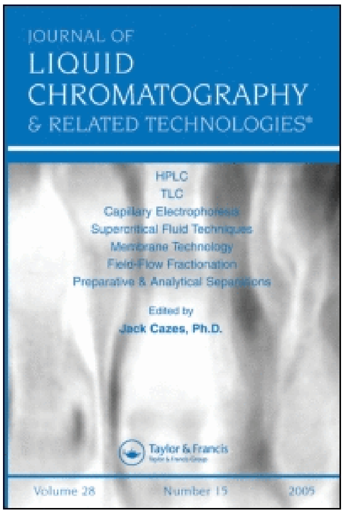

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Analysis of Organochlorine Pesticides in Mollusc Samples by HPLC after Microwave Assisted Micellar Extraction Coupled with Solid Phase Extraction

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Abstract: A mixture of six organochlorine pesticides were determined by HPLC in different types of molluscs after their extraction by microwave assisted micellar extraction (MAME) coupled with a clean-up and preconcentration step by solid phase extraction (SPE).

A non-ionic surfactant (Polyoxyethylene 10 lauryl ether, POLE) was used in MAME followed by SPE for cleaning and preconcentration of the samples. The extracts were determined by HPLC-UV. With the optimum experimental variables established, the analytical parameters of the method were determined with satisfactory results (relative standard desviation values between 3.9 and 9.3% and detection limits between 6 and 38 ng $\cdot$ g^{-1}). The method was validated using the conventional Soxhlet extraction method.

Keywords: Microwave, Micellar solution, Solid phase extraction, Organochlorine pesticides, Molluscs

INTRODUCTION

Organochlorine pesticides have been extensively used for agriculture and vector control purposes. The pesticides applied on the land usually find their way to aquatic environment thus contaminating them.[1] The pesticides

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are transported to aquatic bodies by rain runoff, rivers and streams and associate with biotic and abiotic macroparticles.[2] They are removed from the surface to the benthic layers by settling of the particles into the water column; and during this migration can be captured by filtered organisms.[3] Molluscs are invertebrate organisms with the capacity of filtering huge amount of water with particulate organic matter.[4] On the other hand organochlorine pesticides tend to associate to particulate matter due to their hydrophobicity and persistence, it produces a bioaccumulative effect through food chain[5] with subsequence for human health,[6,7] when contaminated molluscs are incorporated by direct ingest.

Commonly used methods for the extraction and preconcentration of analytes in solid samples are soxhlet and ultrasonic extraction.[8] However, these methods are often too time consuming due to the slow chromatographic elution and the intense preanalytical methods. They usually involve multi-step procedures and the use of high amounts of toxic organic solvents.[9,10] Moreover, with the difficulty for separating matrix components from analytes, frequently they resulted in undesirable interferences which can affect the signal of target analytes.

Microwave assisted extraction (MAE) is a rather new technique, which has been applied to the extraction of organic pollutants for solid samples and which presents an advantageous reduction of extraction time and required less solvent compared to conventional methods.[11–13] An alternative to organic solvents as extractants would be the use of micellar systems. From an analytical point of view, one of the most important properties of these organized structures is their capacity to solubilize solutes of different types found in different environments.[14] Therefore, the combination of the MAE technique with the use of micellar media makes this a simple, fast, low cost, easy handling, and non-toxic procedure (MAME), which could be an alternative for the extraction of different pollutants from solid matrices as has recently been proven.[15–17] However, usually other different components of the analytes of interest can be extracted together and interfere in the determination.[18] For this reason, an intensification of target analytes and a cleanup step could be necessary.

Solid phase extraction is an extraction/preconcentration technique which has been commonly applied to liquid samples.[19] However, another possibility is the intensification of target analyte signals by means of extract cleanup and preconcentration. In this field, the use of solid phase extraction (SPE) has been investigated as a secondary step of several extraction methods, such as MAE.[20]

In this work, we have determined a mixture of organochlorine pesticides in mollusc samples by HPLC-UV after their extraction using microwave assisted micellar extraction (MAME) followed SPE for cleaning and preconcentration of the sample. The experimental parameters of MAME-SPE procedures were studied and the method's precision, recoveries, and linearity were also investigated. To prove the method validity, it was compared with the Soxhlet extraction method and was also applied in different kinds of molluscs.

