

# Optimization of Solid Phase Microextraction Procedure Followed by Gas Chromatography with Electron Capture Detector for Pesticides Butachlor and Chlorpyrifos

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## Abstract

**In this study, headspace solid phase microextraction (HS-SPME) followed by gas chromatography using electron capture detection system (GC-ECD) were developed for the determination of chloracetanilide (butachlor) and chlorpyrifos presented in biological samples. Different parameters affecting the extraction procedure were optimized including extraction time (30 minutes), extraction temperature (80°C), sample volume (3 mL), sample pH (2), added NaCl (0.3 gram) and sample stirring rate (400 rpm). Different concentrations of 1 - 100 ng/ml were applied for butachlor and a linear calibration curve was obtained. Furthermore, a similar linearity was obtained for chlorpyrifos, using a concentration range of 1 - 250 ng/ml. The limit of detection (LOD) obtained for butachlor and chlorpyrifos were 0.088 and 0.53 ng/ml respectively. The optimized methods for both compounds were validated using two concentrations of 25 and 50 ng/ml in spiked urine samples. Obtained recoveries of spiked urine samples were 83.06% - 99.8% with RSD of lower than 11%. Optimized technique was simple, inexpensive, solvent free and fast in comparison with other conventional methods and had compatibility with the chromatographic analytical system. This method offers low detection limits to analyze pesticides in urine samples that are very important**

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**in the exposure monitoring in occupational health.**

## Keywords

**HS-SPME, GC-ECD, Biological Samples, Chlorpyrifos, Butachlor**

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## 1. Introduction

Pesticides are widely used for agricultural purposes around the world. Application of pesticides in agriculture has increased productivity and has led to the increase yields in farming. Pesticides are biologically active and can be harmful to the variety of non-target organisms (including human) when they are applied [1] [2]. Therefore, nowadays, toxic effects of pesticides on humans and the environment are of very important concern [3]. Among the various pesticides available; butachlor and chlorpyrifos are the ones most widely used in developing countries, including Iran by the relevant plant protection organization. Butachlor is structurally belonging to the chloroacetanilide family, and chlorpyrifos is in the organophosphorus pesticide category. It is worth mentioning that, butachlor is a suspected carcinogen [4], so, it needs to be more monitored in the environment, and as a result, monitoring this pesticide in the environment and human is most important for evaluation of the risk hazards associated with such pesticides [5]. Chlorpyrifos as organophosphorus pesticides is very toxic when absorbed by human being because of its property of acetyl-cholinesterase inhibition [6]. To evaluate exposure to these pesticides and the corresponding health risk, measuring the parent pesticide levels in body fluids may be quite useful [7] [8]. There are some traditional techniques to determine the pesticides such as liquid chromatography and gas chromatography with very low detection limits. But the classical gas chromatographic methods are not satisfactory due to the thermal instability of the organophosphorus molecules and a sample preparation or pre-concentration step is needed to determine the analytes at ppb levels. Several analytical methodologies have been developed for sample preparation to facilitate identification of pesticides from biological and environmental samples. Liquid-liquid extraction (LLE) is one of the conventional methods still being used for the extraction of such pesticides from various matrices [9] [10]. Also, solid-phase extraction (SPE) [11]-[17], supercritical fluid extraction (SFE) [18], and matrix solid-phase dispersion (MSPD) [19] is the other more selective approach in this field. Generally, these methods are multistage, expensive, laborious, and consume long time as well as; in these methods, a large amount of organic solvents are applied to extract the analytes of interest [20].

Modern microextraction techniques such as solid-phase microextraction (SPME), that is a rapid, inexpensive, solvent free and quantitative analytical technique was developed by Pawliszyn *et al.* in the early 1990s [21] [22]. SPME has been employed as a sample preparation method for the analysis of pesticides in various matrices [23]-[25]. In SPME, headspace (HS) approach is preferred for extracting analytes from biological samples, including blood and urine because of complexity of these matrices [26]. The previous studies about chlorpyrifos have used HS-SPME-GC-ECD and mass method (MS) in water samples, fruits, vegetable, and milk [27] [28]. Moreover, Direct Immersion (DI)-SPME-GC-MS technique has been performed for determining the chlorpyrifos in water samples [29]. HS-SPME procedure has been applied for pesticide's purification in biological samples such as chloroacetanilide [30] and organophosphorus pesticides [31].

