Chlorella Vulgaris Microalgae as a Green Packing for the Microextraction by Packed Sorbent of Nitrofurantoin in Urine

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A sensitive, relatively selective, and semi-automated microextraction by packed sorbent (MEPS) method was developed for the spectrophotometric determination of nitrofurantoin (NFT) in urine samples using a green sorbent. *Chlorella vulgaris*, a unicellular green microalgae, was used for the first time, as a biosorbent in the developed MEPS method. Effects of several factors such as sample volume, eluent volume, sample pH and number of extraction cycles (draw-ejects) on the performance of the method were carefully studied. It was found that NFT is quantitatively enriched on the packed syringe from a sample adjusted on pH 8, after 14 draw-eject cycles. This procedure was easily performed by a designed reciprocating apparatus. After washing the sorbent for removing matrix interferences, the analyte was eluted by 30% acetone in water as eluent. Under the optimized conditions, a detection limit of 0.039 mg l^{-1} and a linear calibration curve with an R^2 of 0.997 was obtained for NFT determination. The precision of the method, expressed as the relative standard deviation, was 3.54 for six replicates. The method was successfully applied to the determination of NFT in human urine samples.

INTRODUCTION

Sample preparation is a critical step before performing an analysis, especially for samples with a complex matrix. Protein precipitation, liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are common classic sample preparation methods used in bioanalysis laboratories. The classic methods, however, often require large quantities of samples and solvents or solvent mixtures [1-3]. Microextraction techniques such as solid phase microextraction (SPME) and microextraction by packed sorbent (MEPS) are examples of recently developed alternative methods that require no solvent or minimum amounts of solvents [4-6]. The MEPS method, described by Abdel-Rehim et al. for the first time [5], miniaturizes the SPE technique and it can work with small sample volumes

in the range of 10-250 µl. In this method, the packing is a few miligrams of a sorbent inserted into the barrel of a syringe, or between the syringe barrel and the injection needle. Therefore, a separate column is not needed [5-11]. MEPS also reduces the sample preparation time and minimizes the use of organic solvents. The MEPS procedure may be easily automated, thus, it is suitable for the rapid analysis of biological samples [12].

Nitrofurans (NFs) are Schiff's base derivatives of nitrofuraldehyde known to have a broad-spectrum of antimicrobial activity. Nitrofurantoin (NFT), with the chemical name of 1-(5-nitro-2-furfurylidene amino-hydantoin), is a synthetic nitrofuran derivative and antibacterial agent. The effect of NFT depends on its concentration at the site of infection and the susceptibility of the infecting organism [13]. One of the common uses of this drug is in the treatment of urinary tract infections caused by susceptible organisms. *In vitro* bacteria

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inhibition by NFT is usually achieved by concentrations of 1-32 μg ml^{-1} of the drug [14]. Therefore, accurate determination of NFT in urine and other biological fluids is critical.

Biosorption, or the use of algal biomass as adsorbent, has received increasing attention in recent decades because of its potential application in environmental protection and analysis. Many types of such microorganisms have been utilized for removing of metals and nutrients from wastewater or polluted waters [15-17]. However, the use of microalgae in a standard sorption process is not easily possible due to their small sizes, low strength, and difficulties in their separation [18]. *Chlorella vulgaris* is a unicellular green alga, which is known as one of the fastest growing microalgae [19]. It has been used in the free and immobilized forms for the removal of heavy metals [15,16,18], tributyltin[20] and nutrients such as nitrogen and phosphorus [19].

The aim of the present study is to investigate the possibility of using *C. vulgaris* as well as *Dunaliella bardawil* and *Spirulina platensis* algae as green sorbents in a MEPS system. The application of the system to the adsorption of NFT in urine samples are investigated and optimized before the final spectrophotometric determination of the analyte.

EXPERIMENTAL

Reagents and Materials

NFT was purchased from Sigma. *C. vulgaris* UTEX-265 and *Dunaliella bardawil* UTEX-2538, eukaryotic unicellular green algae were obtained from algal collection of Lorestan University, Iran. *Spirulina platensis,* a prokaryotic blue-green multicellular filamentous alga, was obtained from INVE (Thailand) Ltd. Methanol (gradient grade), acetonitrile, acetone, dimethyl sulfoxide (DMF) and sodium phosphate were purchased from Merck (Darmstadt, Germany). Double-distilled water was used throughout. A 100 mg l^{-1} solution of NFT in methanol: DMF mixture (50:50 v/v) was prepared and used as the stock solution for preparation of working standards.

