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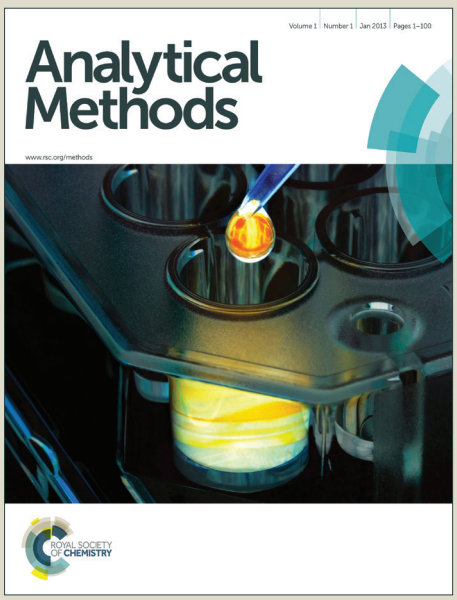

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Molecularly imprinted polymers solid phase extraction of fungicides from wine samples

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Abstract

In order to preconcentrate iprodione fungicide in white wine samples, molecularly imprinted polymers (MIPs) specific for iprodione were synthesized using two polymerization approaches: precipitation (MIPp) and bulk polymerization (MIPb). A comparison of the performance of the MIPs and the corresponding non imprinted polymers (NIPs) was conducted in batch studies. In this case, the MIPp revealed better recognition properties toward iprodione in wine samples than the MIPb. The MIPp and MIPb were then used as sorbent on solid phase extraction cartridges (MISPEp and MISPEb consecutively) in order to pre-concentrate iprodione from white wine samples. The optimization of the MISPE elution step was done using MIPp and acetonitrile was shown to be the best eluting solvent. MISPEp showed better iprodione recovery and pre-concentration factor than MISPEb. The selectivity of the MISPE method for iprodione was evaluated in white wine sample in the presence of two other fungicides pyrimethanil and procymidone. MISPEp was very selective for iprodione compared with the two other fungicides. However, MISPEb was able to preconcentrate iprodione as well as its analogues.

Keywords: fungicide, iprodione, MISPE, wine, selectivity.

1 Introduction

Toxic substances such as pesticides are used for a purpose of increasing the food supply. The residues of such molecules are transmitted to processed foods and are an important source of food-borne diseases.

There are many analytical techniques with satisfactory sensitivity for the detection and quantification of these contaminants often present as traces. However, the complexity of the matrices prevents the direct analysis application on these compounds, and hence a step of pre-concentration is often needed [1].

In the recent years, the food contaminant analysis field has devoted considerable interest to the "molecularly imprinted solid phase extraction" (MISPE) technique. In fact, this technique has been successfully applied to solve several challenging issues especially in the very complex samples where analyte selectivity is required [2-4]. Nowadays, it is by far the most advanced technical application of MIPs [5]. Molecularly imprinted solid-phase extraction (MISPE) has been used as a selective sorbent method in numerous applications where a certain degree of selectivity is required such as sensors[6] and chromatography [7]. MISPE is an attempt to circumvent the drawbacks attributed to the traditional solid phase extraction technique (SPE). On one hand, it has the same advantages as the SPE (economical and rapid), on the other hand it offers additional advantages over SPE such as accuracy and selectivity[1].

The pesticides are linked to a broad spectrum of medical problems such as cancer, neurotoxic effects, reproductive health concerns and endocrine disruption [8-10]. Although problems stemming from the use of pesticides have been known, recently the use of pesticides in viticulture has become an important practice all over the world. While their environmental effects are relatively safe at acceptable doses, some concerns about the toxicity of pesticide residues remain [11]. The possibility exists that residues of these products can pass from grape to must and later to wine with the resulting risk to consumer's health. As a consequence of the widespread use of pesticides, the presence of their residues in both food and the environment has become an important issue in analytical science. Even the generally low concentrations expected for pesticide residues in wines justify the use of sensitive analytical methods. For fungicide in wine, no uniform limits have been established yet, except for procymidone for which the European Union has established maximum residue limit (MRL) of 0.5 mg/kg [12]. Iprodione is a pesticide that inhibits the germination of fungal spores on the surface of fruits and is used also as a four-season fungicide for lawn. It is widely used in vineyards against botrytis for example, but it is a harmful substance for humans (R22, R40), toxic for aquatic organisms (R50/53), and is not readily biodegradable. The MRL of the iprodione in the products to which the MRLs are applied are between 0.02 and 10 mg/kg (Commision Regulation (EU) No 396/2005). The latter fungicide has been detected in more than 90 % of French wine according to a survey by the French Ministry of Agriculture [13]. Because of its poor biodegradability and the risk of accumulation, the use of iprodione for crops will expire on 31 October 2016 (Commission Regulation (EU) No 823/2012 of 14 September 2012).

