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Summary

Continuous exposition to low doses of myco- and phycotoxins poses severe risks to human health. Contemporary analytical methods have the sensitivity required for contamination detection and quantification, but direct application of these methods on real samples can be rarely performed because of matrix complexity. Thus, selective analytical methods, relying on intelligent functional materials are needed. Recent years have seen the increasing use of molecular imprinted polymers in contaminant analysis because these materials seem to be particularly suitable for applications where analyte selectivity is essential. In this review, several applications of molecular imprinted polymers in myco- and phycotoxin contamination analysis will be discussed

Introduction

Food and feed contamination due to natural toxicants can represent a significant source of foodborne illness and it poses severe risks to human health. In fact, besides the well-known food contamination due to the presence of living bacterial cells (e.g. enterotoxins from certain strains of *Escherichia coli* or *Staphylococcus aureus*), several natural contaminants represented by low mass molecules of a non-proteic nature are extremely potent acute toxins (e.g. T2 toxin) or are very strong carcinogens (e.g. aflatoxins) which are officially recognized by the World Health Organization (WHO) as biocontaminants representing a significant source of food-borne illnesses[1].

As in the case of antibiotics, these harmful organic compounds are produced as secondary metabolites and they play a role in the passive defence mechanisms of the producing organism from attacks from other organisms. On the basis of their origin, these biocontaminants can be classified into two main categories:

mycotoxins: this term is usually reserved for the toxic products formed by fungal species. Ergotism was one of the earliest recognised diseases caused by natural toxicants, well known in Europe by the end of the first millennium as “St Anthony's fire”. It was caused by rye crops contaminated with ergot alkaloids produced by the mould *Claviceps purpurea*[2]. Nowadays, mycotoxins which are significant in terms of occurrence in food and feed include aflatoxins, moniliformin, ochratoxin A, deoxynivalenol and related tricothecenes**,** patulin, fumonisins and zearalenone (see figure 1);

phycotoxins: this term is usually reserved for the toxic products formed by monocellular algae in marine environments and bioaccumulated in fish and shellfish. Phycotoxins of significant impact on human health include brevetoxins, cylindrospermopsin, domoic acids, microcystins, okadaic acids and saxitoxins (see figure 2).

While public awareness about synthetic chemicals in food is high, and consumers continue to express concern about the health risks linked to the deliberate addition of chemicals to food, the perception of the health risks posed by food contamination due to natural toxicants is less marked. However, although effects are often difficult to link with a

particular food, it is now largely accepted in academic circles and public health bodies that food contamination from natural toxicants – chiefly mycotoxins and, less frequently, phycotoxins – is a severe public health problem that can deeply affect health not only after a single massive exposure but, more often, after continuous exposure to low doses, and that such exposure can be related to several chronic diseases, including some types of cancer and serious hormonal dysfunctions[3]. Thus, good analytical protocols based on efficient analytical processes – sensitive, selective, fast, inexpensive and suitable for sample mass screenings – are required by legislation, health authorities and companies operating in the food market.

At present, commercially available rapid assays based on the use of immunoanalytical techniques – or a lesser extent biosensing devices – are widely diffused, as these analytical techniques assure the feasibility of fast sample mass screenings in a more affordable fashion compared to the older thin layer chromatographic methods[4]. However, a sample which is positive to toxicant contamination should be validated by using more sophisticated analytical methods. These methods are usually based on instrumental separative techniques coupled with mass spectrometry detectors of varying complexity. They have the sensitivity required for contamination detection and quantification, but direct application of these techniques on food and feed samples can be rarely performed. In fact, contaminants are usually present in food at low concentration (ng-μg/kg) levels, dispersed in highly complex (thousand of different components) and morphologically structured matrices, with an elevated degree of point-to-point and sample-to-sample variability. Thus, such a type of matrix introduces severe disturbances in the analytical separation step. Moreover, very "dirty" samples show the noxious property to influence strongly the background ion current in a MS detector, reducing its sensitivity[5]. Of consequence, quantitative analysis can be performed only after extensive clean-up and preconcentration steps[6].

Current sample pre-treatment methods, mostly based on the solid phase extraction technique, are very fast and economical but not selective, while methods based on immunoaffinity extraction are very selective but expensive and usually not suitable for harsh environments and columns recycling[7]. Thus, economical, rapid and selective clean-up methods based on "intelligent" materials are needed. Solid phase extraction and clean-up methods based on molecularly imprinted polymers (**m**olecularly **i**mprinted **s**olid **p**hase **e**xtraction, MISPE) seem to represent natural candidates to circumvent the drawbacks typical of more traditional solid phase extraction techniques[8]. Recent years have seen a significant increase of the MISPE technique in the food contaminant analysis[9]. In fact, this technique seems to be particularly suitable for extractive applications where analyte selectivity in the presence of very complex samples represents the main problem. In this review, after an introductory overview of the technique, the application of MISPE in the analysis of food contamination by myco- and phycotoxins will be discussed.

Imprinted materials for analytical applications

Molecular imprinted polymers are synthetic materials provided with artificial binding sites able to selectively recognize a target molecule[10]. In brief, these materials are obtained by polymerization around a template molecule of functional and cross-linking monomers chosen taking into the account their ability to interact with the functional groups of the template through non-covalent interactions (non-covalent imprinting), reversible covalent bonds (covalent imprinting) or mixed combinations of the two methods (semi-covalent imprinting). Once polymerisation has taken place, a highly cross-linked three-dimensional network polymer is formed and binding sites with shape, size and functionalities complementary to the template are established in the bulk of the polymer. These artificial binding sites have the same features as the antibody binding sites, showing binding reversibility, enhanced selectivity, high affinity constant and a significant polyclonality (non-covalent approach) or monoclonality (covalent approach). Notwithstanding, as is possible to observe from table 1, imprinted polymers show large differences compared to antibodies. In fact they are

macroscopic objects, stiff, and insoluble in any solvent, whereas their biological counterparts are nanoscopic objects, flexible, and soluble in water.

