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DEVELOPING A METHOD FOR DETERMINATION OF URINARY DELTA-AMINO-LEVULINIC ACID USING MOLECULARLY IMPRINTED POLYMERS

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Abstract. Delta-amino-levulinic acid (ALA) has been introduced as a biological exposure index for workers exposed to lead. In this study, a new analytical method has been developed using molecularly imprinted polymers (MIPs) in microextraction by packed sorbent for urine samples. A spectrophotometric analysis was carried out for urine samples. Fourier transform infrared spectroscopy was used to determine the main constituents of MIPs. The optimized method was fast, sensitive, selective, easy to use and friendly to the environment. The results indicated that the developed method is a suitable and rapid method for the bio-monitoring of individuals exposed to lead.

Keywords: spectrophotometry, microextraction by packed sorbents, molecularly imprinted polymers, delta-amino-levulinic acid, urine sample.

1. Introduction

Lead is a potentially toxic metal commonly used in many industries like the battery industry, paint industries, foundry, construction, and radiator repair [1]. Lead is also used in gasoline, paints, pigments, and inks. In addition to occupational procedures, humans can be exposed to lead through the environment, such as through air, water, food and even smoking [2, 3]. Lead has adverse effects on health, including harmful effects on the cardiovascular system [4] as well as gastric, lung, and bladder cancer [5] and disorders in children's learning [6]. Lead has been classified as a group 2A carcinogen by the International Agency for Research on Cancer [7]. The mechanism of lead poisoning includes the effect on the delta amino-levulinic dehydratase in the heme biological pathway. Lead has occupied a zinc active site in the enzyme and inhibits the activity of delta amino-levulinic dehydratase while increasing the amount of amino-levulinic in plasma and urine. Urinary delta amino-levulinic acid (U-ALA) is used as a biomarker for detecting exposure to lead [8]. The American Conference of Governmental Industrial Hygienists (ACGIH) suggested U-ALA as a biological exposure index (BEI); for workers who have been occupationally exposed to lead, it proposed a limit of 5 mg/l [9].

Thus far, the conventional method for measuring the amount of delta amino-levulinic (ALA) in urine is a colorimetry. In the colorimetry, the solvents such as ethyl acetate, acetic acid, *n*-butanol, acetate buffer, chloroform, and Ehlrich's reagent are used; a color change in the solution is analyzed by a spectrophotometer. These methods have a disadvantage in specificity, as the Ehlrich's reagent reacts with other urinary constituents, such as amino acetone, which forms pyrrole compounds. However, the main problems with this method are the high use of chemical solutions, long and time-consuming process, the possibility of interfering with other materials, high pH in some cases, and the response of a number of solvents at only low concentrations. The lack of selectivity, which has led researchers to use highly selective sorbents such as molecularly imprinted polymers (MIPs), is another disadvantage of this method. In recent years, a new type of efficient adsorbent, MIPs, has been developed in the presence of a target analyte. MIPs will be obtained as a three-dimensional network with a strong cross-linking during a copolymerization reaction between the cross-linking and functional monomer in the presence of a pattern molecule [10]. Given that MIPs is a powerful tool in the development of analytic methods, it is used as a receptor in detecting a large variety of target molecules with high selectivity due to their strong ability to eliminate potential interactions in complex matrices. Molecularly imprinted polymers are used as an absorbent in some methods with solid phase extraction (SPE). The volume of solvents for elution in SPE is about 1–5 ml but in

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chromatographic techniques, few microliters of sample are needed for analysis [11]. To overcome some of these drawbacks of conventional SPE column, Abdel-Rehim [12] introduced a microextraction by packed sorbents (MEPS).

MEPS is a miniaturized SPE technique that is able to process a wide range of samples quickly and efficiently using low adsorbent masses (1–4 mg). It is a new, sensitive, and selective technique in sample preparation. The benefits of MEPS include its direct sample loading with no need for a separate robot, a fully automatic online and fast process, the work with a low volume of samples, and its low cost [12]. A lower mass of adsorber is followed by reducing the volume of the solvent; in addition, the substrate of the absorber is not lost and compared to disposable cartridges of solid phase extraction (SPE), which can be used more than 100 times depending on the sample [13]. Various absorbents such as C2, C8, C18, SCX, SAX, APS, SDVB, HDVB are used in MEPS, but the lack of selectivity, the main disadvantage of these adsorbents, has led researchers to use high-selectivity adsorbents such as MIPs [14]. Therefore, considering the advantages of the MEPS compared to conventional SPE sorbents, as well as the highly selective and low cost of producing MIPs, their combination can be described as a simple, fast, selective, high-sensitivity and environment-friendly method and for use in preparing environmental, pharmaceutical, toxicological, and food samples [15].