In comparison with other methods, there are several more variables affecting the determination of butachlor and chlorpyrifos by HS-SPME method that must be studied and optimized to obtain high analyte recovery and low detection limits. Under the optimum extraction conditions, high extraction efficiency was achieved in a relatively short time. Also, there are very few existing methods to determine butachlor and chlorpyrifos in urine samples specially for monitoring the workers exposure to these compounds. The aim of the study was to develop a HS-SPME procedure by optimizing several extraction parameters, and to validate the optimized method by performing urine sample analysis for simultaneous determination of butachlor and chlorpyrifos. Parameters affecting the extraction efficiency were optimized, *i.e.* extraction temperature, extraction time, salt addition, sample pH, sample volume, and stirring rate. Through this study, optimum conditions were examined in the real samples. The validation of spiked samples was carried out using the optimum conditions. Limit of detection, limit quantification, and linearity range of the method was established for evaluating the pesticides. This method can lead to low detection limits to analyze pesticides in urine samples that are very important in the occupational

exposure monitoring.

## 2. Materials and Methods

### 2.1. Reagent and Materials

Standards of pesticides were purchased from Sigma-Aldrich (Germany) including butachlor (97.7%) and chlorpyrifos (99.9%). A stock solution of butachlor ( $1000 \text{ mg}\cdot\text{L}^{-1}$ ) and chlorpyrifos ( $2500 \text{ mg}\cdot\text{L}^{-1}$ ) was prepared in acetone and methanol, respectively and stored at  $4^\circ\text{C}$ . All solvents (methanol, acetone, and water) were HPLC grade (Merck-Germany). Other reagents with an analytical grade were obtained from Merck (Germany). Working standard solutions of both pesticides were freshly prepared daily by volume dilution in the HPLC grade water.

### 2.2. Biological Samples

After obtaining approval from Tehran University of Medical Sciences (Vice-Chancellor for Research), all participants signed related consent form for the voluntary participation. Then their urine samples were collected for optimization studies. It is worth to mention that a limited number of healthy and non-exposed persons were randomly selected to study. Urine samples were collected in mornings under documented consent, prepared by adding the appropriate volume of the methanol standard solution, and stored at  $4^\circ\text{C}$  until use. These urine samples were adopted to test the recovery and reproducibility of the optimized method. Since one of the aims of this study was to introduce a proper method for determination of trace level of pesticides in biological samples, so the urine samples were selected to approve the obtained extraction efficiency in this kind of samples. To test the method recovery using the biological samples, defined concentrations of pesticides were spiked in urine (related to non-exposed persons) and optimized extraction procedure was used to get the recoveries.

### 2.3. HS-SPME Extraction Procedure

The SPME device for manual extraction purchased from Supelco (Bellefonte, PA, USA). Polydimethylsiloxane (PDMS  $100 \mu\text{m}$ ) fibres were used as extraction phase because of its great sensitivity. Before using, fibers were conditioned in the injection port of the gas chromatograph for 0.5 h at  $250^\circ\text{C}$  according to the recommended instructions by the manufacturer. SPME procedure was performed in biological samples by placing  $500 \mu\text{l}$  of urine sample and appropriate volumes of pesticide solution at two-level concentrations of 25 and 50 ng/ml in 5 volumetric flasks. These levels with defined concentrations were selected as spiked samples to optimize variables of interest. Hydrochloric acid and sodium chloride was added for adjusting the pH at 2 and salt was added to reach the concentration of 10% and then HPLC grade water was used for adjusting. Three ml volume of this solution was transferred to a 10 ml vial. The vial was rapidly sealed with a silicon septum and aluminum cap. Then; the vial was heated up to  $80^\circ\text{C}$  in aluminum block heater. Samples were stirred during the extraction time under the magnetic stirring (400 rpm). SPME needle pierced the vial septum in order to expose the fiber ( $100 \mu\text{m}$  PDMS) on the headspace of the solution. After 30 minutes, the needle was removed from the vial and inserted into the heated injection port of the GC-ECD for thermal desorption of the sample for 5 minutes.