The most common method currently used to obtain molecular imprinted materials suitable for analytical applications is radical bulk polymerisation. It consists in the synthesis of a monolithic polymer that has to be crushed and sieved to obtain particles of the desired size distribution. This method, by far the most popular, presents several attractive properties, especially to newcomers. It is fast and simple in its practical execution, it does not require particular skills, it is widely reported in literature for many different templates and it does not require sophisticated instrumentation. However, the procedure of grinding and sieving is cumbersome, and it causes a significant loss of polymer as a very fine sub-micrometric dust. Moreover, the bulk polymerisation cannot be scaled-up because of dangerous sample overheating due to the exothermic character of the polymerization process.

Several alternative polymerisation strategies to prepare analytical-grade imprinted materials have been proposed in the literature during recent years with the purpose of overcoming the practical problems of the bulk polymerisation[8]. Anyway, each of these alternative methods presents one or more serious drawbacks. Thus, for the moment, their application to prepare imprinted polymers for analytical applications is limited to a few examples, and it is not clear if in the near future some of these techniques will be able to overcome their limitations and will come to represent a valid and widely accepted substitute for bulk polymerization.

Molecular imprinted solid phase extraction

In the last few years, a growing number of papers have been dedicated to the clean-up and preconcentration of analytes of clinical, pharmaceutical or food chemistry interest from several types of matrices. In fact, considering the number of papers published worldwide on peer-reviewed journals, molecular imprinted solid phase extraction (MISPE) is one of the fastest growing applications, with more than 260 papers published since 1994[11].

The MISPE technique is very similar to the traditional solid phase extraction made on non-specific stationary phases. A small amount of imprinted polymer (typically 25–500 mg) is packed in a open column (for off-line applications), in a short HPLC column (for on-line applications) or, less frequently, in a multiwell extraction plate for high throughput analysis. Then, the usual steps of column conditioning, sample loading, column washing and analyte elution are carried out. Usually, the extraction protocol has been previously tested on artificial samples to consider the feasibility of the method, less frequently the same optimised protocol has been validated on real samples against a more commonly used method or published in literature, considering issues such as robustness, accuracy, precision, limits of quantification and determination[12].

Off-line MISPE

Two different approaches can be used to develop the extraction protocol. The extraction column can operate in “normal phase” mode or “reversed-phase” mode. In the first approach the analyte is selectively retained by the extraction column by non-covalent interactions between the analyte molecules and the imprinted binding sites, whereas interfering molecules are not retained by these sites. Then, elution of the analyte is obtained by increasing the eluotropic strength of the mobile phase. In the reversed-phase mode, the analyte and any other interfering substances are retained by the hydrophobic polymeric matrix that acts as a reversed-phase material without any apparent specificity towards the target analyte. The elution of the interfering substances is obtained by increasing the hydrophobicity of the mobile phase, while the target analyte is not eluted because of its ability to bind the imprinted binding sites. Its recovery is obtained by eluting the column with a mobile phase able to interfere with these selective non-covalent interactions, usually methanol containing significant amounts of acetic or trifluoroacetic acid. It should be noted that, even if it has been reported that

the two different approaches show no great differences in terms of significant analytical parameters such as accuracy, precision, extraction efficiency and interferences from the matrix, today, the reversed-phase mode is the preferred by the most of the operators[13].

On-line MISPE

This approach combines the extraction efficiency of reversed-phase SPE with the selectivity of the MISPE. In this format a small column, packed with the imprinted polymer is placed in the loop of the injector or immediately before the reversed-phase analytical column. The imprinted column is loaded with the sample and the interfering substances are washed out while the analytical column is maintained off-line. Then, the analyte is eluted by the mobile phase out of the MISPE column and separated on-line in the analytical column. In an alternative format, a reversed-phase pre-column can be placed before a MISPE column to preconcentrate the analyte and the interfering substances of comparable hydrophobicity. Then, these substances are co-eluted and separated on the MISPE column. It should be noted that this approach is fairly appropriate for automated determinations when used within an appropriate instrumental set-up[14]. At the same time this also represents the main drawback, as an appropriate instrumental set-up needs additional pumps and multiway valves for the automation of the whole system, increasing the complexity and costs of the analytical process.

On-line MISPE with pulsed elution

This approach is based on the use of a small displacing solvent plug to elute the analyte selectively retained on an imprinted polymer packed into a small HPLC column directly connected to the detection system. The choice of a suitable displacing solvent depends on the binding mechanism of the imprinted polymer. When the analyte is retained in the imprinted binding sites by interactions based exclusively on hydrogen bonds, a single pulse of a polar solvent (single pulse elution mode) is sufficient to elute it quantitatively[15]. On the other hand, if the analyte is more strongly retained or interfering substances are retained by the imprinted microcolumn, pulses of polar solvents (differential pulsed elution mode) which contain variable amounts of organic acids (usually acetic or trichloroacetic acid) are needed. The use of sequential pulses of different solvents of increasing eluting power constitutes an improvement of this technique, because it is possible to set up extraction protocols in which one or more washing steps can be performed to efficiently remove any remaining interfering compound before the final analyte elution[16].

MIP-based solid phase microextraction

This approach is based on the partitioning of the analyte between the sample and an imprinted stationary phase, which is coated as a thick layer onto the surface of a fused silica fibre. After fibre equilibration, the analyte is desorbed in suitable solvent for further analysis by chromatography[17]. This format shows the advantage of using much less imprinted polymer than a common MISPE format, and it facilitates the miniaturisation of the analytical system, but its main drawback consists in the fibre coating reproducibility, due to difficulties in controlling the imprinted film thickness. Alternatively, in the so-called “in-tube SPME”, the extraction can be performed by using a capillary tube packed with imprinted beads and coupled on-line to the chromatographic system. The sample is repeatedly drawn and ejected through the capillary for analytes extraction, being directly desorbed by the mobile phase[18]. In a further development of the in-tube SPME technique, the capillary tube is no more filled with preformed imprinted beads, but an imprinted porous monolith is synthesised in situ and used as it is[19] or after dissolution of the silica support[20], avoiding in this manner the need for capillary frits and the cumbersome and difficult step of capillary packing.