Up to now, there are no published studies in the determination of urinary U-ALA using MIP-SPE. In the current study, we developed a novel approach based on MIP-MEPS to determine U-ALA. According to our knowledge, MIP-MEPS analysis has been carried out by analytical methods like GC, HPLC, LC-MS-MS, and GC-MS [14-19]. So far this method has not been performed with a spectrophotometer and for the first time, MIP-MEPS was combined with the spectrophotometric method with a microcell to analyze urinary U-ALA. The selectivity and imprinting effects of synthesized MIP were measured, and to determine the polymers characterization, Fourier transform infrared (FTIR) spectroscopy was used. We optimized the important factors affecting MIP-MEPS performance, including the type of elution, washing solvents, the volume of sample and extraction cycles.

2. Experimental

2.1. Reagents and Materials

All chemicals were of analytical reagent grade. ALA was purchased from Merck (Darmstadt, Germany). Mandelic acid (MA), methacrylic acid (MAA), 2,2'-azobisisobutyronitrile (AIBN), dimethyl sulphoxide (DMSO) and ethylene glycol dimethacrylate (EGDMA) were all supplied by Sigma-Aldrich (Darmstadt or Hohenbrunn, Germany). Methanol, ethanol, hydrochloric acid, acetic acid, and sodium hydroxide were also purchased from Merck (Darmstadt, Germany). Water was obtained from an ultra-water system (TKA, Niederelbert, Germany). A 250 µl gas-tight syringe was used as MEPS syringe (Hamilton, Nevada, USA).

2.2. Instrumentation

The analysis was done using a spectrophotometer (Perkin Elmer Spectrometer UV/VIS, Lambada 950, USA) and microcell (Yixing, China). The flow rate of injection was 10 µl/min, and the system was set at 370 nm. In order to record the FTIR spectra (500–4000 cm^{1}) of MIP and NIP (non-imprinted polymers) a Perkin Elmer Spectrum 65 FTIR spectrophotometer was used.

2.3. Preparation of MIP

There are various methods for preparing MIP. In this study, the bulk polymerization method was used to prepare MIP for ALA on the basis of previously described procedure by Soleimani *et al*. [15]. To do this, we used 4 vials. First, 0.1 mmol (0.018435 g) of ALA was dissolved in 2 ml of dimethyl sulfoxide and then methacrylic acid (0.2, 0.4, 0.8, and 1.5 ml) were added to the vials (Table 1). The resulting solutions were ultrasonicated for 15 min. Then 2 mmol (380 µl) of EGDMA and 0.25 mmol (0.041 g) of AIBN were added and the solutions were ultrasonicated for 20 min. Each of these solutions was subsequently purged with a gentle nitrogen flow for 5 min to eliminate any oxygen present in the mixture. The solution was placed in a paraffin bath for 24 h at 343 K. After 24 h the polymers were cooled to room temperature and the vials were crashed and dried at 328 K. Mechanical grinding and sieving were then done to obtain particles <40 µm in size. Using an ethanol/acetic acid solution with a volumetric volume of 2:8, the samples were then washed on a shaker for 10 h to extract the pattern molecule. The model molecule was withdrawn from the visible-ultraviolet spectrophotometer at 370 nm to monitor the removal of the molecule, and the washing was continued until other absorption at this wavelength was observed. After removal of the pattern molecule, the polymers were dried and kept at room temperature until they were used. Non-imprinting polymers were produced at the same way, with the difference that the template molecule (*i.e*., ALA) was not used in the production process. The process of making this polymer and its application are shown in Fig. 1a.

