sorbent were much higher than that with the NIP (Fig. 19). this result indicated the specific selectivity of
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43 the MIP sorbent to PGN template and its structural analogues. For PGN template, this specific selectivity could be mainly owing
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45 to the predicted hydrogen bonding interactions and the molecular size recognition²²⁻²⁶ and is due to the complementary
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47 binding site available in the sorbent, which was created in the time of polymerization.^{60, 61} This strongly suggests that the
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49 imprinted polymer can be effectively used as an absorber for PGN.
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54 Insert Fig.18
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Insert Table.3

Conclusions

In the present study, a molecular imprinted polymer was prepared and used for the determination of PGN in environmental and biological samples. The obtained results indicated that by combining affinity-based MIP extraction and a simple GC-FID method without applying a derivatization step, a precise, selective and sensitive determination of progesterone in low concentration would be possible. The chemical polymerization method was improved to prepare a novel PGN-PPY/MIP sorbent. The synthesized MIP was homogeneous, highly cross linked, and porous. The extraction amounts of PGN with the MIP were markedly higher than that with the NIP sorbent. An MIP batch extraction method for monitoring PGN was established. The detection limits for PGN was obtained 0.625.00 ng/mL which could meet therapeutic or toxic monitoring of trace PGN in environmental and biological samples. The method was applied to the trace PGN determination at 0.1 mg/lit and the recoveries for the spiked tap water, hospital waste water, urine and blood serum samples were 96.6, 86.2, 101.32 and 88.25%, respectively. This MIP can be considered as a suitable method for the extraction of trace amount of PGN in the complicated biological and environmental samples.

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Figure captions:

Fig. 1 The selection of porogen solvent. Polymerization in constant amounts of functional monomer (4 mmol) and Template (1 mmol).

Solvent volume: 40 mL, oxidizing solution of FeCl_3 (9.2 mmol).

Fig. 2 The effect of template to functional monomer molar ratio. Solvent volume: 40 mL, oxidizing solution of FeCl_3 (9.2 mmol).

Fig. 3 selection of solvent for soxhlet extraction

Fig. 4 time of soxhlet extraction for removing template

Fig. 5 The proposed interaction between PPy and template (PGN)

Fig. 6 SEM micrograph of NIP (a) and MIP (b).

Fig. 7 The effect of the donor phase volume. Extraction condition: volume of acceptor phase: 0.5mL, MIP and NIP 100.0 mg, PGN conc: 1 ppm, pH: 7, time: 30 min in ultrasonic bath. Desorption condition: volume of methanol 0.5 mL time: 30 min, temp: 30 °C. Max Extraction: 32.22%

Fig. 8 The effect of sorbent weight (mg). Extraction condition: volume of acceptor phase: 0.5mL, donor phase: 20 ml 1 ppm PGN, pH: 7, time: 30 min in ultrasonic bath. Desorption condition: volume of methanol 0.5 mL time: 30 min, temp: 30 °C. Max Extraction: 34.58%

Fig. 9 The effect of pH on the extraction. Extraction condition: MIP and NIP 100.0 mg, volume of acceptor phase: 0.5mL, donor phase: 20 ml 1 ppm PGN, time: 30 min in ultrasonic bath. Desorption condition: volume of methanol 0.5 mL time: 30 min, temp: 30°C. Max Extraction: 39.31%

Fig. 10 The effect of extraction time. Extraction condition: MIP and NIP 100.0 mg, volume of acceptor phase: 0.5mL, donor phase: 20 ml 1 ppm PGN, pH: 6.5, desorption condition: volume of methanol 0.5 mL time: 30 min, temp: 30 °C. Max Extraction: 100.06%

Fig. 11 The investigation of salt effect. Extraction condition: MIP and NIP 100.0 mg, volume of acceptor phase: 0.5mL, donor phase: 20 ml 1 ppm PGN, pH: 6.5, time: 35 min in ultrasonic bath. Desorption condition: volume of methanol 0.5 mL, time: 30 min, temp: 30 °C. Max Extraction: 100.08%

Fig. 12 The effect of acceptor phase volume. Extraction condition: MIP and NIP 100.0 mg, volume of acceptor phase: 0.5mL, donor phase: 20 ml 1 ppm PGN, and pH: 6.5, time: 35 min in ultrasonic bath, salt conc: 10 %. Desorption condition: time: 30 min, temp: 30 °C. Max Extraction: 100.06%

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3 Fig. 13 The effect of desorption ultrasonic agitation time. Extraction condition: MIP and NIP 100.0 mg, volume of acceptor phase: 0.5mL, donor phase: 20
4 ml 1ppm PGN, and pH: 6.5, time: 35 min in ultrasonic bath salt, conc: 10 %. Desorption condition: volume of solvent 0.5 mL, temp: 30 C. Max Extraction:
5 100.07%