Instrumentation and Operating Condition

For spectrophotometric determinations, a Spekol, model

2000/1 (Germany) UV-Vis instrument was used. A pair of quartz 350 μl micro-cells (ES-quartz, model Q124, Spain) were utilized for absorbance determinations of test solutions. A pH meter device, Model 211 manufactured by HANNA Co. was used for pH adjustments, and a Hamilton device Model wsc/4D all Pyrex was used for preparation of double-distilled water. For centrifugation of the extracts, a model 5810, Hamburg, Centrifuge was used. Stainless steel laboratory sieves with mesh sizes of 80 to 200 μm were used for sieving and sizing the algae cells.
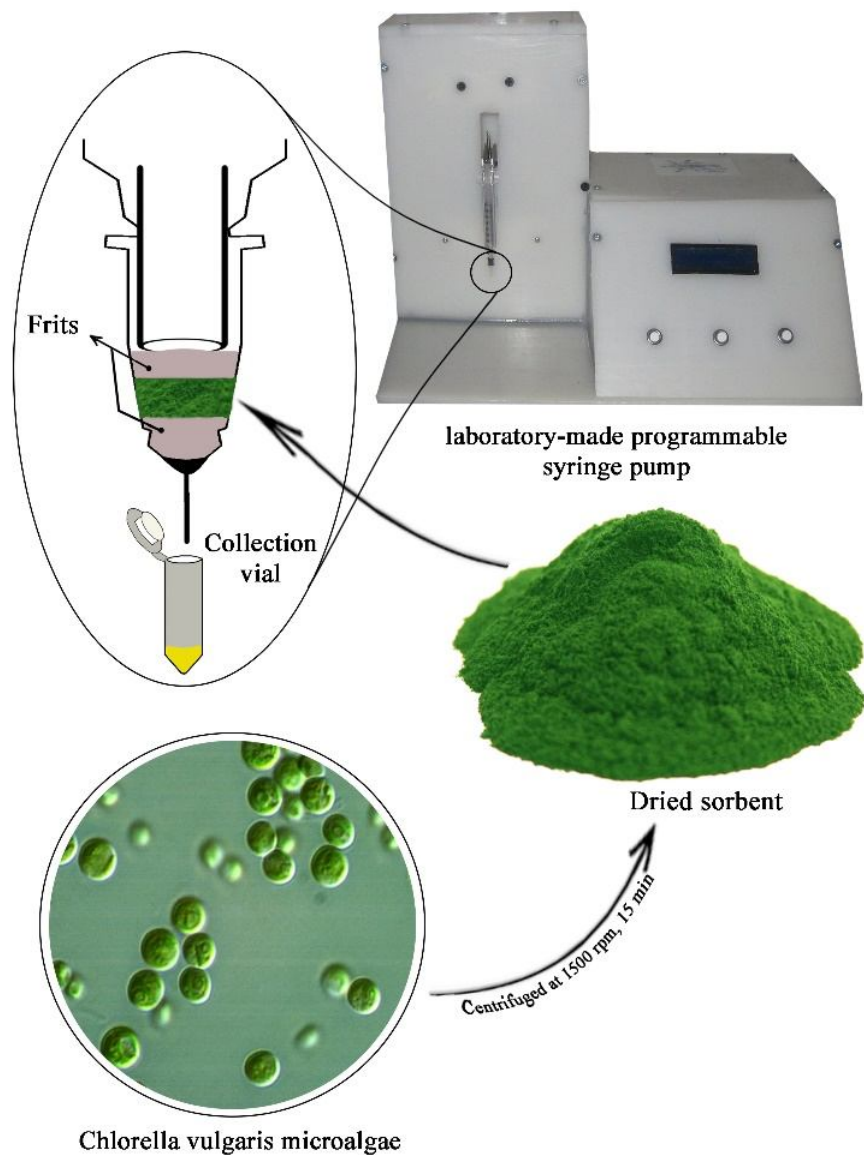
For automation of the MEPS syringe plunger up and down movements, and in order to increase the speed and reproducibility of the extraction, a laboratory-made programmable apparatus (Fig. 1) was designed and used, as described elsewhere[21]. A controller unit was assembled to the system and programmed for the adsorbent activation, analyte adsorption and desorption, absorbent washing, cleanup and column aeration.

