APPLICATION OF SOLID-PHASE MICROEXTRACTION FOR THE DETERMINATION OF PESTICIDES IN VEGETABLE SAMPLES BY GAS CHROMATOGRAPHY WITH AN ELECTRON CAPTURE DETECTOR

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Abstract
A solid-phase microextraction (SPME) method has been developed for the determination of 9 pesticides in 2 vegetables - cucumber and tomato - samples, based on direct immersion mode and subsequent desorption into the injection port of a gas chromatograph with an electron capture detector (GC-ECD). The main factors affecting the SPME process such as extraction time and temperature, desorption time and temperature, the effect of salt addition and fiber depth into the liner were studied and optimized. The analytical procedure proposed consisted of a 30-minute ultrasonic extraction of the target compounds from 1.0 g vegetable samples with 5 mL of distilled water. Then, the samples were filtered and topped up with distilled water to 10 mL. The analytes in this aqueous extract were extracted for 15 minutes with a 100 µm thickness polydimethylsiloxane SPME fiber. Relative standard deviations for triplicate analyses of samples were less than 10%. The recoveries of the pesticides studied in cucumber and tomato ranged from 52% to 82% and the RSD were below 10%. Therefore, the proposed method is applicable in the analysis of pesticides in vegetable matrices. SPME has been shown to be a simple extraction technique, which has a number of advantages such as solvent free extraction, simplicity and compatibility with the chromatographic analytical system.

Introduction
One of the major fields in analytical chemistry is the development of faster and easier methodologies for characterization and quantification of trace compounds in complex matrices. A special attention is given to substances that can compromise food safety, such as pesticide.

Pesticides are widely used for agricultural and non-agricultural purpose throughout the world. Although various methods, using highly efficient instruments such as gas chromatography (GC), high performance liquid chromatography (HPLC), Liquid chromatography (LC) and their combination with mass spectrometry (MS), have been developed for pesticide analysis, most analytical instruments cannot handle the sample matrices directly. In general, the analytical method involves processes such as sampling, sample preparation, separation, detection and data analysis and more than 80% of the analysis time is spent of sampling and sample preparation steps that include homogenization of samples, extraction of the analytes with an organic solvent, and clean up of the final organic extract. Therefore, it is not an exaggeration to say that the choice of an appropriate sample preparation method greatly influences the reliable and accurate analysis of food.

In contrast to conventional techniques, Solid Phase MicroExtraction (SPME) is a solvent-free extraction that minimizes sample preparation allowing the extraction and concentration steps to be focused into a single step. This technique, whose initial concepts were developed by Pawliszyn and co-workers in 1990 [1], is based on absorption of analytes onto a polymeric-coated fused-silica fiber, usually housed in a modified syringe. The total analytes retained in the fibers are thermally desorbed in the injector port and deposited at the head of the GC
column. Due to its advantages over classic extraction methods, SPME has received increasing attention since its commercial introduction in the nearly 1990s [2]. SPME has been applied to the determination of several organic compounds especially in gas and liquid samples, but also in a few solid samples, in combination with both GC and HPLC determination. Two modes of application of SPME have been extensively reported [1]: Direct Immersion (DI-SPME) and Headspace (HS-SPME) extraction. In DI-SPME, the fiber is directly immersed in the liquid sample or in the sample suspension and the analytes are transported from the sample matrix to the fiber coating. In headspace extraction mode, the analytes are extracted in a three-phase system: sample (liquid or solid), headspace, and fiber coating.

SPME has been successfully applied to the determination of pesticide residue analysis in water, soil, food and biological samples as reported in recent reviews published by Beltran et al. [3] and Kataoka et al. [1]. Water samples are by far the most widely analyzed by this technique [4-6].

The number of applications of SPME to complex matrices such as biological fluids is still limited; the headspace mode is the most attractive approach in this field [7-12]. Analysis of other samples such as soil [13-16] or food commodities [1, 17-18] is generally based on a solvent extraction of the analytes before application of SPME.

The low number of references about pesticide determination in food samples by SPME derives from the complexity of these matrices, which makes an extraction of the sample prior to determination by direct immersion SPME necessary in most of cases. This problem can be overcome if headspace SPME is applied, as described in several papers dealing with pesticide residue determination in fruits [19-21] or in a large number of papers related to determination of volatile compounds in food commodities [22-25].