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7 Fig.14 chromatogram of blood serum sample spiked with 0.1ppm PGN after a: MIP extraction and b: NIP extraction
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10 Fig.15 chromatogram of urine sample spiked with 0.1ppm PGN after a: MIP extraction and b: NIP extraction
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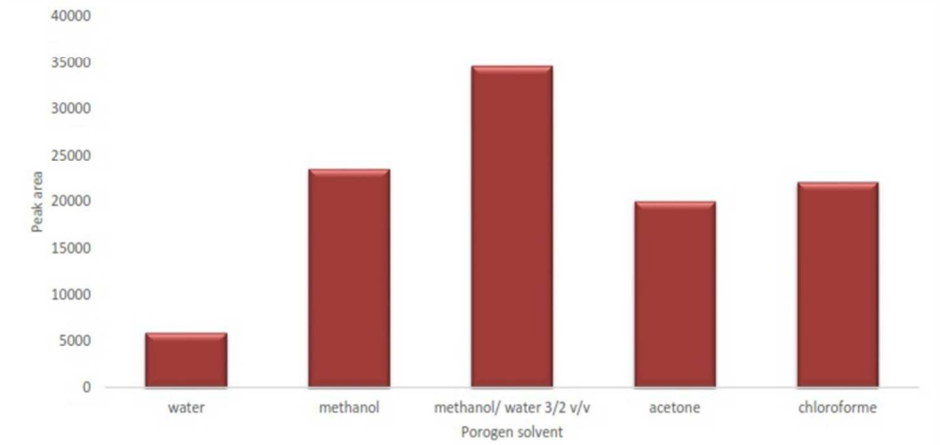
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13 Fig.16 chromatogram of waste water sample spiked with 0.1ppm PGN after a: MIP extraction and b: NIP extraction