EXPERIMENTAL

Reagents

Organochlorine pesticides were obtained from Cerilliant Corporation (provided by LGC Promochem, Barcelona, Spain) and prepared by dissolving appropriate amounts of the commercial products in methanol to obtain a concentration of 1 g · L^{-1} and stored in amber bottles at 4°C. Working solutions were prepared by further diluting these concentrations. The organochlorine pesticides are listed in Table 1 (numbers and abbreviations identify the compounds in figures).

The non-ionic surfactant used, Polyoxyethylene 10 lauryl ether (POLE), was obtained from Sigma-Aldrich (Madrid, Spain) and prepared in ultra-pure water.

The SPE cartridge used in this study was Envirelut-Pesticide 500 mg. It was provided by Varian (Madrid, Spain). Before use, the cartridges were conditioned with methanol and water according to supplier's instructions.

Methanol HPLC grade was obtained from Panreac Química S.A. (Barcelona, Spain).

All solvents and analytes were filtered through a 0.45 μm cellulose acetate membrane filter and ultra high quality water obtained by a Milli-Q water purification system (Millipore, USA) was used throughout.

Apparatus

The chromatograph system consists of a Varian pump fitted with a Varian Autosampler 410 with a volume selector, a Column Valve Module with an internal oven, and a Varian PDA Detector. The system and the data management were controlled by Star software from Varian (Varian Inc., Madrid, Spain). The stationary phase column was a Varian Microsorb-MV 100-5 C_{18}, 150 × 4.6 mm, 4 μm particle diameter. The analytical column was inserted in the column module and thermostated at 30 ± 0.2°C.

Table 1. Target organochlorine pesticides, wavelenghts and retention times

No.	Compound	Abbreviation	λ (nm)[a]	t_R (min)[b]
1	4,4′-Dichlorodiphenyldichloroethane	4,4′-DDD	238	5.4
2	Dieldrin	Dieldrin	220	6.1
3	4,4′-Dichlorodiphenyltrichloroethane	4,4′-DDT	238	8.7
4	2,4′-Dichlorodiphenyltrichloroethane	2,4′-DDT	238	9.6
5	4,4′- Dichlorodiphenyldichloroethylene	4,4′-DDE	238	10.9
6	Aldrin	Aldrin	220	11.9

[a] Detection wavelength.
[b] Retention time.

The microwave oven used in this study was a Multiwave (Anton Paar, Graz, Austria) with a 6 EVAP rotor and 6 MF100 vessels (Anton Paar, Graz, Austria).

Procedures

Preparation of Spiked Molluscs

The mollusc samples were freeze dried and crushed. Spiked molluscs were prepared by mixing them with an organochlorine solution in acetone to obtain a final concentration of 2 μg/g for 4,4′-DDD, 4,4′-DDT, 2,4′-DDT, and 4,4′-DDE, and a concentration for Aldrin and Dieldrin of 4 μg/g. The sample was then stored in amber bottles at room temperature for 24 hours before analysis in order to obtain dry and homogenous samples.

Microwave Assisted Micellar Extraction

Once the mollusc sample was transferred to the vessel (1 g), the optimum volume (10 mL) and concentration of surfactant (5%, v/v) were added, and the sample was subjected to the MAME process at the optimum conditions (300W during 14 minutes). The vessels were then allowed to cool, first 10 minutes with the microwave fan and after another 5 minutes at room temperature, before being opened. The extract solution was filtered with a 0.45 μm syringe driven filter for the clean up process application (SPE).

Solid Phase Extraction (SPE) Process

An Envirelut-Pesticide 500 mg SPE cartridge was used for the cleanup procedure. The cartridges were conditioned with 5 mL of methanol and 5 mL of ultra-pure water before each run.

For the absorption process, 5 mL of the MAME extract plus 20 mL of ultra-pure water were passed through the SPE cartridge at $5\,\mathrm{mL}\cdot\mathrm{min}^{-1}$. For reducing the surfactant remains, a wash step was done with 5 mL of ultra-pure water at the same flow rate. Desorption of the compounds was done with 2 mL of methanol at $1\,\mathrm{mL}\cdot\mathrm{min}^{-1}$.

Soxhlet Extraction

A gram of the spiked sample was extracted with hexane for 24 h at 6-8 cycles/h as proposed by the AOAC Official Method 970.52.[21] The extract was evaporated in vacuo, redissolved in methanol (10 mL), and finally analysed in the HPLC-UV system.

