

Solid phase extraction of food contaminants using molecular imprinted polymers

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Abstract

Food contamination from natural or anthropogenic sources poses severe risks to human health. It is now largely accepted that continuous exposure to low doses of toxic chemicals can be related to several chronic diseases, including some type of cancer and serious hormonal dysfunctions.

Contemporary analytical methods have the sensitivity required for contamination detection and quantification, but direct application of these methods on food samples can be rarely performed. In fact, the matrix introduces severe disturbances, and analysis can be performed only after some clean-up and preconcentration steps. Current sample pre-treatment methods, mostly based on the solid phase extraction technique, are very fast and inexpensive but show a lack of selectivity, while methods based on immunoaffinity extraction are very selective but expensive and not suitable for harsh environments. Thus, inexpensive, rapid and selective clean-up methods, relying on “intelligent” materials are needed. Recent years have seen a significant increase of the “molecularly imprinted solid phase extraction” (MISPE) technique in the food contaminant analysis. In fact, this technique seems to be particularly suitable for extractive applications where analyte selectivity in the presence of very complex and structured matrices represents the main problem. In this review, several applications of MISPE in food contamination analysis will be discussed, with particular emphasis on the extraction of pesticides, drugs residua, mycotoxins and environmental contaminants.

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1. Introduction

Food contamination due to chemicals from natural or anthropogenic sources is a significant source of foodborne illness and it poses severe risks to human health, although effects are often difficult to link with a particular food. Dangerous substances in food may include natural toxicants such as mycotoxins, phyco-toxins and phytotoxins [1–3], environmental contaminants such as polychlorinated dioxins [4] and polycyclic aromatic hydrocarbons [5], and chemicals such as pesticides and veterinary drugs deliberately used to increase the food supply, whose residues can be present in the processed food and potentially affect human health [6].

It is now largely accepted that chemical contamination of food can affect health not only after a single massive exposure

but, more often, after continuous exposure to low doses of toxic chemicals that can be related to several chronic diseases, including some type of cancer and serious hormonal dysfunctions. Public awareness about chemicals in food is relatively high, and consumers continue to express concern about the risks to health due to the deliberate addition of chemicals to food. 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.

Contemporary analytical methods have the sensitivity required for contamination detection and quantification, but direct application of these methods on food samples can be rarely performed. Usually, contaminants are present in food at low concentration ($\text{ng} - \mu\text{g g}^{-1}$) levels, dispersed in highly complex (thousand of different components) and morphologically structured matrices, with an elevated degree of sample-to-sample variability. Thus, such a type of matrix introduces severe disturbances, and analysis can be performed only after some clean-up and preconcentration steps [7–9].

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Current sample pre-treatment methods, mostly based on the solid phase extraction technique, are very fast and economical but show a lack of selectivity while methods based on immunoaffinity extraction are very selective but expensive and not suitable for harsh environments [10,11]. As economical, rapid and selective clean-up methods (relying on “intelligent” materials) are needed, solid phase extraction and clean-up methods based on molecularly imprinted polymers (molecularly imprinted solid phase extraction, MISPE) seem to represent natural candidates to circumvent the drawbacks typical of more traditional solid phase extraction techniques [12–14]. Recent years have seen a significant increase of the “molecularly imprinted solid phase extraction” (MISPE) technique in the food contaminant analysis. 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, several applications of MISPE in the food contamination analysis will be discussed, with particular emphasis on the extraction of pesticides, drugs residues, mycotoxins and environmental contaminants.

2. MISPE formats

The most common method for preparing molecularly imprinted polymers suitable for MISPE consists in bulk thermal- (or photo-) polymerisation that produces 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. It is fast and simple in its practical execution, it does not require particular skills of the operator, it is widely reported in literature for many different templates and it does not require sophisticated or expensive instrumentation. However, the procedure of grinding and sieving is cumbersome, and it causes a substantial loss of useful polymer. Most of the lost polymer is very fine sub-micrometric powder, which could adhere to the bigger particles and cause excessively high backpressures in a SPE column during the extraction procedure, especially with on-line devices. Moreover, the bulk polymerisation cannot be scaled-up. In fact, the process consists of an exothermal radical reaction that can be controlled only when the amount of polymerisation mixture does not exceed a few hundredths of millilitres. After that, heat dissipation becomes difficult and dangerous overheating of the sample can occur, with significant risks for operators. Thus, polymerization by precipitation [15–18], suspension [19–22], multistep swelling and polymerisation [23–25], imprinting in preformed beads [26–31] and grafting onto preformed beads [32–36] have been proposed as alternative polymerisation strategies in literature over recent years – even if only the first two techniques have been used frequently to prepare beads for applications in the MISPE of food contaminants.

Operatively, the MISPE technique is very similar to the traditional SPE performed on non-specific supports. A small amount of imprinted polymer (typically 50–500 mg) is packed in a cartridge (for off-column applications), in a short HPLC column (for on-line applications) or, less frequently, in a 96-well extraction plate for high throughput analysis. Then, the usual steps of

column conditioning, sample loading, column washing and analyte elution are carried out. In most cases, 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 certified food samples [37,38] or in comparison with a more commonly used method, considering issues such as ruggedness, accuracy, precision and limits of quantification and determination [39].

Two different approaches can be used to develop the extraction protocol. As in the traditional SPE approaches, the extraction column can operate in “normal phase” mode or “reverse 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 strength of the mobile phase. In the reverse phase mode, the analyte and any other interfering substance are retained by the hydrophobic polymeric matrix that acts as a reverse 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 disrupting these selective non-covalent interactions. The two different approaches have been carefully compared for the solid phase extraction of propranolol by Martin et al. [40]. In this work significant analytical parameters such as accuracy, precision, extraction efficiency and interferences from the matrix were considered, and no significant differences between the normal phase and the reverse phase mode were found.

