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Design and development of molecularly imprinted polymers for the selective extraction of deltamethrin in olive oil: an integrated computational-assisted approach

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Abstract

This work firstly addresses the design and development of molecularly imprinted systems selective for deltamethrin aiming to provide a suitable sorbent for solid phase (SPE) extraction that will be further used for the implementation of an analytical methodology for the trace analysis of the target pesticide in spiked olive oil samples. To achieve this goal, a preliminary evaluation of the molecular recognition and selectivity of the molecularly imprinted polymers has been performed. In order to investigate the complexity of the mechanistic basis for template selective recognition in these polymeric matrices, the use of a quantum chemical approach has been attempted providing new insights about the mechanisms underlying template recognition, and in particular the crucial role of the crosslinker agent and the solvent used. Thus, DFT calculations corroborates the results obtained by experimental molecular recognition assays enabling one to select the most suitable imprinting system for MISPE extraction technique which encompasses acrylamide as functional monomer and ethyleneglycol dimethacrylate as crosslinker. Furthermore, an analytical methodology comprising a sample preparation step based on solid phase extraction has been implemented using this "tailor made" imprinting system as sorbent, for the selective isolation/pre-concentration of deltamethrin from olive oil samples. Molecularly imprinted solid phase extraction (MISPE) methodology was successfully applied for the clean-up of spiked olive oil samples, with recovery rates up to 94%.

Keywords: Molecularly imprinted polymer, molecular recognition, DFT calculations, solid phase extraction, olive oil, deltamethrin.

1. Introduction

Molecular Imprinting Technology (MIT) has emerged as a versatile technique widely used for the synthesis of "tailor-made" polymeric materials affording the production of highly cross-linked materials, as stable recognition matrices for a wide range of analytes, mimicking the recognition mechanism of antigens and antibodies. These materials are considered to be artificial receptors possessing highly specific sites towards the target molecule presenting additionally improved properties, namely higher physical robustness, strength, resistance to temperature and pressure as well as stability in acid and basic media [1]. During the imprinting process, an in situ formation of the monomer template complex is considered as a key step followed by the use of a crosslinker entity that allows the preservation of the structure of the monomer template complex and, thus the creation of an artificially generated three-dimensional polymer network which possesses binding sites with structural and functional groups complementary to the template molecule. After the polymerization process, the template molecule is removed from the polymer leaving specific recognition sites complementary in shape, size and chemical functionality to the template molecule, allowing the MIP be able to recognize and bind selectively to only the template molecule. Moreover, the less expensive synthesis and the higher storage stability -keeping their recognition ability for several years at room temperature -, and reusability, constitute the major advantages of these imprinting materials [2]. The remarkable properties of these imprinting systems have allowed their widespread application over several fields covering chemistry- chromatography [3,4], catalysis [5,6], sample preparation [7]; biology- drug delivery [8] and engineering- sensor technology [9,10].

Over the last years, the use of molecular modeling methods for the study and characterization of MIPs has emerged as a rational design tool that enables one to

optimize the MIP formulations and is a promising approach for finding highly selective MIPs [11]. This approach improves the tedious and time-consuming conventional method of MIP synthesis especially if the variation in the formulation is performed by trial-and-error. Some reviews covering the computational aspects of MIP study and design recently appeared in the literature [12-15]. However, the main drawback of the computational approach arises from the difficulty in simulating the real recognition process in MIPs being usually restricted to the rationalization of the interactions between the functional monomers and the template during the pre-polymerization stage [16]. A better stability of a given monomer/template complex in the pre-polymerization stage is typically correlated with a better imprinting effect of the functional monomer towards the template. The porogen is typically treated using continuum solvation models [17], and the cross-linker is mostly ignored. Other rational MIP design computational approaches, including atomistic and coarse-grain molecular dynamics methods that can describe the polymerization itself, or tackle issues such as the template aggregation were also reported [18,19]. Nevertheless, most of the studies found in the literature focus on the pre-polymerization stage [20-22].

Hence, computational modeling have proven to be a helpful guide to the selection of the more appropriate formulations contributing to the development of "rationally – designed" selective MIPs for a broad range of templates. Moreover, this approach has also become a powerful tool to elucidate the physical mechanisms underlying the ligand selectivity of the polymeric sorbents prepared by molecular imprinting technology.

In recent years, the use of organochlorides and organophosphorus insecticides has declined owing to their high toxicity being replaced by pyrethroids. Deltamethrin is a synthetic pyrethroid widely used to control insect pests in crops, however, this substance still presents high toxicity affecting the central nervous system of humans and

is also suspected to have endocrine-disrupting effects with a long persistence and a high toxicity to the aquatic environment [23]. Thus, the eventual presence, even at trace levels, of this substance in foodstuffs is a matter of great concern making it necessary the development of robust analytical methodologies that enable high precision and selective detection and quantification.

The use of molecular modeling studies to elucidate the molecular interactions within the imprinting system for the development of deltamethrin selective MIPs has never been attempted. In fact, very few reports on deltamethrin-molecularly imprinted polymers in the literature have been described to date [24-27]. Recently, a chemiluminescense nanosensor has been developed based on a quantum dot MIPs- based and used for the selective detection of trace amounts of deltamethrin in fruits and vegetables [28,29].