Liquid Chromatography Analysis with UV Detection

The analysis of the extracted samples was carried out by using high performance liquid chromatography with UV detection.[22] The separation and determination of the compounds under study were performed by injecting 50 μL of extract into the liquid chromatograph and the absorbance for each analyte, corresponding to the maximum wavelength, was then measured. The retention time and the wavelength for each compound are listed in Table 1. The eluent used for the separation of the six organochlorine pesticides mixture was isocratic methanol:water (84:16 %,v/v) with a flow-rate of 1 $\mathrm{mL \cdot min^{-1}}$.

The corresponding calibration curve was done with the optimum MAME-SPE conditions and the corresponding analyte concentrations ranged between 50-500 $\mu g \cdot L^{-1}$ for 4,4′-DDD, 4,4′-DDT, 2,4′-DDT, and 4,4′-DDE, and the range of 100–1000 $\mu g \cdot L^{-1}$ for Aldrin and Dieldrin. A linear relationship was obtained between peak areas and the analyte concentrations, with high correlation coefficients ($\geq$0.995).

Statistical Analysis

The experimental design was performed using Statgraphics Plus software, version 5.1 (Manugistic, Rockville, MD, USA). It enables obtaining an experimental design where several variables can be studied together without interferences of external or indirect variables.

RESULTS AND DISCUSSION

Optimization of MAME-SPE Methodology

Optimization experiments were performed using mussels with POLE as extractant for the mixture of six organochlorine pesticides under study. Microwave assisted micellar extracts (MAME extracts) can not be directly injected in HPLC systems due to the high interferences produced by several compounds including proteins, which were extractions of the mollusc samples.

Solid phase extraction can be coupled with other extraction techniques to remove these interferences and as a preconcentration step.[23] For coupling the MAME-SPE procedure we used an Envirelut-Pesticide 500 mg SPE cartridge and fixed the volume of MAME extract in 5 mL and the addition of 20 mL ultra-pure water for the absorption, according to previous studies.[24] Before desorption, a washing step was done for cleaning the SPE extract with 5 mL of bidistilled water to remove the surfactant excess.[25] For increasing the preconcentration in MAME-SPE extraction, we used the lower desorption volume which obtained a high signal, but with an acceptable reproducibility of the method. The selected value was 2 mL, which was used for the method's

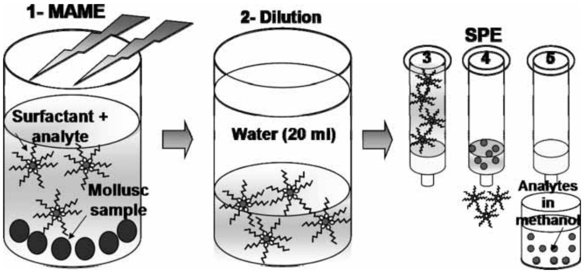

Figure 1. Scheme of MAME-SPE procedure which represents the MAME extract (1) and its dilution (2), the SPE absorption step (3), SPE washing step (4) and desorption and preconcentration of analytes in methanol (5).

application. Figure 1 shows the scheme where the MAME-SPE procedure is represented with all specified steps.

Parameters that can influence the MAME process are amount of sample, extractant volume, and concentration, irradiation time and power.[26, 27] Considering our previous experiences, we fixed the extraction volume in 10 mL and the amount of sample (1 g) and the surfactant concentration (5%, v/v) as the best combination of parameters for the MAME extraction process of organochlorine pesticides.[28]

For the variables optimization in MAME-SPE, we applied a 3^2 factorial design with a duplicate of central points, where the microwave power and time against recovery is represented. It permits obtaining the influence of the variables between them. Figure 2 shows the response surface MAME-

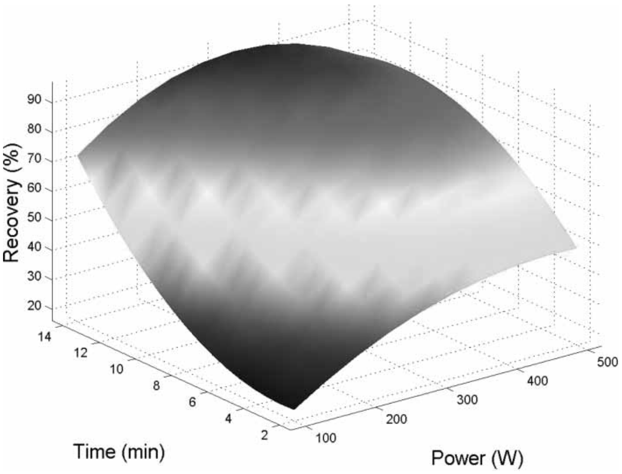

Figure 2. Response surface for the effect of microwave power and time on the extraction of 4,4′-DDT in MAME-SPE procedure.

