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Application of molecularly imprinted polymers in food analysis: clean-up and chromatographic improvements

Review Article

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Abstract: Several natural and synthetic substances have been monitored in analytical laboratories worldwide to ensure food safety. Multiple residue detection (*i.e.*, detection of multiple analytes in a single sample or matrix) is a main weakness of existing analytical methods, when fast and reliable results are required. Multianalyte approaches may save time and money in the food industry, and more importantly, they allow the quick release of food products into the marketplace. In addition, multianalyte approaches notably decrease the time required between sampling and analysis to meet legal requirements.

However, to achieve analytical success, it is necessary to develop thorough clean-up procedures to extract analytes from the matrix. In addition, good chromatographic separation methods are also necessary to distinguish closely related analytes. Molecular imprinting technology (MIT) is an emerging, powerful tool for sample extraction and chromatography. First used for solid-phase extraction, molecularly imprinted polymers (MIPs) are also effective chromatographic phases for the separation of isomers and structurally related molecules. In recent years, a number of analytical methods utilising MIT have been applied for the analysis of residues in food, and existing methodologies have been improved. This review article describes the latest applications of MIT in the development of methodologies to monitor the presence of residues of veterinary products in foodstuff.

Keywords: *Molecularly Imprinted Polymers • Veterinary drugs • Foodstuff • Residues • MRLs*

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1. Introduction

In recent decades, interest in detecting several compounds simultaneously in different matrices such as food and environmental samples has led to the development of a spectrum of analytical methodologies. Official surveillance plans have been implemented in Europe to avoid the presence of residues of veterinary drugs and contaminants in foodstuff to protect the health of consumers. These plans include controls to avoid the misuse of different substances in food-producing animals and to assess compliance with maximum residue limits (MRLs) [1]. Consequently, sensitive and specific analytical screening and confirmatory methods are required to control the illegal presence of veterinary drugs in foods of animal origin.

Because of the great importance of detecting multiple analytes in different edible matrices, many different methods have been developed that are based on chromatographic techniques coupled to common detectors (*e.g.* the UV detector, diode array detector (DAD) and fluorescence detector (FLD)) and more recently, coupled to mass spectrometers (MS) [2,3]. Unfortunately, even after careful optimisation, some matrix components co-elute with the target analytes. Depending on the type of matrix, co-eluting components influence the reproducibility and accuracy of the method [4]. Despite the high selectivity and specificity of modern LC-MS/MS and GC-MS/MS systems, it has only been possible to achieve the very low limits required by the legislation through the application of highly selective extraction procedures. Nowadays, sample preparation is still the most laborious step of the analytical process because several factors must be considered, including the nature of the matrix and the properties of the analyte. The removal of potential interferents and the preconcentration of the analyte are two main objectives of sample pretreatment. In addition, it is necessary to develop a robust and reproducible method that functions independently of the sample matrix.

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Several procedures have been described for preparing samples of different matrices before analysis. New extraction techniques that improve the extraction process have been introduced over the years to analyse foodstuff, including liquid extraction (LE), liquid-solid extraction (LSE), solid-phase extraction (SPE), pressurised liquid extraction (PLE) and ultrafiltration [5]. SPE is a frequently used, low-cost and easily automated method to pretreat food samples and it can be coupled to both liquid and gas chromatography [3,5]. A wide variety of SPE cartridges exist that improve the extraction of analytes from complex matrices. The main drawback of these sample preparation techniques is the lack of selectivity of the sorbents which requires the optimisation of the extraction and clean-up of target analytes, including column conditioning, sample loading, washing and elution.

Molecularly imprinted polymers (MIPs) show promise as SPE sorbents. However, the use of MIPs for SPE is a rather new and scantily explored research domain, particularly for the analysis of certain groups of veterinary drugs. MIPs are synthetic materials with recognition sites that specifically bind target molecules in mixtures with other compounds. In contrast to classical SPE sorbents used for clean-up procedures, MIPs are more selective and allow the elution of analytes from the cartridges nearly free from co-extracted compounds [6]. The superiority of MIPs as selective SPE sorbents (MISPE) for the analysis of residues in food has been extensively demonstrated during the last decade; several studies have compared classical SPE sorbents and MISPE sorbents [7,8]. In addition, the use of MIT for environmental, bioanalytical, food and pharmaceutical applications has been reviewed [9-11]. The application of molecularly imprinted materials to trace even cholesterol and peptides has been reported [12,13]. Novel applications of MIPs include the development of MIP-based sensors, LC stationary phases and magnetic sorbents.

2. Molecularly imprinted polymers

2.1. Synthesis

The challenge of designing and synthesising a molecularly imprinted polymer (MIP) can be discouraging for the uninitiated researcher, mainly because of the large number of experimental variables involved, *e.g.* the nature and amount of template, functional monomer, cross-linker, solvent and initiator, as well as the method of initiation of polymerisation and the duration of polymerisation [14].

2.1.1. Template

The template is of great importance in any molecular imprinting process because it directs the organisation of the functional groups of the functional monomers. The template is selected depending on the type of polymerisation and it should ideally be chemically inert. Different aspects must be considered when selecting an appropriate template: (1) the template must have groups that can polymerise, (2) the template can potentially inhibit or retard a free radical polymerisation (*e.g.* a template with a thiol group or a hydroquinone moiety), and (3) the template must be stable under the polymerisation conditions (*e.g.* temperature and UV irradiation). In addition, employing the analyte directly as a template may be problematic because the complete removal of the target molecule from high-affinity sites of the polymer is not always possible. This is particularly important when MIPs are used for trace analysis because “bleeding” of the residual template during the SPE process can lead to false positives or inaccurate quantification of analytes in actual samples. To address this problem, so-called “dummy” templates that mimic the shape and size of the target analyte have been used for the synthesis of MIPs for the detection of some analytes [15,16]. Ideally, these MIPs will have binding sites with sufficient cross-selectivity for the target analyte and structurally related compounds. The problems of co-elution of the analyte and the “dummy” template during SPE can be avoided with accurate quantification methodologies (*e.g.* HPLC-MS/MS) [15].

2.1.2. Functional monomers

The choice of an appropriate functional monomer is an important step to provide complementary interactions between the template and the substrate [17]. Functional monomers are responsible for binding interactions in the imprinted binding sites. The template and the monomer should be complementary to maximise the complex formation and thus the imprinting effect. The reactivity ratio of the monomers is also important to ensure that copolymerisation is feasible when two or more functional monomers are combined in a “cocktail” polymerisation [8]. To date, one of the most frequently used monomers has been methacrylic acid (MAA): an appropriate monomer for analytes with basic functional groups and for detecting analytes that can form H-bonds. In contrast, vinylpyridine has been frequently used for designing MIPs for detecting analytes containing acid groups. In general, acidic monomers are more appropriate for basic templates and basic monomers are more appropriate for acidic templates (see Fig. 1).

2.1.3. Cross-linkers

The selectivity is greatly influenced by the type and amount of cross-linking agent used in the synthesis of the imprinted polymer. For MIPs, the cross-linker fulfils three major functions: controlling the morphology of the polymer matrix, stabilising the imprinted binding site and conferring mechanical stability to the polymer matrix. Porous (macroporous) materials are usually preferred to generate materials with adequate mechanical stability. Moreover, the amount of cross-linker should be sufficient to maintain the stability of the recognition sites, the three-dimensional structure and the chemical functional groups after template removal. Several cross-linkers can be used for molecular imprinting (see Fig. 1).

2.1.4. Solvents (porogens)

Porogenic solvents play an important role in the formation of the porous structure of MIPs, and they collect all the components (template, functional monomer, cross-linker and initiator) in a single phase during the polymerisation process. The porogenic solvent is also responsible of creating pores in the macroporous polymers, hence the name “porogen”. The nature and the amount of porogenic solvent determine the strength of the non-covalent interactions and influence polymer morphology that directly affects the performance of MIPs. The solvent for non-covalent imprinting polymerisation must also be judiciously selected to maximise the likelihood of forming a complex between the template and the functional monomer. First, the template, monomer, cross-linker and initiator must be soluble in the porogenic solvent. Secondly, the porogenic solvent should produce large pores to ensure good flow-through properties of the resulting polymer. Low solvent polarity is another important feature that reduces interferences during complex formation and confers the high selectivity of MIPs. Thus, the use of a thermodynamically suitable solvent may lead to polymers with well-developed pore structures and high specific surface areas, whereas the use of a thermodynamically poor solvent leads to polymers with poorly developed pore structures and low specific surface areas.

2.1.5. Initiators

Several chemical initiators with different chemical properties can be used as radical sources for free radical polymerisations. The amount of initiator is considerably lower than the amount of monomers. The rate and mechanism of decomposition of an initiator (to form radicals) can be triggered and controlled in a number of ways, including heat, light and chemical/electrochemical means, depending on the chemical nature of the initiator. Free radical polymerisation is retarded with oxygen gas to maximise the rate of monomer propagation and to ensure batch-to-batch polymerization reproducibility. Therefore, the removal of dissolved oxygen from monomer solutions is advisable immediately before polymerisation. Commonly employed initiators are azo(bis)-isobutyronitrile (AIBN), 2,2’-azobis(2,4-dimethylvaleronitrile) (ABDV) and benzoyl peroxide (BPO).