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17 Fig.17 chromatogram of a: blood serum and b: urine samples related to pregnant women after MIP extraction
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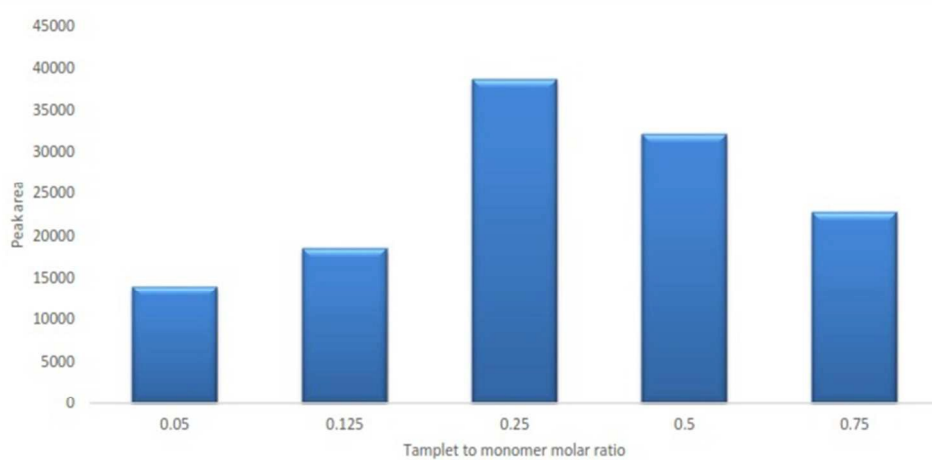
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20 Fig.18 The chemical structure of progesterone, estradiol, testosterone and cholesterol hormones.
