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Tailoring molecularly imprinted polymer beads for alternariol recognition and analysis by a screening with mycotoxin surrogates

Rahma A.G. Abou-Hany[a], Javier L. Urraca[b,c], Ana B. Descalzo[a], Lidia N. Gómez-Arribas[b], María C. Moreno-Bondi[b,**], Guillermo Orellana[a,*]

[a] Chemical Optosensors and Applied Photochemistry Group (GSOLFA), Department of Organic Chemistry, Faculty of Chemistry, Complutense University of Madrid, 28040 Madrid, Spain
[b] Chemical Optosensors and Applied Photochemistry Group (GSOLFA), Department of Analytical Chemistry, Faculty of Chemistry, Complutense University of Madrid, 28040 Madrid, Spain
[c] CEI Campus Moncloa, UCM-UPM, Avda. Complutense s/n, 28040 Madrid, Spain

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ABSTRACT

Molecularly imprinted porous polymer microspheres have been prepared for selective binding of alternariol (AOH), a phenolic mycotoxin produced by *Alternaria* fungi. In order to lead the synthesis of recognition materials, four original AOH surrogates have been designed, prepared and characterized. They bear different number of phenol groups in various positions and different degree of *O*-methylation on the dibenzo[b,d]pyran-6-one skeleton. A comprehensive library of mixtures of basic, acidic or neutral monomers, with divinylbenzene or ethyleneglycol dimethacrylate as cross-linkers, were polymerized at a small scale in the presence of the four molecular mimics of the toxin molecule. This polymer screening has allowed selection of the optimal composition of the microbeads (*N*-(2-aminoethyl)methacrylamide, EAMA, and ethylene glycol dimethacrylate). The latter are able to bind AOH in water–acetonitrile (80:20, v/v) with an affinity constant of $109 \pm 10\,mM^{-1}$ and a total number of binding sites of $35 \pm 2\,\mu mol\,g^{-1}$, being alternariol monomethylether the only competitor species. Moreover, ^1H NMR titrations have unveiled a 1:2 surrogate-to-EAMA stoichiometry, the exact interaction sites and a binding constant of $1.5 \times 10^4\,M^{-2}$. A molecularly imprinted solid phase extraction (MISPE) method has been optimized for selective isolation of the mycotoxin from aqueous samples upon a discriminating wash with 3 mL of acetonitrile/water (20:80, v/v) followed by determination by HPLC with fluorescence detection. The method has been applied, in combination to ultrasound-assisted extraction, to the analysis of AOH in tomato samples fortified with the mycotoxin at five concentration levels (33–110 $\mu g\,kg^{-1}$), with recoveries in the range of 81–103% (RSD $n = 6$). To the best of our knowledge, this is the first imprinted material capable of molecularly recognizing this widespread food contaminant.

1. Introduction

Alternariol (AOH) and alternariol monomethyl ether (AME) (Fig. 1) are two mycotoxins produced by a number of species of the *Alternaria* fungal genus. These molds can be found in soil, plants, food, feed and indoor air and are among the main microorganisms responsible for pre- and post-harvest damage to agricultural products such as cereal grains, sunflower seeds, oilseed rape, pecans and various fruits such as tomatoes, apples, mandarins, olives, peppers, melons or raspberries [1–3]

The main mycotoxin-producing fungal species is *Alternaria alternate* [1]. These fungi may grow naturally under cold-storage [4] or shelf-life conditions, and are responsible for significant financial losses in the food industry. Moreover, the mold *Alternaria* is a recognized allergy-causing fungus [5]. Information on the natural occurrence of AOH or AME in food is still scarce; however, both mycotoxins have been detected in marketed products at concentrations in the 1–1000 $\mu g\,kg^{-1}$ range [1,4]. High values have also been reported in sunflower seeds and food products such as fruit juices, tomato derivatives, beer and wine [6]. The toxicological impact of *Alternaria* toxins on humans and livestock is still under study. Alternariol and AME are not acutely toxic; however some *in vitro* studies have demonstrated that both compounds are

* Corresponding author. Tel.: +34 913 944 220; fax: +34 913 944 103.
** Corresponding author. Tel.: +34 913 945 147.
E-mail addresses: mcmbondi@quim.ucm.es (M.C. Moreno-Bondi), orellana@quim.ucm.es (G. Orellana).