SPE for the case of 4,4′-DDT. The studies carried out for the rest of analytes show similar results for power and extraction time. It can be observed, that the amount of analyte extracted increases with the microwave power up to 300 W. However, a decrease in efficiency extraction is observed at a power higher than this value, probably due to volatilization or degradation of the analytes.

With respect to extraction time, the results obtained show that a time of 14 minutes is the most suitable.

According to the obtained results, we have chosen for the MAME-SPE procedure the following optimum conditions: 1 g of mollusc sample, 10 mL of surfactant volume, 5% (v/v) of surfactant concentration, 300W of microwave power, 14 minutes of microwave time with Envirelut-Pesticide 500 mg SPE cartridge at selected conditions, and 2 mL of methanol for desorption.

Analytical Parameters

The analysis of the samples was carried out using high performance liquid chromatography with UV detection. The chromatogram obtained for the mixture of pesticides (2 $\mu g \cdot g^{-1}$ for 4,4′-DDD, 4,4′-DDT, 2,4′-DDT, 4,4′-DDE, and 4 $\mu g \cdot g^{-1}$ for Aldrin and Dieldrin) extracted from a spiked mussel sample using MAME-SPE procedure is shown in Figure 3. It can be observed, that the mobile phase used (methanol:water, 84:16 v/v) allows a good separation of analytes and a short analysis time.

To obtain the calibration curves for each analyte, MAME-SPE followed by HPLC-UV analysis of the working solutions ranging from 50–1000 $\mu g{\cdot}L^{-1}$ was performed. All pesticides followed a linear relationship between peak areas and the analyte concentrations, with high correlation coefficients (greater than 0.995). Relative standard deviations were calculated using six samples of spiked

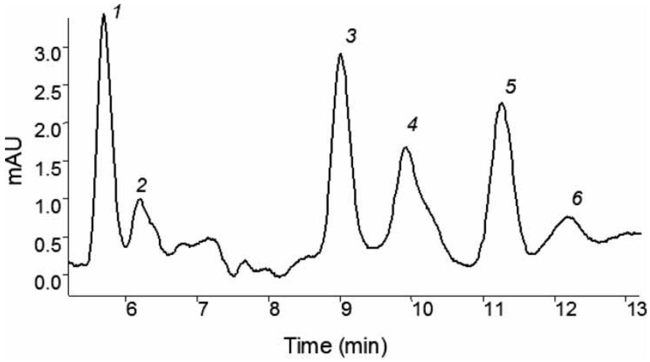

Figure 3. Chromatogram of an extract of a spiked mussel sample after MAME-SPE procedure. Chromatographic conditions as described in the text, λ =238 nm. The numbering refers to Table 1.

Table 2. Analytical parameters of the method in the determination of studied pesticides using MAME-SPE-HPLC-UV[c]

Compound	Recovery (%)	RSD[a] (%)	LOD[b] (ng · g^{-1})
4,4′-DDD	84.6	7.1	19
Dieldrin	92.6	8.0	21
4,4′DDT	97.8	5.4	38
2,4′DDT	94.1	7.7	9
4,4′-DDE	100.4	3.9	6
Aldrin	68.3	9.3	26

[a] Relative standard deviation (n = 6).

[b] Limit of detection.

[c] Concentration of spiked seaweed: 2 μg/g for 4,4′-DDD; 4,4′-DDT; 2,4′-DDT and 4,4′-DDE, and 4 μg/g for Aldrin and Dieldrin.

molluscs with pesticides, to which the MAME-SPE process followed by HPLC-UV were applied, and are shown in Table 2. These results indicate relative standard deviation values between 3.9 and 9.3%, which shows that the proposed method has an acceptable precision. The detection limits (LODs) of each compound[29] were calculated as three times the noise for each pesticide and vary between 6–38 ng·g^{-1} for all compounds studied (Table 2).

In order to probe the validity of the optimized MAME-SPE method, and due to the lack of a certified material available containing these pesticides, it was applied to the extraction of the pesticides present in a mussel sample enriched with a certified mixture (Pesticide-Mix 5, which contain five of the six studied pesticides), obtaining the results shown in Table 3.

