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**Selective enrichment of ailanthone from leaves of ailanthus altissima by tandem reverse phase/molecularly imprinted solid phase extraction**

**This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1742519> since 2020-10-23T13:01:14Z

*Published version:*

DOI:10.1016/j.microc.2020.105198

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(Article begins on next page)

1 **SELECTIVE ENRICHMENT OF AILANTHONE FROM LEAVES OF *AILANTHUS***  
2 ***ALTISSIMA* BY TANDEM REVERSE PHASE / MOLECULARLY IMPRINTED SOLID**  
3 **PHASE EXTRACTION**

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13  
14 **ABSTRACT**

15 The biological activity of extracts from *Ailanthus altissima* is mainly due to the presence of  
16 ailanthone, a compound belonging to the quassinoid class. Recently, attention has been  
17 paid to its strong cytostatic activity. However, the extraction of ailanthone is based on very  
18 long and demanding procedures, which keep the price of the commercial product very  
19 high. Thus, the development of selective adsorbents for the purification of ailanthone from  
20 *A. altissima* leaves extracts could help in reduce the costs of production. In this work, we  
21 describe the rational design of a molecularly imprinted polymer selective for ailanthone  
22 based on the screening of a 96-members not-imprinted polymeric library to rapidly identify  
23 pre-polymerization mixtures able to generate MIPs with enhanced binding properties. A 4-  
24 vinylpyridine-co-trimethylolpropane trimethacrylate polymer showed high binding towards  
25 ailanthone. It was used to prepare an imprinted polymer with interesting binding affinity  
26 ( $K_{eq}=18.3 \times 10^3 \text{ L mol}^{-1}$ ), high imprinting factor (IF= 3.8) and fast binding kinetics  
27 ( $k_{ass}=0.390 \text{ min}^{-1}$ ,  $k_{dis}=0.021 \text{ mol L}^{-1} \text{ min}^{-1}$ ). The imprinted polymer was used to develop a  
28 successful purification protocol of extracts from *Ailanthus altissima* leaves. The purification  
29 was based on the combination of a preliminary clean-up of Soxhlet extracts onto a reverse  
30 phase-C18 cartridge and the subsequent isolation of ailanthone by a molecularly imprinted  
31 solid phase extraction. This approach allowed efficiently purifying the ailanthone contained  
32 in aqueous or methanolic Soxhlet extracts with high yields compared to the quantities  
33 reported in literature (water:  $0.756 \pm 0.027 \text{ mg g}^{-1}$ ; methanol:  $0.770 \pm 0.030 \text{ mg g}^{-1}$ ).  
34 Moreover, it allows processing sample volumes up to 15 mL without significant losses of  
35 the target compound.

36  
37 **ABBREVIATIONS**

38 ACN: acetonitrile; ALA: allylamine; AM: acrylamide; AMO: 4-acryloylmorpholine; AN:  
39 acrylonitrile; DCM: dichloromethane; DEAEM: N,N-diethylaminoethylmethacrylate;  
40 DMAEM: N,N-dimethylaminoethylmethacrylate; DMAM: N,N-dimethylacrylamide; DMPA:  
41 2,2-dimethoxy-2-phenylacetophenone; EDMA: ethylene dimethacrylate; EGMP:  
42 ethyleneglycol methacrylate phosphate; EtOAc: ethylacetate; GDMA: glycerol  
43 dimethacrylate; HEMA: 2-hydroxyethylmethacrylate; MA: methylacrylate; MAA: methacrylic  
44 acid; Me<sub>2</sub>CO: acetone; MeOH: methanol; MISPE: molecularly imprinted solid phase  
45 extraction; NVP: N-vinylpyrrolidone; PEGMA: monomethoxypolyethylene glycol 400  
46 methacrylate; PEGDMA: polyethylene glycol 400 dimethacrylate; PETRA: pentaerythritol  
47 triacrylate; STY: styrene; TRIM: trimethylolpropane trimethacrylate; VIM: 1-vinylimidazole;  
48 4VP: 4-vinylpyridine