Cultivation of *C. vulgaris* and Preparation of the Adsorbent

The alga *C. vulgaris* was aseptically inoculated as pure suspended cultures in 1000 ml flat bottom flasks, containing 500 ml of autoclaved, modified fresh medium, described by Shariati and Lilley [22]. The pH was adjusted on 7.2-7.4 before autoclaving the medium. Cultures were grown under photoperiodic conditions of 16 h cool-white fluorescent light (60 μmol m^{-2} s^{-1})/8 h dark, at 25 °C/18 °C ± 0.5 °C. Cells were sampled during the logarithmic phase of growth (containing 10-13 × 10^{6} cells per ml medium) and were transferred into centrifuge tubs. Cultures were centrifuged at 1500 rpm for 15 min, and the supernatant was removed. Then, the pellet was washed twice with deionized water, and the biomass was dried at room temperature and darkness for about five days.

Preparation of Urine Samples

The method was evaluated for the determination of NFT in urine samples of three volunteers. Filtering and protein precipitation methods are commonly used in preparation of biological samples such as blood and urine. Since there is no protein in the urine of healthy individuals, the protein precipitation step was not necessary in this work and the samples were only filtrated before the extraction [23].

Fig. 1. A graphical representation of the developed MEPS system.

The MEPS Procedure

After packing a microsyringe with 4 mg of the solid sorbent (dried *C. vulgaris* biomass), it was mounted on the reciprocating apparatus for automation of its plunger movement (Fig. 1). In the first use, the sorbent was conditioned by 2×100 µl water. After that, the sample (300 µl, pH 8) was drawn into the syringe up and down (14 times). The adsorbent was then washed once with 100 µl water to remove potential interferences. The analytes were then desorbed with 150 µl of 30% acetone: water (6 times) and the eluate was transferred into the quartz cell for the spectrophotometric analysis. The sorbent was cleaned by 3×100 µl extra volumes of the eluent before the next run.

RESULTS AND DISCUSSION

Effect of Particle Size of the Algae

Effect of size distribution was studied for the alga *C.*

vulgaris. After preparation and drying the alga at room temperature, the alga was classified into three size distributions of under 80 µm, 80 to 125, and 125 to 200 µm, by using laboratory sieves. It should be noted that the sizes of individual algae cells are much smaller 80 µm but they are somewhat agglomerated and stacked together during the drying process. Among the three size distributions, the largest size was found to be the most appropriate. Using the smaller size particles, a substantially higher backpressure was noticed that resulted in a reduced extraction efficiency. According to the observations, the particle size distribution of 125 to 200 µm was used in subsequent experiments.

Selection of the Microalgae

Selection of the sorbent is the first, and probably the most important step in a MEPS procedure. In preliminary studies of this research, three algae of *C. vulgaris*, *Dunaliella bardawil*-UTEX 2538 and *Spirulina platensis* were tested as the packing material for the MEPS syringe. Among the studied sorbents, *C. vulgaris* showed a substantially higher efficiency for extraction of NFT. The adsorption of the analyte may be due to its hydrogen bond formation with the surface groups of the alga such as carboxyl, ether, alcoholic, and amino groups [24]. However, *Dunaliella* is a cell wall-less alga. Possibly because of lacking a cell wall, it showed lower adsorption and extraction efficiencies. Although *Spirulina* has multilayered cell walls, they are completely different from *Chlorella*'s cell walls, in structure and composition.

Effect of the Adsorbent Weight

Another important factor studied in this work was the amount of algae. Values of 2 to 5 mg (dry weigh) of *Chlorella* alga were tested. The results (Fig. 2) showed that the highest extraction efficiency is obtained when 4 mg of the alga was used as the packing. The use of higher amounts of the sorbent increased the column back pressure and hardened the plunger movement of the syringe. Therefore, the extraction efficiency was decreased.

Selection of the Elution Solvent

For eluting the collected analyte on *C. vulgaris,* sorbent mixtures of 70% water and 30% of different organic solvents of methanol (MeOH), ethanol (EtOH), dimethyl-formamid (DMF), acetone and acetonitrile (ACNL) were used. Higher percentages of the organic solvents could damage the microalgae cells and was therefore avoided. For the elution, 6 draw-eject cycles of 150 µl of the elution solvents were used. Blank absorbance measurements at 312 nm (the λ_{max} of NFT) for the tested solvents resulted in absorbances of 0.050, 0.062, 0.073, 0.052 and 0.149 for the mentioned solvents, respectively. Figure 3 compares the recoveries of the analyte obtained for the solvents. Among the solvents, acetone-water and acetonitrile-water mixtures had the highest extraction efficiencies. However, since acetonitrile-water showed a relatively high blank, it was excluded, and 30% acetone in water solvent was selected as the optimum solvent to be used in subsequent experiments.