2.1. On-line MISPE

A molecularly imprinted column for liquid chromatography can be used not only to separate substances, but also to selectively extract analytes from complex samples. This technique is called “on-line Molecularly Imprinted Solid Phase Extraction” (on-line MISPE), and it combines the high extraction efficiency of reverse phase SPE for aqueous samples with the high selectivity of the molecularly imprinted polymers. In this format a small column, packed with the imprinted material is placed in the loop of the injector or immediately before the analytical column (typically a C18 reverse phase). The imprinted column is loaded with the sample and the interfering substances are washed out by maintaining the analytical column off-line. Then, the analyte is eluted by the mobile phase out of the MISPE column and separated on-line in the analytical column. For example, this technique has been used by Theodoridis et al. [41] for the rapid extraction of caffeine from beverages and coffee. More frequently, a reverse phase pre-column has been placed before a MISPE column to preconcentrate the analyte and the interfering substances of comparable hydrophobicity. Then, these substances have been co-eluted and separated on a MISPE column. By substituting the reverse phase with a restricted access material pre-column, this approach shows itself to be suitable for

direct analysis of chemical substances in aqueous fluids [42]. By using imprinted polymers capable of recognizing several structurally related compounds, it has been shown that this kind of approach to on-line MISPE comes out largely appropriate for multianalyte determinations [43].

2.2. On-line MISPE with pulsed elution

This method 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) – usually methanol – is sufficient to elute it quantitatively [44]. 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) containing 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 step can be performed to efficiently remove any remaining interfering compound before the final analyte elution. Several examples of this technique have been reported in literature, principally by Lai's group, some of them concerning analytes of interest in food chemistry, such as ochratoxin A in wine samples [45,46].

3. Template bleeding: a critical point of MISPE technology

A problem associated with the development of a MISPE protocol step is related to the residual template not 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 [47–49]. 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.

To remove all the template molecules from the imprinted polymer is extremely difficult [50]. Several methods have been proposed to overcome this drawback, including thermal annealing of the imprinted polymer [49], parallel extraction on blank solutions [51] and severe washing conditions [50], but the most successful has been revealed to be the use of a mimic of the analyte as a template molecule.

The so-called “template mimic” technique consists of the use of a structural analogue of the molecule of analytical interest as a template (see Fig. 1 for examples in MISPE of food contaminants). The choice of this putative template requires a certain degree of creativity from the chemist, 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 MISPE 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 are frequently minimal and localised far from relevant structural motifs and substituents directly involved in non-covalent interactions with the binding sites. Several different approaches can be used to conceive an efficient template mimic. First of all – once any modification of the target involving structures critical for the molecular recognition has been discarded – a mimic can be directly derived from the target molecule by addition/subtraction of one or more carbon atoms to the molecule skeleton. For example, Andersson et al. [47] describe the MISPE from human serum samples of sameridine – a molecule characterised by a *N*-ethyl-*N*-methanido function – using an *N,N*-dimethanido analogue instead of the analyte, while the same author [52,53] for the extraction of bupivacaine from serum used the analogue pentacaine provided with a longer aliphatic chain. Addition/subtraction of one or more carbon atoms is 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 [54,55] describe the use of propazine (2-chloro-4,6-diisopropylamino-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. In addition, the presence of halogens as substituents offers an interesting possibility to prepare putative templates exchanging one halogen atom with another, as is the case for MISPE for the trace-level analysis of clenbuterol and several related β_2 -agonists in bovine muscle and other biological samples [56–59], where the imprinted polymer was prepared by using the analogue bromclenbuterol.

As seen, the selection of a suitable putative template is mainly driven by the similarity to the analyte, so templates with isosteric substituents not directly involved in the non-covalent interaction with the imprinted binding sites should be preferred. The choice of these templates is usually made by the empirical considerations here reported, 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. This is the case for the preparation of an imprinted polymer for the recognition of the mycotoxin ochratoxin A [60]. A good mimic of this analyte should preserve the general structure of the molecule, including the chirality of the amino acidic sub-structure and the planarity of the benzopyranic 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