49  
50 **1. INTRODUCTION**

51 *Ailanthus altissima*, known as the 'tree of heaven', is native to China and was introduced in  
52 Europe and America around the end of 18th century as an ornamental tree. Extracts of this  
53 plant are used in traditional Chinese medicine to treat cold and gastric diseases. The  
54 biological activity of leaf and stem bark extracts is mainly due to the presence of  
55 ailanthone, a compound belonging to the quassinoid class [1-3]. Over the past three  
56 decades, several studies have clearly shown the strong herbicidal activity of the plant  
57 extracts [4-8]. Besides, anti-tuberculosis, anti-malarial and anti-viral activity has also been  
58 described [9-11]. Recently, a lot of special attention has been paid to the cytostatic activity  
59 of ailanthone itself [12-19]. As many other interesting natural compounds, despite the  
60 potential value as leading compound for pharmaceutical applications, the complex  
61 chemical structure of ailanthone makes its synthesis from natural precursors a very difficult  
62 and expensive task, and, currently, the only available source of ailanthone is represented  
63 by leaf extracts [20,21]. Unfortunately, the isolation of the pure product from these extracts  
64 is based on very long and cumbersome procedures which keep the price of the final  
65 product very high [4,7,10]. Therefore, the development of selective adsorbents for the  
66 extraction of ailanthone from *A.altissima* leaves extracts could help in improving the  
67 compound purity, increase its yield, reduce work-up time minimizing the number of  
68 extraction steps and, as a consequence, the cost of production.

69 Molecularly imprinted polymers (MIPs) are synthetic polymeric materials possessing  
70 cavities homologous to a template molecule, involving a molecular recognition mechanism  
71 based on non-covalent interactions [22,23]. They show binding properties similar to natural  
72 antibodies, like binding reversibility, high binding affinity constant and selectivity for a target  
73 molecule [24]. Imprinted polymers have been successfully used in analytical and  
74 preparative applications where it is necessary to selectively extract target molecules from  
75 complex samples [25,26].

76 In this work, we describe the rational design of a molecularly imprinted polymer selective  
77 for ailanthone based on the screening of a 96-members not-imprinted polymeric library  
78 prepared by combining different functional monomers and crosslinking agents. This  
79 experimental approach is based on the finding that if a not-imprinted polymer (NIP) shows  
80 binding properties toward a given target molecule, the MIP with the same composition of  
81 the NIP will show an enhanced imprinting effect [27]. Thus, the existing connection  
82 between the binding properties of MIPs and NIPs makes possible to use NIP libraries to  
83 rapidly identify pre-polymerization mixtures able to generate MIPs with enhanced binding  
84 properties [28]. The formulation corresponding to the non-imprinted polymer with the best  
85 binding towards ailanthone was considered to prepare an imprinted polymer, and  
86 consequently it was used to develop a successful purification protocol of extracts from  
87 *A.altissima* leaves based on the combination of a preliminary clean-up of extracts onto a  
88 reverse phase-C18 cartridge and the subsequent isolation of ailanthone by a molecularly  
89 imprinted solid phase extraction (MISPE) method.

## 90 91 **1. MATERIALS AND METHODS**

### 92 **2.1 Materials**

93 Ailanthone (purity >98%), 2,2-dimethoxy-2-phenylacetophenone, functional monomers and  
94 cross-linkers were from Sigma-Aldrich-Fluka (Milan, Italy). Polymerization inhibitors  
95 eventually present in monomer solutions were removed by clean-up on activated alumina  
96 columns. Organic solvents and all other chemicals were from VWR International (Milano,  
97 Italy). All the solvents were of HPLC grade, whereas all chemicals were of analytical  
98 grade. Water was deionized on mixed ion exchange columns, and it was ultrapurified in a  
99 Purelab Prima System from Elga (Marlow, UK). Ailanthone stock solutions were prepared  
100 by dissolving 25.0 mg of solid in 5.0 mL of acetonitrile and stored in the dark at -20 °C until

101 use. *A.altissima* leaves were collected in the summer of 2018 from a public park in Torino  
102 and store frozen until use.

