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DESIGN AND APPLICATION OF MOLECULARLY IMPRINTED POLYMERS (MIPs) FOR DETECTION OF VETERINARY RESIDUES AND CONTAMINANTS IN FOOD SAFETY

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Design and application of molecularly imprinted polymers (MIPs) for detection of veterinary residues and contaminants in food safety

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Design and application of molecularly imprinted polymers (MIPs) for detection of veterinary residues and contaminants in food safety

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RESUMO

Hoxe en día a seguridade alimentaria é unha das grandes preocupacións da sociedade debido ás numerosas alarmas sanitarias que se foron xerando co paso dos anos. Entre estas alarmas alimentarias, cabe destacar algunhas das máis relevantes dentro da Unión Europea, como o caso da "enfermidade das vacas tolas" ou encefalopatía esponxiforme bovina (EEB), a contaminación de polos con dioxinas en Bélxica e a retirada do mercado de fórmulas infantís fabricadas en Francia por un brote de salmonelose en nenos do mesmo país, entre outras. A alerta máis recente en España foi a causada por un brote de *Listeria monocytogenes* relacionado con produtos cárnicos fabricados no sur do país que envelenaron a centos de persoas ata o momento, causándolles a morte nalgúns casos. Listeria é unha bacteria perigosa que preocupa moito na industria alimentaria, xa que as cifras demostran que nos últimos anos non deixan de incrementarse os casos. Estas crises son algúns dos moitos exemplos que reflicten a crecente demanda de información sobre a presenza nos alimentos de contaminantes ou residuos de produtos nocivos, tóxicos ou perigosos para a saúde.

Os perigos químicos son unha das principais causas de contaminación dos alimentos asociadas tamén a este tipo de brotes. Dentro dos contaminantes que afectan ós alimentos inclúense os contaminantes ambientais, os referentes ó procesamento, os adulterantes e aditivos alimentarios non aprobados, así coma aqueles procedentes de materiais do propio empaquetado. Ademais, existe unha gran variedade de compostos orgánicos que se atopan nos alimentos como consecuencia non só do seu uso permitido, senón tamén debido a procesos naturais ou á acción do ser humano. Os compostos orgánicos que aparecen nos alimentos debido ó seu uso durante a etapa de produción ou almacenamento (praguicidas, medicamentos veterinarios), clasifícanse como residuos, mentres que os compostos que se foron xerando de forma natural durante a produción, o almacenamento ou a transformación de alimentos (micotoxinas, biotoxinas mariñas) ou debido ás actividades antropoxénicas (dioxinas, bifenilos policlorados similares ás dioxinas), denomínanse contaminantes. Existe gran preocupación tanto por parte dos organismos oficiais como da industria alimentaria, en ter controlado todo o que vai "do campo á mesa", derivando na necesidade de controlar todo tipo de substancias contaminantes ou residuos ó longo da cadea alimentaria. Ademais, é preciso analizalos ós moi distintos niveis nos que se atopan nos alimentos, tanto os residuos de medicamentos veterinarios coma o resto de contaminantes, e que supoñen un risco significativo para a saúde humana.

A gran cantidade de alimentos que están ó alcance do consumidor pode darnos unha idea da gran variedade de análises que se requiren para a súa detección nos laboratorios de control. Ademais, as baixas concentracións ás que se atopan usualmente os residuos de medicamentos veterinarios nas matrices alimentarias, así como os contaminantes, fan indispensable a necesidade de métodos analíticos altamente sensibles para a súa detección, identificación e cuantificación. O método analítico ideal debería combinar o illamento nunha soa fase, a preconcentración e a determinación cuantitativa dos analitos cunha boa relación custo-eficacia. Debido á complexidade das matrices alimentarias, a detección directa de analitos en

mostras reais acostuma ser difícil. Para acadar o éxito analítico, é preciso aplicar protocolos de limpeza eficaces que permitan eliminar as posibles interferencias e extraer posteriormente os analitos da matriz. Ademais, tamén son necesarios bos métodos de separación cromatográfica para distinguir os analitos máis estreitamente relacionados. O desenvolvemento, a aplicación e a validación dos métodos analíticos axeitados a cada residuo/contaminante e a cada alimento son indispensables para poñer en práctica a lexislación vixente. Dispoñer de medios analíticos para un control eficaz de residuos de medicamentos veterinarios e contaminantes nos alimentos, é polo tanto unha parte indispensable de todo o sistema de seguridade alimentaria a nivel mundial.

Os métodos existentes para determinación de substancias perigosas en alimentos divídense en: métodos de rastrexo ou *screening* e métodos confirmatorios. Ámbolos dous tipos de métodos teñen as súas esixencias específicas, que deben terse en conta á hora de seleccionar unha técnica analítica axeitada ó propósito. Os métodos de *screening* son métodos analíticos empregados para detectar a presenza dunha substancia ou grupo de substancias ó seu nivel de interese, que será usualmente un límite máximo legalmente establecido. Este tipo de métodos poden ser cualitativos ou semicuantitativos e están deseñados para filtrar ou cribar un gran número de mostras e así aforrar custos de análise. Entre eles, uns dos métodos máis empregados son as probas de inmunoabsorción enzimática ou probas ELISA, e as probas de inhibidores microbiolóxicos, entre outros. A obtención de resultados precisos é de grande importancia xa que os resultados falsos-negativos e/ou positivos traen grandes consecuencias tanto para o consumidor como para o produtor. Por outra parte, os métodos confirmatorios utilízanse para completar información que permita a identificación da substancia, así como a súa cuantificación, se é preciso. Estes últimos, a diferenza dos métodos de *screening*, utilizan técnicas cromatográficas combinadas con diferentes detectores como os UV ou os espectrómetros de masas (MS). No campo das técnicas de separación, a tendencia durante anos foi mellorar a velocidade e a potencia de separación, o cal se reflicte nunha serie de cambios, como o paso da cromatografía en capa fina (TLC) á cromatografía líquida de alta resolución (HPLC). Hoxe en día, técnicas como HPLC e a cromatografía líquida de presión ultra-alta (UHPLC) xunto coa espectrometría de masas en tándem de triplo cuadripolo (MS/MS) son aplicadas con frecuencia na análise de alimentos, e son moi eficaces para a análise confirmatoria de residuos, contaminantes, ou outras substancias de interese. En certos casos, como pode ser a determinación de substancias volátiles, resultou de grande importancia a introdución no mercado de novos dispositivos de cromatografía de gases acoplada a espectrometría de masas (GC-MS). A pesar de ter moitas aplicacións, este último tipo de métodos confirmatorios presentan unha desvantaxe importante que é o paso previo de derivatización, o cal fai que o proceso de detección sexa máis longo.

O campo da metodoloxía na análise de residuos de medicamentos veterinarios foi evolucionando ata equipos moi sofisticados como a espectrometría de masas de alta resolución (HRMS), a cal permite unha identificación fiable en matrices de alta complexidade. O número practicamente ilimitado de substancias que poden ser analizadas de maneira simultánea, así como a posibilidade de realizar análises de MS e identificar novos biomarcadores, entre outras, son algunhas das vantaxes desta técnica. Aínda que tamén presenta desvantaxes como os altos custos de investimento, ademais da súa sensibilidade reducida en comparación coa espectrometría de masas de baixa resolución (LRMS ou MS). Recentemente, observouse un incremento no uso de métodos máis modernos como os multiclase e multiresiduo, os cales permiten analizar múltiples compostos pertencentes a diferentes grupos de substancias nunha mesma serie e deste xeito, aforrar tempo e solventes.