The propose of this work is the implementation of a highly selective sample preparation methodology based on molecularly imprinted solid phase extraction (MISPE) for the pre-concentration/isolation and further quantification of trace amounts of deltamethrin in olive oil samples. To achieve this goal, the present study addresses the synthesis and chemical characterization of molecularly imprinted polymers selective for deltamethrin by means of chemical and morphological techniques and, furthers the evaluation of the molecular recognition of these imprinting systems. Additionally, computational modeling studies have been used as a tool to understand the molecular imprinting process at the molecular level.

Hence, in this work a molecularly imprinted polymer selective for deltamethrin was successfully used as SPE sorbent for the implementation of the MISPE methodology allowing the pre-concentration/isolation of deltamethrin and further quantification by HPLC-DAD in spiked olive oil samples. High reproducibility's and recovery rates were observed.

2. Experimental

2.1. Chemicals

Acrylamide (AM) and metacrylic acid (MAA) (the functional monomers), ethyleneglycol dimethacrylate (EGDMA; crosslinker), 1,1´-azobisisobutyronitrile (initiator), were purchased from Sigma–Aldrich, dichloromethane for synthesis and acetic acid and methanol for MIP washing were obtained from Merck. All the chemicals were used as received.

HPLC grade acetonitrile and methanol, n-heptane and dichloromethane were purchased from VWR International S.A.S. (Fontenay-Sons-Bois, France). The water used in all experiments was distilled and purified by a Milli-Q system (Millipore, Bedford, MA, USA). The analytical standards deltamethrin, λ-cyhalothrin, fenpropathrin and phenothrin were purchased from Sigma-Aldrich (Bellefonte, PA, USA) and were used without further purification. The 3 mL reservoir glass columns with their frits were supplied by Chromabond-Macherey-Nagel (Germany). Previously to HPLC injection all samples were filtered through 13 mm syringe filters (w/ 0.45 μm PTFE membrane) (VWR, USA). The organic extra virgin olive oil was purchased from a local supermarket.

2.2. Instrumentation

The morphology of the synthesized copolymers was characterized using SEM on a Hitachi S-3700N instrument, with an accelerating voltage set to 10 kV. Samples were mounted on aluminium stubs using carbon tape and were gold coated.

FTIR spectroscopy analysis measurements were performed on a PerkinElmer Spectrum Two IR spectrophotometer.

All the chromatographic measurements were performed using a HPLC Waters Alliance System 2695-series Separation Module equipped with Alliance Series Column Heater and the detection was carried out using a photodiode array detector (2998 PDA Detector) (Waters, USA). Chromatographic experiments were carried out with a LiChroCART C18 Purospher STAR reverse phase column (250×4.6 mm ID, 5 μm) (Merck Millipore, Germany) and the detection has been performed in the range of 190-600 nm. Empower 3 FR2 software was used for management, acquisition and treatment of data.

2.3. Synthesis of the molecular imprinting systems

The synthesis of two different molecular imprinted polymers, MIP1 and MIP2, and their corresponding non-imprinted polymers, NIP1 and NIP2 were carried out using a bulk polymerization method, with the functional monomers MAA (MIP1) and AM (MIP2), respectively, the cross-linker EGDMA, dichloromethane as the porogen and with deltamethrin (template), and in its absence in the case of the non-imprinted polymer (NIP) (Figure 1). Briefly, deltamethrin-MIPs were synthesized using a molar ratio of template, radical initiator, functional monomer, and crosslinker of (1:1.9:4:20). To a 50 mL round-bottomed flask immersed on a ice bath at 0ºC were added successively and under stirring MAA (42.8 μL, 0.5 mmol) or acrylamide (36 mg, 0.5 mmol), EGDMA (0.48 mL, 2.5 mmol), deltamethrin (63.1 mg, 0.125 mmol), and dichloromethane (2.4 mL). The 1,1´-azobisisobutyronitrile (40 mg, 0.24 mmol) was added afterwards to the reaction mixture. The mixture was sonicated under a nitrogen atmosphere for 10 min in an ice bath, and then stirred in an oil bath at 60ºC. After 24 h, the polymer monolith was crushed, ground, and wet sieved with methanol to obtain particles ranging in size from 63 to 125 μm. The particles were washed extensively in a Soxhlet extractor with

methanol/acetic acid solution (1:1, v/v) until no more template was detected by HPLC–DAD analysis of the washing solvent. Subsequently, deltamethrin-MIP was washed in a Soxhlet extractor with methanol for 24 h to remove the residual acetic acid and, then, dried under vacuum at 60ºC. The NIP1 and NIP2 were synthesized using the same procedure but in the absence of template.