Volume of the Elution Solvent

Elution solvent volume should be sufficiently high to completely desorb the analyte from the sorbent and enough low to result in a reasonable enrichment factor. Therefore, different volumes of the eluent (30% acetone in water) were studied. As shown in Fig. 4, the minimum volume for obtaining the maximum recovery was 150 µl that was selected as optimum.
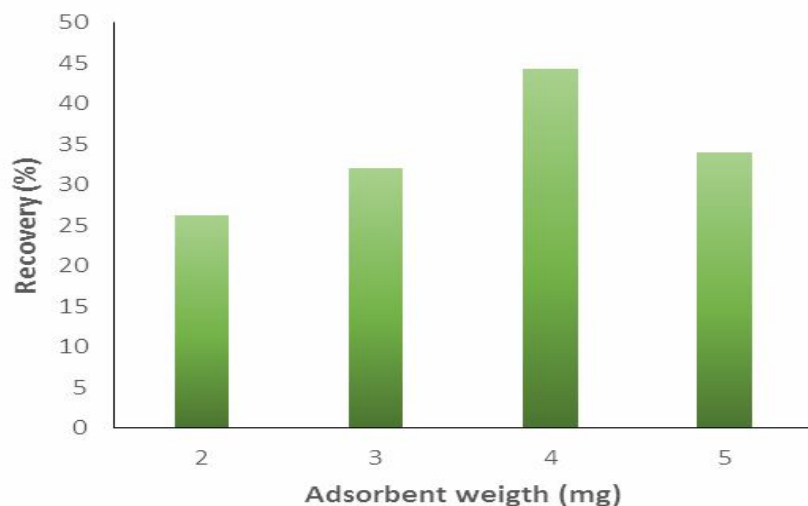
Effect of Sample pH

It is known that the activity and other properties of NFT is pH dependent [25]. Also active groups of the algae have acid/base properties which may be protonate or deprotonated by altering the sample pH. Effect of sample pH on the extraction efficiency of NFT in the MEPS method was investigated in the range of 1-11. As shown in Fig. 5, pH 8 was the optimum pH with the maximum recovery in this case.

Effect of the Ionic Strength

Effect of the ionic strength on the extraction efficiency of NFT was examined by adding different amounts of sodium nitrate (0-5%, w/v) into the aqueous samples. The results indicated that the recovery was moderately decreased by increasing the salt concentration (Fig. 6). This may be explained by the competition of Na^+ ion and the analyte for the active sites of the algae.

Effect of Draw-eject Cycles

Another factor influencing the extraction efficiency in a

Fig. 2. Effect of the adsorbent weight on the efficiency of NFT extraction by the MEPS method. Experimental conditions: sample pH, 5; No. Of adsorption cycles, 10; volume of elution solvent, 100 μl; sample volume, 100 μl; elution solvent, H_2O-ethanol (70:30).
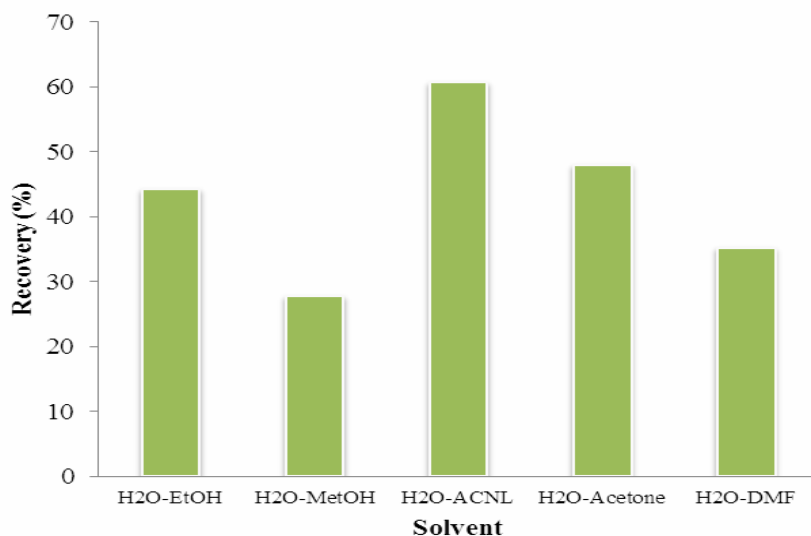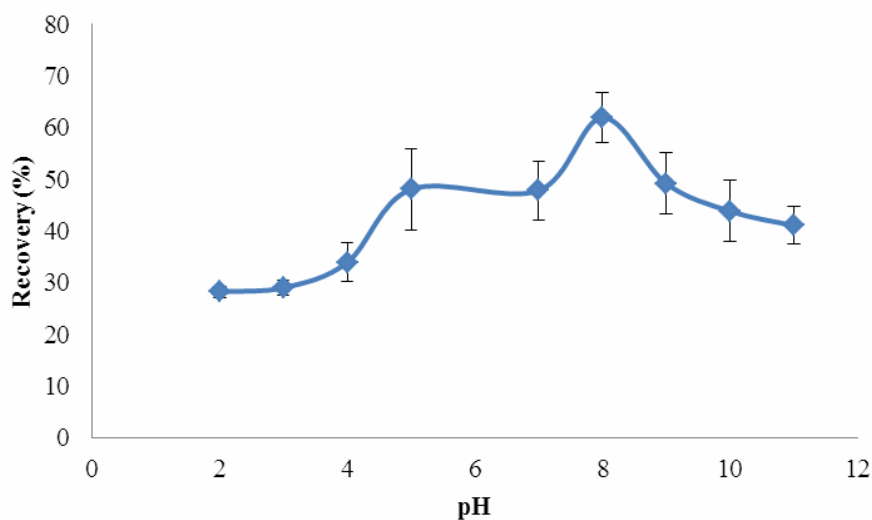