O método analítico ideal debería combinar o illamento nunha soa fase, a preconcentración e a determinación cuantitativa de forma rendible. É importante ter en conta que cada paso adicional pode dar lugar a unha maior probabilidade de perdas de analitos, contaminación das mostras e erros analíticos. A detección directa de analitos en mostras reais é con frecuencia complicada xa que as matrices dos alimentos son complexas. Á vista disto, é necesaria a aplicación de protocolos de limpeza eficaces que permitan a eliminación de posibles interferencias e a posterior extracción dos analitos de interese. Neste sentido, introducíronse gradualmente varias técnicas para facilitar a preparación das mostras de alimentos antes da detección de residuos e contaminantes.

Ata o momento, a extracción en fase sólida (SPE) foi unha das técnicas preparatorias máis empregadas na análise de mostras alimentarias debido ó seu baixo custo e fácil automatización. Esta técnica de preconcentración de mostras substituíu case por completo á tradicional extracción líquido-líquido (LLE), e algunhas das súas variantes están ganando popularidade día a día. En particular, xurdiu a tecnoloxía de impresión molecular (MIT), que ofrece una ampla gama de opcións para apoiar e complementar as técnicas analíticas existentes. O uso de polímeros de impresión molecular (MIP) facilita o proceso de extracción grazas ó seu habitual alto grao de selectividade. Na primeira fase de síntese dos polímeros, o analito e mailo monómero funcional póñense en contacto no disolvente (poróxeno) seleccionado para formar o complexo de prepolimerización. Posteriormente, engádese o *cross-linker* ou polímero entrecruzante e finalmente o axente iniciador para que teña lugar o proceso de polimerización. A natureza da interacción entre o monómero funcional e a molécula molde ou *template* é na actualidade un dos criterios máis empregados para a clasificación dos MIP, xa que afecta considerablemente as propiedades do polímero final obtido. O uso do analito diana directamente como *template* pode ser problemático xa que non sempre é posible a eliminación completa da molécula molde dos puntos de alta afinidade do polímero. É necesario ter en conta este aspecto á hora de utilizar os MIP no análise de trazas, xa que se pode producir o "sangrado" ou "*bleeding*" da molécula molde durante o proceso de extracción, ocasionando falsos positivos ou unha cuantificación inexacta do analito de interese. Para intentar resolver este problema, utilízanse os chamados *"dummy templates"* para a síntese dos MIP. Estas moléculas deben ser similares, na medida do posible, en tamaño e forma ó analito de interese, a fin de lograr a obtención de polímeros altamente selectivos. Estes MIP contarán con sitios de unión moi selectivos para o analito obxectivo e incluso para compostos estruturalmente relacionados. Ademais, o uso deste tipo de *templates* é moi frecuente para evitar a manipulación no laboratorio de analitos perigosos como é o caso do estudo das aflatoxinas incluído nesta tese e no cal se empregou a molécula 5,7-dimetoxi-cumarina (DMC) como *template*.

Os polímeros de impresión molecular poden sintetizarse empregando unha ampla variedade de metodoloxías, utilizando diferentes tipos de polimerización, dependendo a selección normalmente da aplicación final do MIP. A polimerización en bloque ou *bulk*, utilizouse tradicionalmente xa que non require dunha instrumentación sofisticada e as condicións de reacción son facilmente controlables. Este tipo de polimerización foi o método máis empregado para a preparación de MIP dende o seu descubrimento. Pese a que se trata dun método fácil de levar a cabo, a polimerización en bloque é un proceso tedioso que require moito tempo, xa que precisa de diferentes procesos de trituración, moedura e cribado para obter partículas de tamaño homoxéneo na orde de 25-50 μm. Frecuentemente este tipo de polimerización produce partículas de tamaño e forma irregulares, o que dá lugar a unha mestura heteroxénea de nanopartículas impresas. Este tipo de partículas ó empaquetarse en

columnas para o seu uso como fases estacionarias en cromatografía poden causar problemas de contrapresión. Ademais, os procesos de moedura e barutado poden causar unha perda substancial de polímero e polo tanto o rendemento da polimerización vese reducido drasticamente. Para superar este tipo de limitacións, existen outros métodos de polimerización como son a polimerización por suspensión ou a polimerización por precipitación. Neste traballo de investigación, tanto a clásica polimerización en bloque como a polimerización por precipitación foron empregadas no deseño de diferentes MIP para extracción de residuos veterinarios e contaminantes presentes en alimentos.

A polimerización por precipitación é unha técnica relativamente novidosa, utilizada para obter nanopartículas esféricas de MIP. Este procedemento é similar á polimerización en bloque, só que neste caso emprégase unha mestura de reactivos moito máis diluída. Como consecuencia, o crecemento das cadeas de polímeros en formación, non ocupa o volume total do disolvente empregado, producindo unha dispersión das partículas esféricas do polímero dentro da disolución. En xeral, as microesferas ou "boliñas" obtidas teñen un diámetro de aproximadamente 1 µm, dependendo das condicións de síntese. Para chegar a obter partículas de maior tamaño, é preciso axustar os parámetros de solubilidade do *cross-linker* cos dos disolventes poroxénicos empregados. O control da morfoloxía de partícula é, xunto co alto rendemento do polímero obtido, unha das principais vantaxes desta técnica de polimerización. Ademais, está demostrado experimentalmente que as partículas obtidas por precipitación teñen unha maior capacidade de transmisión de fluxo e unha distribución máis homoxénea dos sitios de unión polímero-analito, a diferenza das partículas obtidas mediante polimerización en bloque. Unha vez obtido o polímero, este sométese a un procedemento de limpeza no que se retira o *template*, deixando os sitios de unión libres na rede polimérica que forma o entrecruzante, sendo estes sitios complementarios en forma, tamaño e orientación dos grupos funcionais ó *template* empregado na súa síntese. Esas cavidades permiten que o polímero obtido recoñeza o *template* e outros compostos estruturalmente análogos.

Dende a súa primeira aplicación, o campo dos MIP foi adquirindo unha importancia crecente, xa que constitúen unha poderosa ferramenta analítica que proporciona unha maior selectividade para a súa aplicación en análises tanto químicas como físicoquímicas. Hoxe en día, unha das súas aplicacións máis empregadas é na extracción en fase sólida (SPE), a denominada extracción en fase sólida con polímeros de impresión molecular ou MISPE. Os MIP están deseñados como anticorpos artificiais, capaces de volver a unirse ó *template* empregado durante a síntese, de forma específica. Por outro lado, é posible combinar este tipo de polímeros intelixentes con diferentes materiais, como a sílice, a magnetita, o vidro, o carbono, o polietileno, entre outros materiais. O proceso de *coating* ou revestimento con silano e/ou as técnicas de enxerto ou *grafting* na superficie de soportes físicos, son algunhas das solucións empregadas para combinar este tipo de polímeros con fibras, *quantum dots*, *films*, nanotubos ou perlas de diferentes materiais. Ata a data, os MIP veñen demostrando amplamente a súa idoneidade como absorbentes para a extracción de múltiples residuos e contaminantes nos alimentos. Neste proxecto de investigación de tese de doutoramento, titulado "Deseño e aplicación de polímeros de impresión molecular (MIP) para a detección de residuos veterinarios e contaminantes no campo alimentario", levouse a cabo unha extensa revisión bibliográfica dos avances na ciencia dos polímeros de impresión molecular, incluíndo información sobre os diferentes materiais, técnicas de polimerización, monómeros funcionais, *templates*, *cross-linkers*, iniciadores e/ou outros formatos existentes. Pola súa vez, deseñáronse e sintetizáronse en laboratorio MIP específicos para a extracción de fármacos

veterinarios (corticosteroides, anfenicois e penicilinas) e micotoxinas (aflatoxinas e patulina) en matrices alimentarias (leite, leite en po, produtos para bebés e mazás).