In contrast, iniferter (initiator – transfer agent – terminator) type initiators can act simultaneously as an initiator, chain transfer agent, and terminator in a polymerisation reaction. These molecules generate two free radicals, one of which is able to initiate the polymerisation, and the second one is relatively stable and non-active but capable of terminating the growing polymer chains by a reversible combination. The use of immobilised iniferter-type initiators (normally dithiocarbamates) is useful for obtaining a thin film of polymer grafted on a surface, which controls the degree of polymerization by reaction time [18,19]. Iniferter initiators can avoid solution polymerisation and resulting gelation during the synthesis of MIP composite materials by surface imprinting techniques.

2.1.6. Polymerisation methods

In general, an excess of the functional monomer relative to the template is required to favour template-monomer complex formation and to maintain the integrity of this complex during the entire polymerisation process. Moreover, three different approaches to synthesise MIPs have been reported that use covalent, non-covalent or semi-covalent interactions. The non-covalent procedure is based on the formation of relatively weak, non-covalent interactions (*e.g.* electrostatic interactions, hydrogen bonding, Van der Waals forces, hydrophobic interactions and dipole-dipole bonds) between the template and the functional monomer. The non-covalent approach is most frequently used for the preparation of MIPs because it is relatively simple and because there are several monomers that can interact with almost any kind of template [20]. However, some polymers prepared by this approach can bind the template so strongly that it is challenging to remove all traces of the template (even after repeated washing of the polymer) [21]. The covalent approach involves the formation of covalent bonds between the template and the functional monomer; therefore, the template molecule must be chemically modified. These covalent bonds are broken to remove the template from the polymer matrix, and the covalent bonds are then reformed to rebind to the target molecule. This approach leads to a homogenous population of binding sites because of the high stability of the template-monomer interactions. The semi-

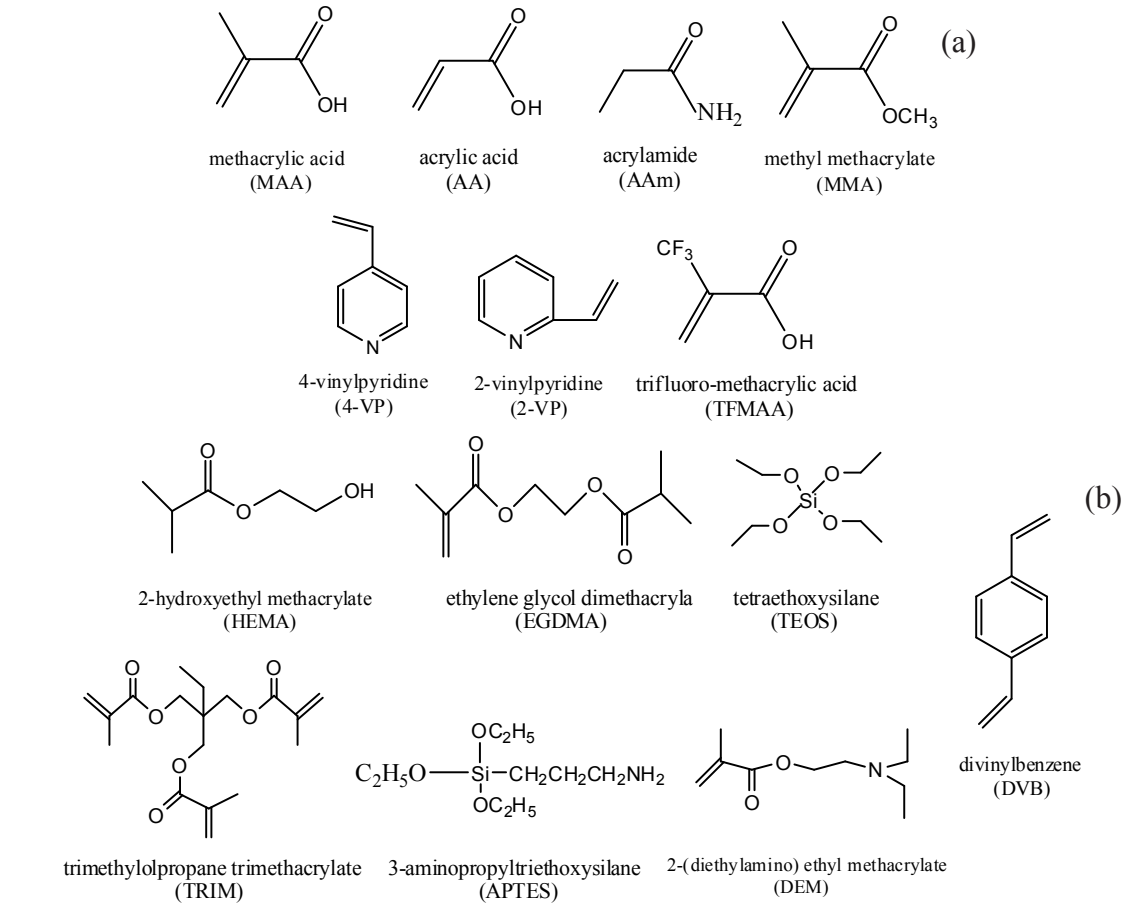

Figure 1. List of the most frequently used functional monomers (A) and cross-linkers (B) in MIP synthesis, including their chemical structures.

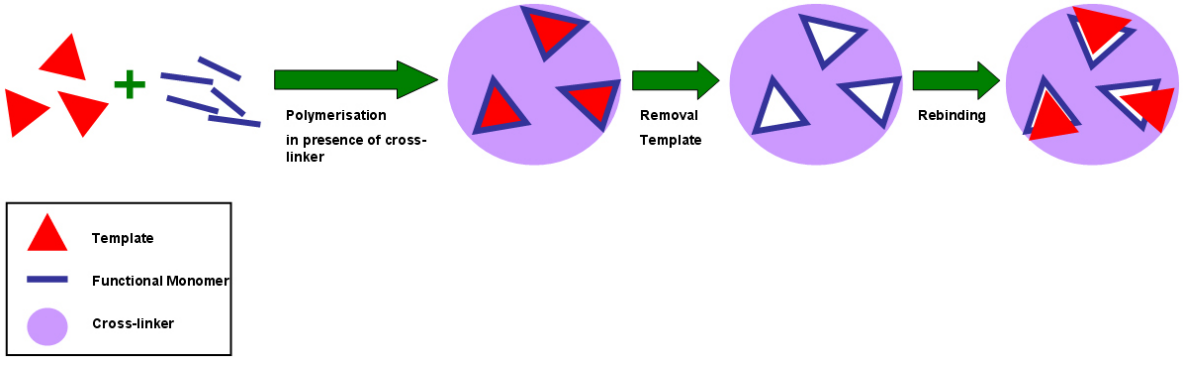

Figure 2. Schematic representation of molecular imprinting process.

covalent approach uses both non-covalent and covalent interactions, covalently binding the template to a functional monomer during the polymerisation and then non-covalently rebinding the target molecule.

Several polymerisation methods can be used to obtain MIPs for SPE. Traditionally, MIPs have been prepared by bulk polymerisation because it does not require sophisticated instrumentation and because the reaction conditions can be easily controlled. Although this procedure is tedious and time consuming, it is the most widely used method for the preparation of MIPs [22]. After polymerisation, MIP particles are obtained by grinding and sieving the resulting monolith to desired size ranges [23]. Although this kind of polymerisation is simple, it has many drawbacks. First, the resulting particles are highly irregular in size and shape, which can cause back-pressure problems when they are packed in columns for use as stationary phases for

chromatography. Secondly, grinding and sieving are tedious and cause a substantial loss of the polymer [24]. Despite these obvious drawbacks, most of the known MIPs have been prepared by bulk polymerisation (see Tables 1, 2 and 3).

In recent years, much effort has been dedicated to develop alternative methods for obtaining imprinted stationary phases. These methods have superior properties, including efficiency and mass transfer. In addition to bulk polymerisation, new techniques have been developed that produce spherical particles and more homogeneous binding sites. These methods include precipitation polymerisation, suspension polymerisation, multi-step swelling polymerisation and *in situ* polymerisation. Precipitation polymerization can be used to prepare MIPs with microspherical shapes of uniform sizes. In addition, the polymerisation yield is quite high for precipitation polymerization because the grinding and sieving steps are not required. Furthermore, MIPs obtained by precipitation polymerisation have an increased rebinding capacity and a more homogeneous distribution of binding sites than those obtained by bulk polymerisation. A different and novel polymerisation method is suspension polymerisation: a heterogeneous procedure for the production of spherical beads of a broad size-range (from a few micrometres to millimeters). In this process, the organic-based polymerisation mixture is suspended as droplets in an excess of a continuous dispersion phase by stirring, and each droplet acts like a mini-bulk reactor. The addition of a stabiliser to the dispersion medium is then necessary because the droplet suspension is not stable. The suspension method is less frequently performed for MIP synthesis, likely because of the need for a polar (water) or expensive (perfluorocarbon fluid) suspension medium. In addition, multi-step swelling polymerisation has recently been developed based on the swelling of uniformly sized particles to more efficiently control size distribution and shape and to decrease material loss. Two-step or multistep methods can produce spherical particles of uniform sizes in the 5–100 µm range [25,26]. These particles have separation properties comparable to those prepared by bulk polymerisation but much better column efficiencies and peak shapes. Finally, *in situ* polymerisation is another recently developed technique that is very simple and rapid compared to other previously described procedures because it enables the preparation of an MIP monolith within 3 hours. The success of the synthesis relies on the presence of both macropores that provide good flow-through properties, and selective binding sites. In a typical *in situ* process, the reaction mixture (containing the template molecule, functional monomer, cross-linking agent, porogenic solvent and initiator) is poured into a stainless-steel tube (sealed at one end) and then degassed ultrasonically. The second tube end is then sealed, and the heating polymerisation takes place. After the removal of the template molecules, the column of the MIP monolith can be connected directly to an HPLC system for on-line SPE of the target molecules. Furthermore, monoliths synthesised in capillaries are potential alternatives to ground monolith columns, polymer bead columns and silica-based columns.