103

## 104 **2.2 Polymeric combinatorial library**

105 The polymeric combinatorial library was made up by 96 different polymer combinations. In  
106 3-mL thick wall borosilicate glass vials, the pre-polymerization solutions with a molar ratio  
107 of 1:5 4:9 between the functional monomer and the cross-linker were prepared by mixing  
108 0.15 mmoles of functional monomer and 1.35 mmoles of cross-linker sampled by weight.  
109 Then, a volume, corresponding to the total volume of the monomers, of dry ACN  
110 containing DMPA (1% of the vinyl groups in the pre-polymerization mixture), was added.  
111 The vials were sonicated in an ultrasonic bath for 10 min and sealed. Then, the mixtures  
112 were photo-polymerized overnight at 4 °C. using a 200 W medium-pressure Hg lamp. The  
113 bulk polymers were grounded in a mechanical mortar, sieved to 15–38 µm, and dried  
114 under vacuum at 70 °C for 2h. Finally, 50 mg of each polymer was packed in a 2-mL solid  
115 phase extraction empty polypropylene cartridge and inserted in a VersaPlate™ 96-well-  
116 SPE system (Agilent, Milano, Italy). The cartridges were sequentially washed with 5x0.5  
117 mL of water, 5x0.5 mL of MeOH-acetic acid 1+9 (v/v) and 5x0.5 mL of ACN, dried under a  
118 gentle stream of nitrogen for 2 h, sealed and stored at room temperature.

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## 120 **2.3 Library screening**

121 Before each measurement, the polymeric combinatorial library was equilibrated with 5x0.5  
122 mL of ACN. Then, 1.0 mL of 100 µg/mL solution of ailanthone in ACN were loaded into the  
123 cartridges and, after 15 min of equilibration in the polymer, vacuum was applied to elute  
124 the unbound fraction. The eluates were evaporated under a gentle stream of nitrogen,  
125 dissolved in 1.0 mL of 78+22 (v/v) water-MeOH and transferred to 1.5-mL HPLC  
126 autosampler vials. The ailanthone in unbound fraction  
127 was measured by HPLC (vide infra). To evaluate the reproducibility of the screening  
128 assay, each elution was repeated three times and the amount of free ailanthone was  
129 estimated as the average of the measured values. The amount of ailanthone bound to the  
130 polymer (B) was calculated by subtracting the amount of free ailanthone (F) from the  
131 known initial amount (total, T).

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## 133 **2.4 HPLC method**

134 Reverse phase HPLC analysis was used for ailanthone determination. The HPLC  
135 apparatus was a LaChrom Elite system composed of a programmable binary pump L-  
136 2130, an auto-sampler L-2200, a UV-Vis detector L-2400, and provided with EZChrom  
137 Elite software for the instrumental programming, data acquisition and data processing was  
138 from Merck-Hitachi (Milano, Italy). The column used was a 125 mm × 4.6 mm i.d., 5 µm,  
139 Li-Chrosphere 100 RP-18 (VWR, Milano, Italy). The mobile phase was composed of  
140 water/MeOH 78+22 (v/v), and the elution was performed in isocratic conditions at a flow  
141 rate of 0.6 mL/min. The sample volume injected was 10 µL, and the detection wavelength  
142 was 254 nm. In these instrumental conditions ailanthone retention time was 2.41±0.05  
143 min.

144 Ailanthone standard solutions at concentrations of 0.25, 0.5, 1, 2.5, 5, 10, 25, 50 and 100  
145 µg/mL were prepared in 78+22 (v/v) water+MeOH immediately before use. The standards  
146 were analysed in triplicate and mean peak areas were plotted against ailanthone  
147 concentration. The calibration plot was drawn by using a weighted linear regression  
148 (weight = 1/conc,  $r^2 = 0.9998$ ). The limits of detection and quantification (LOD = 1.9 µg/mL,  
149 LOQ = 3.6 µg/mL) were calculated as LOD = 3 Sy/b and LOQ = 10 Sy/b, respectively,  
150 where Sy is the standard error of the response and b is the slope of the calibration plot.

151

## 152 **2.5 Molecularly imprinted polymer**

153 In a 5-mL thick wall borosilicate glass vial, a solution with molar ratio template:functional  
154 monomer:cross-linker 1:9:45 was prepared by dissolving 50 mg (0.132 mmol) of  
155 ailanthone (template), 1.18 mmol of 4VP (functional monomer), 5.94 mmol of TRIM (cross-  
156 linker) and 64 mg of DMPA in 2 mL of ACN. The vial was sonicated in an ultrasonic bath  
157 for 10 min and thermopolymerised at 60 °C overnight. The bulk polymer obtained was  
158 broken with a steel spatula, grounded in a mechanical mortar and mechanically wet-sieved  
159 to 15–38 µm. Then, the template was extracted by packing the polymer in polypropylene  
160 SPE columns and exhaustively washing with MeOH-acetic acid 1+9 (v/v) till no ailanthone  
161 was detectable by HPLC analysis of the eluate. No efforts were made to measure the  
162 amount of template recovered. The washed polymer was dried under vacuum at 70 °C for  
163 2 h and stored in a desiccator. A blank polymer was prepared in the same experimental  
164 conditions by omitting the template.