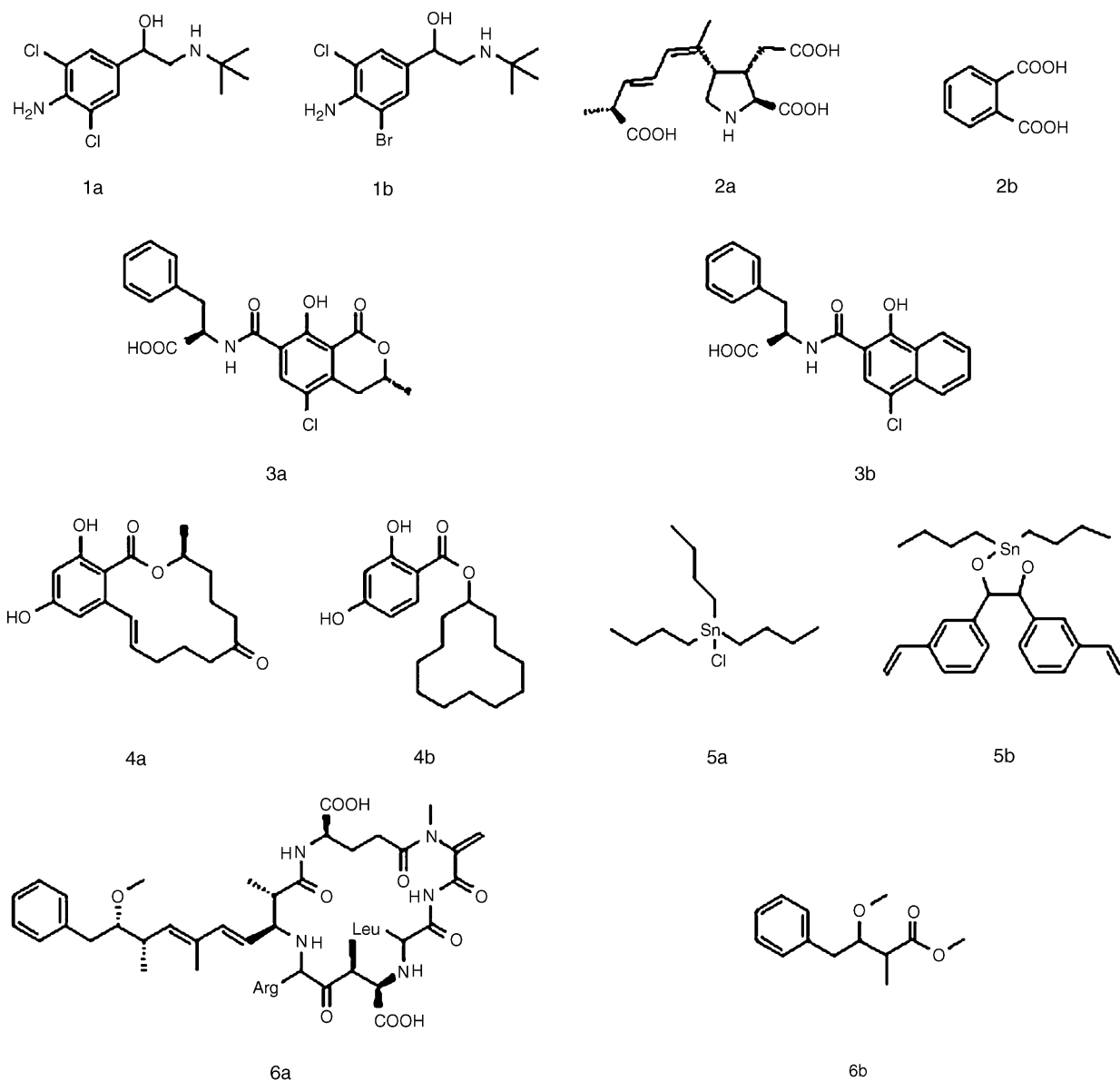


Fig. 1. Examples of molecules used as mimic templates in MISPE of food contaminants. The a-items indicate the analyte molecules, while the b-items indicate the mimic template. clenbuterol (**1a**) and bromclenbuterol (**1b**); domoic acid (**2a**) and *o*-phthalic acid (**2b**); ochratoxin A (**3a**) and *N*-(4-chloro-1-hydroxy-2-naphthoylamido)-(L)-phenylalanine (**3b**); zearalenone (**4a**) and resorcilic acid cyclododecanoyl ester (**4b**); tributylstannane (**5a**) and divinylbenzoin dibutylstannane (covalent imprinting) (**5b**); microcystin LR (**6a**) and 3-methoxy-2-methyl-4-phenylbutyric acid methyl ester (**6b**).

chosen mimic (*N*-(4-chloro-1-hydroxy-2-naphthoylamido)-(L)-phenylalanine) showed quite 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 lipophilic/hydrophilic surfaces. In this case, it was seen that the structure of the mimic 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 [61], whereas a polymer prepared with ochratoxin A as a template recognised the mimic well [62].

In many cases, structural differences between the analyte and the mimic template are significant, and similarity between

molecules remains related 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. [37,63] prepared an imprinted polymer for the isolation of the mycotoxin zearalenone and its main metabolite from corn extracts using a mimic template obtained from the esterification of resorcilic acid with cyclododecanol. 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. Again, Martin-Esteban [64,65] described the use of linuron (*N*-(3,4-dichlorophenyl)-*N'*-methyl-*N'*-methoxyurea) as a mimic template to prepare molecular imprinted polymers for

the MISPE of several related herbicides in vegetables. The herbicide molecules recognized by the imprinted polymer were urea derivatives, differing from linuron for substituents on the aromatic rings. Interestingly, the imprinted polymer was not able to discriminate between ureas containing a methoxy substituent and ureas containing a methyl substituent.

One of the main drawbacks of the mimic template technique is related to the difficulties of practically attaining some optimal templates. In fact, they may be difficult to synthesise, expensive or, simply, not available as commercial products. Thus, 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. Kubo et al. proposed the so-called “fragmental imprinting” approach [66–68], where the mimic template is represented by a significant analyte sub-structure. An interesting example of this approach in the field of food contaminant analysis is reported for the preparation of an imprinted polymer for the recognition of microcystin hepatotoxins [69]. In this case, these toxins are very expensive and not commercially available in the quantities required for a successful imprinting. Moreover, their molecular structures are too complex to be easily modified. The authors solved the problem by using 3-methoxy-2-methyl-4-phenylbutyric acid methyl ester (a mimic of the microcystin side arm) as a template, thus obtaining a polymer able to selectively recognize the main toxins microcystin LR and RR.

4. MISPE application in food analysis

In accordance to several reviews and scientific databases [89–91], in the last 10 years (January 1996–September 2006) more than 1600 papers concerning molecular imprinting have been published worldwide on peer-reviewed journals. Considering the number of papers/year, molecular imprinted solid phase extraction – dedicated reviews excluded – is one of the fastest growing applications, with 223 papers published, of which 128 (57%) from 2004 until today (Fig. 2). Extraction of contaminants from food and beverage samples has been reported in 30 papers. It represents quite a small fraction of papers if compared with the total number (>13%), but it increased faster than other MISPE applications, with 22 papers (73%) published in the last three years (Fig. 3).

4.1. MISPE formats in food analysis

As reported in the initial sections of this review, imprinted polymers for MISPE of food analytes have been prepared using many different techniques. The traditional bulk thermal- or photopolymerization has been used in about 2/3 of the papers, while precipitation polymerization – that is far less used to prepare imprinted polymers for MISPE of environmental or clinical samples – is reported for an unusually high number of cases: polymers for the extraction of thiabendazole, urea- and triazine-based herbicides in vegetables and fruits [15,17,55,64,86] and the endocrine disruptor bisphenol A in shrimp samples [40]. Less used is suspension polymerization, registered for 2 cases only: the preparation of imprinted beads for the extraction of the

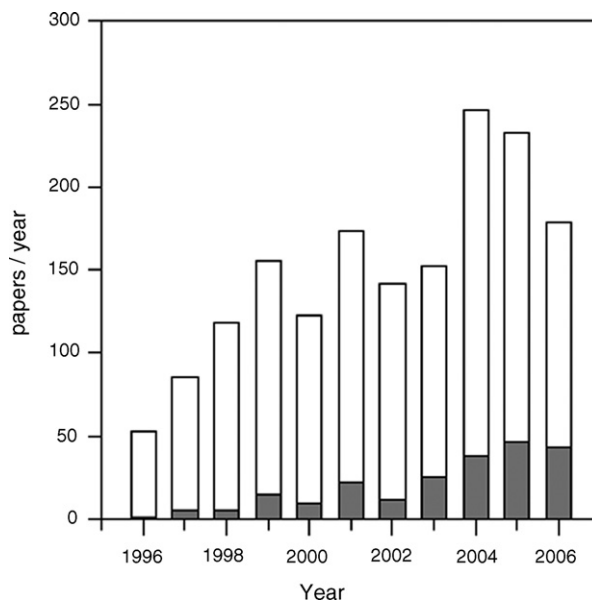


Fig. 2. Original papers on molecular imprinting (open bars) and MISPE application to complex samples (grey bar) published since 1996. The numbers of paper were obtained from [90] and [91] during January 1996–August 2006.