Few analytical methods have been developed for the analysis of pesticides residues, among which iprodione, in some food matrices. These methods are mainly gas and liquid chromatography coupled with powerful detection systems (time-of-flight mass spectrometry, mass spectrometry/selective ion monitoring, tandem mass spectrometry…) and using an extraction method prior to analysis for the enrichment of pesticides in matrices[14-20]. This concentration step is required to reach low concentration levels for trace determination. It has been achieved using SPE method with commercial conventional SPE sorbents such as C8 or C18 bonded silica[17, 18]graphitised carbon[17], or polymeric materials[19] and immunoassay using a commercial ELISA[15] or modified commercial ELISA[16]. Such selective extraction requires a compromise between the selectivity of the immunoassay based methods and the chemical resistance of the conventional SPE sorbents.

Therefore, developing efficient adsorbent materials with high affinity, with low cost, without time consuming and that exhibits high thermal, physical and chemical resistance is essential. Molecularly imprinted polymers (MIPs) have these properties[21] and are used in this study. MIPs resist to the use of organic solvents, strong acidic and basic buffers[22] and to the application of different matrices in any conditions. MIPs are non-biological alternatives to antibodies and then have the same selectivity as the immunoassay method[21] and could be employed in the case of complex matrices[23] such as wine.

The aim of this study was to optimize and verify the extraction performance of iprodione-MISPE in a wine solution. This work investigates the behavior of two MIPs as SPE sorbents. The MIPs were produced using two different polymerization approaches: precipitation and bulk. The selectivity of these MISPE for iprodione in a wine solution and in the presence of other fungicides was also investigated.

2 Experimental section

2.1 Reagents and solutions

Iprodione [CAS number 36734-19-7], methacrylamide (MAM) [CAS number 79-39-0], ethylene glycol dimethacrylate (EGDMA) [CAS number 97-90-5], 2,2-dimethoxy-2-phenylacetophenone (DMPAP) [CAS number 24650-42-8], pyrimethanil [CAS number 53112-28-0], procymidone [CAS number 32809-16-8], toluene, acetonitrile, ethanol and acetic acid were purchased from Sigma Aldrich, France. Solvents were HPLC grade and were used without any purification. High purity de-ionized water, obtained with an Elga Ionic system PURELAB Option, was used to prepare ethanol/aqueous solutions and mobile phase mixture.

2.2 Polymers preparation

MIPs were prepared by using MAM as functional monomer and EGDMA as crosslinker. The molar ratio of EGDMA/MAM/iprodione depending on the polymerization method was 4/1/0.1 for bulk polymerization[24] and 2/0.4/0.1 for precipitation polymerization. The polymer synthesis was carried out with DMPAP as initiator in 1 mL or 10 mL of toluene to obtain a bulk or precipitating MIP particles.
MAM and iprodione were weighed in a test tube and dissolved in toluene. After the addition of EGDMA and DMPAP, the mixture was degassed in an ultrasonic bath for 10 minutes. The tube was then sealed and exposed to ultraviolet radiation overnight. The resulting bulk MIP was ground into powder.
For comparison purposes, corresponding non-imprinted polymers (NIP) were prepared similarly to the process described above, except that the polymerization mixture did not contain the template.

In order to remove the template, the polymer particles were soaked in the mixed solvent of acetic acid and ethanol (30/70 v/v) and subjected to ultrasonic treatment for 10 minutes. This mixture is meant to break the hydrogen bonds between iprodione and the polymer. Washing in ethanol continued until the template could no longer be detected with HPLC in washing solutions. The solvent was then removed by centrifugation, and the fine particles were dried at 65 °C overnight in an oven. These fine particles were used in the subsequent experiments.

2.3 High performance liquid chromatography (HPLC)

Reversed phase high performance liquid chromatography (RP-HPLC) was performed using a C18 column on a Shimadzu LC equipped with a 25 µL loop injector, an SPD 20AT UV-visible absorbance detector and an LC–20AT liquid pump. Acetonitrile/water (60/40 v/v) was used as mobile phase and the flow rate was 1.0 $mL \cdot min^{-1}$. Detection was performed at 220 nm, which is the maximum adsorption wavelength of iprodione.