Table 1

Optimization of conditions for synthesizing NIP and MIP

MIP/NIP	Template molecule ALA, mmol	Functional monomer MAA, mmol	Cross-linker EGDMA, mmol	Initiator AIBN, mmol	Recovery mean RSD*, %
MIPa	0.11	0.2	2.2	0.25	50 (4.3)
MIPb	0.11	0.4	2.2	0.25	64 (3.7)
MIPc	0.11	0.8	2.2	0.25	92 (3.4)
MIPd	0.11	1.5	2.2	0.25	74 (2.7)
NIPa	0	0.2	2.2	0.25	12 (2.7)
NIPb	0	0.4	2.2	0.25	21 (3.0)
NIPc	0	0.8	2.2	0.25	22 (6.3)
NIPd	0	1.5	2.2	0.25	30 (4.3)

Note: *based on triplicate analysis

2.4. The study of Imprinting Effect and Selectivity of MIP

The selectivity of MIP for ALA in the presence of MA as interfering compounds was investigated. MA is a metabolite of ethylbenzene and styrene. This chemical was chosen because the workers were concurrently exposed to its action. For this, 50 mg of NIP or MIP were added to 20 ml of a binary mixture of MA/ALA at concentrations of 1 µg/ml.

After the mixture was stirred at room temperature for 15 min, it was centrifuged at 4000 rpm for 5 min. Its supernatant was collected and analyzed by a spectrophotometer at 370 and 247 nm. The distribution coefficient (K_d), selectivity coeficient of polymers (K), and relative selectivity coefficient (K') were calculated as follows:

$$K_d = \frac{C_i - C_f}{C_f} \cdot \frac{V_s}{m} \quad (1)$$

$$K = \frac{K_{d-analyte}}{K_{d-interferent}} \quad (2)$$

$$K' = \frac{K_{impr}}{K_{non-impr}} \quad (3)$$

where C_i is the initial analyte concentration, ml/g; C_f is the final analyte concentration, ml/g; V_s is the volume of the solution, ml; m is the weight of MIP or NIP, g; K_{impr} is imprinted polymer selectivity coefficient; and $K_{non\text{-}impr}$ is non-imprinted polymer selectivity coefficient.

In addition, the selectivity of MIP was also tested by comparing the extraction recoveries of ALA and MA using the MEPS under the same conditions. Blank urine samples were spiked with 1 µg/ml of each analyte. The pH value was adjusted to the optimized value of 2.0. The sample was percolated through 4 mg of MIP or NIP with 5×100 µl extraction cycle. Using ethanol/acetic acid (80:20, v/v), the analytes were eluted and the elution was injected into the spectrophotometer.

2.5. Standards and Quality Control Samples

A stock solution of 1000 µg/ml was prepared by dissolving 0.1 g of ALA in 100 ml of an ethanol/water (80:20, v/v) solution. Working standard solutions were prepared by diluting the stock solution with water daily. To obtain a calibration curve, blank urine samples were spiked within a range of 0.5–2.5 µg/ml. Samples of quality control (QC) were prepared at concentrations of 0.05, 0.25 and 1 µg/ml.

2.6. Procedure of MEPS

We used the manual MEPS format for the extraction procedure. To do so, 4 mg of MIP were packed between two polyethylene frits inside the barrel of a 250 µl Hamilton syringe. First, the MIP bed was conditioned by 3×100 µl of ethanol, followed by 3×100 µl of water. A spiked urine sample with pH of 2.0 was centrifuged for 2 min at 3500 rpm. It was then percolated through MIP 6 times (6×100 µl) at a speed of 10 µl/s in an extract-discard mode. Then, using 1×100 µl of water, we washed the MIP bed; afterward, the analyte was eluted with 3×100 µl of an ethanol/acetic acid mixture (80:20, v/v). The aliquot was transferred to a microcell analyzed by a spectrophotometer. We cleaned the MIP bed with 3×150 µl of the elution solution, followed by 3×150 µl of the washing solution between extractions in order to avoid "carry over". The same MIP was used about 75 times, without the loss of extraction efficiency and/or MIP becoming clogged. Fig. 1b shows the MEPS procedure.

2.7. Validation of the Method

As discussed in Subsection 2.4, we have investigated the selectivity for samples spiked with ALA and MA. The selectivity was carried out in such a way as to assume that interventional factors such as MA did not disrupt the extraction or reduce recovery. Using a spiked sample of 0.5 to 2.5 µg/ml, a 5-point calibration curve was drawn.