O gran potencial deste tipo de polímeros no campo da química analítica evidénciase na literatura, así como o gran número de factores que se deben ter en conta no seu deseño e aplicación. Así, un primeiro obxectivo logrouse coa publicación de dous artigos de revisión e un capítulo nunha enciclopedia. En base a estes traballos, a eficacia dos MIP como sorbentes selectivos en extraccións de fase sólida de contaminantes e/ou residuos en alimentos foi meticulosamente demostrada. O uso extensivo do ácido metacrílico (MAA) como monómero funcional en síntese de MIP está xustificado pola súa capacidade para interactuar con case calquera *template*. En canto ós *cross-linkers*, o dimetilacrilato de etilenglicol (EGDMA) e o trimetilolpropano (TRIM) son as dúas alternativas globalmente máis populares, mentres que o DVB foi especificamente preferido para polimerización por precipitación. En canto ás aplicacións analíticas, observouse que a extracción MISPE é de lonxe a máis frecuente aplicación destes polímeros intelixentes en análise alimentaria. Por outro lado, levouse a cabo o deseño e a aplicación de diferentes técnicas de síntese de MIP que se empregaron como absorbentes clásicos para a extracción selectiva en fase sólida (MISPE) de diferentes residuos veterinarios: o grupo dos corticoides e os grupos de antibióticos anfenicois e penicilinas. Deste xeito, os resultados dun segundo obxectivo quedan reflectidos en tres artigos orixinais publicados en revistas científicas con revisión por pares. A aplicación dos protocolos MISPE levouse a cabo con éxito para analizar mostras de leite e produtos lácteos con recuperacións satisfactorias (85-150% para os corticosteroides, 91-110% para os anfenicois e 56-88% para as penicilinas). Por outro lado, nun terceiro obxectivo probouse o deseño dos MIP combinados con soportes magnéticos para a extracción selectiva de contaminantes como as aflatoxinas e a patulina en alimentos. O desenvolvemento de métodos rápidos e selectivos fixo posible o illamento dun grupo de aflatoxinas e patulina, grazas á fabricación de "barriñas" de axitación magnéticas que combinan magnetita con este tipo de polímeros, e "barriñas" axitadoras recubertas de vidro con recubrimento MIP (MMIP-SB), respectivamente, mediante a técnica de polimerización en bloque. Estes novidosos protocolos con MMIP-SB foron aplicados a produtos para bebés (lácteos e a base de cereais) e a mazá, cunha recuperación satisfactoria dos analitos (39-60% para aflatoxinas e 70% para patulina). Así, os resultados deste terceiro obxectivo quedaron recollidos en dous artigos ou contribucións orixinais en revistas científicas con revisión por pares. En tódolos traballos experimentais, os materiais sintetizados combináronse con HPLC-MS/MS para a determinación dos analitos de interese.

Os resultados obtidos nesta tese de doutoramento, xunto ca extensa revisión e a discusión das metodoloxías existentes baseadas nos polímeros de impresión molecular, subliñan claramente o enorme potencial dos MIP na seguridade alimentaria. Os MIP demostraron ser soportes axeitados para a extracción selectiva de residuos e contaminantes nos alimentos. O traballo aquí presentado enmárcase dentro dos proxectos de investigación INCITE09 261 380 PR e EM 2012/153 da Xunta de Galicia (España), e AGL2009-14707 do Ministerio de Ciencia e Innovación.

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Abstract

The occurrence of residues and contaminants in food can generate acute or chronic reactions in humans and/or emergence of bacterial resistance and toxicity, among other consequences. Over the years, the great interest in food safety has made it essential to develop analytical methods to be used in laboratories around the world, enabling the detection and quantification of different types of substances in food. In addition, the implementation of official surveillance plans to prevent the presence of residues of veterinary drugs and contaminants in food, and thus protect the health of consumers, continuously demand methods that make it possible. The ideal analytical method should combine cost-effective one-step isolation, pre-concentration and quantitative determination. It is important to consider that each additional step may result in an increased probability of analyte losses, sample contamination, as well as analytical errors. The direct detection of analytes in real samples is often complicated because food matrices are complex. Hence, it is essential to apply effective clean-up protocols that allow the elimination of possible interferences and the subsequent extraction of the analytes of interest. In this sense, several techniques have been gradually introduced to facilitate food sample preparation prior to residue detection. To date, solid-phase extraction (SPE) has been one of the most widely used preparatory techniques in food analysis due to its low cost and easy automation. It can be coupled to both liquid chromatography and gas chromatography and there is also a wide variety of commercial SPE cartridges used in the case of complex matrices. This preconcentration technique has almost completely replaced the traditional liquid-liquid extraction (LLE), and some of its related branches are gaining popularity every day. In particular, molecular imprinting technology (MIT) has emerged, offering a wide range of options to support and complement existing analytical techniques. The use of molecularly imprinted polymers (MIPs) facilitates the extraction process thanks to its usual high degree of selectivity. Today, one of its most widely used applications is SPE, the so-called molecularly imprinted solid-phase extraction (MISPE). Molecularly imprinted polymers (MIP) are designed as artificial antibodies, capable of specifically re-attaching to the template used during synthesis, or analogous compounds. In addition, it is possible to combine this type of smart polymers with different materials, such as silica, magnetite, glass, carbon, polyetilene and others. Coatings, silane chemistry and/or surface-grafting approaches are some of the solutions developed to combine MIP with fibres, quantum dots, films or slides, nanotubes and/or beads of different materials. Also, molecularly imprinted monoliths are finding their way into SPE applications and as stationary phases for LC. As a matter of fact, MIPs have widely proven their suitability as sorbents for the extraction of residues and contaminants in food, and the field is still growing. In this Doctoral Thesis research project, an extensive review on the state of the art in MIP science has been conducted, including information on polymerization techniques and approaches, monomers, templates and/or existing formats. In fact, different approaches to MIP synthesis and design have also been tested and applied, in an attempt to combine modern solutions such as magnetic supports, but also taking advantage of well-established classical formats such as MISPE. Molecularly imprinted materials for selective multi-analyte MISPE extraction of

various groups of veterinary drugs, *i.e.* corticosteroids, amphenicols and penicillins, were obtained by precipitation polymerisation and successfully implemented in the laboratory to analyse milk and milk products with satisfactory recoveries (85-150% for corticosteroids, 91-110% for amphenicols and 56-88% for penicillins). On the other hand, fast and selective methods were developed for aflatoxins and patulin isolation thanks to the fabrication of magnetic molecularly imprinted stir-bars (MMIP-SB) combining magnetite and glass-covered stir-bars, respectively, with bulk polymerisation. These novel MMIP-SBs were applied to baby products (dairy and cereal-based) and apple with satisfactory recoveries (39-60% for aflatoxins and 70% for patulin). In every case, the developed materials were combined with HPLC-MS/MS for analyte determination. The results obtained in this Doctoral Thesis, along with the extensive review and discussion on the existing MIP-based methodologies, clearly underline the tremendous potential of MIPs in food safety.