## 166 **2.6 Calculation of rebinding parameters**

167 To measure the equilibrium rebinding parameters, about 30 mg of polymer were exactly  
168 weighed in 4 mL flat bottom amber glass vials. Then, 0.5 mL of ACN solutions containing  
169 increasing amounts of ailanthone ranging from 5 to 250 µg were added. The vials were  
170 incubated overnight at room temperature under continuous agitation on a horizontal  
171 rocking table. Then, the solutions were filtered on 0.22 µm nylon membranes, 500 µL were  
172 diluted 1+1 (v/v) with water and the free amounts of ailanthone were measured by HPLC  
173 analysis. Each experimental point was assessed as the average of three repeated  
174 measures.

175 To measure the rebinding kinetics parameters, about 30 mg of polymer were exactly  
176 weighed in 4 mL flat bottom amber glass vials. Then, 0.5 mL of ACN solutions containing  
177 20 µg of ailanthone were added and the vials were incubated for time intervals between  
178 0.5 and 60 minutes at room temperature under continuous agitation on a horizontal  
179 rocking table. Then, the solutions were filtered on 0.22 µm nylon membranes, 500 µL were  
180 diluted 1+1 (v/v) with water and the free amounts of ailanthone were measured by HPLC  
181 analysis. Each experimental point was assessed as the average of three repeated  
182 measures.

183 Rebinding isotherms and kinetics were calculated by using SigmaPlot 12 (Systat Software  
184 Inc., Richmond, CA, USA). Non-linear least square fitting was applied to the averaged  
185 experimental data. Rebinding isotherm parameters were calculated by using a Langmuir  
186 binding isotherm model:

187

$$188 \quad B = \frac{B_{max}K_{eq}F}{1 + K_{eq}F}$$

189

190 where B is the ligand bound to the polymer, F the ligand not bound to the polymer,  $K_{eq}$  the  
191 equilibrium binding constant and  $B_{max}$  the binding site density.

192 Rebinding kinetics parameters were calculated by using a 1<sup>st</sup> order kinetic model:

193

$$194 \quad B = B_{max}[1 - \exp(-k_{ass}t)]$$

195

196 where B is the ligand bound to the polymer at time t,  $B_{max}$  the ligand bound to the polymer  
197 at equilibrium and  $k_{ass}$  the association kinetic constant.

198 To assure robust results, weighted (1/y) Pearson VII limit minimization was chosen as the  
199 minimization method. To avoid being trapped in local minima, which would give incorrect  
200 results, minimizations were carried out several times by using different initial guess values  
201 for the binding parameters.

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## 2.7 Development of MISPE protocol.

All of the SPE experiments were made in 3 mL polypropylene SPE cartridges, packed with 100 mg of the MIP. All measurements were carried out in triplicate and recoveries were calculated as the averages of the repeated measures to estimate the method repeatability. Before each experiment, the stationary phase was washed with 3x1 mL of MeOH-acetic acid 1+9 (v/v) and conditioned with 5x1 mL of water.

To measure the effect of different washing solutions on removal of the analyte from the MISPE cartridge, 1 mL of 50  $\mu\text{g mL}^{-1}$  standard solution of aianthone was loaded by applying the vacuum. After sample loading, air was passed through the column for 5 min. Then, the cartridge was washed with 1 mL of water or water containing increasing amounts of organic solvent (10, 20, 30, 40, 50, 75 or 100% (v/v), ACN, Me<sub>2</sub>CO or MeOH). The eluate was immediately dried under a stream of nitrogen at ambient temperature and reconstituted in 500  $\mu\text{L}$  of mobile phase for HPLC analysis

To measure the effect of loading, increasing volumes (1, 2, 3, 4, 5, 8, 10, 15, or 20 mL) of aqueous solutions containing 50  $\mu\text{g}$  of aianthone were loaded by applying the vacuum. After sample loading, air was passed through the column for 5 min. Then, the cartridge was washed with 1 mL of water-MeOH 4+1 (v/v) and eluted with 3x1 mL of MeOH. The eluate was immediately dried under a stream of nitrogen at ambient temperature and reconstituted in 500  $\mu\text{L}$  of mobile phase for HPLC analysis

## 2.8 Extraction of *A.altissima* leaves

Samples of dried *A.altissima* leaves, 10 g, were pulverized in a ball mill, transferred in cellulose thimbles and extracted for 24 hours in a Soxhlet apparatus with an adequate amount of water, MeOH, EtOAc or DCM, respectively. The extracts were evaporated in rotavapor and reconstituted in 250 mL of water under sonication. The solutions were filtered on 0.22  $\mu\text{m}$  nylon membranes and stored at 4 °C in the dark.