2.4 Batch experiments

Equilibrium isotherm data were obtained in a batch wise approach. 10 mg of cleaned imprinted polymer or non-imprinted polymer were suspended in 20 mL of hydro-alcoholic solutions containing from 1×10^{-5} to 1.1×10^{-3} M of iprodione. These isotherms were performed in ethanol/water solutions (50/50, v/v). The mixture was shaken continuously for 2 hours at 25 °C. After removal of the polymer particles and filtration of the resulted solution under 0.45µm membrane, the concentration of free iprodione at equilibrium, F ($mol.L^{-1}$), was analysed by HPLC-UV. The concentration of iprodione bound to the polymer, B ($mol.L^{-1}$), was calculated by subtracting the concentration of free iprodione from the initial iprodione concentration.

2.5 Molecularly imprinted solid phase extraction (MISPE)

MISPE method was applied on white wine samples (Bourgogne Chardonnay 2011 de Charles Renoir)

spiked with iprodione (1×10^{-5} M = 3.3 mg/L). Empty polypropylene SPE tubes (1mL, supelco, Bellfonte, USA) with PE frits (20µm porosity) were connected to a vacuum manifold, and 20 mg of the MIP (or NIP) were slurried with acetonitrile and packed into the cartridges. A second frit was inserted on top of the solvent bed. The SPE cartridges were activated with 5 mL of methanol and conditioned with 5 mL of water/ethanol solution (90/10, v/v). White wine (total of 6 mL) samples were applied to the cartridges and washed with 5 mL of water/ethanol solution (90/10, v/v)[25, 26]. The cartridges were dried for 5 min under vacuum (14psi) after the final stage of the wash step.

In order to get the optimal eluting solution, different eluting compositions were tested using ethanol/water and or acetonitrile/water mixtures with different eluent strength keeping the same conditions for the other steps. SPE steps were controlled by analyzing iprodione in the effluents by HPLC-UV. The behavior of the NIP under these SPE conditions (NISPE) was evaluated and compared with the corresponding MIP.

For the selectivity tests, a solution of the white wine (Bourgogne Chardonnay 2011 de Charles Renoir) spiked with 5×10^{-5} M of three pesticides that have some structural similarity: iprodione, pyrimethanil or procymidone was loaded into the conditioned MISPE and the NISPE. After the loading step, the cartridges were washed with 5 mL of water/ethanol solution (90/10, v/v) and dried for 5 min under vacuum (14 psi). Finally, 2 mL of acetonitrile were used for the elution step. All experiments were performed in triplicate.

3 Results and discussion

3.1 Adsorption isotherm

We synthesized two kinds of molecularly imprinted polymers using the same reagents but varying the polymerization methods: bulk and precipitation. One of the best methods for evaluating the binding sites in MIPs is batch adsorption test. Batch adsorption involves analysis of an MIP in a solution of substrate [27]. Both of the iprodione-MIPs were evaluated by investigating the isotherms adsorption results. For this purpose, batch studies were conducted in a wine model diluted with ethanol (ethanol/water, 50/50, v/v) and the obtained isotherms were outlined. The latter represent a measure of the relationship between the equilibrium concentration of free (F) and bound (B) iprodione over a certain concentration range and can be generated from the breakthrough curves[28]**.** The equilibrium adsorption isotherms of iprodione from the diluted wine model solution onto MIP_b, NIP_b, MIP_p and NIP_p are presented in **figure1**. MIP_p shows larger adsorption capacity than MIP_b and the MIPs have higher affinity for iprodione than the corresponding NIPs. This difference was evaluated by calculating for each MIP the imprinting factor IF = KpMIP/KpNIP [29] where Kp is the partition constant Kp = B/F. The IF of MIP_p was significantly higher (2.40 ± 0.02) than that of MIP_b (1.9 ± 0.02).