Template bleeding: a drawback in the MISPE approach

The main critical point associated with the development of a MISPE protocol is related to the residual template not

being completely removed from the polymeric matrix and slowly leaking during loading, washing and elution operations. Such a template loss (polymer "bleeding") is usually detected at trace levels during the elution step, and it represents a significant source of interferences and systematic errors in trace analysis, as elegantly demonstrated by Martin *et al.* by using a ^{14}C-labelled template for the MISPE of propranolol[21]. Moreover, the concern for the possible contamination of the analytical samples by the residual template released during the analyte elution is one of the main obstacles to a wider diffusion of the MISPE method in current sample treatment methods. Several methods have been proposed to overcome this drawback by efficiently removing the residual template, including thermal annealing of the imprinted polymer[22] and severe washing conditions[23], but, despite all efforts, it seems that to remove all the template molecules from the imprinted polymer will be extremely difficult if not impossible using the current technology.

Thus, the most successful strategy has been revealed to be the use of a mimic of the analyte as a template molecule. The so-called "template mimic" technique was introduced for the first time by Andersson ten years ago[24]. It consists of the use of a structural analogue of the molecule of analytical interest as a template. The choice of this putative template requires a certain degree of creativity from the chemist (and a certain cleverness in organic chemistry), as it should be made in such a way as to obtain imprinted binding sites provided with good selectivity towards the analyte molecules. At the same time, this structural analogue should be different from the analyte in such a way that the analytical separation performed after the extraction step discriminates clearly between the analyte and the residual template molecules released by the imprinted material. Differences in molecular structure between the analyte and the putative template should be minimal and localised far from relevant structural motifs and substituents directly involved in non-covalent interactions with the binding sites. Thus, any modification of the target involving structures critical for molecular recognition should be discarded.

Several different approaches have been described to conceive an efficient template mimic. First of all a mimic can be directly derived from the target molecule by addition / subtraction of one or more carbon atoms to the molecule skeleton, especially if an aliphatic chain is present. For example, Andersson *et al.*[24] describes the MISPE from human serum samples of sameridine – a molecule characterised by a N-ethyl-N-methylamido function – using an N,N-dimethylamido analogue instead of the analyte, while the same author[25,26] for the extraction of bupivacaine from serum used the analogue pentycaine provided with a longer aliphatic chain. Addition / subtraction of one or more carbon atoms seems to be particularly convenient when the target consists of a class of molecules, typically a major analyte and its metabolites, with minimal differences in the molecular structures. For example, Spanish authors[27] describe the use of propazine (2-chloro-4,6-diiisopropylamino-triazine) as a mimic template for the preparation of an imprinted polymer with selectivity towards several 2-chloro-4,6-dialkylamino-s-triazines with herbicidal properties, like atrazine (2-chloro-4-ethyl-6-isopropylamino-s-triazine).

The same approach is valid not only for carbon atoms in aliphatic chains, but also for target analytes provided with substituents not directly involved in non-covalent interactions with the imprinted binding sites. For example, Theodoridis *et al.*[28] used the alkaloid hyoscamine as a mimic template for the preparation of an imprinted polymer for the selective extraction of the related alkaloid scolpolamine in biological samples. In this case, the difference between the mimic template and the target analyte is given by the absence of an epoxide on the tropane ring in the hyoscamine, which is present in the scopolamine molecule. Alternatively, as the selection of a suitable mimic template is mainly driven by the similarity to the analyte, analogs with isosteric substituents not directly involved in the non-covalent interaction with the imprinted binding sites can offer an interesting possibility to prepare putative templates. This is the case for the presence of halogens as substituents, where one halogen atom can be exchanged with another as is the case for MISPE for the trace-level analysis of clenbuterol and several related β2-agonists in biological samples[29], where the

imprinted polymer was prepared by using the analogue bromclenbuterol, where one of the two aromatic chlorine atoms is substituted by a bromine.

The choice of a mimic template is usually made through empirical considerations, but in some cases the selection has been made using computational methods, taking into account not only shape similarity, but also electronic and hydrophobic factors (see figure 3 for some examples of myco- and phycotoxin mimics). This is the case for the preparation of an imprinted polymer for the recognition of the mycotoxin ochratoxin A (**5**)[30]. A good mimic template should preserve the general structure of the target analyte, including the chirality of the amino acidic sub-structure and the planarity of the benzopiranic sub-structure. At the same time, it was necessary to eliminate the α-unsaturated lactone moiety, to which the carcinogenicity of many known mycotoxins is related. Moreover, to assure an efficient imprinting effect the several distinct points of potential interaction with monomers should be maintained: the α-carboxyl of L-phenylalanine, the amido bridge, and the phenolic hydroxyl. A preliminary computational study performed on the molecular structures of ochratoxin A and of the chosen mimic (N-(4-chloro-1-hydroxy-2-naphthoylamido)-(L)-phenylalanine, **14**) showed almost complete overlapping of the two molecules, with a high degree of similarity not only as structures, but also as solvent accessible surfaces, electrostatic potential surfaces and lypophilic / hydrophilic surfaces. In this case, it was seen that the structure of the mimic template fully controls the molecular recognition properties towards related molecules. In fact, a polymer prepared with the same mimic, but with completely different functional monomers showed the same recognition properties towards ochratoxin A[31], whereas a polymer prepared with ochratoxin A as a template recognised the mimic well[32].

One of the main drawbacks of the mimic template technique is related to the difficulties of practically attaining some optimal templates. In fact, as they may be difficult to synthesise, expensive, or simply, not available as commercial products, it could be necessary to use commercially available substances as mimic templates that are less strictly related to the target analyte, paying the price of a more limited molecular recognition effect. As a consequence, in many cases, structural differences between the analyte and the mimic template are significant, and similarity between molecules remains confined to the overall molecular shape and the preservation of substituents able to form non-covalent interactions with the binding sites. For example, Urraca *et al.*[12,33] prepared an imprinted polymer for the isolation of the mycotoxin zearalenone (**7**) and its main metabolites from corn extracts using a mimic template obtained from the esterification of resorcilic acid with cyclododecanol (**15**). In this case, the choice of the mimic template was due not only to the necessity to avoid column bleeding during the MISPE process, but also to the toxicity of zearalenone, which made it unsuitable as a template molecule.