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23 Fig. 19 The extraction amounts (percent) of PGN, cholesterol, testosterone and estradiol with the MIP and NIP sorbent. Extraction condition: MIP and NIP
24 100.0 mg, volume of donor phase: 20 ml 1 PPM, pH: 6.5, time: 35 min in ultrasonic bath. Salt Conc: 10 %. Desorption condition: volume of solvent 0.5
25 mL, time: 40 min, temp: 30 C.
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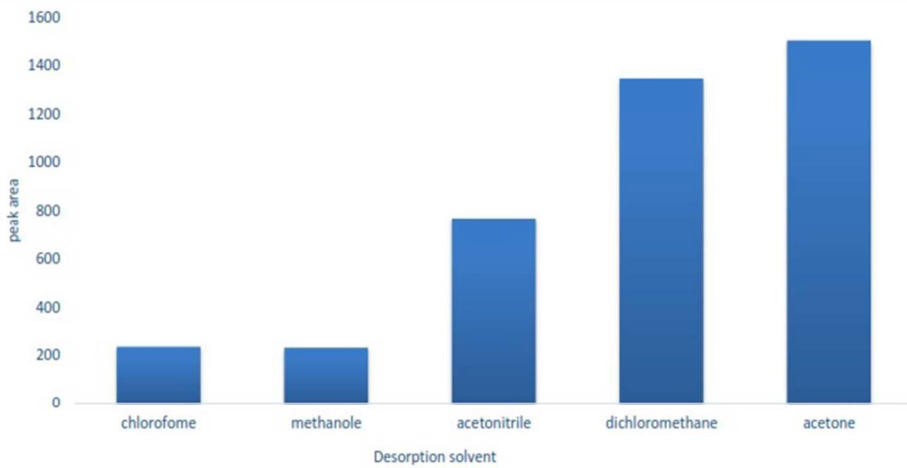
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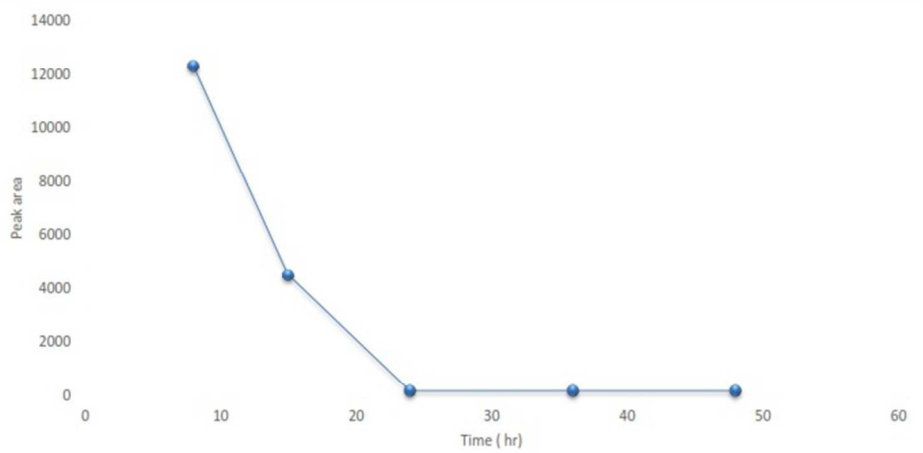