### 2.4. Gas Chromatography

Chromatographic analysis was carried out by a Varian CP 3800 gas chromatograph equipped with an electron capture detector (ECD). Pesticides were separated in a HP-5 ( $30 \text{ m} \times 0.32 \text{ mm}$ ,  $0.25 \text{ m}$  film thickness) column. Nitrogen (99.999%) with a flow rate of  $2 \text{ ml}/\text{min}$  was used as a make-up and carrier gas. Samples were analyzed using the following conditions: oven temperature program: initial temperature:  $80^\circ\text{C}$  hold for 1 min, rate  $30^\circ\text{C}$  to  $200^\circ\text{C}$  hold for 2 min, rate  $10^\circ\text{C}$  to  $250^\circ\text{C}$  hold for 3 min, rate  $20^\circ\text{C}$  to  $280^\circ\text{C}$  hold for 4 min. The injector temperature was  $280^\circ\text{C}$ . The injector was operated in the split less mode. Detector temperature (ECD) was set at  $300^\circ\text{C}$ .

### 2.5. Data Analysis

The statistical calculations and analysis were performed using SPSS version 16. Some tests such as pair T-test and scheffe analysis of variance were used for data analyzing. The level of significance was taken as  $p < 0.05$ . In

order to optimize the method, a constant concentration was used for each pesticide. For each level of intended variables, three samples with defined concentration were prepared and the mean of extraction efficiencies calculated after analyzing the analytes. Then according to obtained data, the optimum level was selected for every variable.

### 3. Results

#### 3.1. Method Optimization

##### 3.1.1. Extraction Temperature

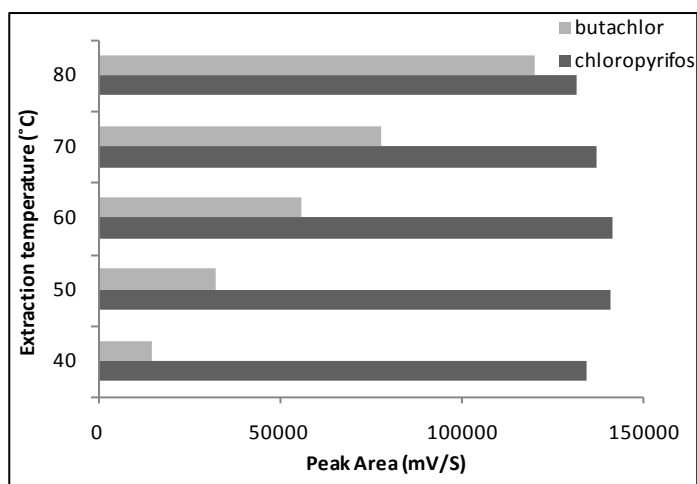
The effect of temperature on extraction efficiency was evaluated from 40°C to 80°C. The highest extraction efficiency for butachlor was obtained at 80°C. This was due to increased transmission of an analyst into the headspace and hence, into the fiber with increasing temperature. The response of chlorpyrifos increased slightly from 40°C up to 60°C. After that, the higher temperature had no significant effect on extraction rate (**Figure 1**). It means that the response is nearly constant in temperatures from 60°C to 80°C. Since the best response for butachlor obtained in the temperature 80°C, for improving the simultaneous extraction of butachlor and chlorpyrifos, temperature of 80°C was chosen as the optimum temperature.