Keywords: MIP, MISPE, corticosteroids, amphenicols, penicillins, aflatoxins, patulin, milk, baby products, apple, stir-bar, HPLC-MS/MS

List of Abbreviations

ABDV: 2´-azobis (2,4-dimethylvaleronitrile)

ACN: Acetonitrile

ADIA: 2,2‘-azobis(N,N‘-dimethyleneisobutyramidine)

AFB1: Aflatoxin B1

AFB2: Aflatoxin B2

AFG1: Aflatoxin G1

AFG2: Aflatoxin G2

AFM1: Aflatoxin M1

AFs: Aflatoxins

AIBN: 2, 2′-azoisobutyronitrile

AIMN: 2,2′-azobis-(2-methyl-butyronitril)

AMP: Ampicillin

AMX: Amoxicillin

AOAC: Association of Official Analytical Chemists

APTS: (3-aminopropyl)triethoxysilane

BLAs: β-lactams antibiotics

BPO: Benzoyl peroxide

BSE: Bovine Spongiform Encephalopathy

CAC: Codex Alimentarius Commission

CAP: Chloramphenicol

CCα: Decision limit

CCβ: Detection capability

CLOX: Cloxacillin

CNT: Carbon nanotubes

COR: Cortisone

CVM: Centre for Veterinary Medicine

DAD: Diode array

DICLOX: Dicloxacillin

2DLC: Two Dimensional Liquid Chromatography
DM: Dexamethasone
DMC: 5,7-dimethoxycoumarin
DON: Deoxynivalenol
DVB: Divinylbenzene
ECD: Electron capture
EGAs: Estrogenic, Gestagenic and Androgenic compounds
EGDMA: Ethylene Glycol Dimethacrylate
ELISA: Enzyme-Linked Immunosorbent Assay
FAO: Food and Agriculture Organization
FDA: Food and Drug Administration
FID: Flame Ionisation
FLD: Fluorescence Detector
FLP: Florfenicol
FLU: Flumethasone
GC-C-IRMS: Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry
GC-MS/MS: Gas Chromatography–Mass Spectrometry
GPS: Glycidoxypropyltrimethoxysilane
HAc: Acetic acid
HCOR: Hydrocortisone
HPLC: High Performance Liquid Chromatography
HRMS: High Resolution Mass Spectrometers
HRMS: High-Resolution Mass Spectrometers
IAC: Immunoaffinity columns
JECFA: Joint FAO/WHO Expert Committee on Food Additives and Contaminants
LC-MS/MS: Liquid Chromatography–Mass Spectrometry
LLE: Liquid-liquid Extraction
LODs: Limits of detection
LOQs: Limits of quantification
MAA: Methacrylic Acid
MeOH: Metanol
MIPs: Molecularly Imprinted Polymers
MISPE: Molecularly Imprinted Solid-Phase Extraction
MIT: Molecular Imprinting Technology

MLs: Maximums levels
MMIPs: Magnetic Molecularly Imprinted Polymers
MMIP-SB: Magnetic Molecularly Imprinted Stir-Bar
MMISPE: Magnetic Molecularly Imprinted Solid-Phase Extraction
MPRD: Methylprednisolone
MPTS: 3-methacryloxypropyl trimethoxysilane
MPTS: Methacryloxypropyltrimethoxysilane
MRLs: Maximum Residue Limits
MRPL: Minimum Required Performance Limit
MS/MS: Tandem Mass Spectrometric
MS: Single Mass Spectrometric
MSPE: Magnetic Solid-Phase Extraction
MWNT: Multi-Walled carbon nanotubes
NAFC: Nafcillin
NIPs: Non-Molecularly Imprinted Polymers
NSAIDs: Non-Steroidal Anti-Inflammatory Drugs
OMS: Organización Mundial de la Salud
OXA: Oxacillin
PAT: Patulin
PEN G: Penicillin G
PEN V: Penicillin V
PRED: Prednisolone
PTFE: Politetrafluoroetileno
QuEChERS: Quick, Easy, Cheap, Effective, Rugged and Safe
SARMs: Selective Androgen Receptor Modulators
SBSE: Stir-Bar Sorptive Extraction technique
SDS: Sodium Dodecyl Sulphate
SEM: Electron Scanning Microscopy
SERMs: Selective Estrogen Receptor Modulators
SFE: Supercritical Fluid Extraction
SOSLE: Salting Out Supported Liquid Extraction
SPE: Solid-Phase Extraction
SPME: Solid-Phase Microextraction
SWNT: Single-Walled carbon nanotubes

TAP: Thiamphenicol

TEOS: Tetraethyl orthosilicate

TLC: Thin Layer Chromatography

TOF: Time-Of-Flight

TOL: Toluene

TRIM: Trimethylolpropane trimethacrylate

UHPLC: Ultra-High Performance Liquid Chromatography

UV: Ultraviolet

VP: Vinylpyridine

WADA: World Anti-Doping Agency

WHO: World Health Organization

ZON: Zearalenone

1 INTRODUCTION

According to the world reference bodies responsible for ensuring food safety in the European Union, together with World Health Organization (WHO) and Food and Agriculture Organization (FAO), drug residues in food of animal origin are considered a risk factor in the economic development of any country. In Europe, official surveillance plans have been implemented in order to avoid the presence of residues of veterinary drugs and contaminants in foodstuff and thus protect the health of consumers [1]. Therefore, food security is a major concern for authorities, and effective methods and analytical technologies have been continuously developed to control food safety and to protect the health of consumers [2]. In addition, there are other substances such as biocontaminants for instance, fungal toxins and bacteria recognised by the WHO, which represent a significant source of foodborne illness as well as a risk to human health [3]. Dangerous substances in food may include natural toxicants, environmental contaminants and chemicals such as pesticides and veterinary drugs, deliberately used to increase the food supply. Moreover, residues of these chemicals may also be present in processed foods, thus affecting human health [4]. Modern analytical methods have the sensitivity required for detection and quantification of contaminants in food and they are crucial for the countries to assure and achieve the required safety and quality of their food products [5]. It is necessary to develop, faster, less time-consuming and more precise analytical procedures every day. However, this is not an easy task since foodstuffs may contain a broad range of components and it is necessary to control all of them, apart from the fact that in some cases those components and hazardous chemicals can be present at trace levels [6]. Therefore, according to the legislation, regulations, prohibitions and authorizations have been established for medicines, anabolic substances and pollutants whose residues have been considered a potential risk factor for human health.

Many different alternatives have been designed to prepare food samples for their analysis, including liquid extractions, liquid-solid extractions, solid-phase sorbents, QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe), molecularly imprinted polymers (MIPs), supercritical fluids, ultrafiltration, etc. [7]. Molecular imprinting technology (MIT) is an emerging powerful tool that leads to the synthesis of polymers with selective molecular recognition properties, both in sample preparation and for chromatography processes [1, 8]. In this context, MIT enables the design of robust molecular recognition materials which are in high demand today in analytical chemistry. These materials are able to mimic natural recognition entities, such as antibodies and antigens, enzymes and substrates, or hormones and biological receptors [9]. Currently, solid phase extraction (SPE) is one of the most frequently used clean-up solutions in food analysis, as it combines the benefits of being a low-cost option and its easy automatization, along with the wide variety of sorbents available on the market. In the last decade, molecularly imprinted polymers have proved their potential as selective sorbents in solid phase extraction (MISPE) or as stationary phases in high performance liquid chromatography (HPLC), demonstrating that they can be a highly versatile tool for the selective detection and quantification of natural and synthetic

contaminants in food matrices. In fact, this technique seems to be particularly suitable for the extraction of complex matrices such as food products, allowing the achievement of high selectivity and exhibiting good extraction efficiency and reusability [10].

1.1 MOLECULARLY IMPRINTED POLYMERS (MIPS)

Molecularly imprinted polymers (MIPs) are highly selective synthetic polymers for a given analyte or group of structurally related compounds, making them ideal materials for use in separation processes [11]. Molecular imprinting is an emerging technology which enables us to synthesize materials with highly specific receptor sites towards the target molecules. These types of materials are a class of highly cross-linked polymers that can bind certain target compound with high specificity. MIPs have been used in a wide range of applications, including liquid chromatography, solid-phase extraction (SPE), capillary electro chromatography, binding assays and biosensors, mostly used in bio-analytical areas [12].