These results were compared with those obtained using the traditional Soxhlet extraction procedure, as proposed by the AOAC Official Method

Table 3. Application of MAME-SPE procedure and Soxhlet extraction to a mussel sample containing a certified mixture of pesticides[a,b]

Compound	MAME-SPE Recovery (%)	Soxhlet Recovery (%)
4,4′-DDD	102.5 ± 5.5	101.6 ± 8.2
Dieldrin	101.3 ± 7.1	89.5 ± 8.9
4,4′DDT	98.8 ± 4.6	91.8 ± 5.5
2,4′DDT	87.9 ± 6.8	84.9 ± 9.3
4,4′-DDE	94.2 ± 5.7	96.7 ± 6.4

[a] Concentration of spiked seaweed: 2 μg/g.

[b] Mean of two determinations.

Table 4. Recoveries (%) and their standard deviation obtained after application of MAME-SPE-HPLC-UV procedure for the pesticides in six different molluscs[a]

Mollusc	4,4′-DDD	Dieldrin	4,4′-DDT	2,4′-DDT	4,4′-DDE	Aldrin
Clam	87.2 ± 7.1	93.0 ± 7.4	98.7 ± 5.3	87.6 ± 6.7	97.5 ± 3.8	72.2 ± 6.7
Cockle	91.8 ± 6.5	103.5 ± 6.5	93.2 ± 5.1	93.7 ± 5.7	101.1 ± 2.7	74.2 ± 7.3
Small Clam	93.0 ± 5.8	97.4 ± 7.1	93.1 ± 4.8	87.7 ± 6.3	95.7 ± 3.4	84.1 ± 6.4
Oyster	96.5 ± 5.2	99.3 ± 5.8	88.4 ± 5.7	103.4 ± 5.1	101.9 ± 2.5	79.0 ± 7.1
Snail	86.8 ± 6.4	95.0 ± 6.2	87.5 ± 4.7	95.7 ± 6.1	106.4 ± 1.8	76.4 ± 7.5
Mussel	84.6 ± 6.8	92.6 ± 7.4	97.8 ± 3.6	94.1 ± 6.7	100.4 ± 2.6	68.3 ± 8.6

[a] Mean of three determinations.

970.52[21] finding an important similarity of results in the two methods; recoveries were around 87–103% (±5%) for MAME-SPE and around 84–101% (±8%) for Soxhlet extraction. However, MAME-SPE offers different advantages over the Soxhlet procedure: a faster method (around 30 min instead 24 hours), easier handling and storage, avoiding the use of organic solvent as hexane, the ratio signal/noise are higher than in Soxhlet, and determination of simultaneous samples.

These advantages conferred by MAME-SPE allow for increased sample throughput, along with decreases in both the exposure and the amount of waste generate. Therefore, we conclude that MAME-SPE is a viable process for the determination of organochlorine pesticides in mollusc samples.

Applications

To evaluate the influence of mollusc's types on the extraction, the optimized method was used to determine the studied mixture of organochlorine pesticides in six different kinds of molluscs. The mollusc samples were spiked with $2\ \mu g \cdot g^{-1}$ of each pesticide after being freeze dried and stored in the dark at room temperature for 24 hours before analysis.

The results obtained for the MAME-SPE-HPLC process are shown in Table 4 and it can be observed that recoveries don't vary significantly depending on the kind of molluscs; obtaining, in general cases, recoveries higher than 85%, except for Aldrin, in all molluscs.

The differences observed between compounds could be essentially due to the variability in their physiological characteristics, which interfere with the interaction with fatty tissue.

But, in general, the proposed method can be applied to different kinds of molluscs for the extraction of organochlorine pesticides.

CONCLUSIONS

Due to the high degree of toxicity of the compound contaminants studied and their persistence in the medium, it is quite clear that there is a need to develop new analytic methods to perfect and facilitate the determination of such contaminants.

With this in mind, we have tried to develop an alternative analytical method for the determination of organochlorine pesticides by HPLC combining microwave assisted micellar extraction and solid phase extraction (MAME-SPE) for improving the quantification limit of the method and the application to different kind of molluscs samples. As main advantages, it is a faster method, relatively simple, and less extractant required, thus lowering the costs dramatically. Moreover, it can be applied to the extraction of simultaneous samples and there are no toxic effects.

Therefore, we conclude that MAME-SPE is a viable and promising process for the determination of organochlorine pesticides in mollusc samples. It may be an alternative tool to the traditional techniques usually employed to extract compounds in these kinds of samples.

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