2.4. Screening of the Molecular Recognition Abilities of the Imprinting Systems

In order to evaluate the suitability of these polymeric materials as sorbents for SPE applications some molecular recognition assays were performed using a molecularly imprinted solid phase extraction (MISPE) methodology according to the following procedure: a slurry of 50 mg of the synthesized MIPs and NIPs in methanol was packed into an empty glass SPE column (3mL) with two polyethylene frits placed on each end to form a regular sorbent bed, and then were placed in a vacuum manifold, connected to a vacuum pump. Firstly, the cartridge was consecutively conditioned with 5 mL of methanol to remove impurities before use, followed by the addition of 5 mL of heptane. In the loading step, 1 mL of pesticide solutions in heptane containing known concentration of the deltamethrin (1.0 mg L^{-1}) were added to the MISPE cartridge, followed by the addition of 2 mL of heptane containing 10% of dichloromethane (washing step). Finally, the elution of deltamethrin was performed with 2 mL of methanol and the fraction was collected and evaporated to dryness. The residue obtained was dissolved in 1 mL of acetonitrile and analyzed with HPLC-DAD employing the following chromatographic conditions: the binary mobile phase consisted of solvents A (water) and B (acetonitrile) with the following gradient: 25–100 % B from 0 to 7 min, then 100 % B from 7 to 14 min, after that 100–25 % B from 14 to 19 min, followed by 25 % B from 19 to 24 min; The flow rate was fixed at 0.5 $mLmin^{-1}$ during

the entire chromatographic process. The injection volume was 25 μL; the temperature of 25ºC; DAD detection was done at 220 nm and the detection was set between 190 and 600 nm to monitor the UV–Vis spectra. All the experiments were conducted in triplicate and the average value taken.

2.5. Molecular modeling

In order to rationalize the design of MIPs with deltamethrin as template molecule, we carried out calculations using density functional theory (DFT) methods. Contrary to most of the previous studies, complexes with more than one monomer unit and, in some cases, the crosslinker were considered in the present work. Calculations were performed with the M06 functional [30], as implemented in the GAMESS–US program [31]. The standard 6-31G(d) basis set was used for N, C, H, O atoms [32,33]. Bromine atoms were described using the SDD effective core potential for the inner electrons and its associated basis set for the outer ones [34]. The SMD method was used in order to take into account the solvent effects [35].

To compare the relative stability of the monomer(s)/template complexes, interaction energies were calculated as:

$$\Delta E = E_{complex} - E_{template} - nE_{monomer}$$

where $E_{template}$ and $E_{monomer}$ refer to the energies of the isolated optimized species and *n* is the number of monomers in the complex. For the cases where the cross-linker was considered, the energy of the isolated optimized cross-linker was also subtracted.

2.6. Screening of MIP toward selectivity with deltamethrin analogues

Attending to the suitability of MIP2 as sorbent for MISPE applications, its binding specificity has been assessed by means of “cross-selectivity” assays towards some

structurally deltamethrin analogues, namely λ-cyhalothrin, fenpropathrin and phenothrin. To carry out this screening study, some binding assays towards the selected deltamethrin counterparts have been undertaken based on MISPE methodology, using standard solutions of those analogues and the extraction procedure and the chromatographic conditions described in section 2.4. The determination of the recovery rates for the different template analogues for the imprinted and non-imprinted system (MIP2 and NIP2) was performed. All the experiments were conducted in triplicate and the average value taken.

2.7. Optimization of the sample preparation methodology based on SPE

In order to optimize the several stages encompassed on the MISPE procedure, the effect of different parameters, namely the flow rate and the solvents used on the loading, washing and elution steps have been carefully evaluated. To perform this optimization, the MISPE and NISPE cartridges were previously conditioned with methanol and, after that with the same solvent used in the respective loading step. To optimize the loading step, the effect of the polarity of the solvents (methanol, acetonitrile, dichloromethane and heptane) on the performance of the imprinting system has been evaluated. So, for carrying out these assays, a solution with known concentration of deltamethrin (1.0 mg L^{-1}) in different solvents was loaded into the MISPE/ NISPE columns and the amount of the unretained pesticide was determined by HPLC-DAD. Since, the occurrence of non-specific interactions could also take place, it is mandatory the optimization of the washing step on the MISPE procedure. To address this particular point, the effect of the use of several solvents (heptane and dichloromethane) and their mixtures were investigated. Finally, in the elution step, the methanol was chosen as the elution solvent however the elution volume was also optimized through the assay (data not shown). A

volume of 2 mL of methanol was selected since it provides efficient recoveries of the template molecule.During all the stages of the MISPE procedure a regular eluent flow rate (approximately 1 drop per second) through the mixed-bed was attained. The chromatographic conditions used in these assays are similar to those described in the section 2.4. Tests were performed in triplicate and the average value taken.

2.8. Implementation of the analytical methodology for the selective extraction of deltamethrin in olive oil samples

Aiming to implement an analytical methodology for the selective extraction and trace analysis of deltamethrin in olive oil samples, some analytical parameters, such as the accuracy, sensitivity and recovery rates have been assessed, using aliquots of the same organic olive oil samples spiked with known concentrations of deltamethrin (1.0, 0.8, 0.6 and 0.4 $\mu g g^{-1}$) in n-heptane. Previously, the MISPE cartridge was conditioned with 5 mL of methanol and then with 5 ml of heptane. After conditioning step, the column was loaded with aliquots of 1 g of the same organic olive oil spiked with the different concentrations of deltamethrin in heptane (1.0, 0.8, 0.6 and 0.4 $\mu g g^{-1}$) diluted with 5 mL of heptane. Immediately, the interfering components present in the sample were removed with 2 mL of heptane followed by 1 mL of heptane containing 10% of dichloromethane (washing step) and, further, the template was eluted with 1 mL of methanol. The eluted fractions were collected, concentrated up to dryness and the residue obtained was dissolved in 1 mL of acetonitrile and analyzed by HPLC-DAD.