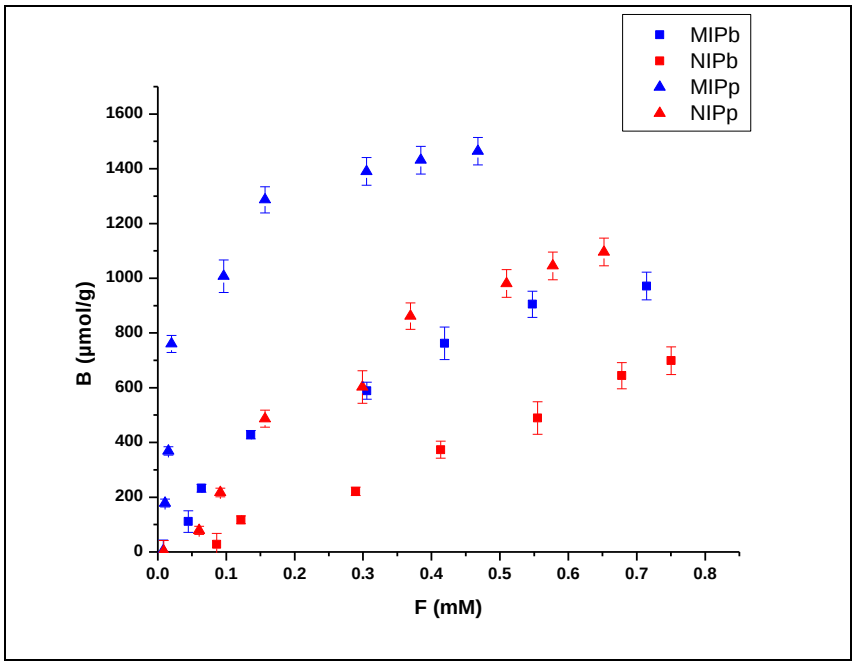


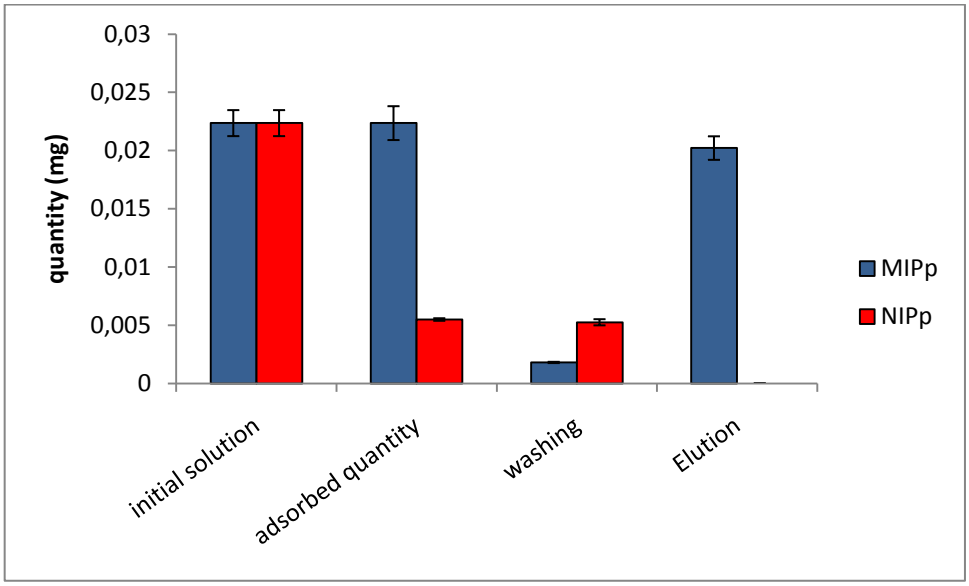
Figure 1 Adsorption isotherms of iprodione on MIPb, NIPb, MIPp and NIPp in ethanol/water (50/50, v/ v).

This reveals that MIP_p has better recognition properties than MIP_b. These differences could be a result of the interactions strength between the iprodione and the functional groups in the polymers. To confirm these results in real samples, the polymers were tested as stationary phases on MISPE and applied on a white wine solution. However, the optimization of the MISPE steps (Conditioning, loading, washing and elution) was performed using MIPp and applied to MIPb.