Determination of non-volatile pesticides has received increasing attention in the recent years in order to solve some of the problems related with the application of DI-SPME in complex matrices. Several papers deal with direct immersion of the SPME fiber into a slurry of fruit with water [19,26]. Complex matrix problems can be solved by prior extraction of pesticide and the subsequent application of DI-SPME over the separated aqueous extract. Once the aqueous extract is obtained, the presence of interfering substances can reduce the efficiency of SPME. This problem can be overcome by simply diluting the extract in order to simplify the matrix complexity [27-28]. Still another problem, closely related with pesticide residue determination in fruits by SPME, is the difficulty of quantification; in most cases it is necessary to use calibration curves prepared using blank matrix, standard addition calibration, and internal standards [20].

The aim of this work is to investigate the feasibility of developing a single-step clean up enrichment procedure for pesticides extracted from vegetables based on SPME prior to gas chromatography with electron capture detection (GC-ECD). Nine pesticides: Carbaryl, Diazinon, Chlorothalonil, Malathion, Chlorpyrifos, Quinalphos, Profenofos, Alpha-Endosulfan, Beta-Endosulfan were selected as the model compounds because residues of these compounds are very often detected in vegetable samples. Table 1 showed the properties of nine selected pesticides. In this study, SPME-GC-ECD conditions have been optimized for the target compounds. The developed procedure was then successfully applied to the analysis of vegetable samples such as cucumber and tomato.

**Table 1:** Name, Molecular Formula, Molecular Weight, Chemical Class of the selected Pesticides.

<table>
<thead>
<tr>
<th>Name</th>
<th>Molecular Formula</th>
<th>Molecular Weight</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbaryl</td>
<td>C_{12}H_{11}NO_2</td>
<td>201.22</td>
<td>Carbamate</td>
</tr>
<tr>
<td>Diazinon</td>
<td>C_{12}H_{21}N_2O_3PS</td>
<td>304.35</td>
<td>OP</td>
</tr>
<tr>
<td>Chlorothalonil</td>
<td>C_8Cl_4N_2</td>
<td>265.92</td>
<td>OC</td>
</tr>
<tr>
<td>Malathion</td>
<td>C_{10}H_{16}O_2PS_2</td>
<td>330.36</td>
<td>OP</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>C_9H_17ClO_3PS_2</td>
<td>350.62</td>
<td>OP</td>
</tr>
<tr>
<td>Quinalphos</td>
<td>C_{12}H_{24}O_2N_2PS</td>
<td>298.18</td>
<td>OP</td>
</tr>
<tr>
<td>Profenofos</td>
<td>C_{11}H_{12}BrC_6O_2PS</td>
<td>373.60</td>
<td>OP</td>
</tr>
<tr>
<td>α-Endosulfan</td>
<td>C_9H_16ClO_2S</td>
<td>406.96</td>
<td>OC</td>
</tr>
<tr>
<td>β-Endosulfan</td>
<td>C_9H_16ClO_2S</td>
<td>406.96</td>
<td>OC</td>
</tr>
</tbody>
</table>

**Experimental**

*Chemicals and Reagents*

All solvents used were HPLC grade. Methanol was purchased from Fischer Scientific, Loughborough, U.K. Deionized water and methanol were filtered through a 0.45 µm membrane filter purchased from Millipore. The
use of high purity reagents and solvents help to minimize interference problems, pesticide standards (carbaryl, diazinon, chlorothalonil, malathion, chlorpyrifos, quinalphos, profenofos, α-endosulfan, β-endosulfan) were > 95% pure and purchased from AccuStandard Inc. New Haven CT, USA. Stock solutions of each pesticide at different concentration level, 50-2000 mg/kg were prepared in methanol and stored at 4 °C. Preparation of different concentration level of stock solution is due to their sensitivity to the ECD detector. Working standard solutions of pesticides mixture were prepared by volume dilution in distilled water. In order to avoid the influence on the results from the possible degradation of pesticides, the working solution was freshly prepared everyday. 1-chloro-4-fluorobenzene (2mg/kg) was used as internal standard to compensate for sample and was added to the vial prior to GC analysis.

**Gas Chromatography – Electron Capture Detector (GC-ECD)**

A Shimadzu GC 17A version 2.21 gas chromatograph with an electron capture detector ECD was used. A SGE BPX5, 30m x 0.32 mm id capillary column with a 0.25 µm film was used in combination with the following oven temperature program: initial temperature 120 °C, then 7 °C/min ramp to final temperature at 250 °C, held for 4.5 min. The total run time was 23.07 min. The injector temperature was at 240 °C and the detector temperature was at 300 °C. Nitrogen gas (99.999%) was used as the carrier gas with a gas flow at 24.4 cm/sec linear velocity and the pressure at 94 kPa and the split mode ratio of 1:36.