1.1.1 General principles and evolution of Molecularly Imprinted Polymers (MIPs)

In the last decades, the progress of molecular imprinting technology (MIT) has been very fast, as evidenced by numerous published studies reporting its application for different purposes. Figure 1 shows the studies compiled by the Society for Molecular Imprinting [13] from 1980 to the present. Since the beginning of this century, a sharp increase in the number of articles published has been observed. In 2018, 1118 articles were published while in the first nine months of this year the total has been 63, as checked on September 18th 2019.

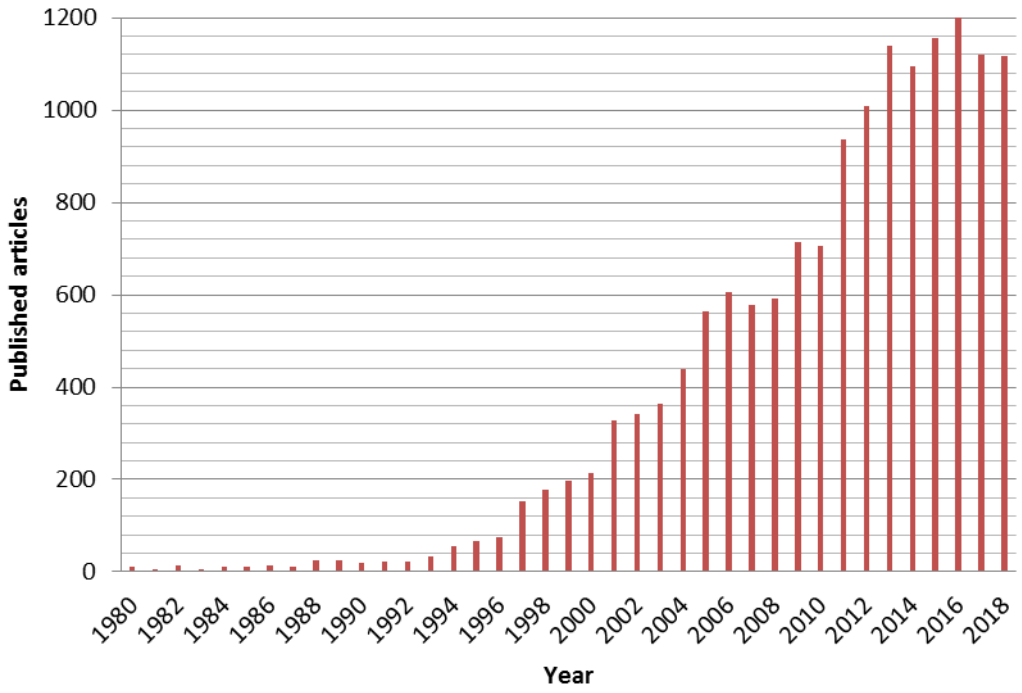


Figure 1. Published works related to molecularly imprinted polymers (MIPs) from 1980 to 2018, compiled from the Society for Molecularly Imprinting [13]

One of the fundamental keys to the functioning of biological systems is the selective molecular recognition. For example, it is involved in the formation of complexes between enzymes and substrates, in the interaction between antigens and antibodies, in the replication of DNA molecules and in the transcription of proteins. These systems have been the starting

point for the synthesis of materials capable of selective interaction with the analytes. The concept of imprinting has been commonly used in Paleontology to refer to the imprint produced by the inclusion of an organism in sediment before its disintegration, giving rise to a fossil. This natural process has some analogy with the technique used for the preparation of MIPs, which are obtained by synthesizing a highly cross-linked macromolecular structure around a template molecule that is extracted after polymerization. In this way, cavities (binding sites) are created in the polymer, being complementary in shape, size and distribution of functional groups to the template, allowing subsequent selective recognition, as if it were an antibody [14]. Initially, a complex forms between the functional monomers and the imprint molecule. Once polymerization has taken place, the functional groups of the complex formed are held in position by the highly cross-linked polymeric structure. Subsequent removal of the imprint molecule reveals binding sites that are complementary in shape and size to the analyte. Thus, a molecular memory is introduced into the polymer, which is now capable of rebinding the analyte with very high specificity [15].

The history of Molecular Imprinting Technology (MIT) goes back to the early 20th century, when Polyakov and his collaborators researched the development of new materials for application in chromatography. They wanted to increase the capacity of silica to be used as a stationary phase in chromatography and to study the influence of certain solvents on the size and structure of the pore. They observed that by adding aromatic molecules such as toluene, benzene, or xylene during the preparation of the silica gel, the synthesized particles were much more selective for these added particles than for similar ones. The observed selectivity of silica was assumed to be a consequence of the chemical properties of the added particles, which was established as the “memory” of the adsorbent [16]. Based on the theory of antibody formation postulated by Pauling [17], Dickey was able to explain Polyakov´s findings by demonstrating silica's recognition of different methyl orange-derived indicators present during the production of the silica gel [18, 19]. However, the low stability of silica greatly limited the progress in this line of research [20]. From the 1950s onwards, molecular printing of materials began to be studied. Nowadays, it is beginning to be applied in the synthesis of specific supports for chromatographic columns, as in the case of thin layer chromatography. In studies using immobilized enzymes, Mosbach investigated a technique for trapping enzymes and microorganisms in a cross-linked synthetic polymer [21]. In the 1970s and 1980s, Wulff [22], Takagishi and Klotz [23] and Mosbach [24] demonstrated the presence of the molecular footprint in organic polymers. Since then, the development of molecular printing technology has followed a progressive growth, as shown in Figure 1. The preparation of MIPs using the covalent model has been based on the work of Wulff [25].

There is currently much interest in the design, preparation and characterization of new synthetic receptor materials capable of “mimicking” (biomimetic materials) to some extent the biological processes of molecular recognition [26]. These materials include crown-ethers, cryptates, cyclodextrins or molecular impression polymers (MIPs), among others. All of them are capable of providing a more or less selective recognition towards a specific group of compounds. With the exception of MIPs, these materials are expensive and very difficult to synthesize in the laboratory. Molecular imprinted polymers have a number of advantages over natural biomolecules. The most relevant are sensitivity, adequate selectivity and possibility of synthesis for a wide variety of analytes, from biomolecules to toxic species. In addition, they have high chemical, thermal and mechanical stability under different conditions of pH, temperature and presence of organic solvents and low cost large-scale production. However, they also have some limitations, especially related to limited selective recognition in aqueous

environments, moderate affinity constants or the difficulty of coupling the recognition stage with the transduction stage for sensor development.

In the molecular imprinting process, the target molecule acts as the template around which the monomers interact. Initially, monomers form a complex with the template through covalent or non-covalent bonds. In a first stage the analyte and the monomer in the selected solvent (porogen) are put in contact to form the pre-polymerization complex. Afterwards, the cross-linker is added and finally the initiator to start the polymerization process. Once the polymer is obtained, it is subjected to an intensive washing process in which the template is removed, thus leaving free binding sites in the polymer network, which are complementary to the template in size, shape and position of the functional group. In essence, a "memory" molecule is imprinted on the polymer, which is now able to selectively reattach the template. Therefore, this type of polymers possesses two of the most important characteristics of biological receptors, the ability to recognize and bind to specific target molecules [27].

1.1.2 Design of Molecularly Imprinted Polymers (MIPs)

The design and synthesis of molecularly imprinted polymers (MIPs) can be discouraging as it involves the control of a large number of experimental variables, such as the nature and quantity of the template, the wide variety of functional monomers and cross-linkers, initiators and solvents, as well as the method of initiation and the duration of the polymerization process [1]. All these parameters can greatly influence the final morphology, properties and performance of the polymers and therefore need to be evaluated. The method of molecular imprinting involves the polymerization of functional monomers around a molecular template, in a cross-linked polymeric matrix, with the subsequent removal of the template to obtain free binding sites for the future target analyte.