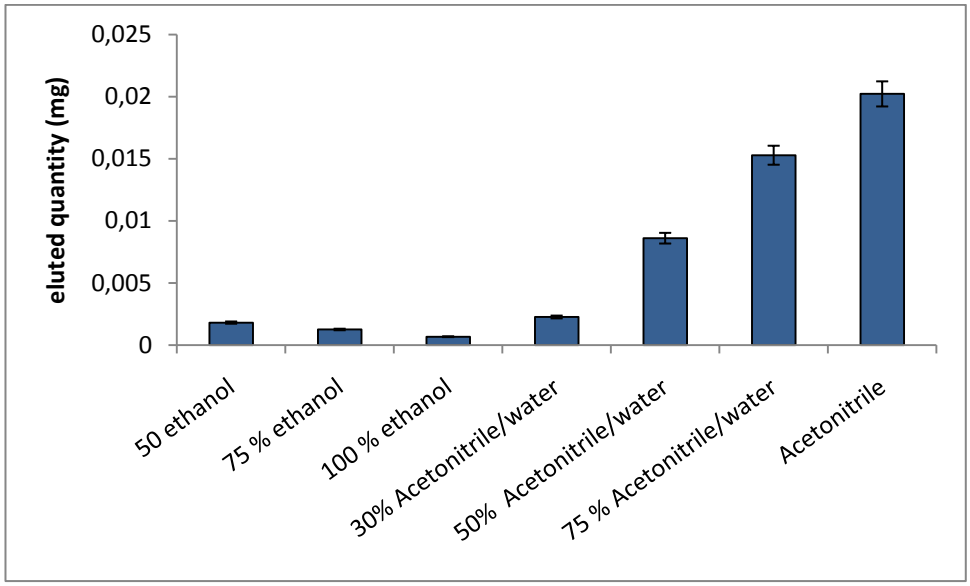
3.2 Optimization of MISPE on white wine sample

White wine samples spiked with iprodione were loaded in the SPE cartridges containing the MIPp and NIPp. **Figure 2** shows the results of the $MISPE_p$ and $NISPE_p$ procedure. By examining the **figure 2a**, we observe 4 times higher adsorption on $MISPE_p$ compared to the $NISPE_p$. The amount of the non-specific adsorbed iprodione (the NIP adsorbed quantity) was lower than the specific one. In this way, specific interactions were developed in the wine matrix. Furthermore, this was confirmed by examining the washing step result in the **figure 2a**, where a lower quantity of iprodione is removed from the MISPE cartridge compared to the NISPE. This result shows that MISPEp offers a good recognition of iprodione in wine sample solution. 90 % of iprodione was retained by specific interactions.

The whole amount of iprodione retained by the NISPEp was due to non-specific interactions, this could be confirmed as well by the washing step where the iprodione was completely eliminated from the NISPE cartridge.



a)



b)

Figure 2 a) Adsorbed and recovered amount of iprodione after the washing step and the elution step (with acetonitrile) from MISPE$_p$ and NISPE$_p$ columns and b) the recoveries of eluted iprodione obtained on the MISPE$_p$ after eluting iprodione with different elution solvents.

The eluting step plays a crucial role in the MISPE technique which is used to preconcentration purpose. The optimal elution solvent should completely elute the target analytes in a one step. An optimization of the eluting step was performed. As shown in **figure 2b** the ethanol eluted 0.6 µg of iprodione which represents only 2% of the residual iprodione. By decreasing the percentage of ethanol, the recovery increases but not sufficiently to remove all the residual iprodione. For that reason, we tried another organic modifier than ethanol which could increase the eluent strength: the acetonitrile. Different

solutions were tested with different compositions of acetonitrile/water (30/70, 50/50, 75/25; v/v) and acetonitrile. The recoveries reached by adding acetonitrile were higher, and the pure acetonitrile solution was able to recover the whole amount of residual iprodione.

To understand this result we compare the hydrophobicity of the solvents by comparing their partition coefficient between octanol and water: LopP (EtOH) = -0.19; LogP (ACN) = - 0.45 and LogP (H_20)= -1.38. We also compare the polarity of the solvents which is evaluated by the dipolar moment of ethanol μ(EtOH)= 1.69 D, of acetonitrile μ(ACN)= 3.92 D and of water $\mu(H_2O)$= 1.85 D. Finally, the dielectric constants (Permittivity) of the different eluting solvents are :ε(EtOH) = 24.55; ε(ACN) = 37.5; $\varepsilon(H_2O)$ = 80; ε(acetonitrile/water 30/70) = 67.25, ε(acetonitrile/water 50/50) = 58.75, ε(acetonitrile/water 75/25) = 48.1. We can conclude that the recoveries are higher when we use a more polar eluting solvent with a quite low hydrophobicity and a quite important dielectric constant.