carcinogenic polycyclic aromatic hydrocarbon benzo[*a*]pyrene from coffee samples [78], and the use of imprinted beads for the isolation of the herbicide carbaryl and its main degradation product, 1-naphtol, from apple extracts [83]. Imprinting in preformed silica beads has been used by Tamayo and Martin-Esteban [65] to prepare polymeric beads suitable for the on-line clean-up and HPLC analysis of six urea-based herbicides (fenuron, methoxuron, chlortoluron, isoproturon, metobromuron and linuron) from potato extracts. An interesting example of use of covalent imprinting has been described by Munoz-Olivas et

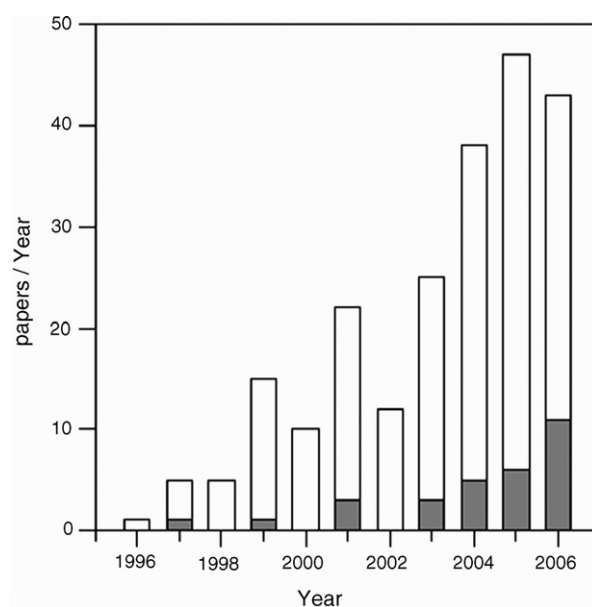


Fig. 3. Original papers on MISPE application to complex samples (open bars) and MISPE of food contaminants (grey bar) published since 1996. The numbers of paper were obtained from [90] and [91] during January 1996–August 2006.

al. [38,79] who prepared a MIP by bulk thermal polymerization with recognition properties towards the environmental contaminant tributylstannane, by using the covalent adduct between divinylbenzoin and dibutylstannane as a template. The polymer was successfully used to extract tributylstannane and other related tin-based organometallics from mussel and oyster samples.

Besides the traditional method of preparing imprinted polymers by using the radical polymerization of vinyl-based monomers, the use of an electropolymerization technique has been described to prepare ochratoxin A-binding thin films of polypyrrole [45,46]. Yu et al. prepared films supported on HPLC frits to set up an on-line extraction device suitable for direct isolation of the mycotoxin in wine samples and its quantification by fluorescence photometry. Such on-line MISPE showed itself to be affordable and competitive with MISPE methods based on conventional polymers for the extraction of ochratoxin A at levels of 50 ng L^{-1} .

4.2. Matrices

The MISPE performed on environmental or clinical matrices differs from the extraction of analytes from food samples, which involve a very large variety of matrices, ranging from vegetables and fruits (apple [83,84], carrot [15,64], grape [17], lemon [17], orange [17], pea [64], potato [55,64,65,86], strawberry [17]), cereals (barley [15,37], corn [54,55,37,64,86], rice [37], rye [37], wheat [15,37,76]), milk and butter [71–73,82], fish [59,74], mussels [38,75,79], shrimps [39], meat (bovine [59], duck [59], rabbit [59], turkey muscle [59], bovine liver [56,59,70,87], pig kidney [88]), beverages (wine [45,46,77], coffee [78], apple and grape juice [81,85]), but also more unusual samples such as red chili pepper [80].

This large diversity implicates that the mainstream use of MISPE is directed towards the matrix clean up, while preconcentration of highly diluted analytes is not the main issue. A significant example of matrix clean up is reported in Fig. 4, where an isoproturon and linuron-imprinted polymers were used for the on-line MISPE of phenylureas herbicides [64]. An interesting exception is represented by the off-line use of a MISPE

column to extract two antibiotics (chloramphenicol [71] and sulphametazine [72,73]) from skimmed milk, full-cream milk and butter before a quantitative analysis performed by square-wave voltammetry. In this case, a sample preconcentration was necessary due to the relatively low sensitivity of the analytical method used.

4.3. Analytes

The MISPE of analytes from food and beverage samples involves both single molecules and groups of analytes. Interestingly, it seems that when a single molecule is the analytical target, authors prefer to use the same molecule as a template (see Table 1), while mimic templates are taken into consideration only in a few papers: the extraction of β -agonist clenbuterol from bovine liver [56], and the clean-up of red wine containing the mycotoxin ochratoxin A [77]. Anyway, the extraction of this mycotoxin is described by other authors by using imprinted polymers prepared without mimic templates [45,46,76]. In this case, no problem of template bleeding and sample contaminations were reported by the authors.

Conversely, there is a widespread use of mimic templates when the analytical target is represented by several different analytes belonging to the same class of substances (see Table 2). Seldom the imprinted polymer was prepared by using a mimic template not present in the real sample, essentially because usually it could be very expensive, difficult to synthesize or not available commercially. Interesting examples are the use of bromclenbuterol as a template mimic to prepare an imprinted polymer for the MISPE of 8 β -agonists [59], and the use of the cyclododecanoyl ester of resorcinic acid as mimic template for the MISPE of mycotoxin zearalenone and its main metabolite α -zearalenol [37,63]. More frequently, the imprinted polymer is prepared by using one of the analytes that can act as a mimic template itself. It is not common that authors claim openly that the template molecule is a mimic template, while it is stated that the template has been chosen because of its molecular structure suitable to prepare an imprinted polymer with class-selective molecular recognition properties. As a significant example, Chiapuis et al. [85] prepared an imprinted polymer with molecular