1.1.2.1 Templates

A wide variety of compounds can be used in the preparation of MIPs; molecules such as amino acids, proteins, hormones, carbohydrates, antibiotics, pesticides, among others, have been successfully used for the preparation of selective recognition matrices [28]. The selection of the template is critical in MIPs synthesis since it directs the organization of the functional groups of the functional monomers. It must be selected according to the future target molecule and the type of polymerization, and it should ideally be chemically inert [1]. Usually, the imprinting molecule is the same compound that will be extracted with the obtained polymer, or a very close structural analogue (e.g. a compound from the same chemical family). However, when choosing the template, the analyst must take into account at least the following aspects: (1) the template must have groups that can polymerize, (2) the template can potentially retard or inhibit a free radical polymerization (templates with a hydroquinone moiety or a group thiol) and (3) the template must be stable under the selected polymerization conditions (*e.g.* UV irradiation and temperature) [29]. Moreover, the use of the future target analyte directly as a template can be problematic because the complete removal of template molecule from the high-affinity sites of the polymer is not always possible. It is very important to take this aspect into consideration when using MIPs in trace analysis, as a possible "bleeding" of the residual template during the extraction process can lead to false positives or inaccurate quantification of the analyte of interest [1]. To try to solve this problem, the so-called "dummy" templates have been used for the synthesis of MIPs [30, 31]. These molecules must mimic as much as possible the size and shape of the target analyte, in order to achieve selective polymers. Ideally, these MIP will have binding sites with sufficient cross-selectivity for the target analyte and even for structurally related compounds.

With the employment of accurate identification and quantification methodologies such as HPLC-MS/MS, problems of co-elution of the analyte and the "dummy" template can be easily overcome [30].

1.1.2.2 Functional monomers

The choice of an appropriate functional monomer is of great importance in order to create highly specific cavities in the polymer [31]. Functional monomers are responsible for binding interactions in the imprinted binding sites. To maximize the complex formation and thus the imprinting effect, the template and the functional monomer should be complementary. It is also important to have into account the reactivity ratio of the monomers to ensure that copolymerization is feasible when two or more functional monomers are combined in a "cocktail" of polymerization [32, 33]. Generally, an excess of functional monomer relative to template is necessary to favour template-monomer complex formation and to maintain the integrity of this complex during the entire polymerization process [34, 35]. Typical functional monomers are carboxylic acids (*e.g.* methacrylic acid, acrylic acid), heteroaromatic bases (*e.g.* vinylpyridine, vinylimidazole) and sulphonic acids (*e.g.* 2-acrylamido-2-methylpropane sulphonic acid). The extensive use of methacrylic acid (MAA) is justified by its capability to act both as hydrogen bond and proton donor and as hydrogen bond acceptor [36]. These characteristics make MAA an excellent candidate for interacting with almost any template. Alternatively, for designing MIPs for isolating molecules containing acid groups, vinylpyridine has been frequently used. Moreover, more strong functional monomers have been developed via metal coordination interactions to bind specific amino acid sequences [37]. Generally, acid monomers are more appropriate for using with basic templates and basic monomers for using with acidic templates [1]. The structure of the most common functional monomers used for molecularly imprinting is shown in Figure 2.

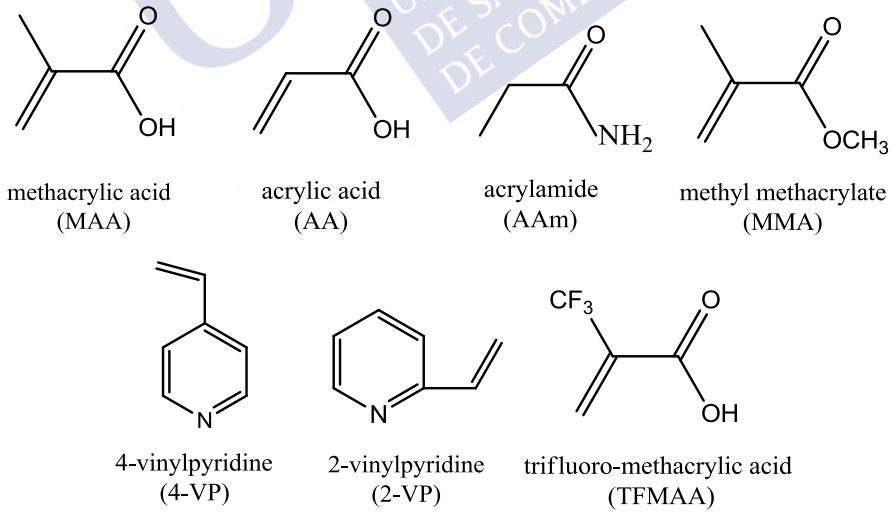


Figure 2. Chemical structures of the most frequently used functional monomers in MIP synthesis. Reproduced from Regal, P. (2012) under open access license [1]

1.1.2.3 Cross-linkers

The type and amount of cross-linker monomer used for the synthesis of any MIP directly influences the selectivity of the synthesized MIP itself. The cross-linker, during the polymerisation process, fulfils very important functions such as stabilizing the imprinted

binding sites, controlling the morphology of the polymeric matrix, and conferring mechanical stability to the polymer, in order to retain its molecular recognition capability [38]. It also provides adequate porosity to guarantee the accessibility of the analyte to the cavities generated [31, 39]. High ratios of cross-linker are generally used in the polymerization mixture, so as to ensure the obtainment of permanently porous (macroporous) materials with adequate mechanical stability. After template removal, it is very important to take into account that the amount of cross-linker is sufficient to maintain the three-dimensional structure, the stability of the recognition sites and the chemical functional groups of the functional monomer in the right position and orientation [1]. According to the literature, the two most commonly employed cross-linkers are ethylene glycol dimethacrylate (EGDMA) and trimethylolpropane trimethacrylate (TRIM) (Figure 3) [36]. TRIM compared to EGDMA results in polymers with more rigidity, structure order and effective binding sites. Moreover, the type of cross-linker has also shown influence in the final size and yield of the MIP nanoparticles in the precipitation polymerization process. According to Yoshimatsu *et al.* (2007), when divinylbenzene (DVB) was used as cross-linker polydisperse MIP particles were obtained in low yield, whereas trimethylolpropane trimethacrylate (TRIM) led to uniform nanoparticles in high yield (90%) [40].

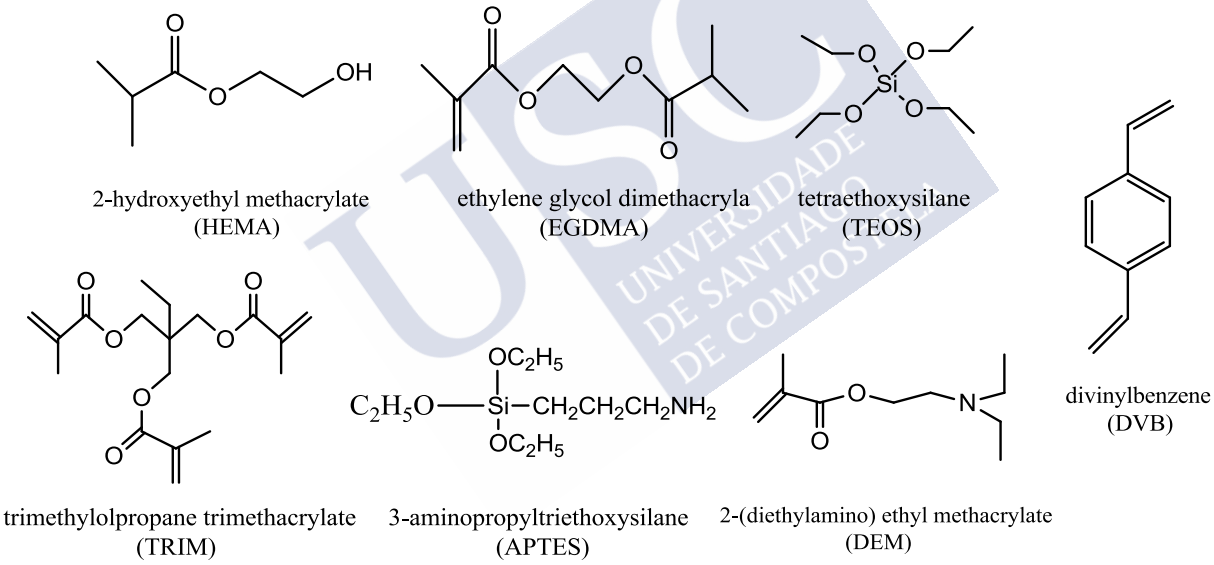


Figure 3. Chemical structures of the most frequently used cross-linkers in MIP synthesis. Reproduced from Regal, P. (2012) under open access license [1]