Nevertheless, there is a growing interest in alternative routes for preparing MIPs to better control morphology and thus explore new applications. Additional polymerisation methods use different materials such as spherical silica or organic polymers to graft or coat thin films of MIP-phases on the surfaces of porous materials [27]. These surface imprinting techniques can be a partial solution to difficulties in generating high affinity binding sites while simultaneously controlling the porous properties, morphology or other structural features. In addition, the use of grafting and coating allow decoupling of the imprinting step from the generation of a particular morphology. Coating techniques consist of grafting a thin layer of MIP to a particular support (fibres, electrodes, silica beads). For example, polymerisation within the pores of preformed silica beads is a simple method, as it simply consists of filling the pores with the polymerisation mixture and subsequently initiating the polymerisation [28]. In contrast, during the grafting process, the polymerisation is initiated from a preformed polymer on a material surface, for example porous modified silica (iniferter-type techniques) [18]. On the other hand, an MIP that can distinguish target molecules from other chemicals in water is of special interest, because many analyses need to be carried out under aqueous conditions. One intriguing strategy to synthesise a water-compatible MIP is to carry out molecular imprinting directly in water. However, water molecules can disrupt the important interactions between the template and monomer (*e.g.* hydrogen bond interaction), causing serious limitations. To solve this problem, recent molecular imprinting methods have been introduced, such as Pickering emulsion polymerisation [29]. With Pickering emulsion, an optimal combination of hydrophobic and electrostatic interactions can lead to successful formation of molecularly imprinted sites in a continuous water phase.

2.2. Applications of MIPs

Molecularly imprinted solid phase extraction (MISPE) has two modes: off-line and on-line. MISPE is typically performed in the off-line mode, whichrequires the conditioning of a cartridge column, sample loading,

interference washing and analyte elution. The eluate can then be analysed with an analytical system, including chromatography (GC, LC) coupled to different detectors. This approach is especially useful for multianalyte detections for which MIPs can recognise several analyte analogues. In contrast, the on-line mode provides automatic sample loading, interference washing, analyte elution and analyte separation and detection by directly connecting the MISPE column to an analytical system. Furthermore, off-line MISPE can also be performed with on-column extraction, solid-phase microextraction and microextraction with a packed syringe. In recent years, MIPs have been used to generate selective LC columns. Because of the high selectivity of MIPs, extraction, enrichment, separation and detection of target analytes can be achieved in one step by directly coupling an MIP column in-line with the detection system [28].

3. Detecting residues of veterinary drugs in foodstuff by molecular imprinting technologies

Substances detected as residues in food have been divided into two major classes according to Council Directive 96/23/EC: group A and group B [31]. Group A includes drugs used as growth promoters in farm animals and substances for which the maximum residue limit (MRL) has not been established. Furthermore, group A substances may be divided into four subgroups: anabolic substances (A1, A3, A4), antithyroid agents (A2), beta-agonists (A5) and Annex IV substances of Council Regulation 2377/90/EEC (A6) [32]. Group B includes authorised veterinary drugs and contaminants. This group can be divided into three subgroups: antibacterial substances (B1), other veterinary drugs such as anthelminthic, sedative and non-steroidal anti-inflammatory drugs (B2), and contaminants (B3). Corticosteroids formally belong to group B (B2f), although they have been banned because of the abuse of these substances in cattle fattening.

The interest in detecting and quantifying drugs in animal tissues and fluids is increasing because it has been shown that drug residues in food of animal origin can cause direct toxic and allergic reactions. Obviously, analytical requirements differ for groups A and B [33]. On the one hand, the identification of very low amounts of drugs from group A is important (zero tolerance policy, legal implications) and several matrices may contain residues. On the other hand, group B consists of substances with a MRL that strongly demands quantitative detection methods. This review describes analytical methods published in recent years that apply molecularly imprinted polymers to extract and detect substances of both group A and B in food.

3.1. Analysis of group A substances

Group A comprises banned substances according to Directive 96/22/EC [34] such as growth promoters abused in animal fattening and “no maximum residue limited (MRL)” substances [2]. The identification of these banned substances is important in a large number of matrices and at minimal concentrations because there is a zero tolerance policy on their presence in food. Thus, there is a need to develop sensitive, rapid and cost-effective analytical methods to detect banned substances. Molecular imprinting technology (MIT) can help to accomplish these requirements because of its high selectivity, stability and affinity, relative ease of use, low cost of preparation and possible use with a wide range of target molecules and matrices (Table 1) [20,22].

According to Directive 96/22/EC, group A substances are subdivided into six main groups: stilbenes and its derivatives (A1), thyreostats (A2), steroids (A3), resorcylic acid lactones (A4), β-agonists (A5) and Annex IV substances of Council Regulation 2377/90/EEC (A6) [32].

3.1.1. Stilbenes and EGAs (groups A1 and A3)

Stilbenes are non-steroidal oestrogenic growth promoters, and their structures are similar to estradiol because they have the same spatial distance between the two OH groups. Therefore, stilbenes are often analysed in conjunction with other steroidal compounds [3]. Stilbenes include diethylstilbestrol, hexestrol and dienestrol. Diethylstilbestrol (DES) is the most studied stilbene because of its endocrine disruptive effect and it is frequently used to increase the heft of flesh of cattle, sheep, chicken and fish and to treat oestrogen-deficiency in veterinary medicine. Another consequence of their use is the frequent occurrence of group A1 and A3 compounds in the environment [35,36].

The synthesis of MIPs using different methods of polymerization has recently been described (using DES as a template) for the detection of stilbenes in fish, river water and tap water. Some of these MIPs have been synthesised using novel types of matrices, including nanosized materials created by grafting with MIP films (nanofibres) that have a larger binding capacity and faster binding kinetics for use in the on-line mode [37]. Moreover, an imprinting process in combination with a sol-gel process has been used to develop an MISPE procedure for the analysis of DES in fish and water samples [38]. Bulk polymerisation has also been applied for the design of sorbents for on-column solid-

Table 1. Application of molecularly imprinted technology to the analysis of group A substances (Council Directive 96/23/EC) in foodstuff.

Target Analyte	Matrice	Template	Monomer/CL/Initiator	Porogen	Polymerisation method	Mode	Analytical system	Ref.
Stilbenes	Fish, water	DES	APTES/TEOS	MeOH	Precipitation in silica gel	MISPE	HPLC-UV	[38]
Stilbenes	milk	DES	MAA/EGDMA/AIBN	ACN	Coating of hollow fiber tubes	MISPE	HPLC-UV	[40]
Stilbenes	Fish, water	DES	AA/MBAA/TEMED+APS	EtOH-water (50:50 v/v)	Grafting in nanofibers	MISPE	HPLC-UV	[37]
EGAs	Water (river)	DES	MAA/EGDMA/AIBN	ACN	Bulk polymerisation	MISPE	HPLC-DAD	[39]
EGAs	milk, yogurt, beef, chicken, pork	E2	4-VP/EGDMA/AIBN	mineral oil–toluene (2:3, v/v)	Precipitation polymerisation	MISPE	HPLC-UV	[43]
EGAs	Milk powder	E2	TFMAA/TRIM/AIBN	ACN	Precipitation polymerisation	MISPE	HPLC and GS/MS	[44]
EGAs	Water (tap and river)	Not avaliable(commercial MIP): AFFINIMIP(POLYNTELL, Val de Reuil, France)				MISPE	LC–MS	[47]
EGAs	Fish, prawn tissue	E2	MAA/EGDMA/AIBN	ACN	Bulk polymerisation	MISPE	HPLC-FLD	[45]
EGAs	Water (tap and river)	MT	MAA/EGDMA/AIBN	ACN	Precipitation polymerisation	MISPE	HPLC-DAD	[46]
Corticosteroids	–	COR	10 different FM/7 CL/DMPA	5 porogen solvents	Bulk polymerisation	MISPE	HPLC-UV	[49]
Corticosteroids	milk	FLU	MAA/DVB/AIBN	ACN-toluene (3:1, v/v)	Precipitation polymerisation	MISPE	HPLC-MS/MS	[50]
β-agonists	Pig urine	SAL	MAA/EGDMA/AIBN	DMSO	Coating of electrodes	MIP-based ISE	Potentiometry	[53]
β-agonists	Pig tissues (muscle, liver)	RAC	MAA/EGDMA/AIBN	MeOH	Bulk polymerisation	MISDSPE	HPLC-UV	[55]
β-agonists	pork, pig liver	RAC	AM+St/TRIM/AIBN	DMSO	magnetic MIP	MISPE	HPLC-FLD	[56]
β-agonists	Pork, liver, feed	RAC	MAA/EGDMA/AIBN	MeOH	Coating of stir bars	SBSE	HPLC-FLD	[54]
β-agonists	Urine	Not avaliable(commercial MIP):MIP4SPE®MIP Technologies (Lund, Sweden)				MISPE	HPLC/MS/MS	[57]
β-agonists	Urine	Not avaliable(commercial MIP):MIP4SPE®MIP Technologies (Lund, Sweden)				MISPE	HPLC/MS/MS	[58]
β-agonists	Bovine muscle	Not avaliable(commercial MIP):MIP4SPE®MIP Technologies (Lund, Sweden)				MISPE	HPLC/MS/MS	[59]
CAP (Annex IV)	Milk	Not avaliable(commercial MIP):MIP4SPE®MIP Technologies(Lund, Sweden)				MISPE	HPLC-MS/MS	[62]
CAP (Annex IV)	Milk samples	CAP	MAA/EGDMA/AIBN	Chloroform	Bulk polymerisation	MIP-based ISE	Voltammetry	[61]
CAP (Annex IV)	Honey samples	FF	2-VP/EGDMA/AIBN	Chloroform/ TFH	Bulk polymerisation	MISPE	HPLC-UV	[64]
CAP (Annex IV)	Honey,urine,milk, shrimp	CAP/DAM/EGDMA/AIBN/TFH (commercial MIP) : SupelMIP SPE cartridges (Sigma-Aldrich, Germany)				MISPE	HPLC-MS/MS	[63]