In order to gain insights about the reproducibility of the implemented analytical methodology for the trace analysis of deltamethrin in olive oil samples, a complementary assay involving the extraction and quantification of deltamethrin contents in spiked organic olive oil samples using three different MISPE cartridges

containing MIP2 as sorbent has been carried out. For this study, samples of organic olive oil spiked with a concentration of deltamethrin of 1.0 $\mu g g^{-1}$, corresponding to the MRL for deltamethrin in olives for olive oil production, have been applied.

During all the stages of the MISPE procedure was attained a regular eluent flow rate (approximately 1 drop per second). Due to the inherent complexity of olive oil, an improved version of the chromatographic method described in section 2.4 has been used in order to ensure an efficient discrimination of the peak corresponding to the target analyte avoiding its eventual co-elution with some matrix interferents. The chromatographic conditions used to perform these studies were the following: a binary mobile phase consisted of solvents A (water) and B (acetonitrile) as follows: 25–100% B from 0 to 80 min, then 100% B from 80 to 85 min, followed by 100–25% B from 85 to 90 min and, after that 25% B until 95 min; The flow rate was fixed at 0.4 $mL min^{-1}$ during the entire chromatographic process. The injection volume was 25 µL; a temperature of 25ºC; DAD detection was done at 220 nm. All the experiments were conducted in triplicate and the average value taken.

2.9. Standard Addition Method

The standard addition method (SAM) has been applied in this study [36] to evaluate the matrix effect. Experimentally, an assay encompassing the extraction of a sample of organic olive oil spiked with a concentration of deltamethrin of 1.0 $\mu g g^{-1}$ using the MISPE procedure has been performed. The eluted fraction (1mL) was splited into five equal volumes (200 µL) in separate vials. The first vial is then diluted to a final volume of 1 mL with acetonitrile. A standard solution of deltamethrin is then added in increasing volumes to the subsequent vials and each vial is then diluted with acetonitrile to the final volume of 1 mL, varying the concentrations of added deltamethrin in the

range 0.95- 1.59 mgL^{-1}. Then, the five solutions are analyzed by HPLC-DAD using the chromatographic conditions detailed in section 2.8. The instrument response is measured and the data is plotted with the standard added concentration in the x-axis and instrument response in the y-axis. Linear regression is performed and the slope and the y-intercept of the calibration curve are used to calculate the concentration of the analyte in the sample. Tests were performed in triplicate.

2.10. Experimental validation (Calibration curves/ Repeatability)

The identification of each pesticide was achieved by comparison of its retention time and UV-Vis spectra with those of the corresponding standards. The quantification was determined by calculating the areas of the relevant chromatographic peaks obtained by UV detection at 220 nm using standard solutions of the pesticides with known concentrations. All experiments were conducted in triplicate, and the average value taken. The analytical parameters for the calibration curves of these standard solutions were presented in Electronic Supplementary Material (Table S1).

3. Results and discussion

3.1. Synthesis, chemical and morphological characterization of the imprinted systems

Two molecularly imprinted polymers selective for deltamethrin were synthesized using MAA (MIP1) or AM (MIP2) as functional monomers and EGDMA as the crosslinker in dichloromethane. Deltamethrin-MIPs (MIP1 and MIP2) and the corresponding NIPs (NIP1 and NIP2) were synthesized using traditional bulk radical polymerization with a thermal free radical initiator, as depicted in Fig. 1 for the MIP2. The imprinting system MIP1 / NIP1, containing MAA as functional monomer, have previously been prepared

by Shi and co-workers [24] and Shingh and co-workers [26]. Nevertheless, in our work some modification of those synthetic procedures have been performed, namely in the use of dichloromethane as porogen and AIBN as radical initiator. The MIP2 and the corresponding NIP2 were prepared using AM as the functional monomer owing to its advantage of having several sites for hydrogen bonding, halogen bonding, dipole–dipole and π-π interactions (Figure 1), leading to significant imprinting and the formation of well-defined imprinted cavities. In this study, the removal of the template from the deltametrin-MIPs was accomplished through Soxhlet extraction with a mixture of methanol/acetic acid (1:1 v/v) to afford the free imprinted cavities for the selective rebinding of the template molecules.

Figure 1

The characterization of the imprinting systems under study encompasses a morphological evaluation by SEM and physicochemical characterization using spectroscopic analysis (FTIR).