## 2.9 Combined solid phase extraction

To eliminate the hydrophobic components of the reconstituted aqueous extracts, these were loaded onto a 250-mg commercial C18 solid phase extraction cartridge pre-conditioned with 3x1 mL of MeOH and 3x1 mL of water. Then, the cartridge was washed with 1 mL of water and the fraction containing aianthone was recovered by elution with 1 mL of water+MeOH 6+4 (v/v). The eluate was diluted 1+1 with water and loaded on MISPE cartridge pre-conditioned with 3x1 mL of MeOH-acetic acid 1+9 (v/v) followed by 5x1 mL of water. The MISPE cartridge was washed 1 mL of water-methanol 4+1 (v/v) and eluted with 3x1 mL of methanol to recover aianthone.

# 3. RESULTS AND DISCUSSION

## 3.1 Screening of the polymeric combinatorial library

The structural characteristics of aianthone make this molecule an ideal target for the synthesis of a MIP. In fact, its rigid structure with multiple condensed rings (figure S1, supplementary informations) guarantees the possibility of forming a well-defined binding site that is not inclined to deform or collapse once the template has been removed, while the presence of several hydroxyl and carbonyl functions ensures the possibility of establishing a sufficiently high number of non-covalent interactions with functional monomers. However, a preliminary test with a classic prepolymerisation mixture consisting of methacrylic acid and ethylenglycole dimethacrylate disappointingly produced a polymer with poor binding properties towards aianthone. Consequently, it was decided to search for a polymerization mixture capable of generating a polymer with adequate binding

252 properties through the screening of a not-imprinted polymeric library prepared by  
253 combining different functional monomers and crosslinking agents.  
254 To ensure a significant degree of molecular diversity, we combined 16 different functional  
255 monomers and 6 cross-linkers in a 96-members polymeric library. Hydrophobic (MA, STY),  
256 hydrophilic (AM, AMO, AN, DMAM, HEMA, NVP, PEGMA), acidic (EGMP, MAA), and basic  
257 (ALA, DEAEM, DMAEM, VIM, 4VP) compounds were used as functional monomers, while  
258 cross-linkers were selected in terms of the number of hydrophobicity and polymerisable  
259 groups: hydrophobic / two (DVB, EDMA), hydrophilic / two (GDMA, PEGDMA), and  
260 hydrophobic / three (PETRA, TRIM). The screening of this polymeric library for ailanthon  
261 binding produced a very variable pattern of binding behaviours (table 1), with a prevalence  
262 of poorly binding polymers ( $B/T < 0.2$ , 81 out of 96 polymers) and very few polymers with a  
263 significant binding ( $B/T > 0.3$ , 3 out of 96 polymers).  
264 The analysis of variance performed on the binding results does not show indications  
265 regarding the effect of the monomers when considered one by one (figure S2,  
266 supplementary informations) ( $p=0.516$ ,  $n=6$ ), nor grouped (figure S3, supplementary  
267 informations) as hydrophobic ( $n=12$ ), acid ( $n=12$ ), basic ( $n=30$ ) or polar neutral ( $n=42$ )  
268 ( $p=0.694$ ). In fact, we generally observed both very low and high binding values for each of  
269 the functional monomers. Conversely, the analysis of variance related to the effect of  
270 cross-linking agents (figure S4, supplementary informations) showed that polymers can be  
271 clustered into three distinct groups: EDMA-GDMA, TRIM-PETRA and DVB-PEGDMA,  
272 where the last binds ailanthon to a significantly smaller extent ( $p < 0.001$ ,  $n=16$ ) than all  
273 others.

274

### 275 3.2 Binding properties of the MIP

276 Based on the results obtained from the screening of the combinatorial library, the mixture  
277 composed of 4VP as the functional monomer and TRIM as the cross-linking agent was  
278 chosen to prepare a MIP. The binding properties of the MIP towards ailanthon were  
279 estimated by measuring binding isotherm (figure S5, supplementary informations) and  
280 association kinetics (figure S643, supplementary informations) in acetonitrile.