**SPME Procedure**

The SPME fiber holder for manual extraction and the fibers of polydimethylsiloxane (PDMS, 100 µm film thickness) were purchased from Supelco (Bellefonte, PA, USA). SPME fibers were conditioned by heating at 250 °C for 0.5 hour in the gas chromatography (GC) injection port according to the manufacturer recommendations in order to remove contaminants and to stabilize the polymeric phase.

Preliminary experiments were carried out to optimize the main parameters affecting the SPME of the pesticides investigated from aqueous solution (i.e. extraction time and temperature, desorption time and temperature, the effect of salt addition, stirring speed of the solution and fiber depth into the liner). In these studies, distilled water samples spiked with the appropriate amount of the standard solution was used.

After optimization, a typical experiment consisted of the direct immersion of the conditioned fiber into the spiked water sample, 10mL in a 15 ml clear glass vial and capped with a PTFE-faced silicone septum (Supelco). The SPME holder needle was inserted though the septum and the fiber was directly immersed in the sample solution for 15 minutes under magnetic stirring at room temperature (25 °C) in order to improve mass transfer from the aqueous sample into the fiber coating. After extraction, the fiber was withdrawn into the holder needle, removed from the vial and immediately introduced into the GC injector port for 7 min at 240 °C for thermal desorption in a split mode injector.

Calibration curves were constructed by SPME of the target compounds from aqueous samples spiked at 7 concentration levels and the constant volume of internal standard under above experimental condition. Three extractions were made for each concentration level of mixture solution. The calibration graph was plotted by the ratio of the peak area of the analyte against the peak area of the internal standard from the spiked samples versus the concentration of the analyte. These calibration lines were used for quantification in subsequent experiments.

**Vegetable Samples**

In order to evaluate the pesticide recoveries, 2 types of vegetables, cucumber and tomato were obtained from pesticide free farms under study. 1.0 g of vegetable samples was finely chopped and placed in a 15ml clear glass vial and capped with a PTFE-faced silicon septum. 5 mL of distilled water was added and spiked with three concentration levels of stock solution. The mixture was shaken for 30 minutes in an ultrasonic bath. Then, the samples were filtered, added with internal standard and topped up with distilled water to 10 mL. Pesticides were then extracted by direct dipping of the PDMS fiber in the solution. Recoveries of pesticides were determined by comparison of the ratio of the peak area of the analyte against the peak area of the internal standard from the spiked samples with that of the standard calibration solutions.
Result and discussions

Method Optimization

Preliminary experiments were performed by direct injection of pesticides for GC-ECD conditions optimization; the temperature program developed was capable of a good separation of the investigated analytes. In order to develop the SPME described method for pesticides selected extraction in vegetables, several parameters such as extraction time and temperature, desorption time and temperature, the effect of salt addition, stirring speed of the solution and fiber depth into the liner were studied.

On the basis of the results previously published for the target compounds, a 100 µm PDMS fiber was chosen as this material has been reported to have satisfactory extraction efficiency for a variety of compounds, including pesticides selected in this study. The 100 µm PDMS fiber (a non-polar phase) is recommended in the literature because it is a rugged liquid coating able to withstand high injector temperature up to 300 ºC. Fibers coated with thicker films required a longer time to achieve extraction equilibrium, but might provide higher sensitivity due to the greater mass of the analytes that can be extracted.

Effects of Extraction Temperature and Time

In order to study the effect of temperature on the extraction process, vials were immersed in a water bath heated by the magnetic stirring unit. A thermometer was used to monitor the water temperature. The temperature effect was evaluated by varying the temperature from 25 to 70 ºC. An increase in extraction temperature causes an increase in the extraction rate and a simultaneous decrease in the distribution constant between the analytes and the fiber [27]. The analysis of the 9 pesticide compounds was performed at room temperature for the subsequent experiments.

The sorption time profile for the selected fiber was obtained by plotting the detector response (peak area) versus the extraction time for each pesticide in order to obtain the partition equilibrium curve (figure 1). Blank aqueous samples (10mL) spiked at 0.1 mL standard solution were analyzed at experimental conditions described in the SPME procedure. Sorption time profiles indicated that a sampling time higher than 30 minutes is necessary to reach the equilibrium. According to the literature [1,12,29-31], the sorption time can be shortened by working in non-equilibrium condition because the amount of analyte adsorbed from the sample onto the fiber is proportional to the initial concentration in the sample matrix, if the agitation and the sampling time are held constants amongst samples. Thus, considering a compromise between the extraction time and the chromatographic analysis time, an extraction time of 15 minutes was selected for further experiments. This time still allowed a good, reproducible extraction response for all pesticides while minimizing analysis time.

