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

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

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ABSTRACT

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

ABBREVIATIONS

ACN: acetonitrile; ALA: allylamine; AM: acrylamide; AMO: 4-acryloylmorpholine; AN: acrylonitrile; DCM: dichloromethane; DEAEM: N,N-diethylaminoethylmethacrylate; DMAEM: N,N-dimethylaminoethylmethacrylate; DMAM: N,N-dimethylacrylamide; DMPA: 2,2-dimethoxy-2-phenylacetophenone; EDMA: ethylene dimethacrylate; EGMP: ethyleneglycol methacrylate phosphate; EtOAc: ethylacetate; GDMA: glycerol dimethacrylate; HEMA: 2-hydroxyethylmethacrylate; MA: methylacrylate; MAA: methacrylic acid; Me_2CO: acetone; MeOH: methanol; MISPE: molecularly imprinted solid phase extraction; NVP: N-vinylpyrrolidone; PEGMA: monomethoxypolyethylene glycol 400 methacrylate; PEGDMA: polyethylene glycol 400 dimethacrylate; PETRA: pentaerythritol triacrylate; STY: styrene; TRIM: trimethylolpropane trimethacrylate; VIM: 1-vinylimidazole; 4VP: 4-vinylpyridine

1. INTRODUCTION

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

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

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

1. MATERIALS AND METHODS

2.1 Materials

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

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

2.2 Polymeric combinatorial library

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

2.3 Library screening

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

2.4 HPLC method

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

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

2.5 Molecularly imprinted polymer

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

2.6 Calculation of rebinding parameters

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

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

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

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

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

Rebinding kinetics parameters were calculated by using a 1st order kinetic model:

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

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

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

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 μg mL^{-1} standard solution of ailanthone 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_2CO or MeOH). The eluate was immediately dried under a stream of nitrogen at ambient temperature and reconstituted in 500 μL 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 μg of ailanthone 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 μL 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 μm 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 ailanthone 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 ailanthone.

3. RESULTS AND DISCUSSION

3.1 Screening of the polymeric combinatorial library

The structural characteristics of ailanthone 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 ailanthone. Consequently, it was decided to search for a polymerization mixture capable of generating a polymer with adequate binding

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

3.2 Binding properties of the MIP

Based on the results obtained from the screening of the combinatorial library, the mixture composed of 4VP as the functional monomer and TRIM as the cross-linking agent was chosen to prepare a MIP. The binding properties of the MIP towards ailanthone were estimated by measuring binding isotherm (figure S5, supplementary informations) and association kinetics (figure S643, supplementary informations) in acetonitrile.
Both MIP and NIP showed relatively low binding sites density (B_{max}) values (MIP = 0.326±0.072 μmol g^{-1}; NIP = 0.369±0.232 μmol g^{-1}), while the equilibrium binding constant was higher in the case of the MIP (K_{eq} = 18.3±7.2 x 10^3 L mol^{-1}) than in the case of to the NIP (K_{eq} = 4.76±3.75 x 10^3 L mol^{-1}), with an imprinting factor (IF = K_{eq}MIP / K_{eq}NIP) equal to 3.8. It must be observed that both the density of binding sites and the equilibrium constant of the MIP are significantly lower than those usually obtained for imprinted polymers. This can be interpreted as a consequence of the fact that the strong hydrophilicity of ailanthone (logP = -0.76 [29]) can hinder any hydrophobic interaction between the molecule and the binding sites, thus limiting the contribution to the binding to the formation of hydrogen bonds between the functional monomers and the polar functions of the molecule.
Slow binding kinetics can hinder the development of an effective MISPE technique, as the analyte may not bind completely to the solid phase. However, the results of the association kinetics for the prepared MIP show that, if a first order kinetic is assumed to be valid, it binds ailanthone speedily and about 4.6 times faster than the corresponding NIP (MIP: k_{ass} = 0.390±0.160 min^{-1}, $t_{1/2}$ = 1.77 min^{-1}; NIP: k_{ass} = 0.0488±0.001 min^{-1}, $t_{1/2}$ = 14.2 min^{-1}). Interestingly, both polymers show to have nearly the same dissociation kinetic constant (k_{dis} = k_{ass}/K_{eq}) (MIP: k_{dis} = 0.021 mol L^{-1} min^{-1}; NIP: k_{dis} = 0.010 mol L^{-1} min^{-1}).

3.3 Development of C18-MISPE mixed protocol

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

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

The ability of the C18 cartridge to retain the coloured pigments was tested by loading ailanthone aqueous solutions and washing the cartridge with water containing increasing amounts of organic polar solvents. Ailanthone recoveries higher than 95% occurred for washing solutions containing 40% (v/v) MeOH, 30% (v/v) ACN or 20% (v/v) Me_2CO (Table 2). The effective release of coloured pigments was visually evaluated in separate experiments by loading the *A.altissima* leaves extracts when washing with 50% (v/v) MeOH, 20% (v/v) ACN or 10% (v/v) Me_2CO. Therefore, the solution containing MeOH 40% (v/v) was considered as the optimal eluent for recovering ailanthone and removing coloured interferences.

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

The washing step was intended for cleaning possible polar components not specifically bound to the column. Thus, water containing increasing amounts of organic polar solvents was tested as the washing.solution. A substantial release of ailanthone from the cartridges was observe when these were washed with water containing quantities equal to or greater than 10% (v/v) of ACN, 20% (v/v) of Me_2CO and 30% (v/v) of MeOH (table 3), while below these levels the release was very limited for ACN and Me_2CO and even absent for MeOH. Consequently, it was decided to use a water-MeOH 4+1 (v/v) mixture as the washing solution, while pure methanol was considered as the ideal eluent for the quantitative recovery of ailanthone from the cartridge in the final step of the protocol.

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

3.4 Combined solid phase extraction of real samples

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

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

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

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

4. CONCLUSIONS

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

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TABLES

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

	DVB	EDMA	GDMA	PEGDMA	PETRA	TRIM
MA	**0.32**	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	**0.36**	0.12	0.11	0.05	0.07	**0.39**

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

water + solvent, v/v	MeOH	ACN	Me_2CO
100 + 0	-	11	8
90 + 10	41	53	**70**
80 + 20	76	**89**	**96**
70 + 30	92	**99**	**100**
60 + 40	98	**100**	**100**
50 + 50	**100**	**100**	**100**
40 + 60	**100**	**100**	**100**

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

water + solvent, v/v	MeOH	ACN	Me_2CO
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

FIGURE CAPTIONS

Figure 1: Preconcentration of ailanthone in the range 5 – 100 μg onto the MISPE cartridge. Data are expressed as the mean of three separate samplings ±1 standard deviation. Regression equation: μg found = 0.934±0.015 μg loaded - 0.186±0.386 (R^2 = 0.998, SEE = 0.218)

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

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