##### 3.1.2. Extraction Time

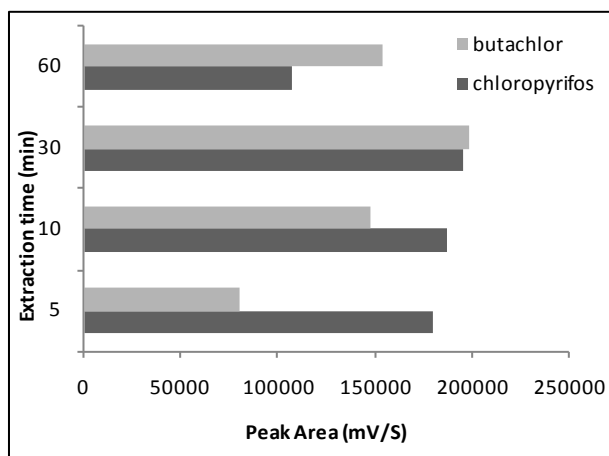
The effect of extraction time was evaluated by exposing the fiber to the headspace of the samples at different extraction times, including 5, 10, 30, and 60 minutes. Equilibrium time for butachlor and chlorpyrifos (from 5 minutes to 30 minutes) was remarkable and showed an increased extraction rate. However, larger extraction time for both pesticides was unsuitable, because the responses of both pesticides were decreased when the extraction time exceeded 30 minutes (**Figure 2**). Therefore, extraction time of 30 minutes was selected at an optimum extraction time, and rms do not have to be defined. Do not use abbreviations in the title or heads unless they are unavoidable.

##### 3.1.3. Salt Addition

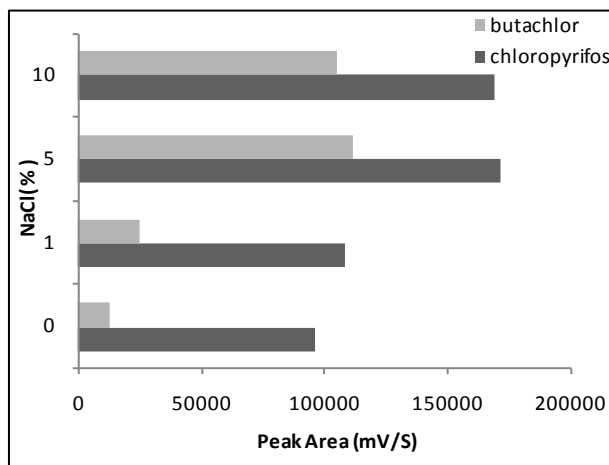
The influence of salt on extraction efficiency was investigated for both pesticides by adding various amounts of NaCl in sample solution, including 0%, 1%, 5%, and 10% (w/v) of sodium chloride. As it can be seen in **Figure 3**, in general, super saturation of the sample with salts is most effective for the extraction of analytes onto the fiber because of the salting-out effect [32]. The response of chlorpyrifos was increased from 0% to 10% of NaCl, and the highest extraction efficiency was obtained in 10% of NaCl. The effect of NaCl on extraction efficiency of butachlor showed that, the amount of NaCl from 0% to 5% increased the efficiency. However, the higher amount of sodium chloride for butachlor showed a slight decrease. Therefore, NaCl 10% was chosen as an



**Figure 1.** Influence of extraction temperature on the extraction efficiency (Extraction time: 30 min, sample volume: 3 ml, sample pH: 2, NaCl: 10% (0.3 gram), stirring rate: 400 rpm).



**Figure 2.** Influence of extraction time on the extraction efficiency (Extraction temperature: 80°C, sample volume: 3 ml, sample pH: 2, NaCl: 10% (0.3 gram), stirring rate: 400 rpm).



**Figure 3.** Influence of salt addition on the extraction efficiency (Extraction time: 30 min, extraction temperature: 80°C, sample volume: 3 ml, sample pH: 2, stirring rate: 400 rpm).

optimum amount of salt added to the butachlor and chlorpyrifos samples. Although according to figure, the optimal salt concentration seems to be 5% for both butachlor and chlorpyrifos, there was no significant difference between 5% and 10% of NaCl for both of them. And since the higher ionic strength had no negative impact on the extraction efficiency and even may improve it in presence of other pesticides, 10% of NaCl was selected as proper salt concentration.