1.1.2.4 Porogenic solvents (porogens)

Porogenic solvents play a very important role in the formation of the porous structure of MIP, collecting all the necessary components (template, functional monomer, cross-linker and initiator) in a single phase during the polymerisation process. The most common solvents used for MIP synthesis are toluene, acetonitrile, chloroform and dichloromethane. The porogenic solvent is responsible of creating pores within the macroporous polymers; hence it is also referred to as "porogen". Both its nature and the amount used in the synthesis will determine the strength of non-covalent interactions and influence polymer morphology that directly affects the performance of MIP. Moreover, the porogen in a non-covalent imprinting approach should be chosen considering its role in maximizing the likelihood of obtaining a

complex between the template and the functional monomer [1]. First of all, necessary components of the synthesis (template, functional monomer, cross-linker and initiator) must be soluble in porogens. Secondly, the porogenic solvents should produce large pores, in order to assure good flow-through properties of the resulting polymer. Thirdly, porogens should be relatively low polarity it is another important feature that reduces the interferences during complex formation as well as providing high selectivity to MIP [31].

The solvent or porogen is chosen according to its ability to stabilize the pre-polymerization complex formed between the functional monomer and the template. Thus, low dielectric constant aprotic solvents, such as toluene, dichloromethane, chloroform or acetonitrile, are chosen to stabilize complexes based on the formation of hydrogen bridge bonds, electrostatic interactions or Van der Waals forces, typical of non-covalent MIPs [41]. Protic solvents such as water or methanol tend to destabilize these joints, making it difficult to form cavities in the polymer matrix, but are suitable when the interactions are hydrophobic [42]. If the polymer is to be used as a stationary phase in separation techniques such as capillary electrochromatography, the porosity of the polymer obtained is controlled by the addition of a small fraction of isooctane to the porogen. Thus, for example, some studies [43] have shown that the addition of a small fraction of isooctane to the toluene commonly used as a porogen does not affect the recognition and considerably increases the porosity of the monoliths obtained by the generation of macropores. The presence of these macropores in the polymer matrix facilitates the conditioning of the capillaries by allowing the hydrodynamic pumping of the electrolyte solution. It can be assumed that the porogen the more polar, the weaker the resulting recognition effect and this behaviour is a result of the influence of solvent polarity on non-covalent interactions between analyte and polymer. In the application of MIPs, the same porogenic polymerization solvent, or at least one with similar characteristics, is generally used as the solvent [44].

1.1.2.5 Initiators

Once the pre-polymerization mixture has been prepared, polymerization begins in the presence of an initiator which, as its name suggests, starts the process by introducing free radicals into the system. The choice of initiator depends on what method of initiation is to be used, ultraviolet radiation or heat and, in the latter case, the temperature at which polymerization will take place. These parameters affect the time required for polymer solidification. Both the speed and mechanism of an initiator to form radicals can be triggered and controlled in different ways such as light, heat and even other types of options (chemical/electrochemical) depending on the chemical nature of the initiator [1]. If the molecule is thermosensitive, it will be done using an ultraviolet radiation source, and if the molecule is photo and thermosensitive, it will be necessary to use materials of the sol-gel type, whose polymerisation is initiated by acid or basic catalysis, and does not require initiation by free radicals [45]. It is recommended to remove dissolved oxygen from monomer solutions immediately prior to polymerization [1]. It is known that photo-initialization at low temperatures allows MIPs to be obtained with better mass transfer properties and superior performance in chromatographic processes. However, there are several monomers and templates that are not compatible with this technique, due to its degradation or radical inhibition [46]. In general, MIP prepared using larger concentrations of initiators possessed a larger surface area. The amount or ratio of initiator in the polymerization mixture is always considerably lower than the amount of monomers [47]. The initiators normally used are 2,2´-azo(bis)-isobutyronitrile or 2,2′-azobis(2-methylpropionitrile) (AIBN), 2,2´-azobis-(2-methyl-butyronitril) (AIMN) 2,2´-azobis(2,4-dimethylvaleronitrile) (ABDV) and benzoyl peroxide

(BPO) [1]. When the beginning of polymerization is produced by heat, the most commonly used initiator is 2,2´-azo-(bis)-isobutyronitrile (AIBN), as it decomposes into radicals by both ultraviolet radiation and heat at temperatures above 60ºC. Hydrogen peroxide, acetyl peroxide, lauryl peroxide and t-butyl peracetate are also used, depending on the method of initiation used, ultraviolet or heat radiation [44].

On the other hand, there is a useful tool for controlling the degree of polymerization by reaction time: living radical polymerization with "iniferter" (initiator-transfer agent-terminator). This type of molecules acts not only as initiators but also as terminator or retarder and transfer agent, inhibiting ordinary bimolecular termination and other transfer reactions. Thus the primary structure of the synthesized polymer and the degree of polymerization can be controlled by reaction time [48]. Iniferters are able to generate two free radicals. One of them is responsible for initiating polymerization while the other is relatively stable and not-active, but capable to complete the growing polymer chains by recombination [49].

1.1.3 Synthesis of Molecularly Imprinted Polymers (MIPs)

In the first stage of synthesis, the analyte and the monomer are put in contact in the solvent (porogen) selected to form the pre-polymerization complex. Afterwards, the cross-linker and finally the initiator are added for the polymerization process to take place. The nature of the interaction between the functional monomer and the template molecule is currently one of the most commonly used criteria for the classification of MIPs, due to it considerably affects the properties of the final polymer obtained. Once the polymer is obtained, it is subjected to a clean-up procedure in which the template is removed, leaving free in the polymer network binding sites that are complementary in shape, size and orientation of functional groups to the analyte template. These cavities will allow the obtained polymer to recognize the template and other structurally analogues compounds (Figure 4). Since their first application in 1972, within the field of organic polymers [50], MIPs have been increasing importance, due to they constitute a powerful tool that provides greater selectivity for their application in chemical and/or physicochemical analysis. The need to selectively extract compounds of interest from complex mixtures is the result of the high use of these new adsorbent phases based on molecular impression polymers.