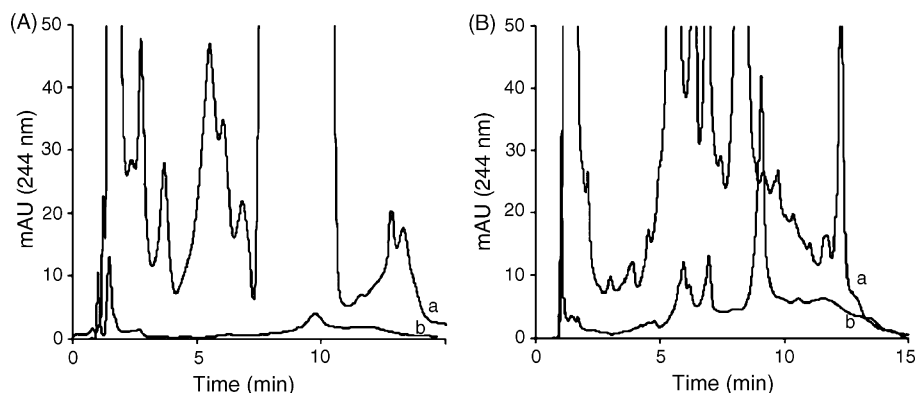


Fig. 4. MISPE of phenylurea herbicides. Chromatograms before (a) and after (b) MISPE of corn sample extract using an isoproturon-imprinted polymer (A) and carrot sample extract using a linuron-imprinted polymer (B). From [64], with permission of Springer-Verlag.

Table 1
Single-analyte MISPE

Analyte	Template	MIP synthesis	Matrix	MISPE mode	Analytical method	Refs.
Atrazine	Atrazine	Bulk	Bovine liver	off-line	HPLC, ELISA	[70]
Fenuron	Fenuron	Precipitation	Wheat, barley, carrot	off-line	HPLC	[15]
Thiabendazole	Thiabendazole	Precipitation	Orange, lemon, grape, strawberries	on-line	HPLC-FLU	[17]
Chloramphenicol	Chloramphenicol	Bulk	Milk	off-line	Voltammetry	[71]
Clenbuterol	Bromclenbuterol	Bulk	Bovine liver	off-line	HPLC-MS	[56]
Sulphametazine	Sulphametazine	Bulk	Milk, butter	off-line	Voltammetry	[72]
Sulphametazine	Sulphametazine	Bulk	Milk	off-line	Voltammetry	[73]
Tetracycline	Tetracycline	Bulk	fish	off-line	FIA	[74]
Domoic acid	<i>o</i> -Phtalic acid	Bulk	Mussels	on-line	HPLC	[75]
Ochratoxin A	Ochratoxin A	Bulk	Wheat	on-line, pulsed	HPLC-FLU	[76]
Ochratoxin A	Synthetic mimic	Bulk	Wine	off-line	HPLC-FLU	[77]
Ochratoxin A	Ochratoxin A	Electropolymerization	Wine	on-line, pulsed	HPLC-FLU	[45,46]
Bisphenol A	Bisphenol A	Precipitation	Shrimps	off-line	HPLC	[39]
Benzo[<i>a</i>]pyrene	Benzo[<i>a</i>]pyrene	Suspension	Coffee	off-line	HPLC-FLU	[78]
Tributylstannane	Tributylstannane	Bulk (covalent)	Mussels	off-line	GC	[38]
Sudan I	Sudan I	Bulk	Red chili pepper	off-line	HPLC	[80]
Butylated phenols	Butylated phenols	Bulk	Apple juice	off-line	HPLC	[81]
Yttrium	Yttrium	Bulk	Milk	off-line	AAS	[82]

AAS: atomic adsorption spectroscopy, ELISA: enzyme-linked immunosorbent assay, FIA: flow injection analysis, FLU: fluorescence detector, GC: gas chromatography, HPLC: high pressure liquid chromatography, MS: mass spectrometry detector.

recognition properties towards the template terbutylazine and 12 other related triazines, and used it for an efficient MISPE of herbicides on potato extracts.

4.3.1. Pesticides

With the exception of some papers related on thiabendazole in fruits [17] and carbaryl in apples [83], there are two type of pesticide whose use in MISPE on food samples has been widely described: triazine-based and urea-based herbicides. In fact, MISPE of a triazine is the oldest reported in literature as regards the extraction of food samples and the only published example of MISPE combined with ELISA assay. Triazine-based herbicides MISPE have been reported for many different food matrices – generally fruits and vegetables –, involving simultaneous extraction of several analytes and related metabolites, ranging from 4 (atrazine, propazine, simazine and terbutylazine, [84]) to

13 (ametryn, atrazine, cyanazine, desethylatrazine, desethylterbutylazine, desisopropylatrazine, hydroxyatrazine, propazine, prometryn, sebutylazine, simazine, terbutryn and terbutylazine [85]) different molecules. In these papers the MISPE method performed well, with >80% analyte recoveries and sensitivity ranging from 0.01 to 0.2 mg kg⁻¹ when matrices were vegetables. A direct comparison with immunoaffinity extraction performed on polyclonal antibody-based columns showed no advantages in using the more traditional immunoaffinity columns compared to the imprinted polymer.

A MISPE of urea-based herbicides has been successfully reported for fenuron alone (Fig. 5) [15] and for six different ureas (chlortoluron, fenuron, isoproturon, linuron, metobromuron and metoxuron), by using isoproturon or linuron as templates, and performing extraction on several different types of vegetables [64,65]. In this case, the authors observed that the MISPE

Table 2
Multiple analyte-MISPE single-analyte MISPE

Analyte	Template	MIP synthesis	Matrix	SPE format	Analytical method	Refs.
Carbaryl + 1 metabolite	Carbaryl	Suspension	Apples	off-line	HPLC	[83]
6 Phenylureas	Isoproturon, linuron	Precipitation	Carrots, corn, pea, Potato	off-line	HPLC	[64]
6 Phenylureas	Isoproturon, linuron	On beads	Potato	on-line	HPLC	[65]
4 Triazines	Simazine	Bulk	Apple	on-line	HPLC	[84]
5 Triazines	Propazine	Bulk	Corn	off-line	MECK	[54]
5 Triazines	Propazine	Precipitation	Corn, potato	off-line	HPLC	[55]
13 Triazines	Terbutylazine	Bulk	Grape juice	off-line	HPLC	[85]
5 Triazines	Propazine methacrylate	Precipitation	Corn, potato	off-line	HPLC	[86]
4 β -Agonists	Clenbuterol	Bulk	Bovine liver	off-line	HPLC	[87]
8 β -Agonists	Bromclenbuterol	Bulk	Bovine, rabbit, duck, turkey muscle, bovine liver, fish	off-line	HPLC-MS	[59]
2 Tetracyclines	Oxytetracycline	Bulk	Pig kidney	off-line	HPLC	[88]
Zearalenone + 1 metabolite	Synthetic mimic	Bulk	Wheat, corn, barley, rye, rice	off-line	HPLC	[37]
3 Alkylstannanes	Synthetic mimic	Bulk	Mussels, oysters	off-line	GC	[79]

GC: gas chromatography, HPLC: high pressure liquid chromatography, MECK: micellar electrokinetic chromatography, MS: mass spectrometry detector.