### 3.1.4. Effect of pH

The effects of pH on extraction efficiency for butachlor and chlorpyrifos have been shown in **Figure 4**. The extraction efficiency of these pesticides was studied at various pHs from 2 to 5. The efficiency of both pesticides was decreased significantly when pH increasing. Hence, a sample pH of 2 was chosen as the optimum pH for each two pesticides.

### 3.1.5. Sample Stirring

The effect of sample stirring was evaluated at different stirring rates (200, 400, and 700 rpm). Stirring rates from 200 to 400 rpm showed that the peak areas of both pesticides were increased with stirring rate. The extraction efficiencies of Butachlor and chlorpyrifos decreased significantly at higher stirring rates (from 400 to 700 rpm)

(Figure 5). Therefore, the velocity of 400 rpm was chosen for subsequent analyses.

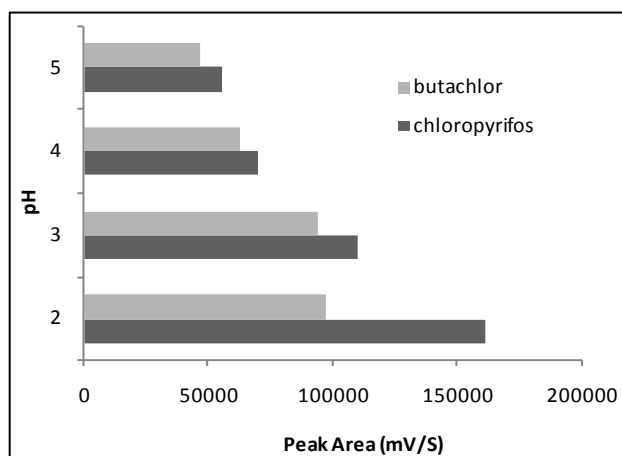
### 3.1.6. Sample Volume

To evaluate the effect of the sample volume on the efficiency of HS-SPME, the sample volumes of 2 to 4 ml were selected. For both pesticides, the maximum response was obtained when 3 ml sample was used. At the higher sample volumes, as it can be seen in Figure 6, extraction efficiency showed a considerable decrease. However, subsequent analyses were performed at 3 ml sample volume.

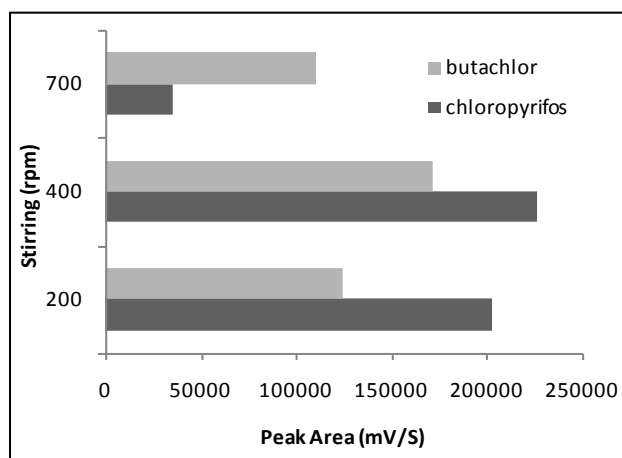
### 3.2. Method Validation

The method was validated using obtained optimized conditions. The detection limits (LODs), quantification limits (LOQs), the inter- and intra-day precisions (RSD), the linearity, and the recoveries were calculated. The results were tabulated in Table 1 and Table 2.