Each concentration was measured five times. The calibration curve was drawn in such a way that the concentrations were shown along the *x*-axis and the absorption along the *y*-axis. To determine within- and between-day precision and accuracy we used QC samples. The experiments were repeated five times within the same day in order to obtain within-day precision. To calculate inter-day precision and accuracy, five replicates of QC samples (low QC, medium QC, high QC) were investigated over 3 consecutive days.

The recoveries of ALA extraction in three QC samples were obtained as follows:

$$Recovery\ of\ extraction = \frac{concentration\ after\ MIP-MEPS\ procedure}{concentration\ before\ MIP-MEPS\ procedure} \cdot 100\%$$

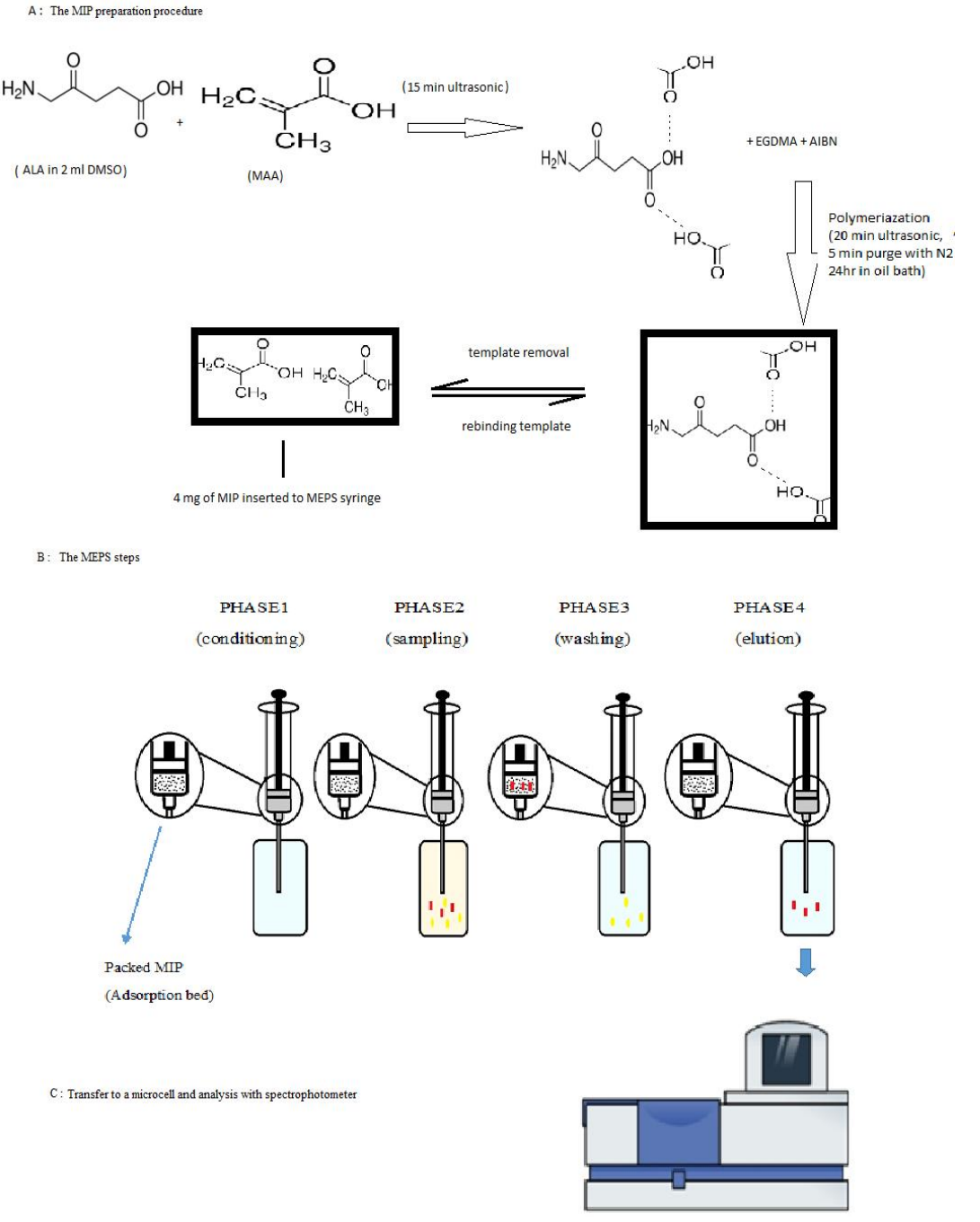

Fig. 1. Schematic drawing of the experimental procedure

3.Results and Discussion

3.1. Characterization of MIP, Study of Selectivity and Imprinting Effect of MIP

The infrared spectrum for ALA was drawn using FTIR (Fig. 2). A broad O–H stretching vibration peak was observed for both MIP and NIP at 3448 cm^{-1}, indicating the presence of O–H bands in the carboxylic group of the methacrylic acid. The tensile peaks associated with the presence of the methyl bands (C–H) in MAA and EGDMA, were observed at 3004 cm^{-1} for MIP and 3001 cm^{-1} for NIP. The tensile peak of the carbonyl group (C=O) was observed for MIP and NIP at 1740 cm^{-1} indicating the carbonyl bond in MAA and EGDMA. The observed peaks between 1395 and 1451 cm^{-1} are related to the bending vibrations of the C–H bands in the methyl group. The observed peak at 1158 cm^{-1} is also consistent with the tensile vibration of O–C(O)–O in