The strategy to use mimic template poorly related to the target analyte is brought to its extreme consequence in the so-called "fragmental imprinting"[34], "epitope imprinting"[35] or "substructure imprinting"[36] approaches, where the mimic template is represented by a molecule largely different from the target analyte as a whole, but similar to one of the sub-structures in which the target molecule could be divided. An interesting example of this approach is given by Kubo *et al.* for the preparation of an imprinted polymer for the recognition of phycotoxins microcystins[37]. In this case, these toxins are very expensive and not commercially available in the quantities required for successful imprinting. Moreover, their molecular structures are too complex to be easily modified. The authors solved the problem by using a mimic of the microcystins side arm as a template, thus obtaining a polymer able to selectively recognize the main toxins related to microcystin LR (**11**).

A recent alternative to mimicking the analyte molecule with fewer or more similar analogues is represented by the use of analyte molecules labelled with stable isotopes as templates in the so-called "isotopic imprinting". As these molecules have the same shape and functionalities of the analyte molecule but a different mass, they can be easily

discriminated by mass spectrometry. In this manner, although bleeding occurs, analyte can be detected and quantified without any interference by the template. Interesting examples are reported for the on-line extraction and detection of bisphenol A in serum and water samples, where perdeuterated[38] and ^{13}C-substituted[39] templates were successfully used. Anyway, it should be remarked that a big limitation to this approach is represented by the commercial availability of isotope-labelled analogues – that are rare and expensive – and the mandatory use of mass spectrometry detectors to discriminate well between analyte and residual template.

Imprinted materials for myco- and phycotoxin analysis

As briefly seen in the introduction, natural toxins derived from fungi and algae are probably the most important food contaminants in terms of toxicity and width of diffusion. Nevertheless, people working in the molecular imprinting field have dedicated their attention only to these analytes in the recent years. As a consequence, compared to the very large number of research papers dedicated to the design, characterization and practical use of imprinted polymers for analytes such as drugs and pesticides, a relatively small number of papers dedicated to natural toxins have been published up to the present day.

There are some reasons for this apparent lack of interest towards molecular imprinting of natural toxins. First of all, immunoextraction represents an extremely valid competitor compared to any clean-up technique based on molecular imprinting[40]; and, in fact, immunoextraction is applied widely in natural toxin analysis to perform extracts clean-up[41]. These immunoaffinity-based materials have sufficient capacity to clean-up heavily contaminated samples, and they usually efficiently remove compounds that could interfere in the determination of natural toxins because the antibodies specifically recognize the toxin of interest. As a consequence, immunoextraction is a consolidated technique in natural toxin analysis from many years, and extraction cartridges are commercially available for many of these substances. Thus, even if imprinted materials are potentially competitive with immunoaffinity-based materials, the acceptance of this technology remains low. Generally speaking, the acceptance of new technology meets resistance from users of old-technology (if my method works well, why change?), and this fact has a practical consequence on the development of imprinted materials competitive with immunoextraction cartridges. Probably, it is not just chance that no imprinted cartridges are commercially available for the extraction of natural toxins, whilst they are for several drugs and pesticides.

Beside the concurrence of the immunoextraction technique, it is a widespread common opinion that natural toxins are very difficult templates compared to other food contaminants[9]. It should be noted that practical difficulties in preparing imprinted polymers for natural toxins do not arise from lack of functional groups suitable to set up non-covalent interactions during the imprinting process. In fact, all the most significant myco- and phycotoxins show many polar groups suitable for hydrogen bond or ion-pair interactions. Again, solubility in organic solvents commonly used in molecular imprinting is not a problem, and usually there is full chemical compatibility with the organic reagents – functional monomers, cross-linkers, radical initiators – used during the polymerization process.

Contrarily, difficulties can arise from the elevated toxicity of this class of food contaminants. In fact, it could be quite dangerous to directly manipulate the amounts of toxin necessary to synthesize a sufficient quantity of imprinted polymer to set-up an extraction protocol. In fact, when working on these materials great care should be taken as such toxins are highly dangerous substances, and that contamination from minute amounts could cause serious health problems, including long-term effects. Another problem is related to the market availability of these compounds. In fact, while there are a lot of companies selling analytical standards of myco- and phycotoxins, it is quite difficult to purchase 100 mg-level amounts of the same toxins at an affordable price, which are pure and in a crystalline state. In the

experience of the authors of this review, besides all other possible considerations on the difficulty of obtaining truly functional imprinted polymers, several interesting metabolites of mycotoxins (see aflatoxin M1 and zearalenols as significant examples) and most phycotoxins of interest cannot be used as templates to prepare imprinted polymers simply because of their inaccessible prices, provided that no alternative sources become be available.

Nevertheless, it has been shown that most of the pitfalls described so far can be avoided through careful design of the imprinting process and, when necessary and feasible, the use of the mimic template approach. Thus, molecular imprinted polymers have been successfully prepared for several natural toxins, as reported in the next sections.

Ochratoxin A

Ochratoxin A (OTA, **5**) was the first mycotoxin for which a successful molecular imprinting has been reported in literature. In fact, in 2001, the same synthetic approach – with minimal differences – was independently reported by our group[30] and Maier *et al.*[31]. As previously described, a good mimic template was rationally designed to preserve the general structure of the target analyte, including the chirality of the amino acidic sub-structure and the planarity of the benzopiranic sub-structure. At the same time, the α-unsaturated lactone moiety characterizing many carcinogenic mycotoxins was eliminated, while the distinct points of potential interaction with functional monomers were maintained: the α-carboxyl of L-phenylalanine, the amido bridge, and the phenolic hydroxyl. The resulting mimic template, N-(4-chloro-1-hydroxy-2-naphthoylamido)-(L)-phenylalanine (**14**), showed almost complete overlapping of the two molecules, with a high degree of similarity not only as structures, but also as solvent accessible surfaces, electrostatic potential surfaces and lypophilic / hydrophilic surfaces. Different polymerization mixtures were considered to prepare the polymers. In fact, while we obtained an imprinted polymer using a traditional methacrylic acid / ethylene dimethacrylate mixture, Maier and coworkers used a more exotic mixture with quinuclidine methacrylamide and *tert*-butylmethacrylamide as functional monomer and ethylene dimethacrylate as cross-linker. Anyway, both the approaches resulted valid, with the presence of specific molecular recognition effects due to hydrogen bond interactions and steric factors and good recognition of OTA compared to several analogs in polar (methanol, acetonitrile) and hydrophobic (chloroform) solvents.