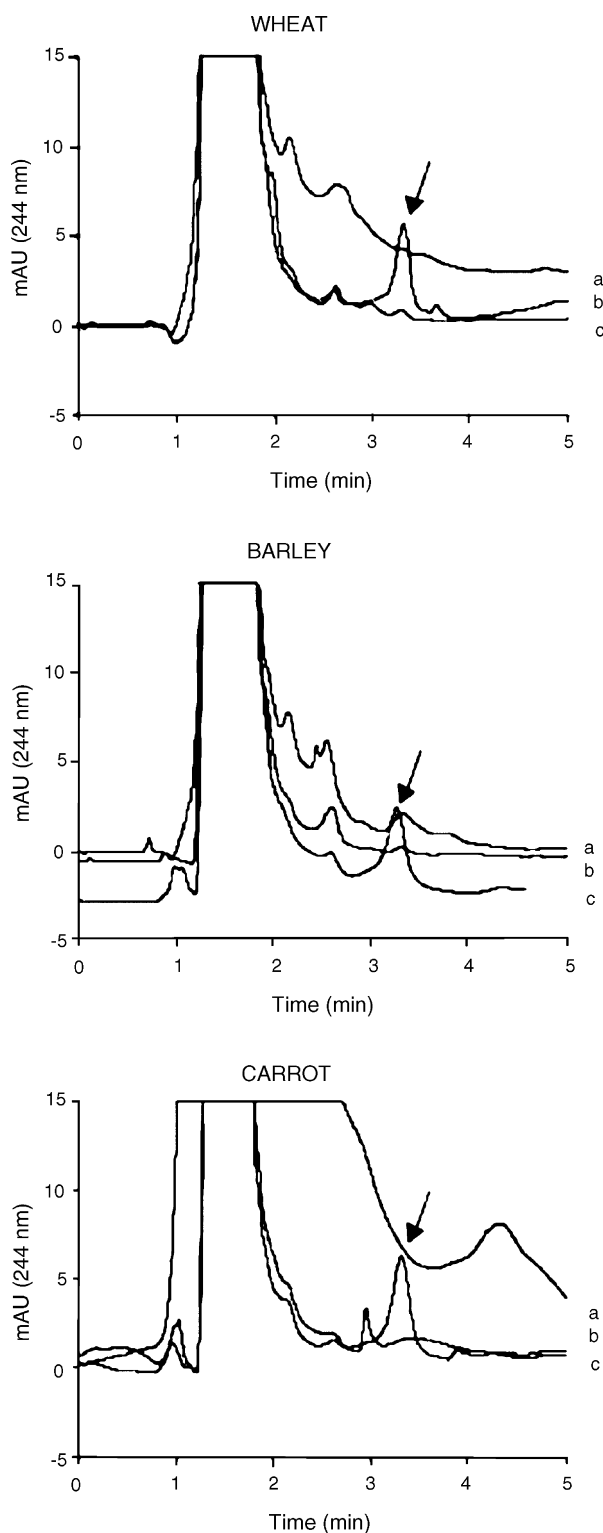


Fig. 5. MISPE of herbicides on a fenuron-imprinted polymer. Chromatograms obtained at 244 nm without (a) and with MISPE on plant sample extracts spiked (b) with fenuron (100 ng g^{-1}) and non-spiked (c). Arrow indicates the peak corresponding to fenuron. From [15], with permission of Elsevier Science.

column showed a progressive loss of extraction performance, presumably due to the interference of several matrix components. An elegant solution was given by coupling a “precolumn” comprising a layer of non-imprinted polymer to ease the removal

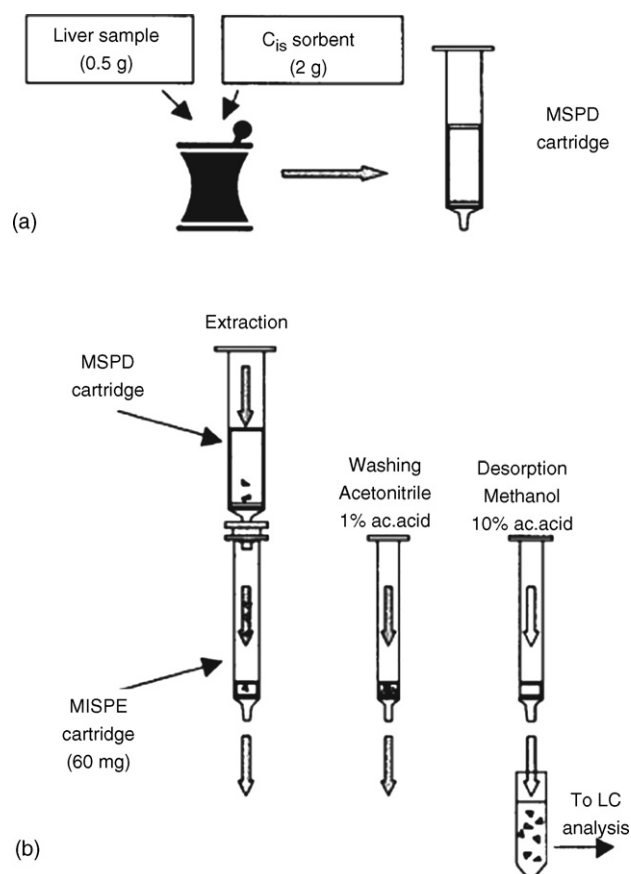


Fig. 6. Beef liver sample preparation by matrix solid phase dispersion; (a) column preparation; (b) tandem extraction of clenbuterol by MISPE. From [56], with permission of Americal Chemical Society.

of interferences without harming the binding properties of the imprinted column.