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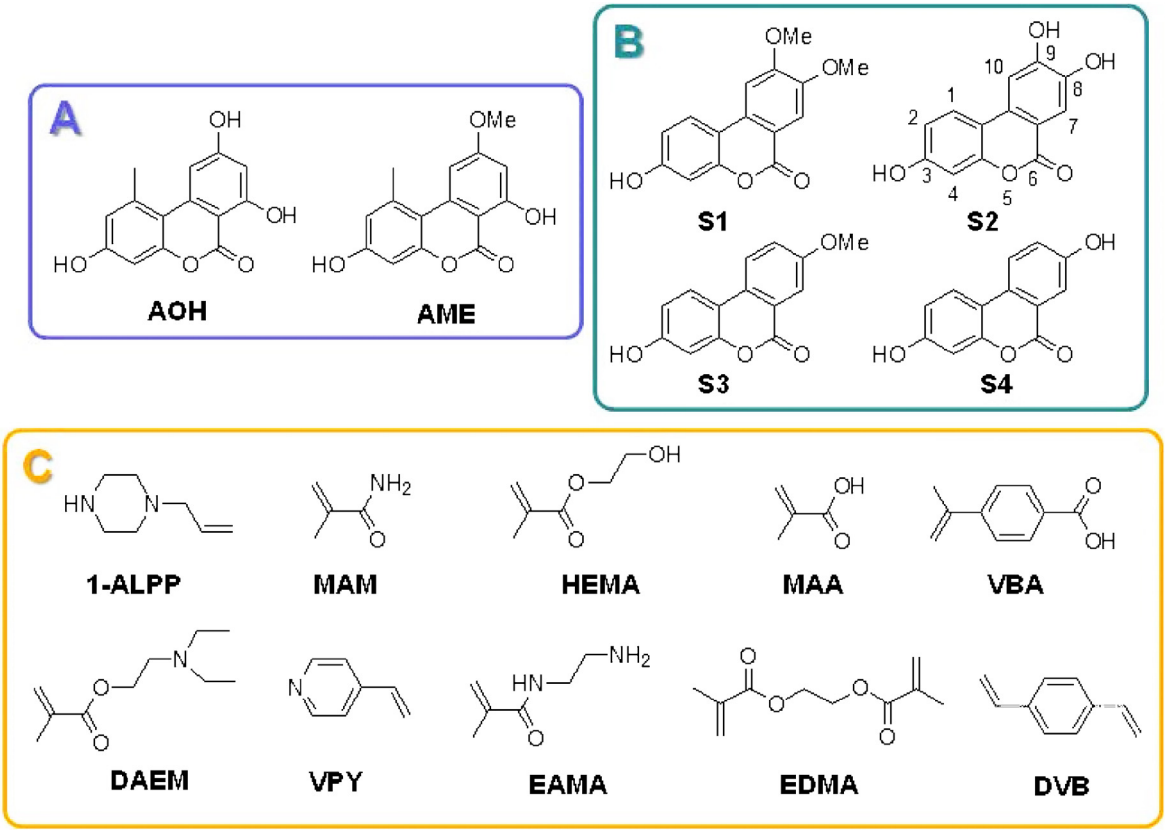

Fig. 1. Chemical structures of (A) alternariol (AOH) and alternariol monomethyl ether (AME) toxins, (B) **S1**–**S4** surrogates and (C) functional monomers and cross-linkers employed for the synthesis of MIPs.

mutagenic and clastogenic [6,7], with AOH showing higher genotoxicity that AME [4]. At present, no formal regulations or limits for any of the *Alternaria* toxins have been set in any country. However, sensitive and selective analytical methods are required to investigate their toxicity and detect their presence in different food matrices, preventing mycotoxin contamination of derived commercial products.

Methods reported in the literature for AOH analysis include gas chromatography, thin layer chromatography and high performance liquid chromatography with UV, fluorescence or mass spectrometry detection [4,8–12]. A couple of immunochemical methods (ELISA-type) have been set for determination of this mycotoxin in apple and tomato [13], corn and feed [14]. Moreover, determination of AOH in complex food matrices usually requires clean-up and pre-concentration steps prior to analysis to improve the method sensitivity and selectivity. Solid phase extraction (SPE), with C_{18}, aminopropyl or Oasis® HLB sorbents [4,15,16], is usually the preferred method for extraction of these toxins from food samples.

In recent years, the use of molecularly imprinted polymers as stationary phases for SPE (also called "molecularly imprinted solid phase extraction" or MISPE) has proven to be a convenient alternative to traditional SPE cartridges, including immunoaffinity solid phases, for selective extraction of mycotoxins in food analysis [17–20]. Advantages of these materials compared to natural receptors comprise their ease of preparation, physicochemical stability, storage convenience, competitive cost, feasibility of manufacturing receptors against toxic substances, and the possibility of performing molecular recognition in organic media [21–24].

This work describes the development of the first MIP selective to AOH. The polymers have been prepared using the "*template mimic*" approach [25], especially useful if the target is toxic, dangerous, expensive or difficult to synthesize [26]. AOH is a toxic expensive chemical, the total synthesis of which is a time-consuming procedure that involves seven synthetic steps [27]. The use of surrogate molecules instead of the analyte itself has an additional advantage: gradual leaching of the remaining template traces will not contaminate the sample, preventing false positives because the surrogate has different physicochemical properties than those of the analyte (chromatographic retention time, optical absorption/emission spectra, or different molecular weight for mass spectral analysis). In the present work, we have employed easy-to-obtain molecular surrogates of AOH and AME (**S1**–**S4**, Fig. 1) [28] in the MIP preparation. A combinatorial screening approach has allowed us selection of the optimal template mimic, functional monomer and cross-linker formulation for the polymer synthesis. Selective rebinding of AOH to the MIP library elements *vs* the non-imprinted (NIP) controls has been evaluated by HPLC, and the best composition was singled out for preparation of microspherical particles using porous silica beads as sacrificial scaffolds in the polymerization [29]. Then, a MISPE method has been optimized for selective extraction of AOH from aqueous solutions of standards and its application to the analysis of the mycotoxin in fortified tomato extracts by HPLC with FLD detection has been demonstrated.

2. Materials and methods

2.1. Reagents and chemicals

Acetonitrile (AcN), methanol (MeOH, HPLC grade) and chlorobenzene (>99.8%) were from Sigma–Aldrich. Extra dry dimethyl sulfoxide (DMSO) was from Acros. Water was purified to type I with a Millipore Direct-Q system. Solvents were