EGDMA. As previously mentioned, both MIP and NIP have the same courier, indicating that they have similar foundations. Due to the formation of hydrogen bands, the electron densities of O–H and C=O are reduced, leading to a decrease in the vibrational frequency. Therefore, the tensile bands for O–H and C=O, which were observed without removing the pattern molecule at 1740 and 3448 cm^{-1}, were observed after removing the pattern molecule at 1737 and 3440 cm^{-1}. These observations indicated that the template molecule was successfully removed and a suitable template polymer was made for ALA.

The results of the selectivity assessment are shown in Table 2. The observations indicate the successful imprinting effect on the polymer. The distribution coefficients (K_d) were 225 and 54 for the MIP and NIP, respectively. Compared with the interferents, the value of K_d for ALA was 225 compared to 72 for MA; the higher value for ALA indicates that MIP has a good ability to discriminate between ALA and MA. The selectivity coefficients (K) were 3.125 and 0.83 for MIP and NIP, respectively. The relative selectivity coefficient, which indicates the polymerization efficiency, is equal to 3.76; as it was greater than 1, the effect of imprinting was successfully performed.

In addition, the extraction recovery for ALA was 92 % and for MA it was 22 %. The higher recovery for ALA is due to the presence of molecular selective sites in the polymer structure. Therefore, it can be concluded that MIPs were successfully synthesized.

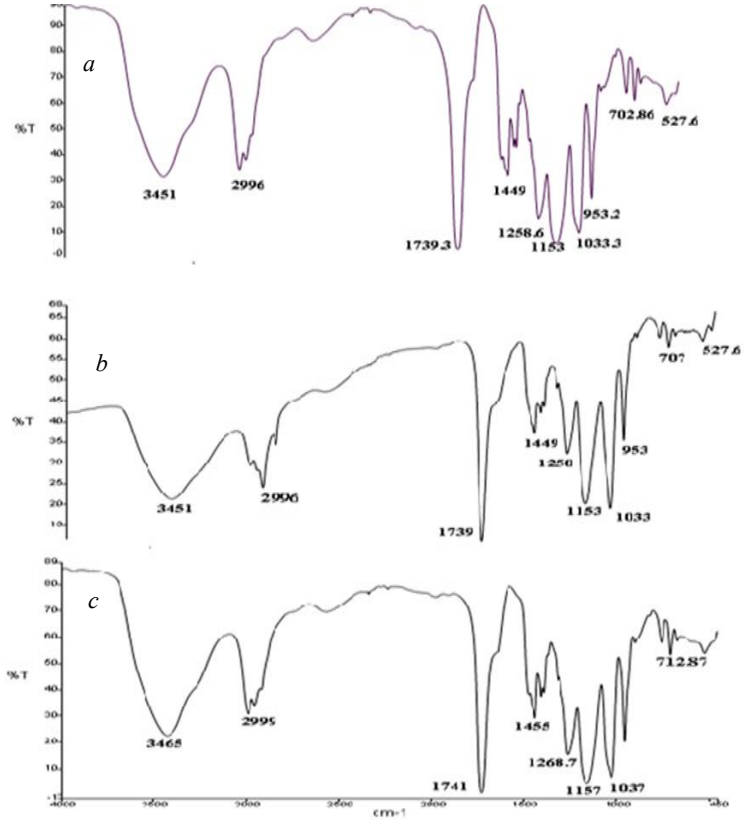

Fig. 2. The FTIR spectra of NIP (a), MIP with template removal (b), and MIP without template removal (c)