4.3.2. Drugs residua

Several papers describe the MISPE of veterinary drugs present in food samples as residua.

β -Agonists extraction has been described on many occasions, both as a single-analyte MISPE for clenbuterol in bovine liver [56], and as a multiple-analyte MISPE for clenbuterol and related substances in different matrices (bovine liver and muscle, rabbit, duck and turkey muscle, fish) [59,87]. In clenbuterol MISPE from bovine liver, Crescenzi et al. described the use of a double cartridge tandem system based on a matrix solid phase dispersion (MSPD) approach to directly extract the analyte from the liver tissue without any matrix pre-treatment (see scheme in Fig. 6). A reverse-phase sorbent was added directly to a sample of homogenized liver and accurately mixed with it; the solid mixture was packed in a SPE column and eluted in a MISPE column with acidified acetonitrile. Finally, clenbuterol was recovered by eluting the MISPE cartridge with acidified methanol and quantified by HPLC-ion trap mass spectrometry (Fig. 7). This extractive method turned out to be very affordable, free from interferences and with a detection limit below $0.1 \mu\text{g kg}^{-1}$. Extraction of several β -agonists by MISPE was validated for screening purposes by Kootstra et al. using

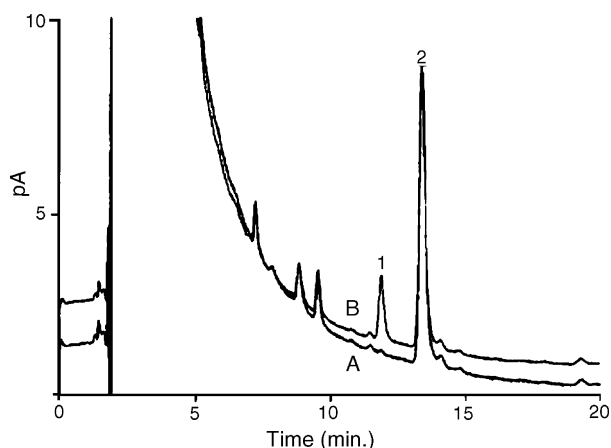


Fig. 7. MISPE of β -agonists in beef liver. Chromatograms of extracts after tandem MSPD-MISPE: (A) blank liver; (B) spiked sample (1 ng g^{-1}), (1) clenbuterol, (2) bromoclenbuterol (mimic template). From [56], with permission of American Chemical Society.

an HPLC-MS technique [59]. The validation results showed that nine compounds (brombuterol, cimbuterol, cimaterol, clenbuterol, clenproperol, isoxsuprine, mabuterol, mapenterol and ractopamine) meet the requirements for screening quantitative determination at $1 \mu\text{g kg}^{-1}$ using MISPE for sample clean up.

MISPE of antibiotics has been described for chloramphenicol [71] and sulphametazine [72,73] in skimmed milk, full-cream milk and butter samples. As previously reported in this review, the aim of the extraction was directed mainly towards analyte preconcentration, before a quantitative analysis was performed by square-wave voltammetry. Good selectivity and nominal preconcentration factors of 500 (chloramphenicol) and 45 (sulphametazine) were obtained, with nearly quantitative analyte recoveries for both the analytes.

Tetracycline and oxytetracycline were extracted from pig kidney by using an oxytetracycline-imprinted polymer described by Caro et al. [88]. Selectivity towards four antibiotics (doxycycline, epichlorotetracycline, oxytetracycline and tetracycline) was observed, and an efficient clean-up of the pig kidney tissue extract was attained, with good recoveries near 100% and clean HPLC chromatograms with a detection limit of $0.1 \mu\text{g kg}^{-1}$ for tetracycline. The same approach was used by Xiong et al. to extract tetracycline and oxytetracycline from fish samples [74]. The MISPE method was combined with a flow-injection chemiluminescence detection system and tetracycline was measured with a detection limit of $1 \mu\text{g L}^{-1}$ through its good enhancing effect on the chemiluminescence reaction between Ce(IV) ions and rhodamine B.

4.3.3. Mycotoxins

Mycotoxins are probably the most important food contaminants in terms of toxicity and widespread diffusion [2], but they can be considered very difficult analytes in MISPE technology and few papers have been published up to the present day. Difficulties do not arise from lack of functional groups suitable to set up non-covalent interactions during the imprinting process. Aflatoxins, ochratoxin A, nivalenols, zearalenone; all these

molecules show many polar groups suitable for hydrogen bond interactions – neither they do arise from the extraction process itself, that potentially cannot differ from MISPE performed on other analytes. On the contrary, difficulties arise from the elevated toxicity of this class of food contaminants, the high costs to purchase quantities of template suitable to prepare imprinted polymers, and the necessity to isolate and detect very low concentrations of contaminants.

Some examples of on-line MISPE of ochratoxin A in wheat and wine have been reported by Lai and co-workers. As previously reported in this review, ochratoxin A was extracted from red wine by using polypyrrole imprinted thin films supported on HPLC frits, and directly analysed by HPLC with an analyte recovery of 40% and a detection limit of 50 ng L^{-1} [45,46], whereas a bulk polymer prepared with the unusual functional monomer *N*-phenylacrylamide was used for the on-line MISPE of wheat extracts, with an analyte recovery of 100% and a detection limit of $5 \mu\text{g kg}^{-1}$ [76]. Unfortunately, the authors do not state if template bleeding was present or not.