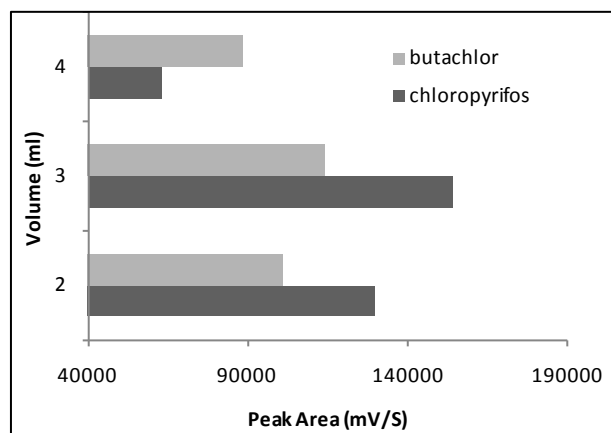
Linear standard curves in the range of 1 - 100 ng/ml and 1 - 250 ng/ml for butachlor and chlorpyrifos were obtained each day for 6 consecutive days, respectively. The obtained correlation coefficients ( $r^2$ ) were 0.9928 or greater (Table 1). Intra- and inter-day precisions of the method were determined by two spiked extracted samples



**Figure 4.** Influence of sample pH on the extraction efficiency chlorpyrifos and butachlor (Extraction time: 30 min, extraction temperature: 80°C, sample volume: 3 ml, NaCl: 10% (0.3 gram), stirring rate: 400 rpm).



**Figure 5.** Influence of stirring speed on the extraction efficiency (Extraction time: 30 min, extraction temperature: 80°C, sample volume: 3 ml, sample pH: 2, NaCl: 10% (0.3 gram)).



**Figure 6.** Influence of sample volume on the extraction efficiency (Extraction time: 30 min, extraction temperature: 80°C, sample pH: 2, stirring rate: 400 rpm, NaCl: 10% (0.3 gram)).

**Table 1.** Analytical performance of the optimized SPME method for two pesticides.

Analyte	Linear rang (ng/ml)	Correlation coefficient ( $R^2$ ) (ng/ml)	LODs (ng/ml)	LOQs (ng/ml)
Butachlor	1 - 100	0.992	0.0888	0.269
Chlorpyrifos	1 - 250	0.997	0.053	0.161

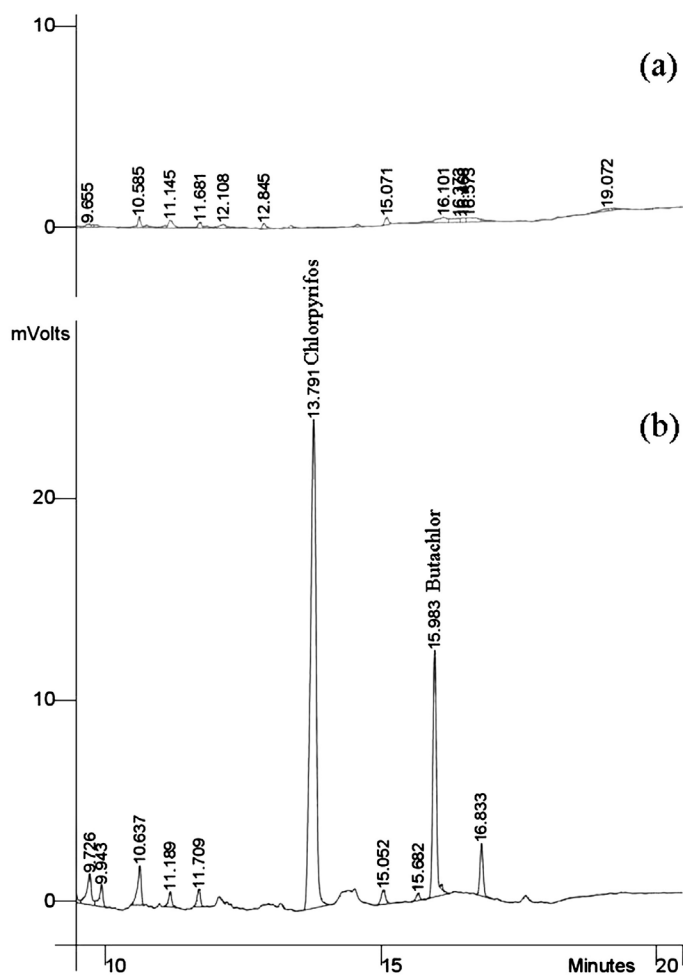
**Table 2.** Inter-day and intra-day reproducibility of method for spiked urine sample.