Table 2

Selectivity parameters for NIPs and MIPs

Polymer	K_d, ml/g		K^1	K'^2
	Δ-ALA	MA		
MIP	225	72	3.125	3.76
NIP	54	65	0.83	–

Notes: K^1 is the ratio of K_d for ALA to K_d for MA; K'^2 is the ratio of K_{impr} to $K_{non\text{-}impr}$

3.2. Optimization of Experimental Parameters

3.2.1. Optimization of MEPS procedure

Approximately 4 mg of the synthesized MIP were placed in a 250 µl syringe body between two layers of polyethylene. An optimum concentration of 1 µg/ml was selected. The entire experiment was repeated 3 times.

3.2.2. Sample volume and extraction cycles

Five extraction cycles (2, 4, 6, 8, and 10 extraction cycles) and 3 sample volumes (25, 50, and 100 µl) were investigated. To allow more contact, the sample was passed through a bed at a rate of 10 µl/s. The recovery of the ALA extraction increased from 49 % (extraction recovery of 25×2) to 92 % (extraction recovery of 6×100). No significant changes were found in the recovery after increasing the number of sample loadings to 8×100 and 10×100. Therefore, the value of 6×100 was considered the optimal amount and was selected to continue the process (Fig. 3a).

3.2.3. Washing solutions

The type and volume of the washing solution are important for removing the unwanted components from the bed, decreasing the loss of analyte, and achieving a clean extraction. In this study, a water-ethanol (90:10; 80:20 and 70:30 v/v), water-methanol (90:10; 80:20 and 70:30 v/v), and water (100×1 µl) solutions were investigated to eliminate the interfering factors. When the water-ethanol solution (70:30, v/v) was used, the recovery was reduced to 81 %. The most observed recovery was due to water alone, that is why water was selected as the optimal washer solution (Fig. 3b).

3.2.4. Optimization of elution solution

The elution solution is used to separate the analyte from the absorbent bed, and our goal was to optimize it to reach the highest extraction recovery with the minimum volume of consumption. In this study, the effect of acetic acid-ethanol solution (10:90; 20:80 and 30:70 v/v) and acetic acid-methanol solution (10:90; 20:80 and 30:70 v/v) on the recovery of ALA extraction were investigated. The solution of acetic acid-ethanol (20:80, v/v) with a cycle of 3×100 has the highest recovery. Therefore, acetic acid-ethanol (20:80, v/v) solution was selected with 3×100 cycles as the optimum amount (Fig. 3c).

3.2.5. pH value

The value of pH is a very important parameter for obtaining repeatable data. Therefore, in the present study, the effect of pH in the range of 2–10 on the recovery of ALA was investigated. To adjust pH in the desired amount, hydrochloric acid and sodium hydroxide were used. The highest extraction efficiency for ALA was obtained at pH = 2 (Fig. 3d).

3.3. Carry Over

The term "carry-over" is commonly used to describe a process by which materials are carried into a reaction mixture to which they do not belong. These materials can be either parts of a specimen or reagents, including diluent or wash solution. In this research "carry over" means the amount of analytes or washing solution which remains in the memory of MIP bed after elution of the sample [20]. In MEPS, the absorbent is washed several times to prevent the memory effect. Therefore, MIP can be used several times and cannot be disposable. It is important to choose a suitable solvent for doing this. In this study, a urine sample of 1 µg/ml was used to study the effect of memory. For this, the extraction and then washing was done in 3 different cycles (1×150, 3×150 and 5×150). The results showed that the extraction cycle of 3×150 reduced the memory effect to 0.16. When using 5×150 cycle, the memory effect reached 0.06. Therefore, the 5×150 cycle was selected as the optimal amount.