APTES: 3-aminopropyltrimethoxysilane; AA: acrylic acid; AM: acrylamide; APS: ammonium persulfate; St: styrene; MAA: methacrylic acid; MBAA: N,N-methylene-biacrylamide; TEMED: N,N,N',N'-tetramethylethlenediamine; EGDMA: ethylene glycol dimethacrytlate ; AIBN: azo(bis)-isobutyronitrile; DMPA: 2,2-dimethoxy-2-phenylacetophenone; TEOS:tetraethoxysilicane; VP: vinylpyridine; DES: diethylstilbestrol; E_2: 17β-estradiol; MT: methyltestosterone; TFMAA: 2-(trifluoromethyl) acrylic acid; TRIM: trimethylolpropane trimethacrylate; DAM: (diethylamino)ethyl methacrylate; ACN: acetonitrile; MeOH: methanol; DMSO:dimethyl sulfoxide; EtOH: ethanol; TFH: Tetrahydrofuran; EGAs: estrogens, gestagens, androgens; SAL: salbutamol; RAC: ractopamine; COR: cortisol; FLU: flumethasone; CAP: chloramphenicol; FF: Florfenicol; ISE: ion selective electrode; DSPE:Dispersive SPE; SBSE: Stir bar sorptive extraction

phase extraction of DES, hexestrol and oestrogens in water using DES as a template [39]. An innovative DES molecularly imprinted (coating) polypropylene hollow fibre tube (connected at one end to the needle tip of a micro-syringe) has been used for the detection of stilbenes in milk samples in an on-line mode [40].

Steroid hormones (*i.e.*, steroids that act as hormones) can be divided into four groups: oestrogens, gestagens, androgens (EGAs) and corticosteroids. These hormones have many applications in the veterinary field; however, in addition to their regulated use, they are illegally used in animal fattening. Steroid hormones may increase the weight gain of treated animals and induce other changes that are generally characterised by lower fat content and leaner mass [41]. Animals can be treated with EGAs in the feed; however EGAs are typically injected when they are used as growth promoters. Improper or illegal use of steroid hormones may leave residues in food products derived from these animals (*e.g.* meat, milk, eggs and fish). Because of their possible toxic effects, European Union Council Directive 96/22/EC prohibited the administration of steroid hormones in livestock breeding and aquaculture [34]. A naturally occurring oestrogen, 17β-estradiol (E_2), the most active oestrogen, plays an important role in the oestrous cycle. The use of 17β-estradiol to promote animal growth has been banned by Directive 2003/74/EC because of recent evidence that E_2 is a complete carcinogen because of its tumour-initiating and tumour-promoting effects [42].

E_2-imprinted polymers prepared by precipitation polymerisation have been used for off-line MISPE to detect oestrogens in dairy and meat samples such as milk, yogurt, beef, chicken and pork [43] and in milk powder [44]. MIT has also been successfully applied to extract E_2 from fish and prawn tissues using off-line MISPE and the same template, but with polymers prepared by bulk polymerisation [45]. Oestrogens are released into aquatic ecosystems from human and animal urine and they contribute to endocrine-disrupting activity in aquatic environments. To assess the ecological risk associated with the presence of these compounds in river and tap water, a recent study used methyl-testosterone as a template and MIPs (synthesised by precipitation polymerisation) as selective sorbents in SPE cartridges [46]. Moreover, several recent studies have used commercial MIPs for SPE; most of these studies have been performed in the off-line mode. For example, Lucci *et al.* described an MISPE procedure for the group-selective extraction of natural and synthetic oestrogens at concentrations as low as ng L^{-1} using commercial SPE cartridges [47].

3.1.2. Corticosteroids

Corticosteroids are steroid hormones used in human and veterinary medicine as antipyretic, anti-inflammatory and anti-allergy drugs. Formally, they belong to group B2f, but they are illegally used as growth-promoting agents, synergistically combined with other molecules such as β-agonists or anabolic agents [48] in order to increase weight gain and reduce the feed conversion ratio. To protect consumer health, the European Union has banned the use of corticosteroid hormones in livestock breeding and aquaculture [32]. In 2010, Baggiani *et al.* developed several MIPs based on bulk polymerisation by combining different functional monomers, cross-linkers and porogenic solvents [49]. Cortisol was finally selected as a template molecule because it is a natural corticosteroid very similar to most synthetic ones. However, there is only one published report on the application of MIT for the analysis of corticosteroids in actual foodstuff [50]. This report describes the use of flumethasone as a template for the synthesis of imprinted polymers, which proved to be better suited for MISPE in comparison to commercial SPE cartridges for the extraction of corticosteroids from milk [50].

3.1.3. β-agonists (group A5)

Beta-adrenergic agonists (BAAs) constitute a class of illegal growth promoters that decrease the fat content of cattle and other farm animals in favour of a higher percentage of muscle [2]. They are also used to treat pulmonary diseases such as bronchitis, asthma and emphysema. The abuse of these substances has caused adverse effects in humans, including food poisoning (related to the presence of residues in the liver), cardiovascular disease and central nervous disease [51]. The most frequently used BAAs are clenbuterol (CLEN), salbutamol (SAL) and ractopamine (RAC). In recent years, RAC and SAL have been used as templates for the design of MIPs because they are used to substitute for clenbuterol as growth-promoting agents. RAC has been approved as a feed additive for pigs and cattle in the United States and other countries [52]; thus, it is also necessary to establish fast and accurate methods to detect SAL and RAC residues because of the potential health risks for individuals consuming animal products contaminated with these substances.

A recent trend in MIT is to develop novel sensors based on MIPs. Some researchers have used MIPs to prepare electro-active materials (using SAL as a template molecule) for the detection of BAAs in food samples. For example, an ion-selective electrode (ISE) was directly synthesised on screen-printed electrodes based on molecularly imprinted membrane-coated electrodes. This type of sensor has been designed and fabricated for the detection of SAL in pig urine [53]. Moreover, an MIP-coated stir bar with RAC as a template molecule has been designed for the detection of beta-agonists in spiked pork, liver and animal feed [54]. Classic MIPs and magnetic MIPs have been designed for MISPE to detect beta-agonists in pig tissue (muscle and liver). These MIPs were developed using classical bulk polymerisation methods and RAC as a template, but they showed cross-selectivity with isoxsuprine and fenoterol [55,56].

Some companies have launched new commercial MIP-based SPE cartridges for different applications. These cartridges are highly selective in the extraction of certain analytes and have been used for the quantification of drugs at very low concentrations. For example, several authors have described the use of MIP4SPE® cartridges for multi-residue off-line clean-up of β-agonists in calf urine and bovine muscle [57-59].

3.1.4. Annex IV substances (A6)

The substances included in Annex IV of Council Regulation 2377/90/EEC are banned for use in all food-producing animals [30]. The most important substances in this group are chloramphenicol (CAP) and the nitrofurans. CAP is an antibiotic used in veterinary medicine for the prevention and treatment of typhoid fever and numerous bacterial infections. It was first produced in the bacterium *Streptomyces venezuelae* but it is now mainly prepared by chemical synthesis. CAP has several harmful side effects in humans, including

aplastic anaemia, Grey's syndrome and bone marrow suppression. CAP belongs to the amphenicol group that also includes thiamphenicol (TAP) and florfenicol (FF), approved for use in all food-producing animals [3]. FF is a synthetic, fluorinated analogue of CAP that has been widely used in food-producing animals to prevent and treat infections in pigs, vibriosis in fish and bovine respiratory disease in cattle [60].

Many MIT studies have focused on the detection of CAP in milk using CAP as a template molecule in the polymerisation reaction. A recent report described the generation of CAP-MIP by bulk polymerisation and subsequent addition to carbon paste for the construction of an IES. This method is an alternative to traditional methods for reliable CAP detection in complex actual food samples (such as milk) [61]. To develop confirmatory methods with detection limits matching those required by regulatory agencies, some researchers have used commercial cartridges to selectively extract CAP in the off-line mode. Commercial MIP-SPE sorbents and classical SPE preparation procedures have been compared for their use with complex food matrices such as milk (raw, skimmed and powder) [62], honey, urine and shrimp [63]. In 2009, Shirmer *et al.* described the preparation of CAP-imprinted polymers synthesised by bulk polymerisation, with different functional monomers and templates such as FF, TAP and azidamphenicol and two different porogens. The imprinted polymer synthesised with FF as a template and applied in off-line MISPE provided higher retention capabilities for honey samples than MIPs prepared with TAP and azidamphenicol [64].