Fig. 3. Effect of the type of elution solvent on the extraction efficiency of NFT by the MEPS method. Experimental conditions: sample pH, 5; adsorbent weight, 4 mg; No. of adsorption cycles, 10; volume of elution solvent, 100 μl; sample volume, 100 μl.

MEPS system is the number of times that the sample is passed through the sorbent or the number of draw-eject cycles. Moving the plunger of the syringe up and down repeatedly, increases the contact time between the sample and sorbent commonly resulting in a higher recovery. In order to evaluate this factor, using different adsorption cycles were evaluated. As shown in Fig. 7, using 14 draw-
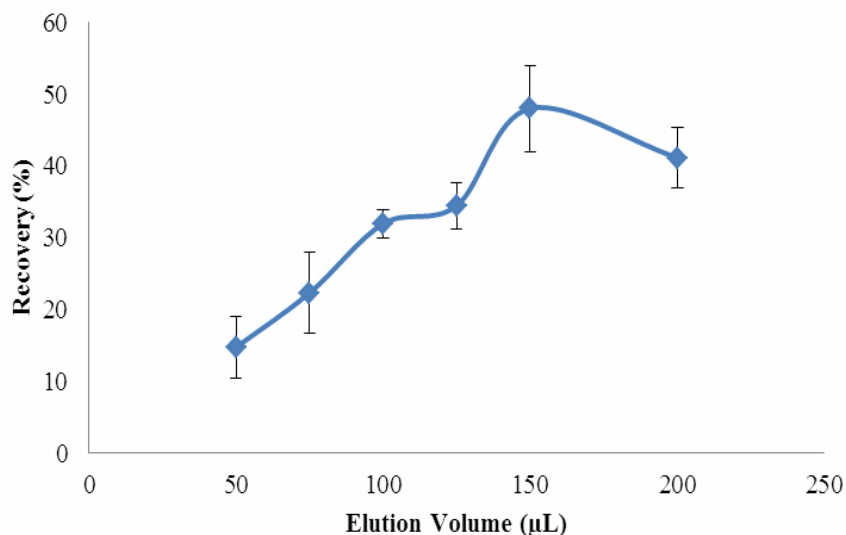
Fig. 4. Effect of elution volume on the extraction efficiency of NFT by the MEPS method. Experimental conditions: sample pH, 5; adsorbent weight, 4 mg; No. of adsorption cycles, 10; elution solvent, H_2O-aceton (70:30); sample volume, 100 µl.
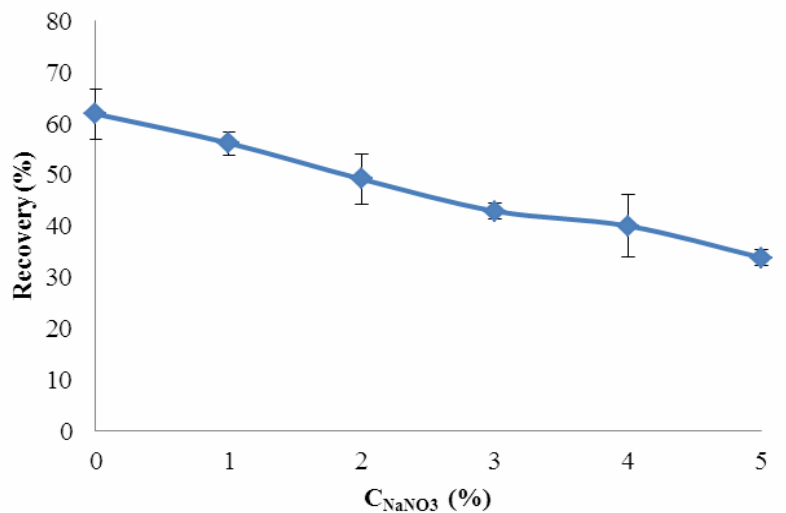