The same procedure was applied to $MISPE_b$ and $NISPE_b$ using the acetonitrile as the elutionsolvent. The results are presented in **figure 3**. It shows that the MIPs prepared with two different polymerization methods do not exhibit the same behaviour. The extraction efficiency of the NISPEb was better than the NISPEp : this is a result of a higher number of non-specific interaction sites in the polymer prepared by bulk polymerization. The difference between the amount of iprodione adsorbed on MISPE and NISPE is higher when the precipitation method is used. The latter result is in accordance with the batch results. $MISPE_b$ showed high recovery in the washing step which is consistent with the high amount of non-specific bound iprodione. Furthermore, the amount of iprodione removed from MIPSPE cartridge in the eluting step, which corresponds to iprodione retained by specific interactions, was lower compared to the iprodione removed in the washing step. Only 40 % of the total amount of the adsorbed iprodione was due to specific interactions between the sorbent and the analyte in the real wine sample. This suggests that the polymer synthesized by precipitation polymerization has been more successfully imprinted or that the crushing step of the MIP synthesized in bulk destroys sites of specific recognition (no specific cavities on the surface of MIPb particles).

The concentration factor of the $MISPE_b$ was 3 while the $MISPE_p$ concentration factor was 5.8 (almost 2 times higher).

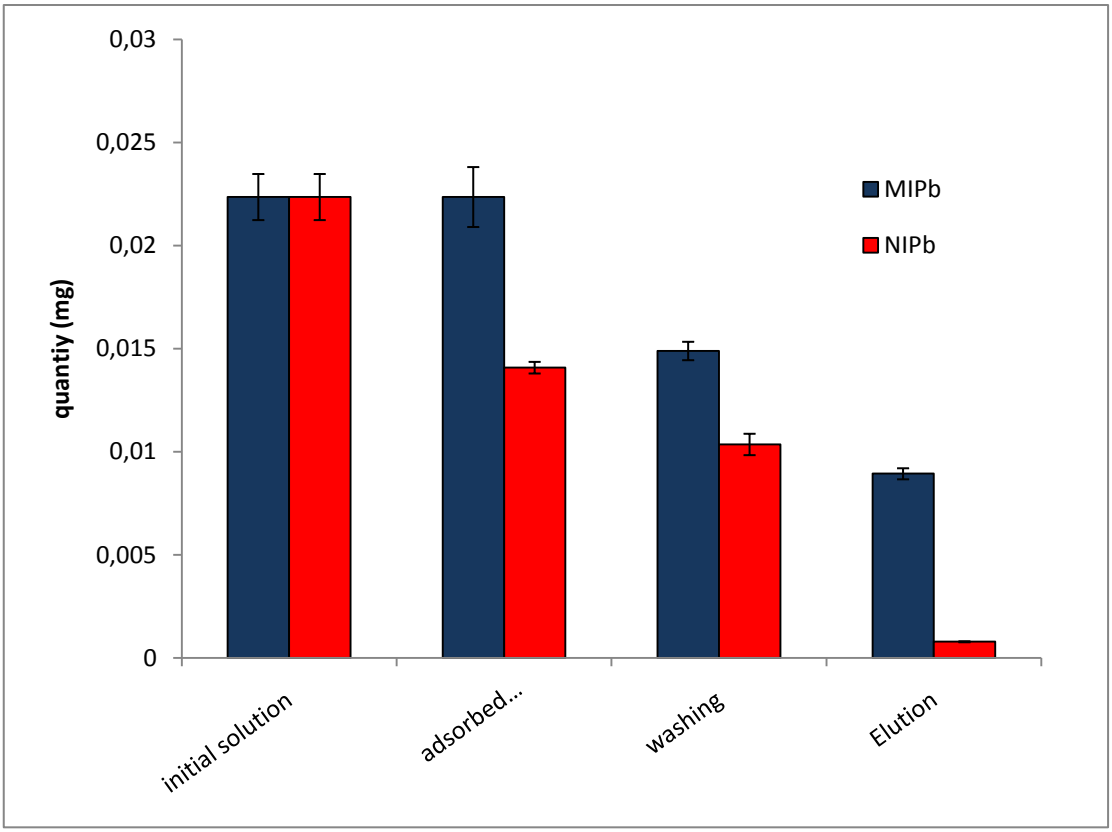


Figure 3 Adsorbed and recovered amount of iprodione after the washing step and the elution step (with acetonitrile) from MISPEb and NISPEb cartridges.

3.3 Selectivity

The MIPs were evaluated for their binding selectivity toward two other fungicides structural analogues of iprodione: pyrimethanil and procymidone **(figure 4)**. In order to study the selectivity of MIP_p and MIP_b sorbents toward iprodione and its structural homologues, MISPE using MIP_p and MIP_b were performed in a white wine sample spiked with the three fungicides. For this purpose, the optimized SPE procedure was used.