The quinuclidine methacrylamide polymer reported by Maier *et al.* was used in a subsequent work[42] to extract OTA from red wine before quantification by HPLC-fluorescence detection. The approach involved a two-stage sample clean-up protocol on coupled reversed-phase (C18-silica) and MISPE cartridges, where the use of the reversed-phase cartridge was crucial for the removal of the interfering acidic matrix compounds (see figure 4). The method provided recovery >90% and R.S.D. < 10%, with detection and quantification limits of 10 and 33 ng l^{-1} in spiked and commercial red wines. However, authors raised doubt on the effectiveness of the MISPE protocol, as similarly favourable performances were observed in control experiments in which the imprinted polymer was replaced by the corresponding non-imprinted material. These findings provided some evidence that under the employed experimental conditions analyte binding was mainly due to non specific interactions with the polymeric matrix of the MISPE cartridge.

An imprinted polymer prepared by using OTA directly as a template molecule is described by Turner *et al.*[32]. In this case good functional monomers were identified by molecular mechanic simulations between the OTA molecule and 20 different functional monomers. From these simulations a equimolar mixture of methacrylic acid and acrylamide was selected as functional monomers, while ethylene dimethacrylate was used as cross-linker. The use of N,N-dimethylformamide as a porogenic solvent – uncommon in molecular imprinting – generated an imprinted polymer with excellent affinity and specificity for OTA in buffered aqueous solutions, while polar organic solvents such as acetonitrile suppressed the molecular recognition effects. Interestingly, as an effect of conformation change of the polymer matrix, low buffer concentration or basic pH caused a loss of the polymer recognition properties, while high

buffer concentration or acidic pH enhanced the specific binding. Unfortunately, no applications in food clean-up were reported by the authors for this polymer.

An on-line MISPE with pulsed elution has been described by Lai *et al.* for the rapid analysis of OTA in wheat extracts[43]. The imprinted polymer was synthesized in chloroform by using N-phenylacrylamide as functional monomer and trimethylolpropane trimethacrylate as cross-linker, crushed and packed in a 0.8x40 mm HPLC micro-column for fluorescence detection. MISPE conditions were optimized for the loading of OTA extracts in methanol/acetic acid (99:1 v/v) and pulsed elution with methanol/triethylamine (99:1 v/v, 20 μl). Nearly quantitative binding could be achieved from one 20-μl injection of sample containing up to 30 ng of OTA, with a detection limit of 0.1 ng/injection (5.0 ng ml^{-1}) and a recovery of OTA from wheat extracts was of 103±3%. However, as in the case of the quinuclidine methacrylamide polymer reported by Maier *et al.*[42], a non-imprinted polymer prepared with N-phenylacrylamide shows binding properties towards OTA and a significant level of selectivity compared to mycotoxins other than OTA, casting doubts on the real effectiveness of the imprinting process[44].

In a completely different approach, on-line MISPE miniaturised devices based on thin layers of OTA-imprinted electropolymerised polypyrrole were described by Lai *et.al.* for detection and quantification of OTA in wine. The imprinted polypyrrole layers were supported on stainless-steel frits, directly fixed onto the porous steel surface (see figure 5) or adsorbed onto a layer of single-wall carbon nanotubes[16]. When relatively large amount of wine samples, up to 3 ml, were loaded onto the extraction devices, recovery up to 40% was obtained, with detection limits of 50 ng l^{-1} and 12 ng l^{-1} for a polypyrrole-imprinted layer supported by steel and single-wall carbon nanotubes respectively. This approach was subsequently used by the same authors to set up a miniaturized surface plasmon resonance device for OTA sensing in wine and wheat extracts with sensitivity down to 50 μg l^{-1} [45].

Zearalenone

A first attempt to obtain imprinted polymers for mycotoxin zearalenone (ZON, **7**) was down to Weiss *et al.*[46]. As the molecular structure of this mycotoxin brings a carbon-carbon double bond potentially interfering with the polymerization process, the authors used the flavonoid quercetin as putative template. Unfortunately, the polymer obtained showed some molecular recognition properties towards ZON, but a limited overall binding capacity, making it unsuitable for the development of a MISPE method.

In a different approach, Urraca *et al.*[33] used the cyclododecanoyl ester of resorcilic acid as a mimic template to prepare imprinted polymers based on allylpiperazine as functional monomer, trimethylolpropan trimethacrylate as cross-linker and acetonitrile as porogenic solvent. As previously described, the choice of cyclododecanoyl resorcilate (**15**) as mimic template was made on the basis of its commercial availability and its resemblance to ZON in terms of size, shape and functionality, aiming to preserve the resorcilic sub-structure directly involved in the hydrogen bond interaction with functional monomers. The resulting imprinted polymer was evaluated by liquid chromatography, and good molecular recognition properties were observed not only towards the target molecule, but also towards its metabolites α- and β-zearalenol, zearalanone, α- and β-zearalanol, while resorcylic acid and several steroidal estrogens were very poorly recognized. This imprinted polymer was successfully applied for the clean-up of ZON and its main metabolite α-zearalenol from cereal and swine feed sample extracts[12]. The analysis of these mycotoxins from the food samples were very efficiently accomplished using pressurized liquid extraction in organic solvent (methanol/acetonitrile 1+1 v/v). Clean-up was performed through the MISPE cartridge and quantitative determination by liquid chromatography with fluorescence detection (see figure 6). The method – validated using a corn reference material for ZON – gave recovery of 85-97% (RSD 2.1-6.7%) and 87-97% (RSD 2.3-5.6%) for α-zearalenol and ZON, respectively. The detection limits for the different matrices tested, based on a signal-to-noise ratio of 3:1, ranged between 1.7 and 2.4 ng g^{-1} for ZON and

from 0.7 to 1.3 ng g^{-1} for α-zearalenol. These values are similar to those reported in the literature based on the application of liquid chromatography with mass spectrometric detection[47] and immunoaffinity extraction[48], and are much lower than the maximum levels allowed by the European Union for ZON in unprocessed cereals[49].