FTIR

Concerning the imprinting system MIP1 / NIP1, the FTIR spectra are very consistent with those reported in the literature [26]. The FTIR spectra of MIP2 and NIP2 are shown in Supplementary Material (Figure S1). The spectra of MIP2 showed peaks at 3408, 3462 and 1680 cm^{-1} due to the stretching and bending vibrations of the N-H and C=O bonds of acrylamide. Also, the characteristic peak at 1393 cm^{-1} is attributed to the stretching of the C-N bond of AM. The band for the C–H stretch appeared at 2957 and 2991 cm^{-1}, respectively. The band at 1154 cm^{-1} was probably due to a C–O stretch

whilst the band at 1728 cm^{-1} is attributed to the C=O stretch. The presence of a band at 1638 cm^{-1} probably assigned to a C=C stretch of the unreacted EGDMA is also observed in the spectra. As expected, the FTIR spectra of MIP2 and NIP2 were very similar.

SEM

The morphology of the imprinting systems under study were assessed by SEM since the overall morphological feature of these materials, including the distribution and texture of the porous, affects greatly the performance of the MIPs in terms of molecular recognition abilities. The SEM micrographs of the imprinting system MIP2 / NIP2 are shown in Figure 2, exhibiting different morphologies for the imprinted and non-imprinted polymers. The NIP2 shows a smooth and compact (homogeneous) featured image while the imprinted polymer is more heterogeneous, showing fractures and an irregular surface, which seems to indicate that the presence of the template molecule influence the morphology of these tailor made materials. SEM images also show that the imprinted polymer have a more uniform dispersion and quantity of imprinting cavities than the non-imprinted polymer.

Figure 2

3.2. Evaluation of the molecular recognition of the imprinting systems

The molecular intrinsic affinity of these imprinting systems is a crucial feature on the development of selective sorbents for SPE applications. In this work a preliminary study encompassing the molecular recognition abilities of the imprinting systems MIP1 / NIP1 and MIP2 / NIP2 has been performed, in order to select the most appropriate polymeric porous material to be further used as MISPE selective sorbent. This screening

assay has been conducted by means of a MISPE-based procedure for the extraction of deltamethrin using standard solutions of this target analyte with a concentration similar to the maximum residue limit established for olives for olive oil production [37]. As summarized in Table 1, these preliminary results have indicated that the MIP2 displays the highest imprinting factor showing that AM-based polymer binds deltamethrin better that the MAA-based polymer.

Table 1

3.3. Computational modeling studies of the imprinted systems

Deltamethrin, a pyrethroid ester, is a highly flexible template. The presence of seven torsion angles makes the number of possible conformations particularly high. In addition, there are several possible sites of interactions between the template and the monomers including the nitrile group, the ester, the -CBr_2, and the ether. Given that calculating the huge number of conformations was virtually impossible, we decided to focus on two conformers, one extended conformation in which deltamethrin is somewhat linear, and another more compact conformation in which deltamethrin is folded, with the biaryl group close to the -CBr_2 group (Figure 3). These two conformations were used to evaluate the interactions with the monomer. In order to mimic the experimental conditions, the calculations were performed in CH_2Cl_2. Both conformers have similar energies, with the “compact” conformer approx. 0.9 kcal/mol lower than the “extended” one. Similar conformers were already reported in studies using semi-empirical techniques [38,39]. Since the energy of the “compact” conformer is lower, we used this conformation for our studies. It should also be noted that a full

conformational study of the template does not guarantee that the conformational minimum would be the best one for the interaction with the functional monomer.

Figure 3

The two functional monomers tested experimentally (MAA and AM) were inspected for this computational study. AM has the best imprinting effect towards deltamethrin, while MAA, despite its wide applicability in the design of MIPs, has showed lower molecular recognition abilities. Different possibilities of interaction between the monomers and deltamethrin were studied with one monomer located at various sites on the deltamethrin template, as depicted in Figure 4. The carbonyl and the singly bound oxygen of the ester function, the nitrogen atom of the nitrile, and the oxygen of the ether were selected, because these heteroatoms are more likely to form hydrogen interactions with the monomers. Optimizations with a monomer around the -CBr_2 group always converged with the monomer moving towards the nitrile or the ester. The template-monomer complexes were optimized and their interaction energies in CH_2Cl_2 were calculated and are reported in Table 2.

Table 2

All the complexes formed between MAA and the template are more stable than the ones with AM, which seems to contradict the experimental data showing that AM is the best functional monomer for deltamethrin imprinting. Calculating a relatively small number of complexes does not make this study conclusive, and looking simply at the interaction between one monomer and the template is not sufficient to rationalize the better

399 templating effect of the acrylamide based MIP for deltamethrin. Due to the various
400 potential sites of interaction, we thus decided to compute complexes with four
401 monomers simultaneously around the template, instead of one. We placed two
402 monomers (Monomer 1 and Monomer 2 in Figure 4) around the ester function, one
403 monomer close to the nitrile (Monomer 3), and the last monomer (Monomer 4) next to
404 the oxygen between the two phenyl rings. We optimized the complex formed for both
405 functional monomers. The full optimizations afford the complexes Deltamethrin-
406 $(MAA)_4$ and Deltamethrin-$(AM)_4$, represented in Figure 4, together with their respective
407 interaction energies.
408
409 **Figure 4**
410
411 The optimized structures are significantly different between both monomers. In the case
412 of MAA, the four monomers stay close to where they were initially located, *i.e.* close to
413 the interaction sites (see Deltamethrin-$(MAA)_4$), as it can be seen on the left of Figure 4.
414 In contrast, Deltamethrin-$(AM)_4$ shows a different orientation of the monomer around
415 the template. Monomer 1 remains roughly at the same position, *i.e.* close to the C=O of
416 the ester (with a NH---O distance of 2.00 Å). Monomer 3 also stays close to its starting
417 position, around the C≡N bond. On the contrary, Monomer 2 slightly moves from the
418 oxygen atom of the ester to form interactions with Monomer 3. Similarly, Monomer 4
419 moves from the ether function towards Monomer 3. If we compare the stability of the
420 complexes, the one formed between the template and the four MAA monomers is less
421 stable by around 7.5 kcal/mol. This follows the experimental data which reports a better
422 affinity of the MIP2 for deltamethrin. Nevertheless, such stability is likely to be due to
423 the interactions between the AM themselves, and therefore do not really reflect the