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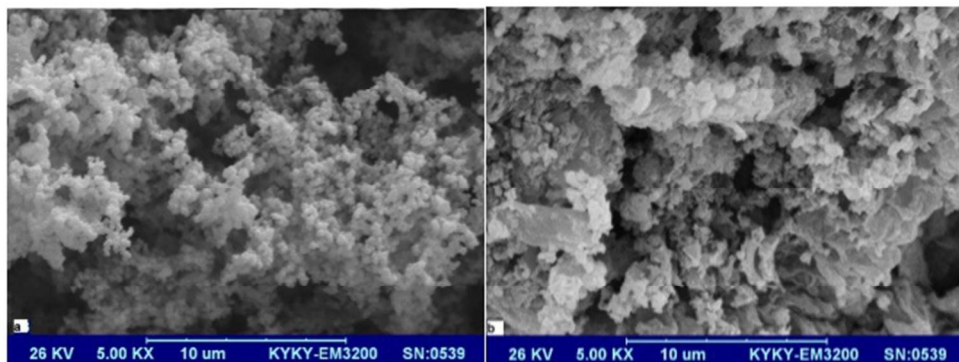
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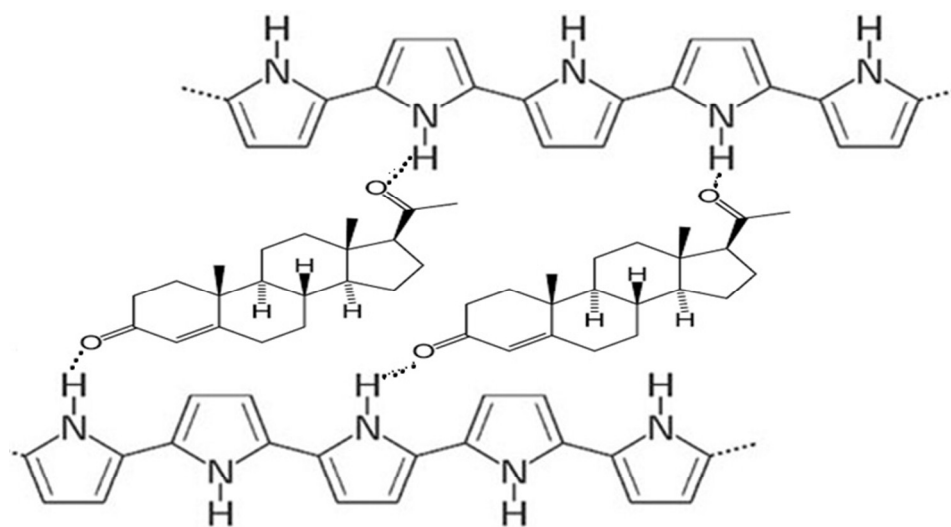
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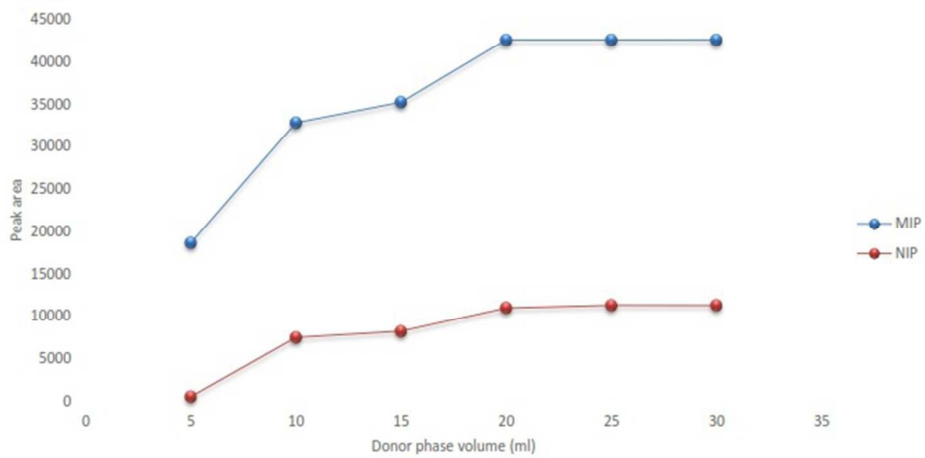
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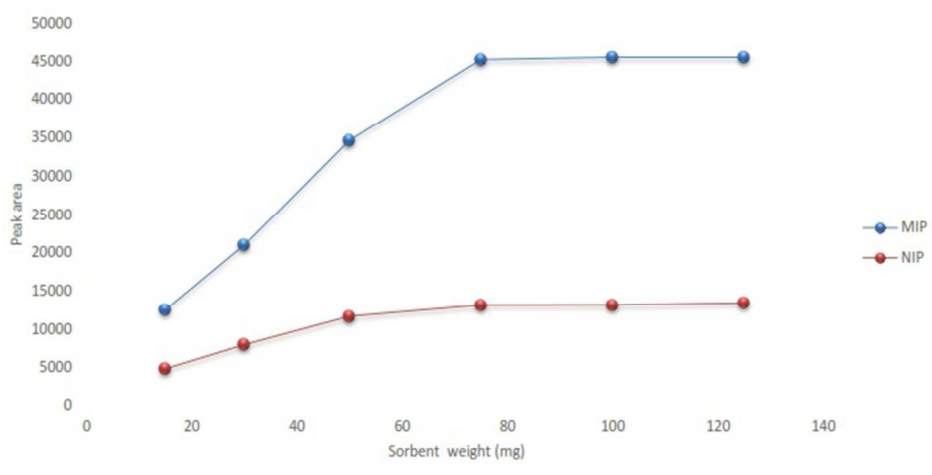
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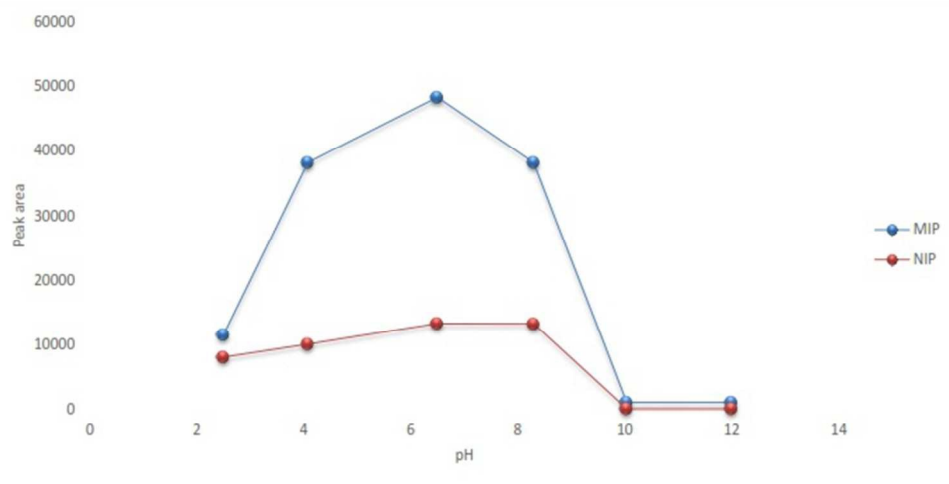