3.1.5. Thyreostatics and resorcylic acid lactones (groups A2 and A4)

Thyreostatic drugs constitute a complex group of substances that disturb the normal metabolism of the thyroid gland by inhibiting the synthesis and secretion of the thyroid hormones triiodothyronine (T3) and thyroxine (T4). The administration of thyreostatics to livestock leads to considerable weight gains through increased water retention and filling of the gastro-intestinal tract [65]. Consequently, the meat from these treated animals is of lower quality. Thyreostatics have been banned in the EU since 1981 because of their potential teratogenic and carcinogenic effects that pose possible human health risks [66]. There have been no reports regarding the application of molecular imprinting technology (MIT) for the analysis of thyreostatics in food.

Resorcylic acid lactones are non-steroidal oestrogenic growth-promoting compounds, the structures of which are similar to estradiol. They are illegally used as implants and feed additives for animal growth promotion [67]. This group of molecules includes zeranol, β-taleranol, zearalenone, α-zearalanonle, β-zearalanonle and zearalanone. To our knowledge, MIPs have not yet been employed for the analysis of resorcylic acid lactones in food.

3.2. Analysis of group B substances

Group B includes substances for which a MRL has been established [1]. The control of pharmaceuticals in edible matrices is based on the need to avoid reaching the recommended maximum acceptable daily intake (ADI) in humans. All pharmacological substances intended for use in food-producing animals have to undergo a safety assessment based on the ADI value for humans who would consume their residues through food. For drugs with established MRL values, the most suitable tissues for analysis are those that are intended for human consumption, including muscles, organs (liver, kidney) and milk. Existing methodologies for the surveillance of group B substances can be divided into *screening* and *confirmatory* methods. Screening methods are used to detect the presence of an analyte or analyte class (*e.g.* a group of antimicrobial agents) in the concentration of interest. Confirmatory methods provide unequivocal identification, and if necessary, quantification of analytes. Analytical methodologies must be validated and fulfil the legal criteria described in Commission Decision 2002/657/EC [33]. However, food samples are usually very complex matrices, and a pre-treatment step is thus required. Hence, several solid-phase extraction (SPE) sorbents have been developed for clean-up of edible matrices because of their low cost, time savings and simplicity. In the last few years, MIPs have been increasingly exploited as selective sorbents for MISPE of food samples (Tables 2 and 3).

3.2.1. Group B1: antibacterial substances including sulphonamides and quinolones

Numerous antibacterial substances are used in animal husbandry, not only for the prevention or treatment of disease, but also for growth promotion. The potential effects of their residues in edible products include the development of allergies and drug-resistant bacterial strains that reduce the efficacy of antibiotic treatments in humans. Therefore, the application of antibacterial agents in veterinary medicine has been subjected to regulation and maximum residue limits have been set. Because of the emergence of microbes resistant to antibiotics that are used to treat human and animal infections, the European Commission has decided to phase out (January 1, 2006) the marketing and use of antibiotics as growth promoters in animal feed [68]. This political decision is based on the precautionary principle

Table 2. Application of molecularly imprinted technology to the analysis of group B1 substances (Council Directive 96/23/EC) in foodstuff.

Target Analytes	Matrice	Template	Monomer/CL/Initiator	Porogen	Polymerisation method	Mode	Analytical system	Ref.
CTC	Fish	CTC	MAA/EGDMA/BPO	ACN	bulk polymerisation	MIP-based ISE	potentiometry	[78]
TCs	Pig kidney	OTC	MAA/EGDMA/AIBN	DMSO	bulk polymerisation	MISPE	HPLC-UV	[69]
TCs	egg, fish, animal feeds	TC	MAA+GMA/TRIM/AIBN	MeOH/ACN (30:35 v/v)	precipitation polymerisation	Hydrophilic MIP-based IES	Voltammetry	[80]
TCs	serum, urine	TC	2-VP/EGDMA/BPO	ACN	bulk polymerisation	IESs	Potentiometry	[81]
TCs	chicken feed and muscle, milk	TC	AA/TRIM/AIBN	acetone	coating of silica fibers	SPME fibers	HPLC-FLD	[76]
TCs	milk and honey	TC	MAA/EGDMA/AIBN	MeOH/cyclohexanol/ dodecanol	Monolithic polymerisation	MISPE-On line	HPLC-DAD	[77]
TCs	eggs, chicken	OTC	MAA/DVB/AIBN	etanol:water (9:1, v/v)	magnetic MIP	MISPE	HPLC-MS/MS	[71]
TCs	fish	TC	MAA/EGDMA/AIBN	MeOH	bulk polymerisation	MISPE-On line	FI-CL	[75]
TCs	egg	TC	MAA/TRIM/AIBN	MeOH/ACN (30:35 v/v)	precipitation polymerisation	MISPE-On line	HPLC-DAD	[72]
TCs	lobster, duck, honey, eggs	TC	MAA/TRIM/AIBN	MeOH/ACN (30:35 v/v)	precipitation polymerisation	MISPE	HPLC-MS/MS	[70]
TCs	Lobster, milk and honey	OTC+CTC	MAA/TRIM/AIBN	MeOH/ACN	precipitation polymerisation	MISPE-On line	HPLC-UV	[73]
TCs	egg samples	TC+OTC	MAA/TRIM/AIBN	MeOH/ACN (25:40 v/v)	precipitation polymerisation	MISPE-on line	HPLC-DAD	[74]
TCs	water	TC	MAA+$La(NO_3)_3$/EGDMA/AIBN	MeOH/water (9:1; v/v)	PVC membrane coating	MIP-based ISE	Potentiometry	[79]
BLAs	urine (human)	AMX	MAA/EGDMA/AIBN	ACN	bulk polymerisation	MISPE	LC-UV	[87]
BLAs	river and tap water	PEN G	Urea-based+MAA/EGDMA/ ABDV	ACN	bulk polymerisation	MISPE-On line	HPLC-DAD	[85]
BLAs	milk	PEN V	MAA/EGDMA/AIBN	water:ethanol (9:1, v/v)	magnetic MIP	MISPE	HPLC-MS/MS	[82]
BLAs	river and tap water	Nafc	MAA/EGDMA/ABDV	MeOH	bulk polymerisation	MISPE	HPLC-DAD	[86]
BLAs	yogurt, milk, water	PEN G	MAA/EGDMA/AIBN	DMF	bulk polymerisation	MISPE	Spectrophotometry	[83]
BLAs	fish	AMX	MAA/EGDMA/BPO	MeOH	PVC membrane coating	MIP-based ISE	Potentiometry	[84]
MACs	pork, water	ERY	MAA/EGDMA/AIBN	MeOH:ACN (2:3, v/v)	bulk polymerisation	MISPE	HPLC-FLD	[90]
MACs	chicken	ERY	Acryloyl-β-CD+MAA/EGDMA/ AIBN	ACN	grafting	MISPE	HPLC-UV	[91]
Qns	urine (human)	CIP	MAA/EGDMA/AIBN	DCM	bulk polymerisation	MISPE	HPLC-UV	[96]
FQs	chicken tissue	OFLO (dummy template)	2-HEMA/EGDMA/AIBN	MeOH:water (9:1, v/v)	bulk polymerisation	MISPE	HPLC-DAD	[16]
Qns	milk	OFLO	2-HEMA/EGDMA/AIBN	MeOH:water (10:1, v/v)	bulk polymerisation	MISPE	HPLC-UV	[92]
Qns	fish, chicken muscle	ENR	APTES/TEOS	N-dimethylformamide	precipitation in silica-gel	MISPE-on line	HPLC-FLD	[101]
FQs	baby foods	Not avaliable(commercial MIP): SupelMIP™ SPE-FQs cartridges, from Sigma-Aldrich				MISPE	LC-FLD	[104]
FQs	water	ENR	MAA/HEMA/ABDV	ACN	precipitation polymerisation	MISPE-on line	HPLC-FLD	[103]
FQs	water	CIP	MAA/EGDMA/AIBN	MeOH	precipitation polymerisation	MIP-MEPS off line	HPLC-MS/MS	[98]
Qns	water	ENR	URE-based+MAA/EDMA/ABDV	ACN	bulk polymerisation	MISPE	HPLC-FLD	[99]
FQs	urine	ENR	MAA+HEMA/EGDMA/ABDV	ACN	bulk polymerisation	MISPE	HPLC-FLD	[100]
Qns, FQs	milk	PEF	MAA/DEGDMA/AIBN	MeOH:water (10:3, v/v)	monolithic polymerisation	PMME off line	HPLC-FLD	[95]
Qns	urine	OFLO	MAA/TRIM/AIBN	MeOH:water (9:1, v/v)	bulk polymerisation	MISPE	HPLC-DAD	[97]
FQs	human urine and pig liver	ENR	MAA/EGDMA/AIBN	Dichloromethane	bulk polymerisation	MISPE	HPLC-UV	[6]
Qns	baby foods	CIP	MAA/EGDMA/AIBN	MeOH	precipitation polymerisation	MISPE	HPLC-UV	[105]
FQs	Bovine raw milk, pig kidney	Not avaliable(commercial MIP): SupelMIP FQs SPE column, from Supelco				MISPE	CE-LIF	[93]
Qns	cow milk(UHT)	Not avaliable(commercial MIP): SupelMIP Qns SPE column, from Supelco				MISPE	capillary HPLC-LIF	[94]
Qns	pork muscle	NOR	MAA/EGDMA/AIBN	DMF:DMSO (1:5 v/v)	Monolithic polymerisation	MISPE-on line	HPLC-DAD	[102]

Continued Table 2. Application of molecularly imprinted technology to the analysis of group B1 substances (Council Directive 96/23/EC) in foodstuff.