3.4. Validiation of the Method

As previously mentioned, the MIP selectivity was investigated in the presence of MA as an intervener and the results showed that MIP has a good chance for ALA and has the ability to eliminate interferents. The calibration curve was drawn at 5 concentrations from 0.5 to 2.5 µg/ml and a good relationship was found between the surface of the curve and the concentration (R^2 = 0.9902). The accuracy and precision of inter- and intra-day were determined using three quality control samples at concentrations of 0.05, 0.25 and 1 µg/ml. In order to calculate the accuracy of intra-day, each quality control sample was analyzed 5 times a day. For inter-day, each

quality control sample was analyzed 5 times for 3 consecutive days. The precision was reported as a percentage of standard deviation and accuracy was reported as the percentage of deviation of the measured values from nominal values. The accuracy of the method was -8 to -12.8 %. The precision for inter-day was 2.13 to 8.04 % and for intra-day, it was 1.66 to 5.4 %. Blank urine samples were analyzed 5 times to determine the limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ were determined based on the same guidelines using these formulas LOD = 3.3 SD/*b* and LOQ = 10SD/*b*, where SD is equal to the standard deviation of 5 blank samples and *b* is the slope of the calibration curve. The LOD was 0.036 µg/ml and LOQ was 0.12 µg/ml.

After optimization of all available parameters, the urine samples were spiked with concentration of 5 µg/ml, and extraction recovery was 93 %. Extraction recovery was also estimated for qualitative control samples and it ranged from 90.2 to 92 % (Table 3).