424 interactions between the template and AM. To counteract the attraction of Monomer 4
425 to Monomer 3, we froze the O---H distance between the oxygen atom (the one between
426 both phenyl groups) of the template and the hydrogen atom of Monomer 4 closest to the
427 template, allowing all monomers to be in close contact with their given interaction site.
428 We thus obtained Deltamethrin-$(AM)_4^*$, structurally more similar to Deltamethrin-
429 $(MAA)_4$. This structure cannot be considered an optimized structure due to the
430 constraints imposed, but gives an indication of the stability of such a complex. The
431 complex is indeed destabilized by 9.3 kcal/mol compared to Deltamethrin-$(AM)_4$,
432 suggesting again that the monomer/monomer interactions play an important role in the
433 interaction energy of the complex. Nevertheless, we presumed that such
434 monomer/monomer interactions might be impeded by the other reagents present in
435 solution. We thus considered the possible role of the crosslinker (EGDMA). The
436 crosslinker makes polymer formation possible, and is the main species in solution
437 (proportions for the template/monomer/crosslinker are 1:4:20). Such high ratio suggests
438 that interactions between the crosslinker and the other species present in solution are
439 very likely, even in the early pre-polymerization stage. We thus decided to compute the
440 template with the four monomers located close to the four most likely sites of
441 interaction with the template, together with a crosslinker molecule. The crosslinker was
442 placed between Monomer 3 and 4, in order to avoid monomer/monomer interactions,
443 and thus forcing monomer/template interactions (Figure 5). The impact of the
444 crosslinking reagent such as EGDMA prior to the establishment of the polymer network
445 has been largely overlooked. Only recently, molecular dynamics studies emphasizing
446 the potential role of the crosslinker in the pre-polymerization stage were reported
447 [13,40,41].
448

Figure 5

The crosslinker (represented in black in Figure 5) forms interactions with Monomer 3 and Monomer 4, for both MAA and AM cases. They consist of weak hydrogen interactions between the oxygen and the hydrogen atoms of both the functional monomer and the crosslinker. The crosslinker might thus impede monomer/monomer interactions, and also facilitate the monomer/template interaction. Assuming that these clusters that have been optimized in CH_2Cl_2 are a good representation of the MIP's structure, we then decided to compute their corresponding interaction energy in different solvents. When computed in CH_2Cl_2, the complex formed with the AM is less stable than the one formed with MAA, this time by around 4.0 kcal/mol (see Table 3). However, this situation is reversed when employing heptane as a solvent. In this case Deltamethrin-$(AM)_4$EGDMA, is more stable than Deltamethrin-$(MAA)_4$EGDMA by 1.9 kcal/mol. Also, both complexes are more stabilized by around 10 kcal/mol compared to the ones computed in CH_2Cl_2. On the opposite, the interaction energies are lower in methanol. This follows the trend observed experimentally where heptane is indeed the solvent affording the best binding capacity of the MIP towards deltamethrin, followed by CH_2Cl_2, and methanol (see Figure S2 in Supplementary material). Also, the weakest binding energies observed for methanol are consistent with the fact that it is the best eluting solvent (see experimental conditions in 2.4 and 2.7).

3.4. Optimization of the SPE-based analytical methodology

As discussed in the previous sections 3.2 and 3.3, the results obtained in the molecular recognition assays for the imprinting systems under evaluation are corroborated by computational modeling studies enabling to select the MIP2 as the more promising sorbent for the selective extraction of deltamethrin from olive oil samples using a

MISPE - based methodology. Hence, the optimization of the MISPE methodology using MIP 2 as selective sorbent has been carried out encompassing the selection of the most appropriate solvents for the loading and washing SPE steps.

3.4.1- Selection of the loading solvent

The effect of the polarity of the loading solvent on the binding of deltamethrin to MIP2 has been assessed by the measurement of the quantity of retained template on the polymeric material using HPLC-DAD, and the results summarized in Figure S2 (see Supplementary material). The data has evidenced that the binding of the target molecule is strongly dependent of the polarity of the loading solvent In fact, the use of polar solvents, like acetonitrile (dielectric constant (ε) = 37.5; dipole moment= 3.44 D) and methanol ((ε) = 32.6; dipole moment= 1.70 D), aprotic and protic solvent, respectively, leads to relatively low binding of the template (less than 50%). In the case of dichloromethane ((ε) = 9.1; dipole moment= 1.60 D), an aprotic polar solvent, a poor binding of deltamethrin has been also achieved. However, the binding capacity increased significantly usingheptane ((ε) = 1.9; dipole moment= 0.0 D),as loading solvent, which suggested that the strongest interactions between MIP2 and the target molecule were obtained in apolar solvents. Hence, heptane has been chosen as loading solvent in further experiments.