Microcystin

As previously seen for ochratoxin A, an imprinted polymer for cyanobacterial phycotoxin microcystin-LR (MC-LR, **11**) has been developed by Chianella *et al.*[50] by using a computational approach. A virtual library of 18 functional monomers was designed and screened against the target phycotoxin. The monomers giving the highest binding energy were selected and used in a simulated molecular dynamics process to investigate their interaction with the template. To prepare the imprinted polymer, a stoichiometric ratio MC-LR : 2-acrylamido-2-methyl-1-propansulfonic acid : ethyl-3-(2H-imidazol-5-yl)acrylate 1:1:6 was selected as template - functional monomer composition, while ethylene dimethacrylate was used as cross-linker and dimethylsulphoxide as porogenic solvent. A batch-binding competitive assay with MC-horseradish peroxidase conjugate was optimized and used to evaluate the molecular recognition properties of the polymer. The performance of the artificial receptor was compared to the performance of commercial monoclonal and polyclonal antibodies raised against the phycotoxin. It was found that imprinted polymer has affinity and sensitivity comparable to those of polyclonal antibodies (detection limit for microcystin-LR using the MIP-based assay was found to be 0.1 μg l^{-1}). Interestingly, recognition towards other phycotoxin analogues (nodularin, microcystin-YR and –RR) was low for the imprinted polymer, in contrast to the results achieved for antibodies. The MC-LR binding polymer was used by the same authors as a support for MISPE and the sensing element in a piezoelectric sensor[51]. Using a combination of the two devices it was possible to selectively preconcentrate and measure the phycotoxin in tap water with a detection limit of 0.35 μg l^{-1} (see figure 7) and recovery between 89±13% (50 μg l^{-1}) and 65±16% (0.5 μg l^{-1}).

The needs for imprinted polymers able to recognize not only MC-LR, but several other microcystins of similar toxicity, has been solved by Kubo *et al.* through a fragmental imprinting approach[37,52]. In this approach the templates are represented by different molecules mimicking the side arm common to all the existing microcystins, represented by the peculiar amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (ADDA). This side arm was well mimicked by using complex templates such as 3-methoxy-2-methyl-4-phenylbutyric acid methyl ester (MMPB, **16**) and less expensive and commercially available 2-methoxypropylbenzene (2MPB) and octylbenzene. Polymers prepared in the presence of these mimic templates showed good recognition properties towards the main microcystin homologues. Interestingly, recognition and group-selectivity properties were further implemented by using as trapping device for microcystins MISPE which is a mixture of two imprinted polymers prepared with different templates. Unfortunately, at present, no applications for water clean-up have been reported by the authors for this system.

Domoic acid

Lotierzo *et al.* prepared a 2-(diethylamino)ethyl methacrylate-*co*-ethylene dimethacrylate imprinted polymer for the cyanobacterial phycotoxin domoic acid (DA, **10**) by photografting onto a gold chip suitable for a surface plasmon resonance device[53]. To assure the stability of the layer of imprinted polymer and control its thickness, the gold surface was first functionalised with a self-assembled monolayer of 2-mercaptoethylamine and subsequently reacted with a carboxylic photoinitiator. The surface grafting resulted in the formation of thin and homogeneous imprinted film with a thickness of about 40 nm. A competitive assay with DA-horseradish peroxidase conjugate was optimized and used to evaluate the molecular recognition properties of the imprinted layer. The performance of the sensor was compared to the performance of an analogous device prepared with a commercial monoclonal antiserum raised against DA. It was found that both the sensors had comparable selectivity, while sensitivity was better for the antiserum-based one, with a

detection limit for DA of 0.4 μg l^{-1} against a detection limit of 2.0 μg l^{-1} for the polymer-based sensor. However, in contrast to monoclonal antibodies, the regeneration of a polymer-based sensor did not affect its recognition properties and continuous measurement was possible over a period of at least 2 months.

As previously seen for microcystin LR, the fragmental imprinting approach has been used by Kubo *et al.* to develop an imprinted polymer for DA[54]. Several commercial cyclic dicarboxylic compounds related to phthalic acid were tested to mimic molecular sub-structures of DA. The highest selective molecular recognition ability towards DA in the tested polymers was found when *o*-phthalic acid was used as the mimic template. The authors speculated that the recognition properties were due to the acidity of the carboxylic acids in the DA and the similarity of the molecular shape around the carboxylic functions for DA and the templates. DA from blue mussel extracts was effectively separated by using a chromatographic column packed with the imprinted polymer, but no data on selectivity nor preconcentration efficiency were given by the authors.

Moniliformin

Imprinted polymers for the mycotoxin moniliformin (MON, **4**) have been described by Appell *et al.*[55]. A small library of polymers was prepared in dimethylformamide by varying template – functional monomer molar ratio, template structure, functional monomer and cross-linker. It was found that significant differences in MON binding by the polymers were dependent on polymer composition, and these differences were highly dependent on the template used to imprint the polymer. The best binding polymer was obtained by using 3,4-diethoxy-3-cyclobuten-1,2-dione (MON diethylester, **17**) as mimic template, N,N-dimethylaminoethyl methacrylate as functional monomer and trimethylolpropan trimethacrylate as cross-linker in molar ratio 1:8:40. This polymer was evaluated as a sorbent for MISPE of acetonitrile corn extracts. the method was not optimized, but clean chromatograms by liquid chromatography with UV detection at 229 nm and quantitative recoveries with samples containing 0.5 μg l^{-1} of MON were obtained.

Cylindrospermopsin

An imprinted polymer for the cyanobacterial phycotoxin cylindrospermopsin (CYN, **9**) has been described by Kubo *et al.*[56]. As CYN is usually present in its zwitterionic form, it is very difficult to prepare an imprinted material using this highly hydrophilic substance as a template. The authors approached the problem using the fragmental imprinting method, using tributyl-(4-carboxybenzyl)ammonium chloride (TCBA, **18**) as mimic template. The functional monomers were chosen to form ion pair interaction with CYN in a polar porogenic solvent. In this way, the sulphonic function of CYN was mimicked by the carboxyl function of TCBA, forming an ion pair with diethylaminoethyl methacrylate, while the guanidyl function was mimicked by the tributylammonium group of TCBA, forming an ion pair with 4-styrylsulphonic acid. The resulting polymer showed good molecular recognition properties towards CYN. Unfortunately, no analytical applications were reported by the authors for this polymer.