281 Both MIP and NIP showed relatively low binding sites density ( $B_{max}$ ) values (MIP =  
282  $0.326 \pm 0.072 \mu\text{mol g}^{-1}$ ; NIP =  $0.369 \pm 0.232 \mu\text{mol g}^{-1}$ ), while the equilibrium binding constant  
283 was higher in the case of the MIP ( $K_{eq} = 18.3 \pm 7.2 \times 10^3 \text{ L mol}^{-1}$ ) than in the case of to the  
284 NIP ( $K_{eq} = 4.76 \pm 3.75 \times 10^3 \text{ L mol}^{-1}$ ), with an imprinting factor ( $IF = K_{eqMIP} / K_{eqNIP}$ ) equal  
285 to 3.8. It must be observed that both the density of binding sites and the equilibrium  
286 constant of the MIP are significantly lower than those usually obtained for imprinted  
287 polymers. This can be interpreted as a consequence of the fact that the strong  
288 hydrophilicity of ailanthon ( $\log P = -0.76$  [29]) can hinder any hydrophobic interaction  
289 between the molecule and the binding sites, thus limiting the contribution to the binding to  
290 the formation of hydrogen bonds between the functional monomers and the polar functions  
291 of the molecule.

292 Slow binding kinetics can hinder the development of an effective MISPE technique, as the  
293 analyte may not bind completely to the solid phase. However, the results of the association  
294 kinetics for the prepared MIP show that, if a first order kinetic is assumed to be valid, it  
295 binds ailanthon speedily and about 4.6 times faster than the corresponding NIP (MIP:  $k_{ass}$   
296 =  $0.390 \pm 0.160 \text{ min}^{-1}$ ,  $t_{1/2} = 1.77 \text{ min}^{-1}$ ; NIP:  $k_{ass} = 0.0488 \pm 0.001 \text{ min}^{-1}$ ,  $t_{1/2} = 14.2 \text{ min}^{-1}$ ).  
297 Interestingly, both polymers show to have nearly the same dissociation kinetic constant  
298 ( $k_{dis} = k_{ass}/K_{eq}$ ) (MIP:  $k_{dis} = 0.021 \text{ mol L}^{-1} \text{ min}^{-1}$ ; NIP:  $k_{dis} = 0.010 \text{ mol L}^{-1} \text{ min}^{-1}$ ).

299

### 300 3.3 Development of C18-MISPE mixed protocol

301 The extracts of *A. altissima* leaves were strongly coloured. When they were loaded onto a  
302 cartridge packed with the NIP, this resulted irreversibly discoloured. Hence, to avoid

303 damaging the cartridges packed with the MIP, it was decided to develop a two-step mixed  
304 protocol. A preliminary step was devised to eliminate the coloured pigments and the more  
305 hydrophobic components by a C18 cartridge, and, in the successive step, the resulting  
306 eluate was extracted onto the ailanthone-selective MISPE cartridge. It should be noted  
307 that this approach is not new in the MISPE technique, as a preliminary clean-up before the  
308 extraction on an Ochratoxin A-imprinted column has been reported for wine samples. The  
309 clean-up successfully eliminated high hydrophobic components that interfered with the  
310 MISPE-based protocol [30].

311 The ability of the C18 cartridge to retain the coloured pigments was tested by loading  
312 ailanthone aqueous solutions and washing the cartridge with water containing increasing  
313 amounts of organic polar solvents. Ailanthone recoveries higher than 95% occurred for  
314 washing solutions containing 40% (v/v) MeOH, 30% (v/v) ACN or 20% (v/v) Me<sub>2</sub>CO (Table  
315 2). The effective release of coloured pigments was visually evaluated in separate  
316 experiments by loading the *A. altissima* leaves extracts when washing with 50% (v/v)  
317 MeOH, 20% (v/v) ACN or 10% (v/v) Me<sub>2</sub>CO. Therefore, the solution containing MeOH 40%  
318 (v/v) was considered as the optimal eluent for recovering ailanthone and removing  
319 coloured interferences.

320 To setup the MISPE protocol, in a preliminary experiment, increasing volumes (0.5 – 20  
321 mL) of a solution containing 50 µg mL<sup>-1</sup> of ailanthone were loaded onto the MIP-cartridge,  
322 and no analyte leaching was observed. Thus, the loading step in aqueous solution was  
323 deemed safe for a complete retention of ailanthone onto the cartridge.