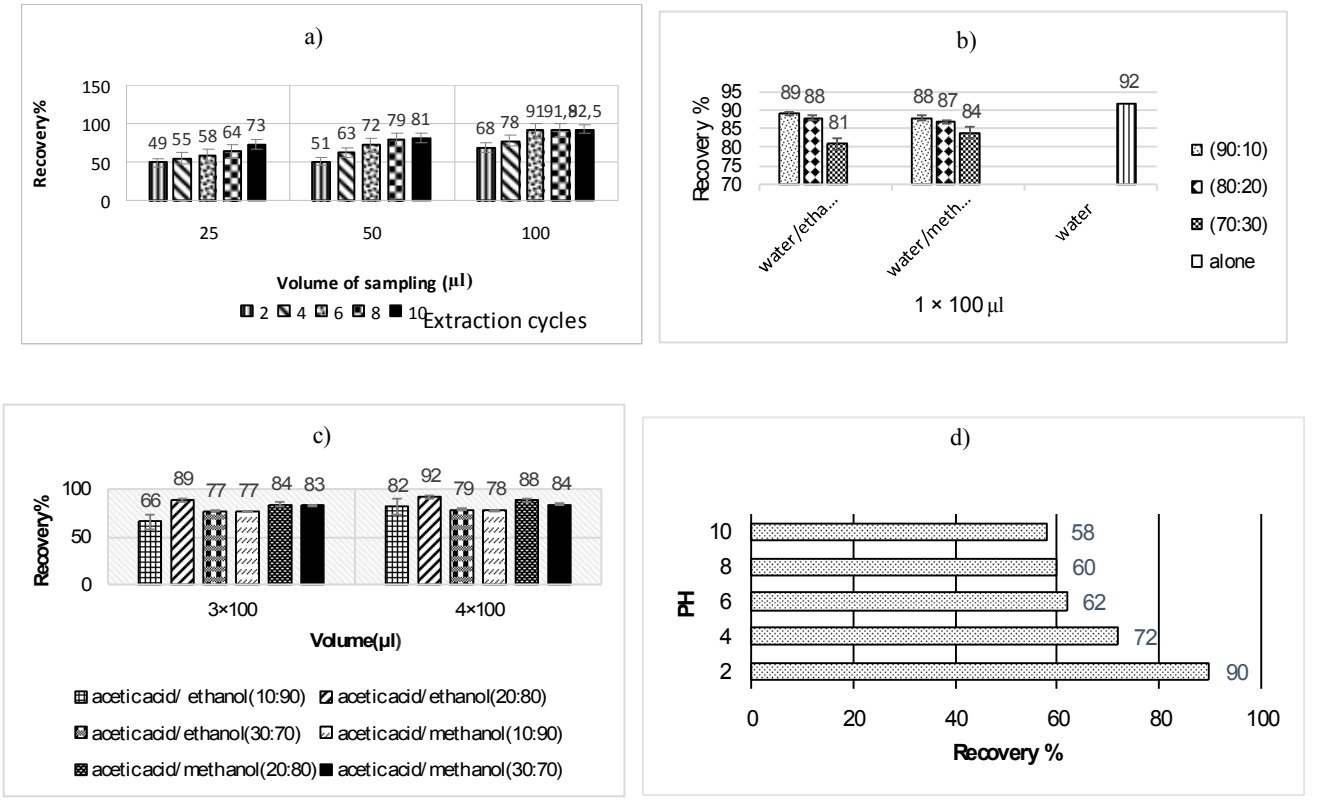

Fig. 3. Effects of experimental parameters on MIP-MEPS performance: number of extraction cycles and sample volume (a); volume and type of washing solutions (b); volume and type of ellution solution (c) and sample pH (d)

Table 3

Validation method results

QC, mg/ml	Linearity	LOD, mg/ml	LOQ, mg/ml	Recovery, %	Mean concentration, mg/ml ($n = 6$)	Accuracy, %	Precision, % RSD	
							Inter-day	Intra-day
0.05	$Y = 0.0045X + 0.0043$	0.036	0.12	87.0	0.0436	-12.8	8.04	5.40
0.25	$R^2 = 0.9902$	–	–	92.0	0.230	-8.0	3.61	3.07
1.00	–	–	–	90.2	0.902	-9.8	2.13	1.66

Table 4

Comparison of method with other methods

Analysis method	LOD, mg/ml	LOQ, mg/ml	Recovery, %	Solvent consumption	Selectivity	Time consuming	Ref.
Colorimetrically and ion-exchange column chromatography	N.M	N.M	91–93.9	high	low	high	[21, 22]
Colorimetrically and fluorometric HPLC	N.M	N.M	90–98	high	low	high	[23-26]
MIP-MEPS (our method)	0.036	0.12	> 90.2	low	high	low	–

Note: N.M is not mentioned

3.5. Comparison of Methods

By comparing different methods (Table 4), the method presented has a recovery similar to the previous ones. The main problem of the previous methods is the high use of chemical solutions, long process and time consuming, the possibility of interfering with other materials, high pH in some cases, and the response of their number only at low concentrations. The provided method has a high recovery rate, LOD and LOQ values were the same and even more than in the previous methods. In addition, the recovery and sensitivity were similar to other methods. The proposed method has advantages such as less amount of absorbent, less organic material consumption, less sample preparation time (about 5 min), high selectivity and recyclability of up to 80 extractions. Moreover, there are small volumes of sample, washing and elution solutions, short extraction cycles. Therefore, the MIP-MEPS method can be used as a valid method for determining the ALA in urine samples.

4. Conclusions

In this study, a new method of sample preparation was introduced to determine the ALA in urine samples using MIP-MEPS. This method is sensitive, selective, inexpensive, fast and friendly to users and the environment. For the first time we successfully combined MIP-MEPS with the spectrophotometric analytical method. Since MIP-MEPS is faster, simpler, and needs much smaller volumes of organic solvents compared with colorimetry-spectrophotometry, it is suggested to apply this method as an alternative for the biomonitoring of exposed individuals with lead.

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РОЗРОБЛЕННЯ МЕТОДУ ВИЗНАЧЕННЯ УРИННОЇ ДЕЛЬТА-АМІНОЛЕВУЛІНОВОЇ КИСЛОТИ З ВИКОРИСТАННЯМ ПОЛІМЕРІВ З МОЛЕКУЛЯРНИМИ ВІДТИСКАМИ

Анотація. *Дельта-амінолевулінову кислоту (ALA) використано як індекс біологічної дії для працівників, які піддаються впливу свинцю. Розроблено новий аналітичний метод з використанням полімерів з молекулярними відтисками (ПМВ) у мікроекстракції упакованими сорбентами для зразків сечі. Проведено спектрофотометричний аналіз зразків сечі. За допомогою Фур'є спектроскопії визначені основні складові ПМВ. Показано, що розроблений метод є швидким, чутливим, селективним, екологічним та зручним у використанні. Він ж придатним для біомоніторингу осіб, які піддаються впливу свинцю.*

Ключові слова: *спектрофотометрія, мікроекстракція упакованими сорбентами, полімери з молекулярними відтисками, дельта-амінолевулінова кислота, зразок сечі.*