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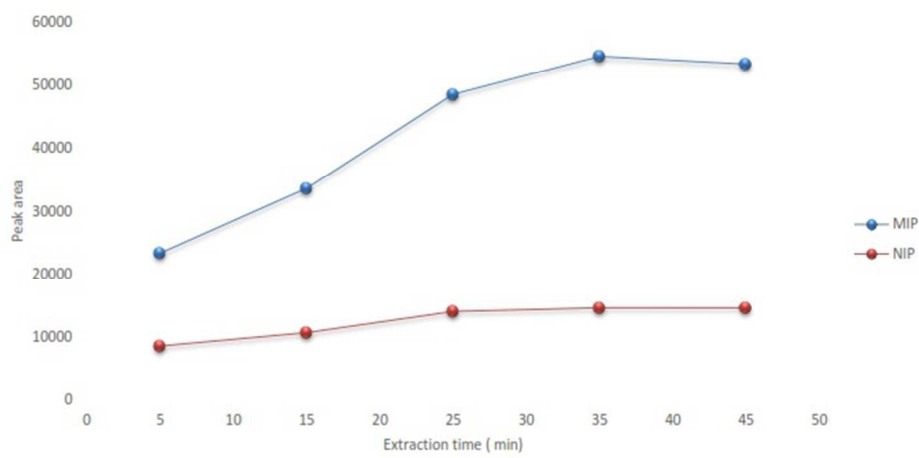
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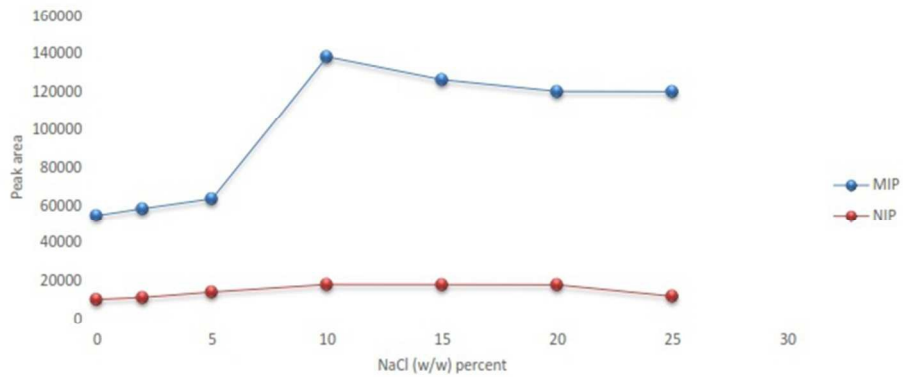
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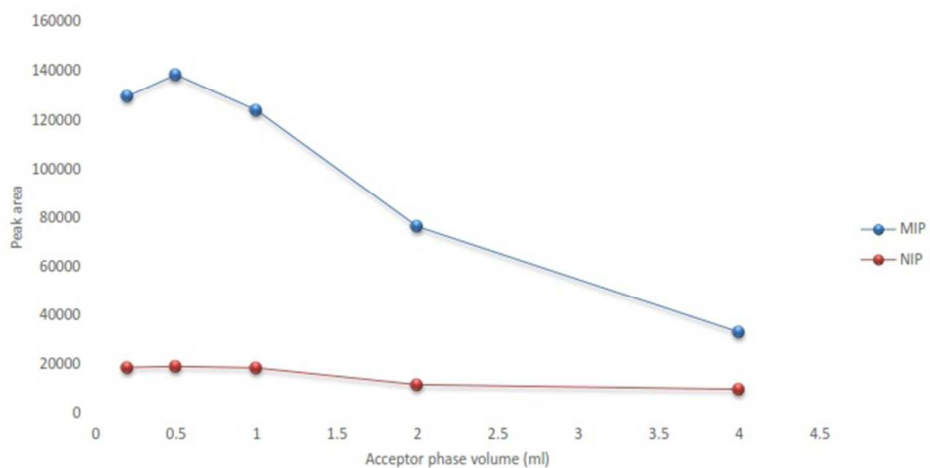
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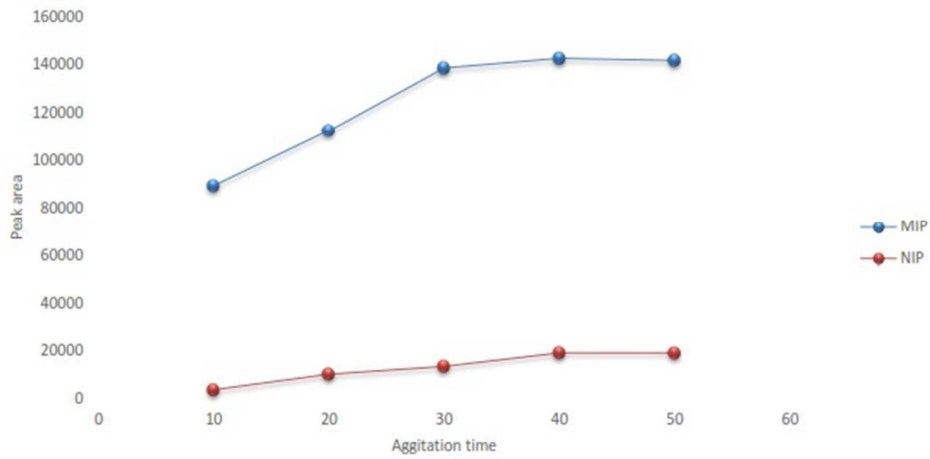
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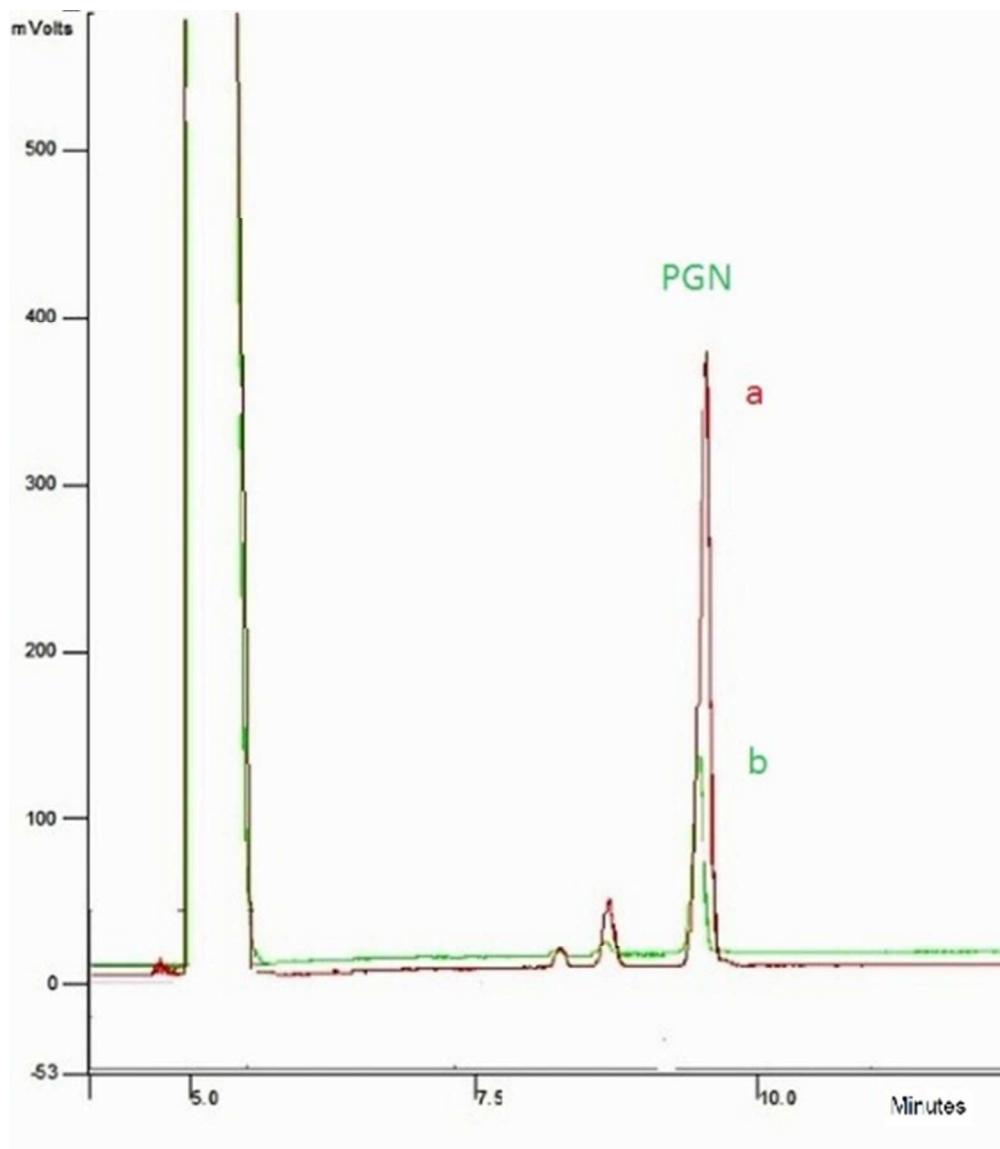
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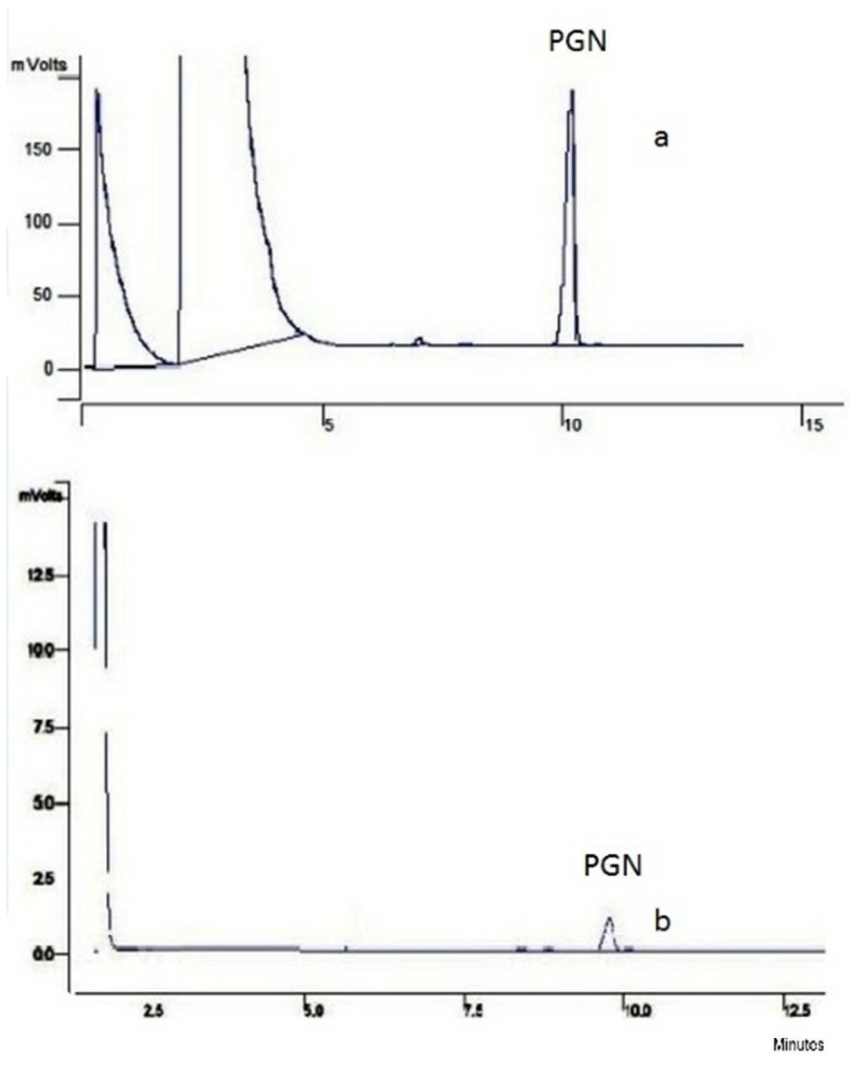