Target Analytes	Matrice	Template	Monomer/CL/Initiator	Porogen	Polymerisation method	Mode	Analytical system	Ref.
SAs	shrimp and fish	SDM	MAA/EGDMA/AIBN	ACN	bulk polymerisation	MISPE	HPLC–UV	[111]
SAs	milk, buttermilk	SMZ	MAA/EGDMA/AIBN	ACN	bulk polymerisation	MISPE	Voltammetry	[107]
SAs	eggs and milk	SMO	AA/EGDMA/AIBN	ACN	Si-NP: Silica gel nanoparticles SMO-MIP: bulk polimerization	MISPE	HPLC-UV	[106]
SAs	honey	SMD	MAA+Fe_3O_4/EGDMA/AIBN	DMSO	precipitation polymerisation	MSPE: magnetics MIPS SPE	LC-MS/MS	[110]
SAs	pork and chicken muscle	SMZ	APTES/TEOS	ACN	precipitation in silica-gel	MISPE-on line	HPLC-UV	[108]
SAs	pork, liver and chicken	SMZ	MAA/ EGDMA/AIBN	MeOH	MIP-coated stir bar	MISPE	HPLC-UV	[109]

AA: acrylic acid; MAA: methacrylic acid; EGDMA: ethylene glycol dimethacrylate ; AIBN: azo(bis)-isobutyronitrile; ABDV: 2,2'-azobis(2,4-dimethylvaleronitrile); DVB: divinylbenzene; GMA:Glycidilmethacrylate; BPO: benzoyl peroxide; Urea-based:1-(4-vinylphenyl)-3-(3,5-bis(trifluromethyl)phenyl)urea;TRIM: trimethylolpropane trimethacrylate; DEGDMA: di(ethylene glycol) dimethacrytlate; DCM:dichloromethane; 2-HEMA: 2-hidroxyethylmethacrylate; APTES: 3-aminopropyltriethoxysilane; TEOS: tetraethoxysilicane; VP: vinylpyridine; TC: tetracycline; CTC: chlortetracycline; OTC: oxytetracycline; BLA: β-lactam; AMX: amoxicillin; PEN: penicillin; Nafc: nafcillin; Qns: quinolones; FQs: fluoroquinolones; ENR: enrofloxacin, CIP: ciprofloxacin; NOR: norfloxacin; OFLO: ofloxacin; PEF: pefloxacin; SAs: sulphonamides; SDM: sulfadimethoxine; SMO: sulfamethoxazole; SMZ: sulfamethazine; SMD: sulfamethoxydiazine; MACs: macrolides; ERY: erythromycin; ACN: acetonitrile; MeOH: methanol; IES: ion selective electrode; MISPE: molecularly imprinted solid-phase extraction (off-line); SPME: solid-phase microextraction; FLD: fluorescence detector; MEPS: microextraction by packed sorbent; PMME: polymer monolith microextraction; FI-CL: flow-injection chemiluminescence

(Principle 15 of the Rio Declaration, 1992): 'Where there are threats of serious or irreversible damage, lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation'.

According to Council Directive 96/23/EC, group B1 substance residues are to be detected in bovine, ovine, caprine, porcine and equine animals as well as in poultry and aquaculture and in milk, honey, eggs and rabbit meat, including feed stuff, drinking water and primary products. Therefore, several analytical methods (mainly confirmatory liquid chromatographic separations) have been proposed to monitor antibacterial drugs in biological matrices [2]. However, these methods usually involve preliminary extraction and clean-up steps by liquid-liquid extraction (LLE) or solid phase extraction (SPE). In recent years, SPE has emerged as a simple alternative to LLE due to its low cost and easy automation as well as the availability of a wide variety of commercial cartridges of different materials. SPE extraction is generally applied to remove matrix-interfering substances from complicated samples such as edible products and for the concentration of the target analytes. To reduce the influence of the matrix and to improve the sensitivity of current methodologies, novel and rapid pretreatment methods are required for the analysis of group B1 substances.

Molecular imprinting technology (MIT) has appeared as an interesting solution to solve the problem of recognition ability using conventional SPE materials. In addition, MIT presents a higher specificity and selectivity than conventional SPE materials. In recent years, several methods using MIP materials have been reported for the analysis of antibacterial agents in edible matrices of animal origin (Table 2).

3.2.1.1. Tetracyclines

Tetracyclines (TCs) are applied in veterinary medicine for the treatment and prevention of microbial infections because of their broad antibacterial spectrum against both Gram-positive and Gram-negative bacteria. The most widely used TCs are tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC). Although these compounds are beneficial in animal husbandry, edible products derived from treated animals (*e.g.* meat and milk) may contain residues of tetracycline. These residues could pose a health threat to consumers depending on the type of food and the amount of residue present in the matrix. The European Union has established TC MRL values of 100 μg kg^{-1} in muscle, 300 μg kg^{-1} in liver, 600 μg kg^{-1} in kidney, 100 μg kg^{-1} in milk and 200 μg kg^{-1} in eggs when using these antibacterial chemicals as anti-infectious agents [1] (all values correspond to the sum of the parent drug and its epimers). Additionally, TCs have been forbidden for use as growth promoters (zero tolerance). Numerous analytical methods for the screening, identification and quantification of TCs in animal products have been developed over the years. Most methods use high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS) and tandem mass spectrometry (HPLC-MS/MS) because of their superior sensitivity and selectivity [2].

Several MIT-based applications for the analysis of TCs in edible matrices have been described in the recent years. The use of TCs may leave residues in animal tissues, eggs and fish (complex matrices).Several off-line and on-line MIP applications have been described in recent years for clean-up procedures of these samples. The specificity of off-line MISPE (composed of polymers synthesised by both bulk and precipitation

polymerisation) of TCs has been already described for tissue samples (*e.g.* pig kidney, lobster and duck), honey and eggs [69,70]. In addition, sample preparation may be simplified because of the development of magnetic MIPs [71], consisting of magnetic particles encapsulated by organic polymers. On-line clean-up procedures based on MIT have also been reported for the analysis of TCs in edible matrices such as eggs, fish and lobster [72-75]. In these on-line approaches, whole sample extracts are transferred to the analytical column, improving the sensitivity of the HPLC detection and decreasing the time required for sample pretreatment.

Novel applications of MIT for the solid-phase extraction of milk samples have recently been published. In 2009, Hu *et al.* prepared and evaluated MIP-coated silica fibres for solid-phase micro-extraction of various TCs using TC as the template molecule [76]. These fibres provide very low detection limits that are significantly lower than the MRLs established by the EU and that are suitable for multi-residue analysis of trace amounts of TCs in milk, chicken feed and muscle. In contrast, MIPs can be prepared by an *in situ* molecular imprinting technique and directly used as a monolithic column for HPLC systems. In fact, the selectivity of molecularly imprinted monolithic columns for TC detection has been investigated, and these columns can be employed as precolumns in MISPE-HPLC methods [77]. Results with milk and honey samples indicate that the method can be applied to simultaneously analyse multi-residue samples for very low amounts of TCs. Furthermore, Jing *et al.* reported the preparation of novel MIPs using mixed templates for on-line solid phase extraction of trace amounts of TCs in foodstuff, such as lobster, milk, honey and eggs [73,74]. Their results suggested that polymers derived from mixed templates provide better selectivities and affinities for a group of TCs than those derived from a single template.

Ion-selective electrodes (ISEs) may be advantageous alternatives for highly specific and sensitive measurements of residues in food samples. In addition, ISEs are much less expensive than chromatographic methods for routine purposes. Their selectivities may be enhanced by using MIT to improve analyte recognition, and MIPs acting as carriers provide a means to enhance the selectivity of ISEs membranes. Therefore, some researchers have used MIPs to prepare electro-active materials for the detection of TC antibiotics based mainly on the use of MAA as a functional monomer, EGDMA as a cross-linker and TC as a template molecule [78-81]. These sensors have been successfully applied for the analysis of TCs in fish, eggs and animal feeds as well as in biological and aqueous samples, achieving generally good sensitivity, stability, response time, detection limits and precision.

In summary, tetracycline is the most frequently applied template for MIP synthesis for the analysis of TCs in foodstuff, even when mixed templates appear more appropriate. In addition, MAA appears to be the most interesting functional monomer, whereas EGDMA and TRIM appear to be the most frequently used cross-linkers.