Fig. 5. Effect of sample pH on the extraction efficiency of NFT by the MEPS method. Experimental conditions: adsorbent weight, 4 mg; No. of adsorption cycles, 10; elution volume, 150 µl; elution solvent, H_2O-aceton (70:30); sample volume, 100 µl.

eject cycles resulted in the highest recoveries and was chosen as optimum. The decrease in recovery in the higher number of draw-eject cycles may be due to desorption of the adsorbed analyte by the sample medium.

Effect of Sample Volume

Different sample volumes in the range of 50-400 µl were tested for the analysis by the MEPS method containing the same amount of the analyte (0.2 µg). The extraction
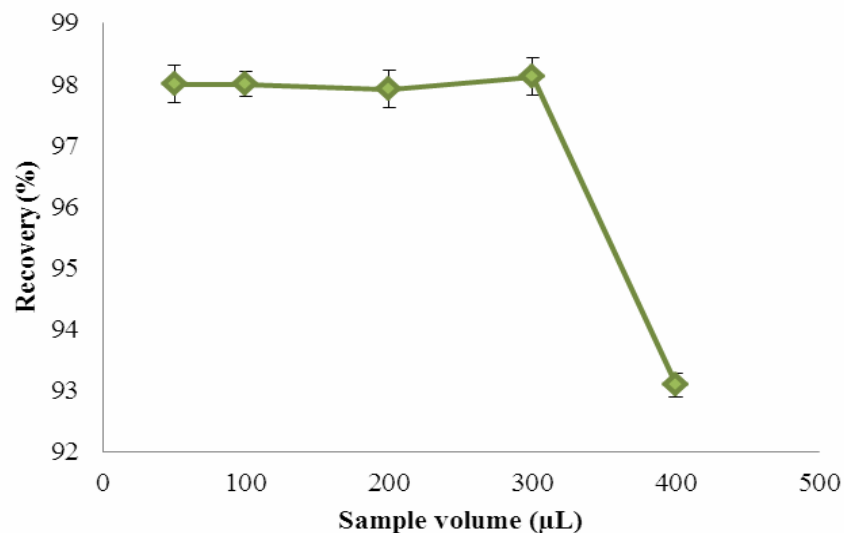
Fig. 6. Effect of ionic strength on the extraction efficiency of NFT by the MEPS method. Experimental conditions: sample pH, 8; adsorbent weight, 4 mg; No. of adsorption cycles, 10; elution volume, 150 µl; elution solvent, H_2O-aceton (70:30); sample volume, 100 µl.
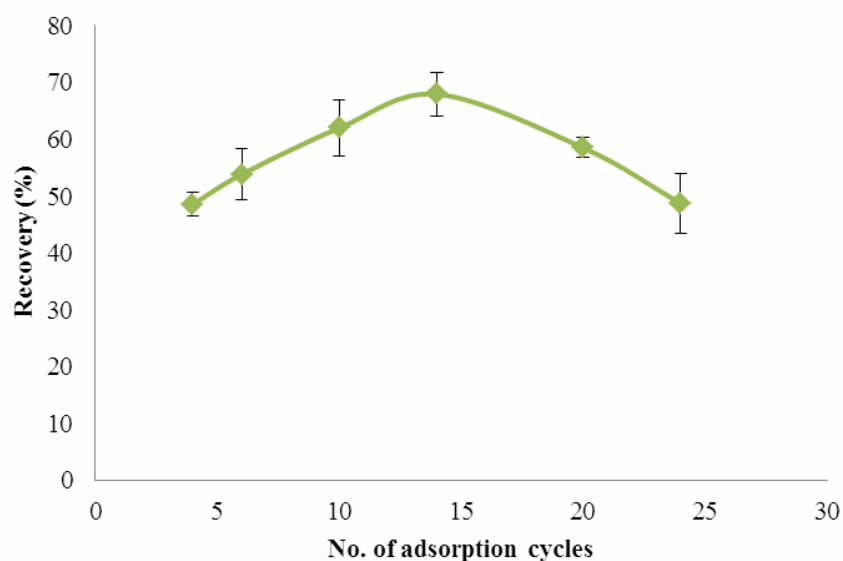