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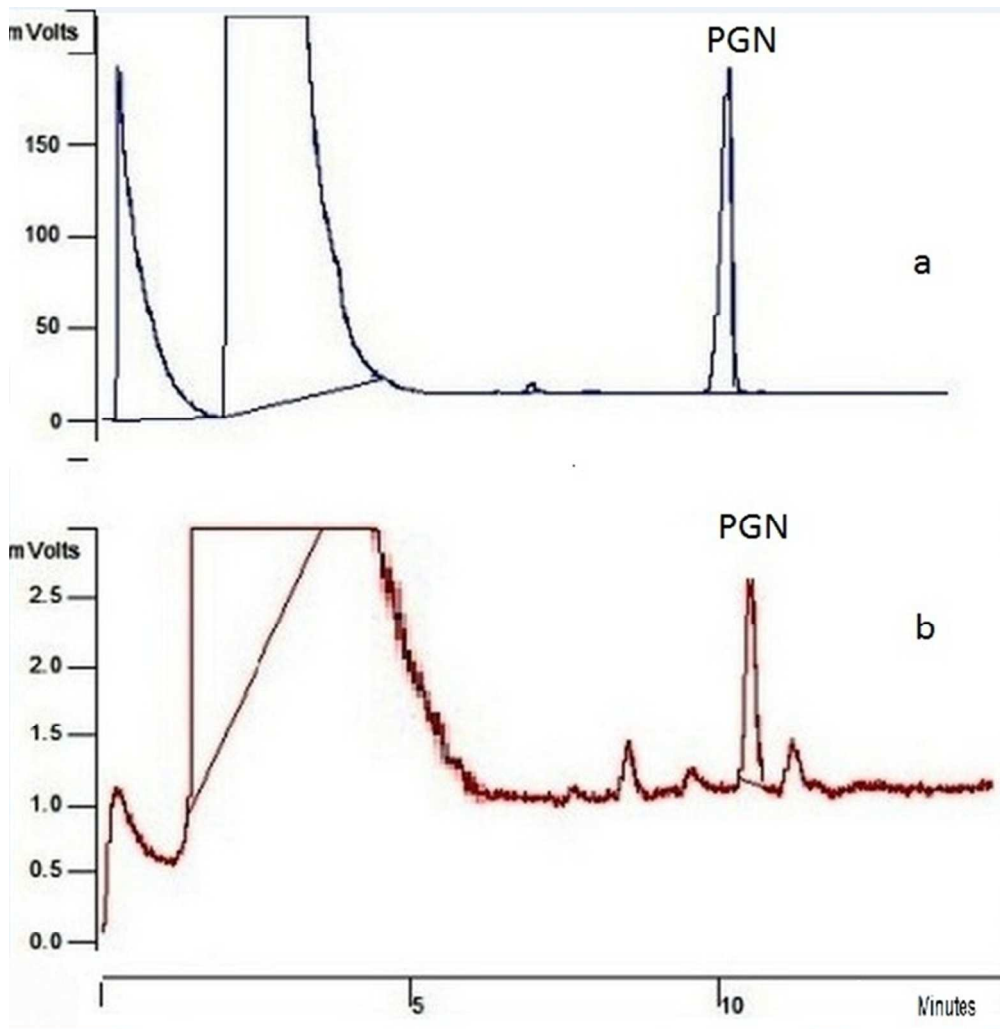
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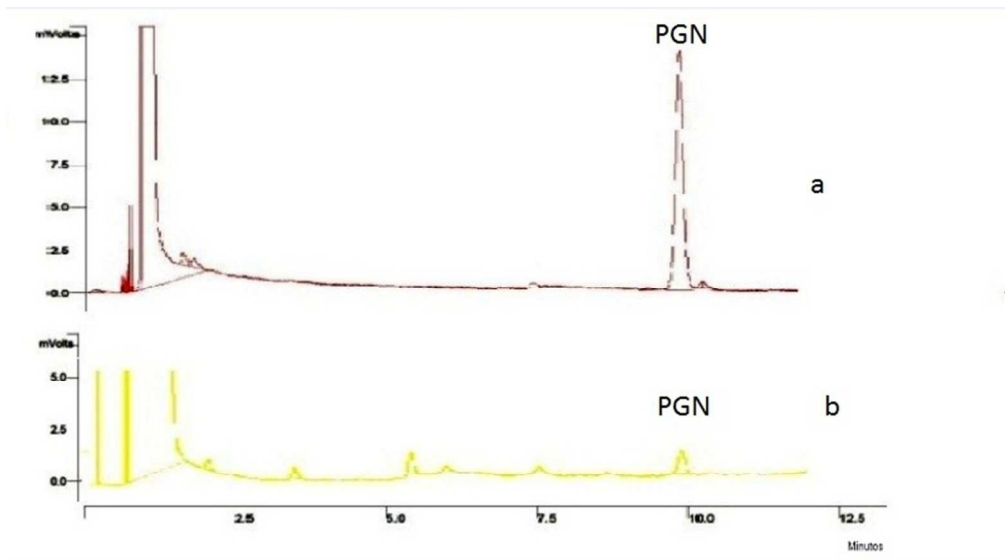
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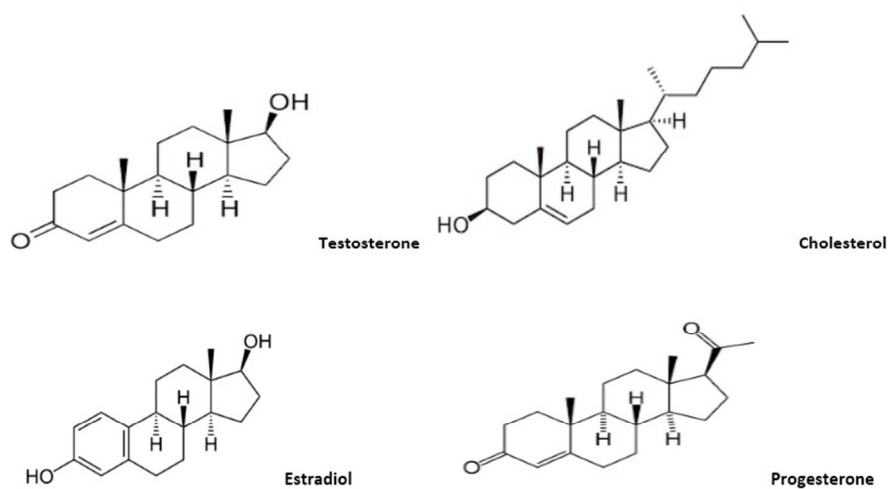
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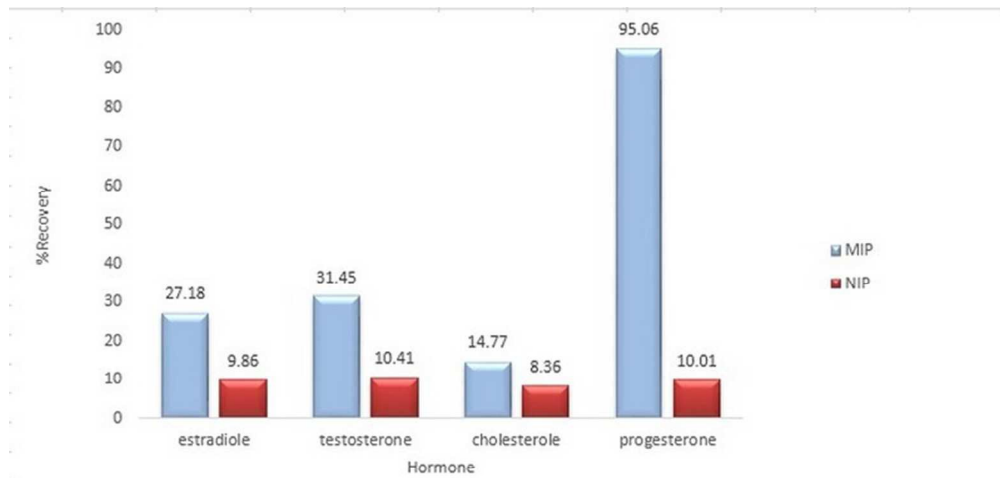
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Table.1 Optimized PPy-MIP preparation, progesterone (PGN) extraction and desorption conditions