3.2.1.2. Beta-lactams

Beta-lactam antibiotics (BLAs) constitute one of the most widely used anti-microbial drug classes in veterinary medicine, especially for the treatment of dairy cattle. The most important classes of molecules in this antibiotic group are the penicillins and the cephalosporins. The European Union has established MRLs for the presence of these chemicals in the muscle, fat, liver, kidney and milk of all food-producing animals [1]. Therefore, rapid, selective and simple multi-residue methods for the analysis of BLAs in milk are required. The applicability of MIPs as selective sorbents for the extraction of BLAs in milk has already been reported [82,83]. In comparison to conventional SPE methods, magnetic MIPs (MMIPs) provide a selective, relatively rapid and convenient method to extract trace amounts of BLAs form complex matrices by applying an external magnetic field. This approach has been successfully used for the extraction of BLAs from milk samples with high affinity and selectivity and with good recoveries and low variability [82]. Moreover, a novel amoxicillin MIP-based ISE was recently reported by Guerreiro *et al.* for the analysis of amoxicillin in fish samples [84]. This new sensor was designed as a screening tool; however, its detection limit was higher than desired.

The broad application of BLAs and their emergence as contaminants in the environment, particularly in water, have raised concerns regarding their environmental impact. One of the main limitations of using MIPs for SPE is their poor performance in aqueous media. However, a few analytical methods based on MISPE protocols have been published recently for selective and reliable detection of trace amounts of BLAs in water samples [83,85,86]. Reported MISPE sorbents designed for the selective pre-concentration of β-lactam antibiotics in water allowed high recovery, precision and sensitivity with the HPLC method, despite the low concentrations of BLAs in water samples [85,86]. Furthermore, MIPs perform well as SPE sorbents for biological aqueous samples, such as urine [87]. For urine samples, molecularly imprinted SPE sorbents, designed to extract amoxicillin in an off-line mode, show a useful cross-

selectivity with structurally related antibiotics [87]. In addition, MISPE cartridges provided cleaner extracts in comparison to commercial SPE cartridges, which allows easy quantification of AMX in urine at clinically relevant concentrations.

In summary, penicillins G and V, amoxicillin and nafcillin (pseudo-template) are BLAs that have to date been used as templates in the design of MIPs for analytical purposes. MAA appears to be the most appropriate functional monomer, and EGDMA appears to be the most commonly used cross-linker.

3.2.1.3. Macrolides

Antibiotics of the macrolide-lincosamide group have been used in the worldwide treatment of food-producing animals for several decades. The most frequently used macrolide agents are lincomycin and tylosin for controlling dysentery and *Mycoplasma* infections in swine, and spiramycin for treating mastitis in cattle [88]. Tylosin has also been the most frequently used agent for growth promotion in swine production, whereas spiramycin has been the most frequently used agent in poultry. The use of macrolides for growth promotion has been banned in all European Union countries since July 1999. High erythromycin (ERY) resistance in pigs may be related to extensive veterinary use of macrolides [89]. The European Union has established different MRLs for the presence of these chemicals in the muscle, fat, liver, kidney, milk and eggs of all food-producing animals.

Few reports exist on the application of MIT to analyse macrolides in foodstuff. Recently, Song *et al.* reported the synthesis of MIPs for MISPE of ERY from pork muscle and tap water. These polymers showed a moderate cross-reactivity for the extraction of other macrolides [90]. Conversely, a new surface-imprinting technique has been reported to synthesise multi-walled carbon nanotube MIPs for use as SPE sorbents in the analysis of chicken muscle that provide very good recoveries [91]. In summary, ERY is to the best of our knowledge the only macrolide thus far used in the synthesis of MIPs for the analysis of macrolide drugs in edible matrices.

3.2.1.4. Quinolones

Quinolones (Qns) and fluoroquinolones (FQs) are among the most important classes of synthetic antibiotics for human and veterinary medicine worldwide. They are used for the treatment of a wide variety of infections in food-producing animals, leaving residues of the antibiotics in foodstuff. These residues may adversely affect humans, causing allergic reactions or antibiotic resistance. Thus, Commission Regulation 37/2010 established MRLs for different Qns and FQs in raw foods when they have been used as anti-infective agents, but not when they have been used as growth promoters [1]. These MRLs have been established for different target matrices such as muscle, fat, liver, kidney and milk at different maximum residue amounts depending on the animal species and the target compound. Several animal species de-ethylate enrofloxacin (ENR) to its primary metabolite ciprofloxacin (CPX); therefore the MRL for ENR has been set based on the sum of ENR and CPX.

MRLs in milk range from 30 µg kg^{-1} (for danofloxacin) to 100 µg kg^{-1} (for the sum of ENR and its metabolite CPX) [1]. Recently, several applications of MIT for the analysis of milk and other aqueous samples (*e.g.* water and urine) have been reported. Among these applications, MISPE methodologies are promising alternatives to classic SPE for providing purer extracts. Water-compatible MIPs designed to extract Qns and FQs allow the matrix compounds present in milk samples to be effectively removed for further analysis of the extract [92-94]. For MISPE procedures, commercially available MIPs have become a common alternative for the treatment of complex matrices such as milk [93,94]. In addition, polymer monolith micro-extraction (PMME) is a novel approach that has been applied to the analysis of various FQs in milk samples and that allows simple and rapid sample preparation [95]. Furthermore, several methods for the analysis of Qns and FQs in other aqueous matrices, including water and urine, have been successfully developed with MIT [6,96-100].

Residues from Qns and FQs can be found in animal tissues; subsequent MRLs have therefore been established for these kinds of matrices, depending on the target tissue (muscle, fat, liver, kidney skin and fat) and the animal species [1]. Several MISPE procedures have been described for the analysis of Qns and FQs in tissue samples at sub-MRLs [6,16]. Novel applications of MIPs include their use as enrichment sorbents for on-line MISPE-HPLC methods for the analysis of Qns in fish and chicken muscle [101], pork muscle [102] and FQs in water samples [103]. This on-line procedure is effective for the detection of trace amounts of these pharmaceuticals in fish, chicken and water (with good recoveries), demonstrating the application of these MIPs as reliable and robust sorbents. In addition, it is important to stress that babies and infants are more sensitive to exposure to these chemicals. Consequently, analytical methods have been developed (using MISPE clean-up) that are suitable for the detection of Qn and FQ residues in baby food [104,105].

Table 3. Application of molecularly imprinted technology to the analysis of group B2 and B3 substances (Council Directive 96/23/EC) in foodstuff.

Target Analyte	Matrice	Template	Monomer/CL/Iniciator	Porogen	Polymerisation method	Mode	Analytical system	Ref.
Anthelmintics (BZ)	Water	TBZ	MAA/DVB/AIBN	ACN:TOL (75:25 v/v)	Precipitation polymerisation	MISPE Off-line/ On-line	HPLC-DAD	[113]
Anthelmintics (BZ)	Water	TBZ	MAA/DVB/AIBN	MeOH/Acetic acid (50:50 v/v)	Precipitation polymerisation	MISPE On-line	HPLC-UV	[114]
Anthelmintics(AVM)	Aqueous samples	AVM	MAA/EGDMA/AIBN	ACN	Bulk polymerisation	MISPE Off-line/In-line	HPLC-UV	[115]
Sedatives	-	PZ	MAA/EGDMA/AIBN	Toluene	Polymerisation in silica beads	MISPE In-line	HPLC	[28]
Sedatives	Fish	CPZ	MAA/EGDMA/BPO	EtOH	Bulk polymerisation	MIP-based ISE	Potentiometry	[116]
Sedatives	Pig urine	CPZ	MAA/TRIM/AIBN	DCM	Bulk polymerisation	MISPE	HPLC-UV	[117]
NSAIDs	River water	FFA	MAA, 2-VP, 4-VP/EDMA/ABDV	TOL	Multi-step swelling	MISPE On-line	HPLC–MS/MS	[118]
Dyes (MG)	Fish	MG	MAA/ EGDMA/AIBN	ACN	Bulk polymerisation	MISPE	ECL	[119]
Dyes (MG)	Fish water, fish feed	MG	MAA/ EGDMA/AIBN	Chloroform	Bulk polymerisation	MISPE	HPLC-UV	[120]
Dyes (MG)	Salmon	Not avaliable (commercial MIP): ExploraSep™, from Supelco				MISPE	LC-MS/MS	[121]

MAA: methacrylic acid; EGDMA: ethylene glycol dimethacrytlate ; AIBN: azo(bis)-isobutyronitrile; ABDV: 2,2'-azobis(2,4-dimethylvaleronitrile) ; DVB: divinylbenzene; BPO: benzoyl peroxide; TRIM: trimethylolpropane trimethacrylate; DCM:dichloromethane; VP: vinylpyridine; TBZ: thiabendazole; MG: Malachite green; FFA: Flufenamic acid; NSAIDs: Non-steroidal anti-inflamatory drugs; BZ: Benzimidazoles; AVM: Avermectin; CPZ: chlorpormazine; PZ: promazine; ; ACN: acetonitrile; MeOH: methanol; EtOH: ethanol; TOL: toluene; IES: ion selective electrode; MISPE: molecularly imprinted solid-phase extraction (off-line); ECL: electrochemiluminescence

In conclusion, ofloxacin (OFLO) and ENR have been the best options as template molecules and MAA is the most appropriate functional monomer in the design of MIPs for the analysis of Qns and FQs in foodstuff.

3.2.1.5. Sulphonamides

Sulphonamides (SAs) are antibacterial agents that are applied worldwide to prevent and control numerous bacterial diseases in farm animals and aquaculture. However, SAs cause antibiotic resistance in bacterial strains with potential implications for human health and the environment. Therefore, the EU has established that the combined total residue from all substances of the sulphonamide group should not exceed 100 μg kg^{-1} in the milk, muscle, fat, liver and kidney of any food-producing animals [1]. Consequently, several quantitative analytical methods have been developed to detect the presence of SAs in different edible matrices.