Analytes	Concentration	Inter-day (n = 6)		Intra-day (n = 6)	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
<b>Butachlor</b>	25	90.65	8.098	87.70	8.266
	50	96.70	6.594	97.47	4.601
<b>Chlorpyrifos</b>	25	83.06	10.06	85.59	10.206
	50	99.60	4.833	99.81	5.523

at concentrations of 25 and 50 ng/ml followed by analysis each day for 6 consecutive days to estimate inter-day (day-to-day) reproducibility as well as six times a day to estimate intra-day (within the day) reproducibility. Totally, 72 analyses were performed to determine intra and inter reproducibility. In within day experiments evaluation, one urine samples per day were analyzed six times for three consecutive days (36 analyses at two intended concentrations). In day-to-day reproducibility, experiments were performed every day for 6 consecutive days with three replications (36 analyses at two intended concentrations). **Figure 7** shows the chromatograms of urine sample with and without spiked pesticides. The optimized extraction protocol was followed along with optimum chromatographic conditions. The measurement of butachlor and chlorpyrifos on each day of the analysis was calculated by comparing it with the extracted calibration curve generated on that day. Intra-day variation or reproducibility of the method was assessed by calculating the relative standard deviation (RSD) (**Table 2**). As indicated by RSD, the precision of the optimized method was high enough (RSD less than 11%). The relative standard deviation of 50 ng/ml showed lower RSD (higher precision). Experiments were also undertaken to assess Inter-day reproducibility. The results are shown in **Table 2**. The obtained RSD indicates that the method was much more precise at two concentrations. The recoveries obtained by SPME-GC-ECD method was between 83% - 99% that shown in **Table 2**.

#### 4. Discussion

In this study, headspace solid phase microextraction (HS-SPME) method in combination with Gas Chroma-



**Figure 7.** Chromatograms of urine sample (a) without and (b) with spiked sample of chlorpyrifos and butachlor.

Tography-Electron Capture Detector (GC-ECD) was used for the extraction and quantification of butachlor and chlorpyrifos. This technique is simple, inexpensive, and fast compared to other conventional methods [21] [22] [33] [34]. Through this study, different parameters affecting the extraction procedure were optimized. Temperature is the main parameter that can affect the extraction of the analyte in HS-SPME. By raising temperature, the partition constant of the analyte was increased in favour of the gaseous sample; therefore, partitioning of the analytes between the sample and headspace can reach equilibrium more quickly. Thus, the analyte can exist in the headspace of the sample and can be extracted by the SPME fiber. Increasing temperature causes an increase in the amount of the analyte extracted. The obtained results indicated that, temperature up to 60°C could increase the amount of the analyte extracted for chlorpyrifos, where it reached a plateau and remained constant. No significant effect was seen at higher temperatures ( $p > 0.05$ ) and highest extraction efficiency for butachlor was obtained at 80°C. These results are consistent with previously reported data [30] [35].

The effect of extraction time of 5 - 60 minutes on extraction efficiency indicated that, up to 30 minutes there was a significant effect in all experiments for both pesticides. It means that based on the peak obtained from the GC, the higher amounts of analytes could efficiently extract from the samples with increasing the time up to 30 minutes. After this time, analytes were desorbed from the fiber to the headspace and the amounts adsorbed on the fiber were slightly decreased. The more extraction time can decrease the distribution coefficient of the analyte [36].

NaCl is also a parameter that can affect extraction efficiency, because the partition constant of the analyte can be changed in its presence, and therefore, more analyte may be exist in the headspace of the sample. In fact, the



solubility of the analyte decreases when NaCl is added.