324 The washing step was intended for cleaning possible polar components not specifically  
325 bound to the column. Thus, water containing increasing amounts of organic polar solvents  
326 was tested as the washing solution. A substantial release of ailanthone from the cartridges  
327 was observed when these were washed with water containing quantities equal to or greater  
328 than 10% (v/v) of ACN, 20% (v/v) of Me<sub>2</sub>CO and 30% (v/v) of MeOH (table 3), while below  
329 these levels the release was very limited for ACN and Me<sub>2</sub>CO and even absent for MeOH.  
330 Consequently, it was decided to use a water-MeOH 4+1 (v/v) mixture as the washing  
331 solution, while pure methanol was considered as the ideal eluent for the quantitative  
332 recovery of ailanthone from the cartridge in the final step of the protocol.

333 The effect of loading increasing volumes of an aqueous solution containing 50 µg of  
334 ailanthone confirmed that using the water-MeOH 4+1 (v/v) mixture in the loading and  
335 washing steps, and MeOH in the elution step allowed for a quantitative recovery of the  
336 analyte in the range 50 µg – 1 mg (figure 1). This result envisages the application of the  
337 MISPE technique to large sample volumes, up to 20 mL, thus allowing treating leaf extracts  
338 in relatively large quantities.

### 340 **3.4 Combined solid phase extraction of real samples**

341 The chromatograms corresponding to the extraction in Soxhlet of the leaves with water or  
342 organic solvents are shown in figure 2. When water or MeOH were used, the  
343 chromatograms were characterized by large complexity with many overlapping peaks,  
344 while in the case of EtOAc this complexity was lower, and disappeared in the case of  
345 DCM, whose chromatogram showed low and isolated peaks. The extraction on C18  
346 cartridges considerably simplified the chromatographic patterns for all extracts, but in no  
347 cases it was possible to observe an isolated peak corresponding to the retention time of  
348 the ailanthone. Consequently, the further extraction on MISPE cartridges proved to be  
349 necessary to isolate the target molecule from real samples.

350 The eluates (2 mL) from the extraction on C18 cartridges were then repeatedly (n=8)  
351 loaded onto the MISPE cartridges, and the quantity of ailanthone recovered each time was  
352 measured with respect to the initial weight of the extracted leaves. When water or MeOH  
353 were used in the Soxhlet extraction step, the quantity of recovered ailanthone



354 corresponded approximately to the aianthone present in the leaves according to the  
355 literature *i.e.* about  $1\text{ mg g}^{-1}$  of leaves (water:  $0.756\pm 0.027\text{ mg g}^{-1}$ ; MeOH:  $0.770\pm 0.030\text{ mg}$   
356  $\text{g}^{-1}$ ) [7]. Instead, when EtOAc was used the quantity of aianthone isolated was significantly  
357 lower ( $0.591\pm 0.072\text{ mg g}^{-1}$ ), and minimal when DCM was used ( $0.083\pm 0.024\text{ mg g}^{-1}$ ). This  
358 result confirms the previous literature [4,7], and indicates that polar solvents such as water  
359 or methanol are very effective in extracting aianthone from the leaves, while with  
360 decreasing polarity of the solvent, this capacity decreases sharply.

361 As shown in the figure 3, loading increasing volumes (1-15 mL) of methanolic leaf extract  
362 previously cleaned on the C18 cartridges on MISPE cartridges resulted in a yield of  
363 aianthone proportional to the volume of the cleaned extract. This further demonstrated the  
364 possibility of purifying relatively large volumes of leaf extracts without loss of the target  
365 compound., The peak corresponding to the retention time of the aianthone is clearly  
366 visible in the chromatograms, even if always accompanied by a secondary peak  
367 corresponding to a substance of unknown nature, slightly less polar (retention time 3.1  
368 min), and present in smaller quantity compared to the target compound (about 10%,  
369 estimated from the ratio of peak areas). Since this compound was well recognized by the  
370 MISPE cartridge, it is plausible that its molecular structure is similar to that of the  
371 aianthone. However, we did not investigate whether this substance was present in the  
372 leaf extracts or was formed by degradation during the extraction process.