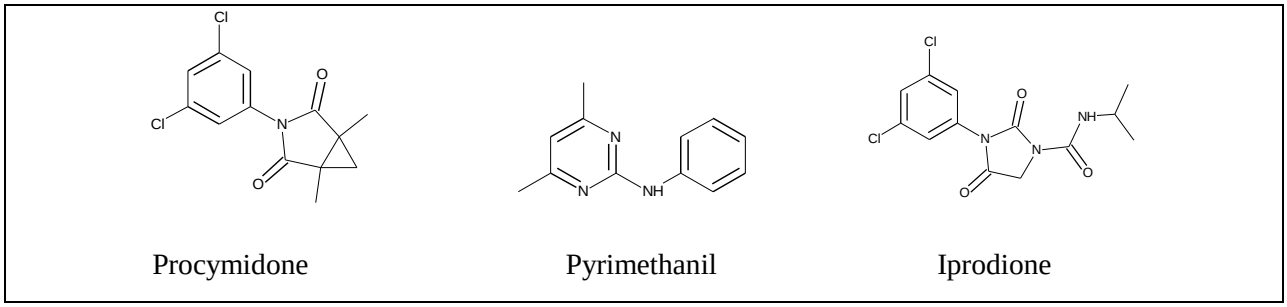


Figure 4 The chemical structures of the fungicides: Iprodione, pyrimethanil and procymidone.

The chromatograms of the white wine sample spiked with the three fungicides are presented in figures 5 and 6 respectively (green lines). The blue and the red lines are chromatograms of the three fungicides in

the eluting solvent after the enrichment step using MISPE and NISPE respectively. The chromatograms corresponding to the final elution step show that the MIPp **(figure 6, blue line)** is more selective than the MIPb **(figure 5, blue line)**. This could be explained by the more important non-specific interactions of the MIPb compared to MIPp, MIPp preconcentrated only iprodione whereas MIPb was able to preconcentrate iprodione as well as its analogues. Moreover, another observation demonstrates that MISPEp was very selective; the iprodione had a stronger retention than pyrimethanil and procymidone. On the other hand, the three fungicides had the same strength of retention on the MIPb and were eluted simultaneously in the eluting step **(figure 5, blue line).**

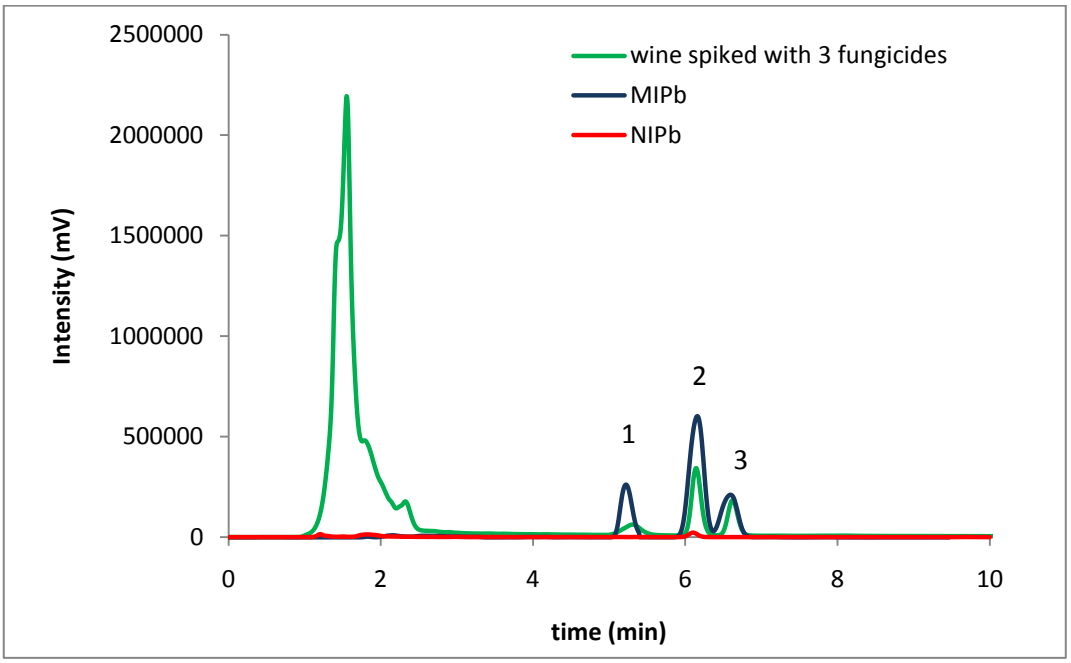


Figure 5 Overlay of chromatograms of : a white wine solution spiked with three fungicides : (1) pyrimethanil, (2) iprodione and (3) procymidone and the eluted solutions from the $MISPE_b$ (MIP_b) and $NISPE_b$ (NIP_b).