Recently, a selective extraction procedure for SAs present in milk and eggs was reported based on the use of silica-coated MIP nanoparticles [106]. This novel and off-line MISPE-HPLC method permits the efficient enrichment of SAs at low concentrations with good recoveries from spiked milk and eggs. The synthesis and evaluation of MIPs as selective sorbents for on-line solid-phase extraction of SAs in milk have also been reported [107]; these methods allowed recoveries of nearly 100%. A few methods combining MIT with different analytical platforms have been described in the past few years to detect the presence of SAs in animal tissues. For instance, He *et al.* described the synthesis of a highly selective, imprinted, amino-functionalised silica gel sorbent for the on-line MISPE extraction of pork and chicken muscle [108]. This sorbent appears to be useful for the selective adsorption and subsequent detection of SAs in trace amounts in these matrices with good linearity and precision. Additionally, the selective extraction of sulpha drugs with an MIP-coated stir bar offers a simple method with satisfactory recoveries from pork, liver and chicken samples [109]. A different approach utilises magnetic MIPs: a good alternative to traditional SPE techniques that can be used to efficiently extract SAs from honey [110]. In 2011, another analytical method was described, which employs group-selective MISPE cartridges for the simultaneous detection and quantification of SAs in aquaculture products [111].

Overall, sulfadimethoxine (SDM) and sulfamethazine (SMZ) appear to be the most appropriate template molecules, and acrylic acids are the best functional monomers for the design of MIPs to detect SAs in edible matrices.

3.2.2. Group B2: other veterinary drugs

Group B2 consists of veterinary drugs or veterinary medicinal products (VMPs) that are not antibacterial substances: anthelminthics, coccidiostats, (*e.g.* nitroimidazoles), carbamates, pyrethroids, sedatives, non-steroidal anti-inflammatory drugs (NSAIDs) and other pharmacologically active substances [31]. In this group, some substances are regulated by established MRLs [1]; others do not have established MRLs.

Because of the importance of studying and monitoring the presence of these drugs in foodstuff, several analytical techniques have been developed for their analysis, including LC coupled to photo-diode array detection (DAD), LC-MS and GC-MS. However, the lack

of volatility and the thermal instability of many group B2 chemicals make LC-MS the method of choice for their analysis. With the rapid development of the MIT, MIPs have been widely applied (*e.g.* solid-phase extraction, chemical sensors and artificial antibodies) because of their high selectivity, physical robustness, thermal stability, as well as low cost and easy preparation [112]. Consequently, various methods using MIP materials have been reported for the analysis of group B2 drugs in food products of animal origin (Table 3).

3.2.2.1. Anthelminthics(B2a)

An important aspect of efficient animal production is the effective control of helminthic infections. This parasitic infection has long been of major economic importance in animal husbandry. Anthelminthic drugs are chemicals used to treat these parasitic infections and include the benzimidazoles (BZs), flukicides (FCs), levamisole, macrocylic lactones (MLs) and morantel.

A number of specific methods can detect residues from drugs used to treat anthelminthic infections in several matrices [3]. However, the use of MIPs for the detection of anthelminthic chemicals in foodstuff is rather scarcely reported [2]. The best-studied matrices are water samples, using thiabendazole (TBZ) or avermectin as template molecules for MIP synthesis [113-115]. The synthesised MIPs can be applied for the analysis of a wide range of benzimidazole compounds in aqueous samples using off-line, on-line and in-line approaches. Based on previous results, MAA appears to be the most suitable monomer for the development of MIPs to analyse benzimidazoles.

3.2.2.2. Anticoccidials (B2b)

Coccidiostats are used for the treatment and prevention of coccidiosis, a contagious amoebic disease (*Eimeria spp.*). Because of the intensive nature of the poultry industry, it is economically essential to control this disease. Several compounds have been used as anticoccidial drugs, including sulphonamides, pyridones, 4-hydroxyquinolines, carbanilides, thiamine analogues, quinazolinones, guanidine derivatives, ionophores and nitroimidazoles. To our knowledge, there are no reports on the application of MIPs for the analysis of these substances in foodstuff.

3.2.2.3. Carbamates and pyrethroids (B2c)

Pyrethroids are synthetic insecticides (derived from naturally occurring pyrethrins) that combine efficacy, safety, low environmental hazard and photostability [3]. Pyrethroid insecticides are typically highly lipophilic esters of chrysanthemic acid. Carbamate insecticides exist as esters of carbamic acid. To our knowledge, there are no published reports on the application of MIT for the analysis of carbamates and pyrethroids in foodstuff.

3.2.2.4. Sedatives (B2d)

Veterinary tranquilisers or sedatives are often used in animal production to prevent mortality during handling and transport to the slaughterhouse, especially with pigs that are particularly sensitive to stress. In pigs, stress leads to high mortality and poor meat quality (Pale, Soft, Exudative; PSE). Since the 1970s, sedatives have been extensively used to calm down pigs before transport to the slaughterhouse. The use of certain substances (derived from phenothiazine: acepropionyl and chlorpromazine) is prohibited, although other substances, including butyrophenone (azaperone) and β-blockers (carazol), are permitted but regulated by established MRLs [1].

Several studies have described the application of MIT for the analysis of sedatives. Recently, Felismina *et al.* designed host-tailored sensors for the analysis of chlorpromazine (CPZ) in fish samples [116]. Chlorpromazine-based MIPs have also been applied for the solid-phase extraction of CPZ in pig urine [117]. Additionally, other reports describe the use of promazine as a template in the synthesis of molecularly imprinted stationary phases [28] for the separation of triazine herbicides. MAA appears to be the most appropriate monomer in the design of polymers for the analysis of sedatives in foodstuff.

3.2.2.5. NSAIDs (B2e)

Non-steroidal anti-inflammatory drugs (NSAIDs) comprise a heterogeneous group of drugs that includes mostly acidic chemicals such as salicylic acid and pyrazole derivatives. In food-producing animals, the use of these drugs is restricted to registered products for which an MRL has been established. The NSAIDs with established MRLs are carprofen (bovine, equine), vedaprofen (equine), flunixin (bovine, equine, porcine), tolfenamic acid (bovine, porcine) and meloxicam (bovine, equine, caprine, porcine, rabbit). Two NSAIDs do not have established MRLs (ketoprofen and salicylates), and their use is not permitted in farm animals [1].

Restricted access media — molecularly imprinted polymer (RAM-MIP) for flufenamic acid, prepared by multi-step swelling and polymerisation followed by a hydrophilic surface modification, has been developed for the detection of NSAIDs in river water samples. The simultaneous detection of five NSAIDs (flufenamic acid, mefenamic acid, indomethacin, etodolac and ketoprofen) was performed accurately and with good reproducibility by LC-MS/MS using RAM-MIP in the pretreatment step with an on-line column [118].

3.2.2.6. Other pharmacologically active substances (B2f)

The most important substances in the B2f group are the corticosteroids. The application of MIT in the analysis of corticosteroids in edible matrices was discussed in the the context of group A substances (see section 3.1.2. Corticosteroids)

3.2.3. Group B3: Other substances and environmental contaminants

Group B3 includes organochlorine compounds (*e.g.*, PCBs), organophosphorus compounds, chemical elements, mycotoxins and dyes.

The triphenylmethane dyes (TPMs) include malachite green (MG) that is used illegally in aquaculture to treat and prevent fungal and parasitic infections. MG is not registered as a veterinary drug and has potential toxicity, teratogenicity and carcinogenicity. A few methods have recently been published on the analysis of MG in different matrices using MISPE procedures in an off-line mode (see Table 3). For example, Guo *et al.* successfully developed an electrochemiluminiscence (ECL) inhibition method combined with MISPE for the quantitative detection of MG in fish [119]. Furthermore, Li *et al.* prepared MIPs as an alternative SPE sorbent for the group-selective extraction of not only MG but also leucomalachite green (LMG) and crystal violet (CV) from fish water and fish feed samples [120]. Overall, MAA appears to be the most appropriate monomer in the design of polymers for the analysis of these substances. In contrast, it has been found that commercial MIPs in combination with solid-liquid extraction (SLE) can be used to detect MG in salmon by HPLC-MS/MS [121]. This procedure reduces matrix effects and provides good precision and accuracy with salmon samples, achieving very low limits of detection for MG.

4. Conclusions

At present, MIT is considered a powerful tool in the development of highly selective analytical methods to detect residues of veterinary drugs in foodstuff. The successful performance of MIPs as selective sorbents in SPE (MISPE) has been thoroughly described in this review. Although bulk polymerisation is one of the most frequently used polymerisation methods, additional novel techniques are emerging for polymerisation in the MIT field. For example, precipitation polymerisation has been frequently reported in recent applications of MIPs for the analysis of residues in foodstuff. Precipitation polymerisation is simpler and easier than bulk polymerisation and provides particles with controlled morphologies. Furthermore, several companies already sell MISPE cartridges for the extraction of certain analytes, and their use is increasing. In addition, novel applications of MIPs are emerging and include the development of MIP-based sensors, LC stationary phases and magnetic MIP-based sorbents. The direct coupling of MIP-LC columns in-line with the analytical detection system will lead to very simple analytical methods for routine laboratory use.

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