PPy-MIP preparation conditions		template removal conditions		PGN extraction conditions						
porogen solvent	template to monomer molar ratio (mmol)	extractor solvent	Soxhlet Time (Hr)	pH	donor phase volume (mL)	Time (min)	Concentration of NaCl (w/v)%	acceptor phase volume (mL)	ultrasonic aggitation desorption time	amount of sorbent (mg)
mixture of methanol /water (3/2) v/v	1/4 mmol	methanol	24 Hr	6.5	20mL	35 min	10.0% (w/v)	0.5 mL methanol	40 min	100 mg

Table. 2 The concentrations (ng/mL) and relative recoveries (%) of PGN in the environmental and biological samples.

Real sample	Concentration (ng/mL)	(MIP) Relative	(NIP) Relative
		Recovery(%)±%RSD	Recovery(%)±%RSD
		(n=5)	(n=5)
Tap water	0.100 ^a	96.6±3.5	48.2±5.1
Hospital waste water	0.100 ^a	86.2±3.8	45.3±6.2
Urine	0.100 ^a	101.32±4.5	50.3±7.1
Urine	0.026 ^b		
Blood	0.100 ^a	88.25±3.8	45.8±5.3
Blood of pregnant women (7-9months)	0.072 ^b		

^aSpiked amount of analytes

^bFounded amounts of analytes without spiking

Table 3 Comparison with other extraction methods.

Sample type	Method ^a	Sample volume	Extraction time	Detection	LOD	References
Water	SPE	2L	48h	HPLC/UV	0.041–0.16 mg/L	11
Water	CPE	10	1h	HPLC/UV	5 ng/mL	12
Water Urine	SBSE	30 mL	2h	HPLC/DAD	1ng/mL	14
Urine Water	SBSE	50 mL	2.5h	HPLC/DAD	0.12 ng/mL	13
Water	2P-HF-LPME	5 mL	1h	GC-MS	0.02 ng/mL	18
Water	SPME	18 mL	135 min	GC-MS	0.12 ng/mL	19
Rabbit plasma	LLE	0.2 mL	3min	GC-FID	100 ng/mL	32
Water Serum Urine	MIP batch extraction	20 mL	35 min	GC-FID	0.625 ng/mL	This work

^a SPE, solid-phase extraction; CPE, Cloud point extraction; SBSE, stir bar sorptive extraction; 2P-HF-LPME, 2 phase hollow-fibre liquid phase microextraction; SPME, solid-phase microextraction; LLE, liquid–liquid extraction.