3.4.2- Selection of washing solvent

The optimization of the washing solvent is crucial for the development of the MISPE-based analytical methodology aiming to avoid the occurrence of non-specific binding on the imprinting material and the removal of some bonded interfering compounds contained in the complex olive oil matrix. As depicted in Figure S3 (see supplementary

material), a heptane /dichloromethane (90:10 (v/v)) mixture gave the best results and was thus chosen as the washing solvent.

3.5. Screening of MIP toward selectivity with deltamethrin analogues

For the development of selective sorbents for SPE applications the evaluation of their selectivity towards some template analogues is mandatory. Thus, the cross-selectivity of MIP2 into several deltamethrin analogues, such as λ-cyhalotrin, fenpropathrin and phenothrin were assessed. Table 4 summarizes the data obtained for the selectivity studies comprising the recovery rates (%) of the selected deltamethrin derivatives using a MISPE- based methodology.

Table 4

As shown in Table 4, the λ-cyhalotrin and fenpropathrin analogues displayed moderate binding on MIP2 since these compounds share an equivalent basic structure with deltamethrin. Nevertheless, MIP2 provides a selective entrapping of deltamethrin even in the presence of some structurally related compounds proving its potential usefulness as SPE sorbents in the selective preconcentration and extraction of deltamethrin from olive oil samples.

3.6. Implementation of the MISPE methodology to spiked organic olive oil samples

Furthermore, the implementation of the MISPE methodology for the isolation/pre-concentration of spiked organic olive oil samples with deltamethrin at concentration of 1.0 $\mu g g^{-1}$, which corresponds to the maximum residue limit (MRL) for this pesticide in olive products [37], has been successfully attempted since a high recovery rate has been achieved with good accuracy and precision. In order to gain insights about the

performance of the MISPE, the implemented methodology has been applied to olive oil samples spiked with concentrations of deltamethrin slightly below the MRL (until 0.40 $\mu g\ g^{-1}$), as shown in Table 5.

Table 5

The results demonstrated that, even at levels below the limits imposed by legislation, the suitability of the MISPE methodology for the trace enrichment of deltamethrin in spiked organic olive oil samples has been proven, since higher recovery rates (around 94%) with good accuracy and precision were obtained. In figure 6 is depicted the chromatogram of the MISPE extraction from spiked olive oil samples at a concentration of deltamethrin corresponding at MRL.

Figure 6

In order to evaluate the column-to-column MISPE reproducibility, three different SPE cartridges containing the MIP2 as sorbent were prepared and the recovery rates assessed using the experimental MISPE procedure optimized in this work (for each MISPE cartridge the assays were performed in triplicate using a concentration of deltamethrin similar to the MLR). The results obtained showed that the percentage recovery rates (± RSD %) was 90.33 (± 2.8%) proving a high column-to-column reproducibility.

3.7. Matrix effect

Owing to the complex composition of olive oil, some components of the sample matrix could interfere with the analyte signal leading to ion suppression/enhancement effects -

548 a situation known as the matrix effect, hindering a comparison of the analytical signal of
549 the sample and standard using the traditional calibration curve approach, thus causing
550 inaccuracies in the quantification of the target compound. Often, these matrix effects
551 occur during quantitative analysis in mass spectrometry detection hyphenated to liquid
552 chromatography separation, like LC-ESI-MS/MS affecting the accuracy, the precision
553 and the limit of detection [42].
554 Aiming to evaluate the matrix effect, the standard addition method (SAM) has been
555 used in our studies which have been performed by the analysis of the unspiked sample
556 followed by the consecutive standard addition solutions, as described in detail in section
557 2.9. The number of standard additions used in SAM is the most often recommended in
558 the literature and, as also suggested, the validation for the matrix effect has been
559 performed choosing a concentration of the analyte as close as possible to that expected
560 in the real samples [42]. With the experimental data, a regression line was applied in the
561 normal way and the line equation $y=144799x + 32393$ ($R^2=0.998$) was calculated. By
562 comparing the concentration of the "unspiked" sample extract with the concentration of
563 the analyte in the test sample extract obtained by extrapolation using the standard
564 addition approach it is clear that the matrix effect is absent since those concentrations
565 are coincident.
566

567 **4. Conclusions**

568 A novel extraction and determination method for the trace analysis of deltamethrin was
569 developed based on MISPE methodology using as selective sorbent a MIP possessing
570 recognition abilities for deltamethrin, which was further successfully validated for the
571 isolation and pre-concentration of this target pesticide in olive oil samples. In this work,
572 the molecular interactions within the imprinting systems under study and the role of the

crosslinker, the functional monomers, and the solvent on the template recognition were elucidated by computational modeling studies. The crosslinker plays a critical role balancing the monomer-monomer and the template-monomer interactions and it is thus expected that it might have an impact on the pre-polymerization stage of other MIPs. Even more important is the inclusion of the solvent in order to rationalize the binding affinity of the MIP. It is important to differentiate between the solvent used for the synthesis, the loading, and the elution steps, since the binding affinity of the MIP towards the template is highly dependent on the solvent. All in all, considering all the possible interaction sites on the template, the inclusion of the crosslinker, and taking into account the proper solvent were crucial to understand the better binding capacity of MIP2 compared to MIP1.