373

#### 374 **4. CONCLUSIONS**

375 The isolation of aianthone from the leaves of *A.altissima* presents considerable difficulties  
376 due to the complex nature of the leaf extracts. The use of a MISPE cartridge preceded by  
377 a cleaning of leaf extracts from pigments and hydrophobic compounds through the use of  
378 a C18 cartridge, made it possible to develop an extraction protocol simpler than those  
379 previously reported in the literature, reproducible and with a high yield in aianthone  
380 compared to the mass of leaf material used. Moreover, the use of a polymeric library to  
381 identify the optimal combination of functional monomers and cross-linking agents  
382 demonstrate that it is possible to operate successfully a rational protocol to rapidly identify  
383 a polymerization mixture optimal for the efficient molecular imprinting of complex organic  
384 molecules.

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- 477

478 **TABLES**

479

480 **Table 1:** B/T ratio for ailanthon binding by the 96-members polymeric library. Polymers  
481 with a significant binding (B/T>0.3) are reported in bold

482

	DVB	EDMA	GDMA	PEGDMA	PETRA	TRIM
MA	<b>0.32</b>	0.29	0.23	0.00	0.08	0.12
STY	0.11	0.20	0.11	0.06	0.14	0.14
AM	0.01	0.16	0.18	0.00	0.14	0.09
AMO	0.01	0.14	0.14	0.07	0.10	0.18
AN	0.00	0.13	0.16	0.06	0.12	0.21
DMAM	0.06	0.16	0.20	0.17	0.17	0.16
HEMA	0.00	0.18	0.13	0.10	0.11	0.18
VPO	0.04	0.16	0.14	0.01	0.08	0.12
PEGMA	0.07	0.09	0.12	0.00	0.02	0.10
EGMP	0.08	0.10	0.12	0.05	0.12	0.05
MAA	0.13	0.13	0.20	0.14	0.09	0.07
ALA	0.00	0.06	0.25	0.02	0.11	0.00
DEAEM	0.00	0.11	0.26	0.12	0.15	0.06
DMAEM	0.09	0.14	0.24	0.00	0.30	0.08
VIM	0.02	0.29	0.27	0.11	0.12	0.05
4VP	<b>0.36</b>	0.12	0.11	0.05	0.07	<b>0.39</b>

483

484

485

486

487

488 **Table 2:** effect of washing solution composition on the recovery of 50 µg of ailanthon  
489 from a C18 cartridge. Recovery is expressed in % units. Washing solutions that caused  
490 release of coloured components from the cartridge are marked in bold

491

water + solvent, v/v	MeOH	ACN	Me <sub>2</sub> CO
100 + 0	-	11	8
90 + 10	41	53	<b>70</b>
80 + 20	76	<b>89</b>	<b>96</b>
70 + 30	92	<b>99</b>	<b>100</b>
60 + 40	98	<b>100</b>	<b>100</b>
50 + 50	<b>100</b>	<b>100</b>	<b>100</b>
40 + 60	<b>100</b>	<b>100</b>	<b>100</b>

492

493

494 **Table 3:** effect of washing solution composition on the recovery of 50 µg of ailanthon  
495 from a MISPE cartridge. Recovery is expressed in % units.

496

water + solvent, v/v	MeOH	ACN	Me <sub>2</sub> CO
100 + 0	-	-	-
90 + 10	-	7	2

80 + 20	1	15	8
70 + 30	11	51	38
60 + 40	43	98	88
50 + 50	77	100	100
75 + 25	97	100	100
0 + 100	100	100	100

497 **FIGURE CAPTIONS**

498

499 **Figure 1:** Preconcentration of ailanthonone in the range 5 – 100 µg onto the MISPE  
500 cartridge. Data are expressed as the mean of three separate samplings ±1 standard  
501 deviation. Regression equation: µg found = 0.934±0.015 µg loaded - 0.186±0.386 (R<sup>2</sup> =  
502 0.998, SEE = 0.218)

503

504 **Figure 2:** HPLC chromatograms of the samples obtained by Soxhlet extraction of the  
505 *A. altissima* leaves with water, MeOH, EtOAc and DCM, respectively. Black  
506 chromatograms: samples evaporated and back-dissolved in water. Red chromatograms:  
507 the same solutions after C18 extraction. The grey bar indicates the position of the peak  
508 related to ailanthonone

509

510 **Figure 3:** HPLC chromatograms of samples of increasing volume (0.5 - 15 mL) obtained  
511 by Soxhlet extraction of the *A. altissima* leaves with MeOH and clean-up on C18/MISPE. In  
512 the inset: correlation between the sample volume and the peak height (mV = 2.447±0.125  
513 mL - 1.272±0.482, R<sup>2</sup> = 0.9845, SEE = 0.409)

514