Deoxynivalenol

Weiss *et al.* described an imprinted polymer for mycotoxin deoxynivalenol (DON, **2**)[46]. The synthesis was performed in acetonitrile, by using DON directly as a template, methacrylic acid as functional monomer and ethylene dimethacrylate as cross-linker. Interestingly, when selectivity was measured in acetonitrile, the polymer recognized nivalenol slightly better than the template, while the analogs fusarenon-X, 15-acetyl-DON and 3-acetyl-DON were recognized only marginally. No analytical applications were given.

Conclusions

As shown in this review, molecular imprinting can be successfully used to prepare intelligent materials for detection, clean up and preconcentration of natural toxins in complex samples. From the examples reported, imprinted polymers

are potential competitors with traditional solid phase extraction materials for their selectivity, and with immunoaffinity extraction for their stability and low cost of preparation. The main problem affecting imprinted polymers for analytical purposes, *i.e.* the residual template bleeding, can be successfully addressed through the template mimic approach. However, it should be noted that – up to now – there are several natural toxins of relevant practical interest that show a lack of hypothetical mimicking templates. As a significant example, notwithstanding aflatoxins being probably the most searched mycotoxins in food analysis, and their analytical significance in terms of food safety being of primary relevance, no imprinted polymers have been described, and no mimicking templates have been proposed to approach this issue. On this premise, to make the molecular imprinting a mature technique in natural toxin analysis, it would be opportune for new approaches to the template bleeding problem to be studied and introduced into literature.

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Table 1: a comparison between antibodies and molecular imprinted polymers

	antibodies	***molecular imprinted polymers***
low-mass molecules binding (< 5000 Da)	yes, but necessity of a spacer arm to raise antibodies could change the specificity	yes
high-mass molecules binding (>5000 Da)	yes	yes
binding mechanism	well known	known, but some aspects under debate
binding affinity spectrum	narrow for monoclonal, broad for polyclonal	narrow for covalent imprinting, broad for non-covalent imprinting
mean affinity constant	frequently above 10^9 M^{-1}	rarely exceeds 10^6 M^{-1}
binding site density	low for polyclonal	high
binding kinetics	slow dissociation	slow dissociation
specificity	high, fine tuning for monoclonals feasible	high, fine tuning very difficult
batch-to-batch reproducibility	limited	very high
non-specific binding	negligible	variable
resistance to harsh conditions (pH, temperature, organic solvents, ultrasounds)	limited	high
resistance to microorganisms	no	yes (can be autoclaved)
needs of a solid phase as support	yes, this involve use of coupling reactions	no, the MIP itself is the support
reuse	difficult	yes
cost for single run	medium to high	medium to high (myco- and phycotoxins are expensive when used as template molecules)
commercial availability	large	rare
in-house feasibility	no, well trained people and a dedicated laboratory are necessary	polymers prepared by non-covalent approach are simple to make. Otherwise, good skills in organic chemistry are needed
health risks (excluded the use of common chemicals for chromatography)	not significant	grinding produces sub-micrometric particles, dangerous if inhaled. Some monomers (acrylamide, styrene) are toxic
literature	very large	large and rapidly growing
state of the art	mature	in continuous evolution

Figures

Figure 1: examples of mycotoxins of significant impact on human health and food safety: aflatoxin B1 (**1**), deoxynivalenol (**2**), fumonisin B1 (**3**), moniliformin (**4**), ochratoxin A (**5**), patulin (**6**), zearalenone (**7**)

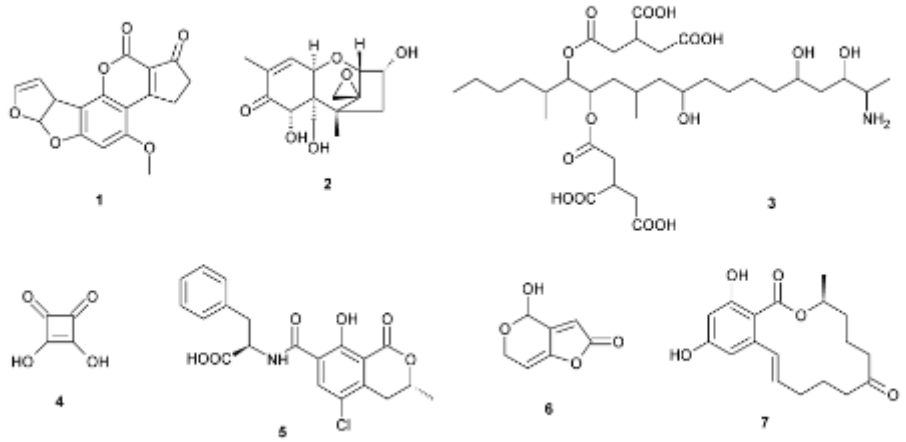

Figure 2: examples of phycotoxins of significant impact on human health and food safety: brevetoxin B2 (**8**), cylindrospermopsin (**9**), domoic acid (**10**), microcystin LR (**11**), okadaic acid (**12**), saxitoxins (**13**)