Maier et al. observed poor recovery of ochratoxin A from a MISPE performed on untreated red wine samples as an effect of matrix interference [77]. The problem was solved by an initial clean up of the matrix from acidic components by reverse phase SPE, with a subsequent MISPE step performed on the eluate from the reverse phase column (Fig. 8). Quantitative analysis by HPLC with fluorescence detection on spiked and commercial samples provided recoveries $>90\%$, with good reproducibility and a limit of detection of 10 ng L^{-1} . However, the authors raised doubt on the effectiveness of the MISPE protocol as similarly

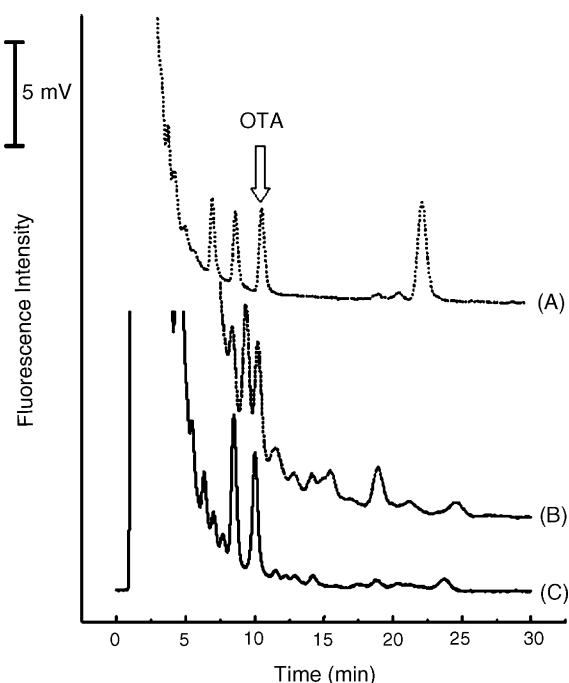


Fig. 8. MISPE of ochratoxin A in wine. Chromatograms of contaminated samples (0.22 ng mL^{-1}) after different SPE steps: (A) red wine sample after MISPE clean-up (analyte recovery $<70\%$); (B) after C18-based SPE clean-up; (C) after combined C18-MISPE clean up (analyte recovery $>90\%$). From [77], with permission of Elsevier Science.

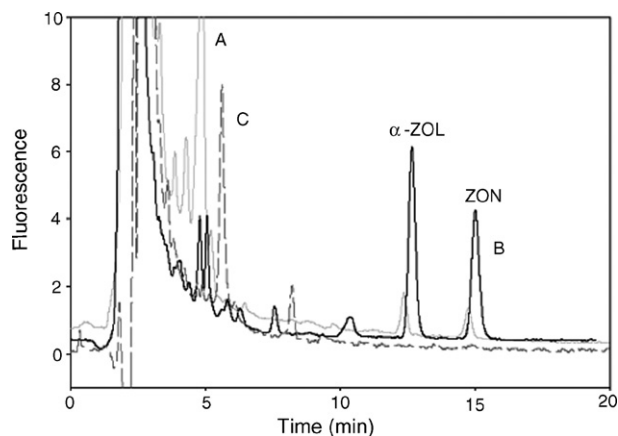


Fig. 9. MISPE of zearalenone (ZON) and α -zearalenol (ZOL) in wheat extracts. Chromatograms of contaminated samples (100 ng g^{-1}): (A) without and (B) with clean-up on the MISPE column. (C) Chromatograms resulting from clean-up on NIP. From [37], with permission of Springer-Verlag.

favourable performances were observed in control experiments in which the imprinted polymer was replaced by the corresponding non-imprinted materials.

The resorcinic acid derivate zearalenone is another mycotoxin considered to set up a MISPE method for food samples. Urraca et al. [37] prepared an 1-allylpiperazine-*co*-trimethylolpropantrimethacrylate polymer imprinted with a mimic of zearalenone – cyclododecanoyl ester of resorcinic acid – to clean-up the mycotoxin and its main metabolite α -zearalenol from cereal samples previously extracted with pressurized liquid extraction. Quantitative analysis of the MISPE eluate was carried out by HPLC with fluorescence detection on spiked and certified samples and it gave reproducible recoveries of 85–97% for both the analytes and a limit of detection of 10 ng L^{-1} (Fig. 9).

4.3.4. Environmental contaminants

Lai et al. prepared imprinted beads for the extraction of the carcinogenic polycyclic aromatic hydrocarbon benzo[*a*]pyrene from coffee samples [78]. 4-Vinylpyridine-*co*-divinylbenzene beads suitable for the MISPE were selected from several different kinds of imprinted polymers, differing for functional monomer (4-methacrylic acid, trifluoromethacrylic acid, vinylpyridine) cross-linker (divinylbenzene, ethylene glycol dimethacrylate) and polymerization technique (bulk or emulsion). Even if the selected polymer showed broad group selectivity, recognizing well several polycyclic aromatic hydrocarbons, it was used for direct MISPE of benzo[*a*]pyrene only in coffee samples. Quantitative analysis of the MISPE eluate by HPLC with fluorescence detection on spiked samples gave reproducible recoveries of >70%, with a limit of detection of 1 ng L^{-1} .

Bisphenol A-imprinted beads prepared by precipitation polymerization were used by Zhang et al. for the MISPE of this endocrine disruptor from acetonitrile extract of shrimps [39]. Samples containing bisphenol A over a concentration range of $5\text{--}50 \mu\text{g L}^{-1}$ were successfully cleaned-up, with reproducible recoveries of >75%. In direct comparison with a reverse-phase SPE, MISPE showed a better baseline, HPLC separation efficiency and recoveries.

As previously stated in this review, covalent imprinting with a synthetic mimic has been used by Munoz-Olivas et al. in the only known example of organometals extraction by MISPE [38,79] for the extraction of several alkylstannanes (dibutylstannane, monobutylstannane, tributylstannane and tripropylstannane) in certified mussels and oyster samples. Analysis of the MISPE eluate by gas chromatography gave reproducible results, with quantitative recoveries of the analytes and a detection limit of $3 \mu\text{g kg}^{-1}$. Moreover, chromatograms came out showing the complete removal of the interfering components that affects gas-chromatographic detection of organostannanes.

5. Conclusions

As shown in this review, MIPs can be successfully used as selective sorbents to clean up and preconcentrate contaminants of natural or anthropogenic origin from food samples. From the examples reported, it shows that MISPE is a potentially competitive technique with traditional solid phase extraction for its selectivity, and with immunoaffinity extraction for the stability and low cost of preparation of the imprinted materials. Many polymerization methods and extraction formats make MISPE a very versatile technique, suitable for analytes of different origin and nature. A careful choice of the template structure offers the possibility to develop MISPE methods suitable for the simultaneous extraction of many related analytes, opening the way to sample screening for whole analyte classes. The main problem affecting MISPE, i.e. the undesirable template leakage during the analyte elution step, can be avoided using the mimic template method. On these premises, extraction of food contaminants is destined to become a relevant application of MISPE as well as the extraction of analytes from clinical, environmental and pharmaceutical matrices.

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