![Figure 1: Peak area versus extraction time for 9 investigated pesticide](image)

Effects of Desorption Temperature and Time

The temperature of GC injector and desorption time were tested in order to guarantee the complete desorption of pesticides to avoid carryover. For the PDMS fiber, temperatures ranging between 200 and 280 ºC were tested (selected according to the recommended temperature range indicated by the manufacturer). High desorption temperature can enhance the process but they can also degrade analytes. Desorption at 200 and 230 ºC was not
capable of desorbing completely the analytes; they were completely removed from the coating at 240 – 280 °C and not much significant differences were observed within this range of temperature. Hence a temperature of 240 °C was selected since high temperatures can shorten the coating lifetime and can result in the bleeding of the polymer, causing problems in the separation and quantification [29].

Figure 2: Peak area versus desorption time for 9 investigated pesticide.

Desorption profiles of the pesticides were obtained by plotting the detection response versus different desorption times, 1 – 10 minutes. Desorption profiles showed that a 6 minute-period was sufficient to desorb pesticides in the GC injector port (Figure 2); therefore a 7 minute-period was chosen to guarantee a reproducible desorption.

Effects of Salt Addition
The addition of salts into the samples can modify the extraction efficiency, because the partition coefficients are partially determined by matrix-analyte-fiber interactions [29]. Pesticides that are more soluble in water have a lower affinity for the fiber coating. The amount of these analytes extracted by the fiber can be increased if the solubility of the analytes in water is decreased by adding sodium chloride to alter the ionic strength [27]. The effect of increasing the ionic strength of the sample was determined with samples containing no salt, 5, 10, 15, 20 and 25% (w/v) of sodium chloride. Result showed that the amount of compounds extracted decreased when the salt concentration increased. The best results were obtained when no salt was added; this could possibly be due to the formation of a thin layer of salt around the fiber, which decreases the extraction efficiency [29].

Effects of Stirring Speed
The use of a magnetic stirrer allows the control of the stirring speed as well as the mode of stirring, and hence a cyclic change in stirring direction. The results showed the responses increased if the stirring speed is increased which agrees with the fact that SPME is a technique based on equilibrium and that good diffusion through the phases is essential to reach equilibrium faster. Although the equilibrium time progressively decreases with increasing agitation rate, faster agitation tends to be uncontrollable and the rotational speed might cause a change in the equilibrium time and poor measurement precision. A constant gentle stirring speed was selected in this study to increase the rate of extraction.

Effects of Fiber Depth into the Liner
The effect of the fiber depth into the liner was also checked, and the results showed that peak areas increased when the depth of the fiber into the injector glass-liner was higher, which is closer to the column entrance and the center of the hot injector zone.

Method performance
After optimization of all the variables considered, the recommended procedure was established as follows: extraction of 10 mL of water sample containing no salt under magnetic stirring for 15 min at room temperature using a PDMS, 100 µm fiber coating and subsequent desorption at 240 °C over 7 min. The optimum procedure developed was applied to the extraction of nine pesticides in spiked water samples. With the selected conditions for the SPME procedure, quality parameters of the SPME-GC-ECD method such as linearity, limits of detection and quantitation, and recovery were calculated.
The linearity of the method was tested using a series of aqueous solution (distilled water) in the difference concentration range (7 levels, three replicates for each level). After plotting the ratio of analyte peak area relative to that of the peak area of internal standard versus the analyte concentration to generate the calibration curves, a statistical regression model was applied to obtain the corresponding values for slope and intercept for each compound. The SPME procedure showed a linear behavior in the ranges tested with r² values > 0.9900. Linear ranges and determination coefficients (r²) obtained for each pesticide are given in Table 2. The loss of linearity observed at higher concentrations can be justified due to overloading of the SPME fiber capacity.

The detection limit (LOD) was calculated by comparing the signal-to-noise ratio (S/N) of the lowest detectable concentration to a S/N=3. A S/N of 10 was applied for the calculation of the quantification limit (LOQ). The results obtained are shown in Table 2.