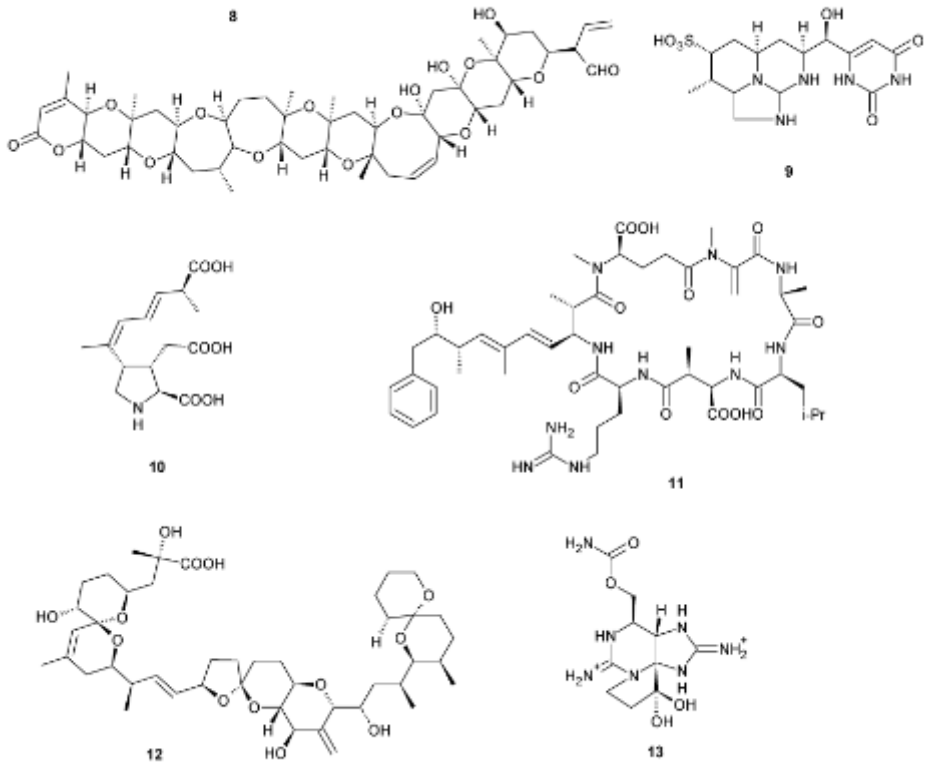

Figure 3: mimic templates for myco- and phycotoxins. N-(4-chloro-1-hydroxy-2-naphthoylamido)-(L)-phenylalanine (**14**) as mimic for ochratoxin A (**5**); cyclododecanoyl resorcilate (**15**) as mimic for zearalenone (**7**), 3-methoxy-2-methyl-4-phenylbutyric acid methyl ester (**16**) as mimic for microcystin LR (**11**), moniliformin diethylester (**17**) as mimic for moniliformin (**4**), tributyl-(4-carboxybenzyl)ammonium (**18**) as mimic for cylindrospermopsin (**9**)

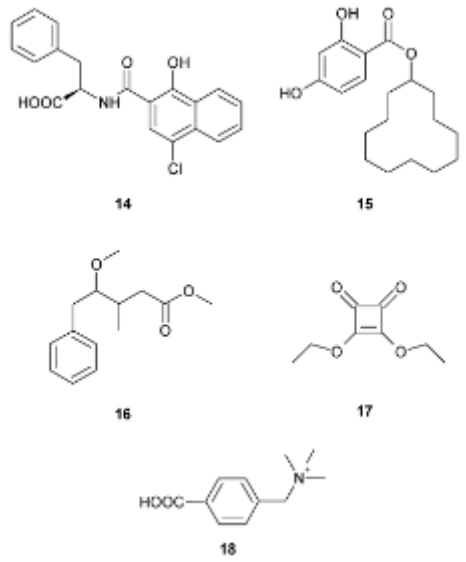

Figure 4: chromatograms of OTA-contaminated red wine (0.22 μg l^{-1}) after different extraction steps. Trace A: red wine sample after clean-up with a MIP cartridge only. Trace B: after C18-based SPE clean-up. C: after combined C18-MIP SPE clean-up (analyte recovery >90%). From ref.41, with permission of Elsevier Science

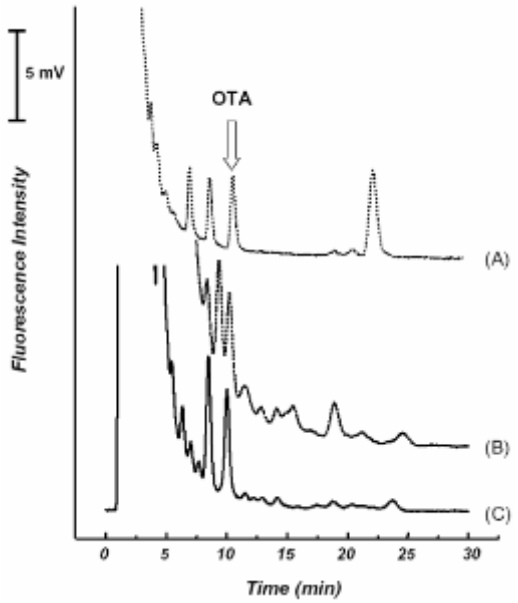

Figure 5: on-line pulsed solid phase extraction of OTA. Images of stainless steel frit before (a) and after (b) deposition of a layer of OTA-imprinted polypyrrole by electropolymerisation. From ref .16, with permission of Springer-Verlag

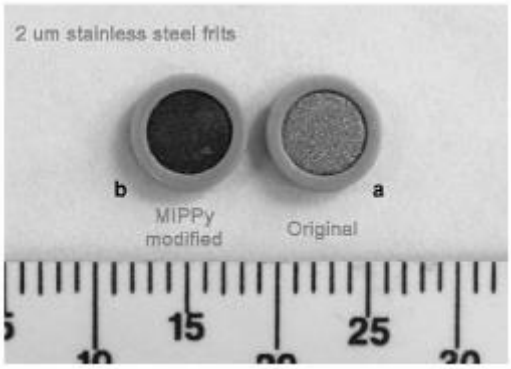

Figure 6: chromatograms of a wheat extract (5 g sample spiked with 100 ng g^{-1} of ZON and α-ZOL) without (A) and with clean-up on the MISPE (B) and not-imprinted (C) cartridge. From ref.11, with permission of Springer-Verlag

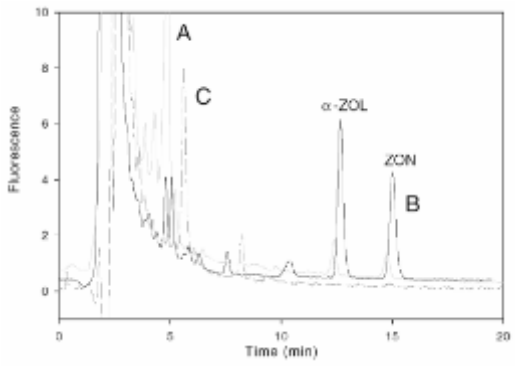

Figure 7: responses of an MC-LR-imprinted sensor to different phycotoxin concentrations in tap water. From ref.50, with permission of Elsevier Science