Fig. 7. Effect of the number of adsorption cycles on the extraction efficiency of NFT by the MEPS method. Experimental conditions: sample pH, 8; adsorbent weight, 4 mg; elution volume, 150 µl; elution solvent, H_2O-aceton (70:30); sample volume, 100 µl.

efficiency did not significantly change for sample volumes up to 300 µl, but it reduced for higher volumes as shown in Fig. 8. Therefore, this volume may be considered as the breakthrough volume of the system. Using larger sample volumes causes desorption of some of the adsorbed analyte. Further tests indicated that the sorbent is not able to adsorb more amounts of the analyte. According to the results, the capacity of the algae for NFT was calculated to be

Fig. 8. Effect of sample volume on the extraction efficiency of NFT by the MEPS method. Experimental
conditions: sample pH, 8; adsorbent weight, 4 mg; No. of adsorption cycles, 10; elution volume,
150 µl; elution solvent, H_2O-aceton (70:30).
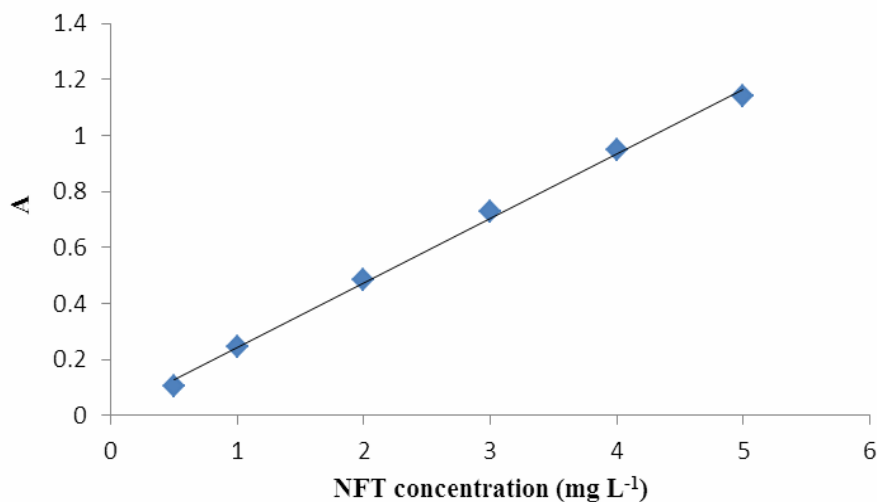
Fig. 9. The calibration curve for measuring NFT by the MEPS method under the optimized conditions.

50 µg g^{-1}.

Selection of the Regeneration Solvent

For a complete cleanup of the sorbent and its
regeneration, several 100 µl volumes of 30% acetone were
passed through the syringe successively, and each time
the effluent was analyzed by UV spectrophotometer. The

results showed that for a complete cleanup and regeneration
of the adsorbent, one volume of the 100 µl solvent was
sufficient.

Analytical Performances

Replicated experiments in the optimal conditions
obtained by the one-at-a-time method for the extraction of

Table 1. Comparison of some Analytical Performances of the Developed MEPS Method with the Results of some
Reported Methods for the Quantitation of NFT