<table>
<thead>
<tr>
<th>Name</th>
<th>R²</th>
<th>Linear Range (mg/kg)</th>
<th>LOD (mg/kg)</th>
<th>LOQ (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbaryl</td>
<td>0.9976</td>
<td>0.2 – 200</td>
<td>0.01</td>
<td>0.05</td>
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<tr>
<td>Diazinon</td>
<td>0.9968</td>
<td>0.05 – 50</td>
<td>0.005</td>
<td>0.02</td>
</tr>
<tr>
<td>Chlorothalonil</td>
<td>0.9988</td>
<td>0.02 – 20</td>
<td>0.001</td>
<td>0.005</td>
</tr>
<tr>
<td>Malathion</td>
<td>0.9965</td>
<td>0.05 – 50</td>
<td>0.005</td>
<td>0.02</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>0.9986</td>
<td>0.005 – 5</td>
<td>0.0005</td>
<td>0.001</td>
</tr>
<tr>
<td>Quinalphos</td>
<td>0.9985</td>
<td>0.05 – 50</td>
<td>0.005</td>
<td>0.02</td>
</tr>
<tr>
<td>Profenofos</td>
<td>0.9941</td>
<td>0.01 – 10</td>
<td>0.001</td>
<td>0.005</td>
</tr>
<tr>
<td>α-Endosulfan</td>
<td>0.9952</td>
<td>0.005 – 5</td>
<td>0.0005</td>
<td>0.001</td>
</tr>
<tr>
<td>β-Endosulfan</td>
<td>0.9972</td>
<td>0.005 – 5</td>
<td>0.0005</td>
<td>0.001</td>
</tr>
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</table>

**Vegetable Samples**

The developed method has been applied to the vegetable samples, cucumber and tomato, treated as described in the Experimental section. From Figure 3, it is clear to show that all the target analytes were detectable in the sample and appeared completely separated from interfering peaks. The extraction efficiencies were calculated by comparing the chromatogram (Figures 3) obtained from the extracts of the spiked samples by SPME with those obtained by direct GC injection of non-extracted (Figure 4). It is shown that SPME is effective in the extraction of all the pesticides investigated without absorbing any other unwanted compounds from the samples.

![Figure 3: Chromatogram on recovery of spiked cucumber and extracted by SPME.](image-url)
three concentration levels. The peak areas obtained when these samples were analyzed by the same procedure were compared with the standard calibration curves. Mean recoveries and RSD obtained in the analysis of fortified cucumber and tomato samples are listed in Table 3. For fortified cucumber samples, the recoveries were between 53 – 75 % and for the tomato, the recoveries were between 53 – 82 % with is quite similar to that of the fortified cucumber. The precision determined using the same conditions was good, with the vast majority yielding relative standard deviations (RSDs) below 10 %.

![Figure 4: Chromatogram on recovery of spiked cucumber and direct injection](image)

Table 3: Average recoveries and relative standard deviations (RSDs) from three representative commodities fortified vegetable samples using the optimized SPME extraction method.

<table>
<thead>
<tr>
<th>Name</th>
<th>Cucumber (n=3)</th>
<th>Tomato (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recovery (%)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>Carbaryl</td>
<td>74.10</td>
<td>8.22</td>
</tr>
<tr>
<td>Diazinon</td>
<td>53.96</td>
<td>1.43</td>
</tr>
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<td>Chlorothalonil</td>
<td>58.26</td>
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<td>Malathion</td>
<td>75.03</td>
<td>2.11</td>
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<td>Chlorpyrifos</td>
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<tr>
<td>Quinalphos</td>
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<td>Profenofos</td>
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<td>α-Endosulfan</td>
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<tr>
<td>β-Endosulfan</td>
<td>65.27</td>
<td>4.39</td>
</tr>
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</table>

Conclusions

A fast, simple, solvent free screening method based on a 30 min ultrasonic extraction of a 1.0 g vegetable samples with distilled water and subsequent SPME of the analytes from the aqueous solution and detected by gas chromatography with an electron capture detector has been developed for the determination of nine pesticides. The main experimental parameters affecting the SPME step were optimized. This method offers very low detection limits for all nine pesticides. The recoveries of the pesticides studied in cucumber and tomato ranged from 53% to 82% and the RSD were below 10%. Therefore, the proposed method is applicable in the analysis of pesticides in vegetable matrices. SPME has been shown to be a simple extraction technique, which has a number of advantages such as solvent free extraction, simplicity and compatibility with the chromatographic analytical system.
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