Because of the salting-out effect, super saturation of the sample with salts can cause the extraction of analytes onto the fiber [32]. Also, previous studies have shown that ionic strength can improve extraction efficiency [37]. Therefore, the effect of extra NaCl on the extraction efficiency of both pesticides was shown. There is a competition among compounds with different polarity for the fiber in SPME. Also intensities of competition in presence of various pesticides are different. So, there are some differences in various studies due to different existing pesticides. For butachlor, the amount of NaCl from 0% to 5% increased the extraction rate; after that, there was no significant difference ( $p > 0.05$ ) between 5% and 10% of NaCl and the extraction remained constant.

The pH of the sample was the main parameter which could theoretically affect extraction efficiency. The ionic strength of the analytes could be changed by increasing or decreasing pH. The extraction efficiency of both pesticides was decreased significantly when pH was increased. In general, the sample is acidified for the extraction of acidic analytes and is made alkaline for the extraction of basic analytes, because, compounds for extraction should be in the molecular form [38].

Sample agitation is another important parameter that has to be optimized. It can increase extraction efficiency. The stirring process accelerates the transfer of an analyst into the headspace and hence, into the fiber coating. Although the equilibration time decreases with the increasing stirring rate, quick agitation depends on being rotational speed might cause a change in the equilibration time [38]. Higher stirring rate may cause a negative effect on the reproducibility of the sorption condition because of spattering.

For optimizing the volume of the sample, since the size of headspace vials was 10 ml, 2 - 4 ml of the sample volume was tested. By increasing the volume of the sample up to 3 ml, the amount of the analyte extracted can be increased. Gorecki and Pawliszyn in their recent paper have reported that, a large sample volume can provide the best results [39]. At the higher sample volumes, extraction rate showed a considerable decrease due to lower headspace. For rapid extraction, a large capacity of the headspace is required. It is achievable when there is a large headspace-to sample volume ratio. On the other hand, increasing the sample volume in a constant vial size leads to the lower head space capacity. In present study, one vial size was selected because of the poorer precision using variable vial volumes. Therefore, increasing the sample volume decreased the capacity of headspace, resulting lower extraction efficiency. Also the effect of sample volume on the magnitude of extraction depends on the analyte (how much is the analyte soluble and volatile). Gorecki and Pawliszyn have worked on the partition coefficient (K) values on their study and express that the large sample volumes is used to determine K values, as they enable broader ranges of values with good accuracy. On the other hand, they have emphasized that a large headspace capacity may lead to the loss of sensitivity. These reasons may explain the difference between the results of studies.

Finally, validation of the method was performed using spiked samples and standards to determine the pesticides in urine samples. The recoveries and RSD values in the present study were compared with those values in the existing methods [27] [32] [40] and the significant differences were found between them. These mentioned studies expressed the different RSDs and Extraction recoveries (like  $RSD < 19\%$ ), but there were significant differences between them and present study. The obtained recoveries in this study, ranging from 88% to 97%, were higher than the mentioned investigations. The optimized method is sensitive enough for all concentrations (RSD less than 11%). Based on results, the proposed method is easy, fast, precise, and reproducible with wide linear range and it does not require any toxic solvent for extraction. In addition, it can be seen a successful application of the HS-SPME technique to analyze the pesticides at a low concentration level in urine samples.

## 5. Conclusion

The SPME procedure seems to be a useful method for qualitative and quantitative evaluation of butachlor and chlorpyrifos in biological samples. This technique is simple, inexpensive, fast compared to the more conventional methods. To implement HS-SPME-GC-ECD, it was necessary to optimize the parameters affecting the extraction of the analytes. The optimum extraction conditions were achieved. Finally, validation of methods for determining butachlor and chlorpyrifos in the spiked urine sample was performed. Inter-day and intra-day relative standard deviation of method was evaluated by spiked urine sample with butachlor and chlorpyrifos. The results indicated that, the developed SPME-GC-ECD method was suitable for biological monitoring of butachlor and chlorpyrifos.

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