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Table 1

Polymers	Amount of bound template ± SD[a] (mg)	IF ± SD[a]

MIP 1	0.28±0.01	
		2.0±0.1
NIP 1	0.140±0.005	
MIP 2	0.930±0.007	
		4.65±0.01
NIP 2	0.20±0.01	

734 All experiments were conducted in triplicate (n=3); [a]Average ± Standard deviation (SD);

735 IF (Imprinting factor) = MIP/NIP.

736

737

738 **Table 2**

Interaction sites	MAA	AM
Carbonyl group	-8.8	-2.0
Singly bound oxygen of ester	-9.0	-7.0
Nitrile	-9.3	-4.0
Ether	-7.8	-5.9

739

740 **Table 3**

Solvent	Deltamethrin-$(AM)_4$ EGDMA	Deltamethrin- $(MAA)_4$ EGDMA
CH_2Cl_2	-39.6	-43.4
Heptane	-48.7	-46.8
Methanol	-37.2	-41.0

741

742

743

744 **Table 4**

745

Analytes	Recovery rates ± SD[a] (%)	
	MIP2	NIP2
Deltamethrin	94.0±0.7	20.0±0.1
λ-Cyhalotrin	55.0±2.1	11.0±0.10
Fenpropathrin	67.0±0.7	9.3±0.2
Phenothrin	10.0±1,4	2.0±0.1

746 [a]Average ± Standard deviation (SD); Tests were performed in triplicate (n=3).

747

748 **Table 5**

749

Spiked concentration (µg g^{-1})	Concentration of bound pesticide ± SD[a] (µg g^{-1})	RSD[b] (%)	Recovery rates ± SD[a] (%)
1.00[c]	0.90±0.03	3.33	90.00±2.50
0.80	0.69±0.01	1.45	87.00±1.70
0.60	0.54±0.01	1.85	90.00±1.50
0.40	0.38±0.01	0.88	94.00±1.70

750 [a]Average ± Standard deviation (SD); [b]Variant coefficient (RSD); [c]Concentration of

751 deltamethrin corresponding to the MLR; Tests were performed in triplicate (n=3).

752

753 **Table Captions**

754

755 **Table 1**. Retention of deltamethrin on the different molecularly imprinted systems

756 under study.

757

Table 2. Comparison of stability for various monomer/template complexes using AM and MAA at different sites on the template. (Interaction energy values in CH_2Cl_2 are in kcal/mol.)

Table 3. Comparison of stability of complexes Deltamethrin-$(AM)_4$EGDMA and Deltamethrin-$(MAA)_4$EGDMA depending on the solvent. (Interaction energy values are in kcal/mol.)

Table 4. Recovery rates (%) of different deltamethrin structural analogues in the MIP2 and NIP2 MISPE columns obtained after loading with 1mL of 1.0 mgL^{-1} of the corresponding pesticide solution in n-heptane, washing with 1 mL of n-heptane containing 10% of dichloromethane and elution with 2 mL of MeOH.

Table 5. Precision and accuracy of the MISPE column for the extraction of deltamethrin from spiked organic olive oil samples.

Figure Captions

Figure 1. Representative scheme for the controlled formation of the imprinted cavity through appropriate hydrogen bonding, halogen bonding, dipole-dipole and π-π interactions between the AM, EGDMA and deltamethrin (template).

Figure 2. SEM micrographs of MIP2 (left) and NIP2 (right).

Figure 3. Representations of the two selected conformers of deltamethrin.

784 **Figure 4.** Schematic representation of the template in the compact form, with four
785 monomers (labeling of the monomers is shown in the upper part of the Figure). In the
786 lower part the complexes with both AM (on the right and in the middle), and MAA (on
787 the left) are depicted. On the left an optimized structure with the template surrounded by
788 four molecules of MAA is depicted. The optimized structure obtained with AM is
789 shown in the middle. The complex on the far right is a constrained structure where the
790 O---H distance between the oxygen atom of the template and the hydrogen atom of
791 Monomer 4 of AM has been frozen. The interaction energy values are in kcal/mol. The
792 template is shown with the Van der Waals surface, and the monomers are represented
793 using a ball-and-stick model.
794
795 **Figure 5.** Representations of the template with four monomers (AM for the complex
796 depicted on the left, MAA for the complex on the right) and the cross-linker (EGDMA).
797 Interaction energy values are in kcal/mol. The template is shown with the Van der
798 Waals surface, the monomers and the cross-linker are represented as ball-and-stick
799 models. The crosslinker is further highlighted in black.
800 **Figure 6**. HPLC/DAD chromatogram after MISPE pretreatment of olive oil sample
801 spiked with deltamethrin at a concentration corresponding to the MRL.
802