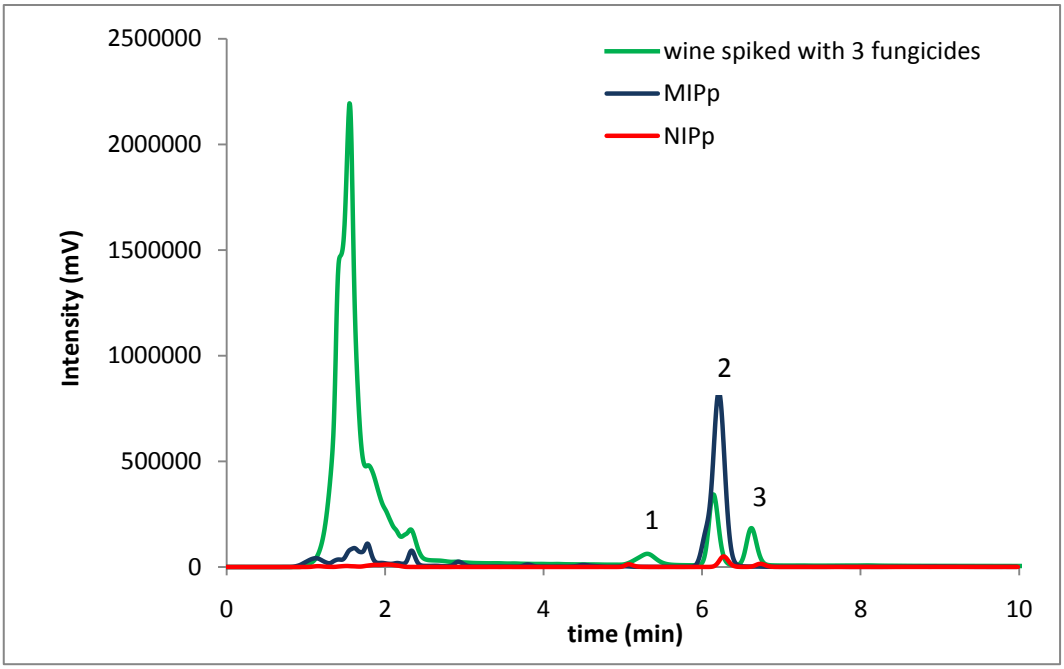


Figure 6 Overlay of chromatograms of: a white wine solution spiked with three fungicides : (1) pyrimethanil, (2) iprodione and (3) procymidone and the eluted solutions from the $MISPE_p$ (MIP_p) and $NISPE_p$ (NIP_p).

3.4 Preliminary validation of the method

The HPLC calibration curve of the analyzed iprodione was constructed at different concentrations, in the range 0.16 to 33 mg/L. The correlation coefficient was R^2 = 0.9998. The limit of detection (LOD) and the limit of quantification (LOQ) of the MISPE method were calculated using the following equations: LOD = (3.3/5.8)(SD/S) ; LOQ = (10/5.8)(SD/S) where SD is the standard deviation of the HPLC response and S is the slope of the calibration curve. 5.8 corresponds to the preconcentration factor of the MISPE method. By considering the HPLC calibration curve and the iprodione MISPE preconcentration procedure, we found a LOD of 139 µg/L and a LOQ of 422 µg/L. The iprodione recovery rate was 98.5 % using MIPp and 106.6 % using MIPb. The relative standard deviation of the method (n=4) was 13 %.

4 Conclusion

This study demonstrated the potential of an iprodione imprinted polymer for the preconcentration of iprodione in wine samples. This very selective MIP for iprodione fungicide was prepared via precipitation polymerization and subsequently applied to MISPE. It has higher selectivity, specificity, imprinting factor and enrichment capability compared with MIP prepared by bulk polymerization. The MIP prepared by precipitation polymerization provides an alternative SPE sorbent for the selective extraction of iprodione from wine samples and potentially from beverages and water. The MIP prepared by bulk polymerization could be a subject of future research to preconcentrate a family of pesticides based on the result obtained

from the selectivity test where the preconcentration of the three pesticides was possible. MIP presents potentiality for routine detection of forbidden substances in wine such as fungicide.

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