Method	Detection method	RSD (%)	LOD (mg l^{-1})	Recovery (%)	Ref.
Biosensor	Electrochemical	1.15	0.65	96.3-97.2	[26]
Extraction	HPLC	<10.2	1.3	72.2-107.4	[27]
Flow injection-spectrophotometry	UV-Vis	<1	4.8	100.164	[28]
Supercritical fluid extraction	HPLC	0.73	2.88	Not reported	[29]
MEPS	UV-Vis	3.53	0.039	98.1	This work
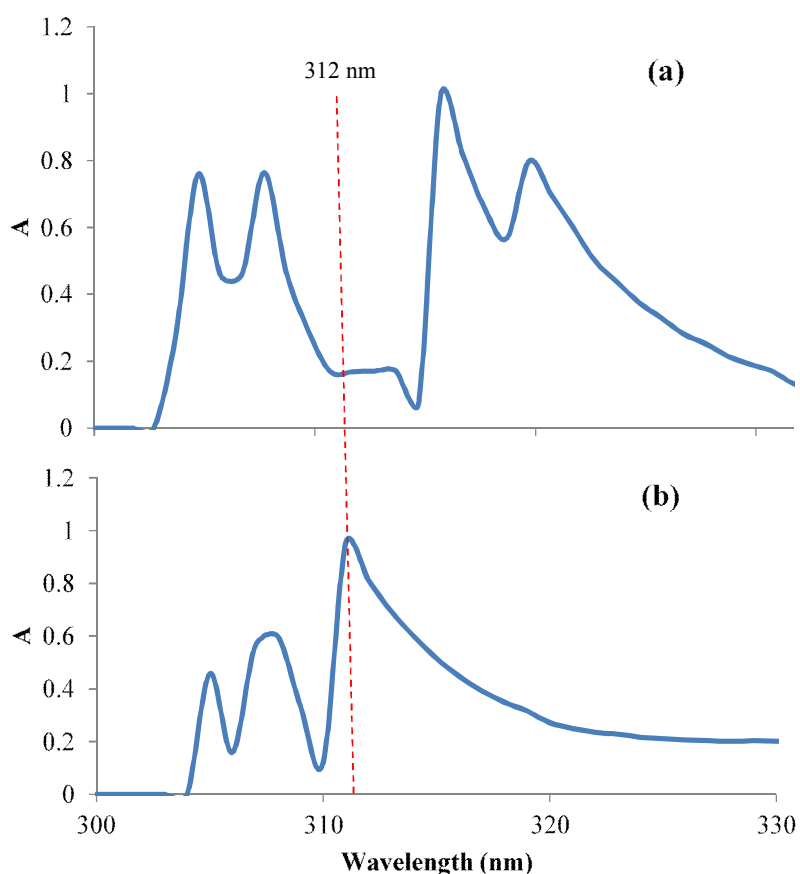
Fig. 10. Spectra of a urine sample (a) and a 5 mg l^{-1} of NFT sample (b) after being extracted by the MEPS method.
The dashed line shows the wavelength selected for the quantitation of NFT.

Table 2. The Results Obtained for the Spectrophotometric Determination of NFT by the MEPS Method in Spiked Urine Samples ($\lambda = 312$ nm)

| Added | Sample 1 | | Sample 2 | | Sample 3 | |
| | Found | Recovery | Found | Recovery | Found | Recovery |
$(mg\ l^{-1})$	$(mg\ l^{-1})$	(%)	$(mg\ l^{-1})$	(%)	$(mg\ l^{-1})$	(%)
0	-	-	-	-	-	-
1	1.1	111.7	1.1	113.6	1.0	103.8
3	2.8	91.8	3.1	103.3	2.9	95.5
5	4.8	95.4	4.6	92.3	4.6	91.9
10	10.2	101.8	10.1	101.5	10.2	102.1

NFT resulted in an extraction recovery of 98.1% with a relative standard deviation (RSD) of 3.54% for n = 6. The limit of detection (LOD) of the method, calculated from 3σ, was 0.039 mg l^{-1} of NFT. The calibration curve was linear over a wide range from 0.5 to 5 mg l^{-1} with an R^2 value of 0.997 (Fig. 9). Table 1 compares the analytical performances of the developed MEPS method with the results of some reported methods for the quantitation of NFT. As the results show, better or comparable LOD, RSD and recovery values have been obtained in this work.

NFT Measurement in Urine Samples

The proposed extraction method was used for measuring the amount of NFT in three urine samples. For this purpose, the urine samples were extracted by the MEPS technique, before and after being spiked by different amounts of NFT. As shown in Fig. 10a, NFT has a sharp peak at 312 nm, after being extracted by the method, but the urine matrix in this region is free from interfering peaks (Fig. 10b). Therefore, this wavelength could be used for quantitation of the analyte with no significant interferences from the matrix.

Three urine samples from volunteer students were extracted by the MEPS method. The samples where then spiked by different amounts of NFT and their absorbances at 312 nm were measured. Table 2 summarizes the results obtained for the urine samples. As indicated in this table, quantitative results were obtained for all the spiked samples.

CONCLUSIONS

The first use of dried algae cells as green sorbents in the MEPS method was successful. *C. vulgaris* alga was efficiently used for the adsorption of NFT from urine samples and its elution by a mixture of aceton-water for its simple spectrophotometric measurement. The proposed biosorption method was rapid, environmentally friendly, efficient, and relatively selective. Moreover, the unicellular alga could be used as a cost effective and efficient alternative to more costly materials such as nanoporous sorbents in the extraction process. In this technique, a packed biosorbent could be used several times and the required sample volume was small.

Further studies may be planned for the removal or enrichment of different species such as medicine residuals, organic pollutants, dyes and heavy metal ions by the use of different biomass materials in the MEPS technique.

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