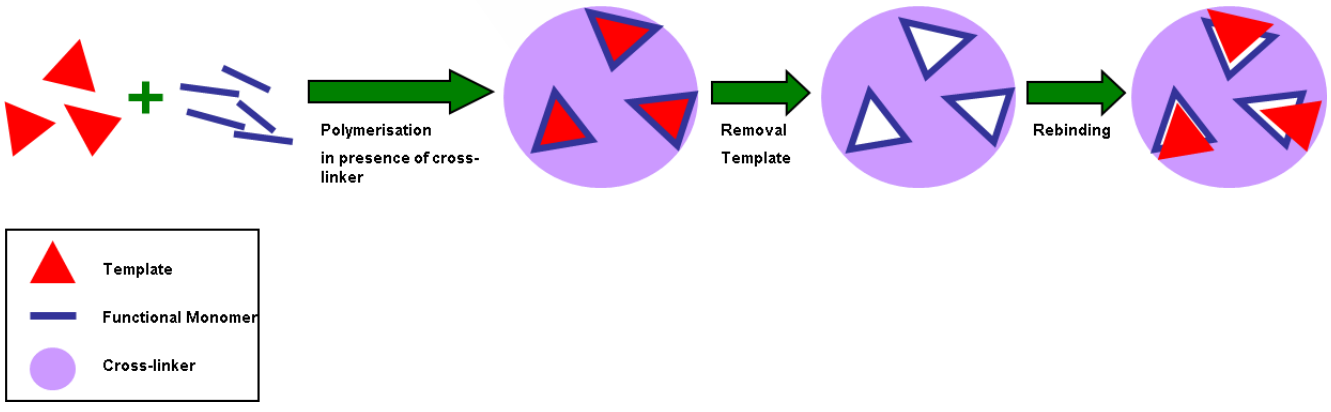


Figure 4. Schematic representation of molecularly imprinting process and rebinding of the template. Reproduced from Regal, P. (2012) under open access license [1]

1.1.3.1 Polymerization approaches

There are three different approaches to prepare MIPs: covalent (pre-organized approach), semi-covalent approach and non-covalent (self-assembly approach) [51]. Their main characteristics are described below.

1.1.3.1.1 Covalent model

The first molecular printing studies with synthetic organic polymers were based on the covalent model and were presented in 1972 by Gunter Wulff's group [22]. The covalent approach involves the formation of reversible covalent bonds between the template and functional monomers before polymerization. Latter, the template is removed by cleavage of the covalent bonds, which will be re-formed upon rebinding of the target molecule. This approach leads to a homogenous population of binding sites due to the high stability of the template-monomer interactions. However, it is restrictive since the cleavage of covalent bonds always requires a rather harsh chemical condition. This high degree of homogeneity is a very important advantage as it allows its use in certain applications, such as its use as a stationary phase in HPLC or in the development of chemical sensors. The advantages of this model used in the synthesis of MIPs are that the monomer-template conjugates are stable, stoichiometry can be controlled so that there are no functional monomers outside the cavities, which considerably minimizes the presence of non-specific interactions [34]. In addition, due to the stability of the conjugates formed by covalent bonds, they can be used in a wide variety of polymerization conditions such as from high temperature, high or low pH as well as highly polar solvents. On the other hand, this type of polymerization also presents a series of disadvantages such as the limited number of existing monomers capable of forming a reversible covalent bond with the analyte template, as well as the difficulty of extraction of the analyte that sometimes requires severe conditions that may affect the structure of the polymer, by imposing serious restrictions on the spatial arrangement of the functional groups of both compounds.

In addition, the synthesis of these polymers is considerably more complex than that of non-covalent polymers, since prior synthesis of the template-monomer compound is required. Finally, another important limitation of this type of polymerization is that most of the covalent bonds used in this type of polymerization are unstable in aqueous media, which considerably limits the available polymerization procedures. Due to these limitations and despite being the first polymers to be developed [52-54] their analytical application have been rather limited in comparison to non-covalent models.

1.1.3.1.2 Semi-covalent model

In this approach, the template is covalently bound to a functional monomer, but the rebinding is based on non-covalent interactions. Semi-covalent polymers can be considered as a particular case of covalent polymers. In this sense, although the initial interaction formed between the template and the monomer is through a covalent bond, the interaction involved in the subsequent recognition of the analytes is of a non-covalent nature. This is of considerable importance, since by eliminating the need to form a reversible covalent bond during the use of the polymer, other types of monomers and/or insole analytes may be used that result in the formation of a stronger and more stable covalent bond during the synthesis of the MIP [34]. This approach was initially described by Sellergren and Andersson in 1990 [55], who synthesized a polymer imprinted from p-aminophenylalanine acetate. After hydrolysis of the amide bond template formed between the monomer and the template, the carboxylic acid

residues present in the polymer matrix were able to interact with the amino acid by forming hydrogen bridge bonds.

Since they share a common first stage during synthesis procedure, semi-covalent polymers have several advantages analogous to those described above for covalent polymers, e.g., their extraordinary selectivity and greater homogeneity in the distribution of binding points in the polymer matrix. The main disadvantage of this type of polymer derives from the steric differences between covalent and non-covalent bonds, since a greater distance is needed to form the non-covalent bond than a covalent bond. This limitation in some cases of semi-covalent imprinted polymers was solved by Whitcombe [56], who synthesized in 1995 a imprinted polymer using the carbonyl group as a spacer between the functional monomer (methacrylic acid) and the template analyte (cholesterol). This spacer group was subsequently removed as CO_2 during hydrolysis of the bond formed between the analyte template and the monomer. Comparison of this polymer with a synthesized polymer following a non-covalent polymerization demonstrated its greater efficiency when used as a stationary phase in chromatographic systems [57].

1.1.3.1.3 Non-covalent model

Finally, the non-covalent or self-assembly approach is based on formation, before polymerization process, of relatively weak non-covalent interactions (*e.g.* Van der Waals forces, hydrogen bonding, hydrophobic interaction, electrostatic interactions, and dipole-dipole bonds) between the template and functional monomers [1]. Up to date, this approach has been the most frequently used method for the preparation of MIP, due to its simplicity and the availability of different monomers able to interact with almost any kind of template [58]. Some polymers of this type of approach are able to bind the template so tightly that it is challenging to remove all traces of the template, even after several washing steps of the polymer [1].

The first work carried out by Arshady and Mosbach in 1981 [59] described the preparation of a synthetic imprinted polymer of non-covalent type, using methyl methacrylate as a functional monomer that, by means of electrostatic forces, forms a pre-polymerization complex with N,N'-phenyldiacrylamide. The selectivity of the polymer created was demonstrated by a comprehensive chromatographic evaluation. The main advantage of the non-covalent model is that it is not necessary to synthesize a monomer-template covalent conjugate. The template is easily removed from the polymer under soft conditions because non-covalent interactions are weak bonds. Among the main disadvantages of this type of material are its lower selectivity, which leads to the co-elution of interfering species in complex samples when used as stationary phases in HPLC or capillary electro chromatography [60], and the lower homogeneity in the distribution of the binding points present in the polymer matrix [61], which results in a widening of the chromatographic peaks or worsens the linearity of the sensors [35]. In addition, polymerization conditions must be carefully chosen to maximize the formation of the non-covalent complex, and the existence of a large excess of functional monomers to displace the equilibrium and form the complex often provides non-specific binding sites and thus decreases selectivity. Non-covalent imprinting is generally easier to achieve and is applicable to a wide range of templates. The choice of one of the methods depends on the need and operating situations, such as the type of compound to be imprinted, the selectivity required of it, as well as the cost and time of preparation [44].

1.1.3.2 Polymerization techniques

Molecularly imprinted polymers can be synthesized in a variety of physical forms, using different methods of polymerization, usually depending on their final application.

1.1.3.2.1 Bulk polymerization

This type of technique has been used traditionally because it does not require sophisticated instrumentation and the reaction conditions can be easily controlled [34]. It has been the most widely used method for the preparation of MIP, so far although this kind of polymerization is tedious and time consuming [51, 62, 63]. Bulk polymerization is popular because it is simple to perform, yet not everything is advantages in this technique. This method consists of radical polymerization of a concentrated solution of monomers around the template molecule. Polymerization can be initiated both photochemically and thermally, and once completed a monolith of insoluble polymeric material is obtained. This polymeric block is subjected to different crushing, grinding and sieving processes to finally obtain homogeneous particles in the order of 25-50 μm [64]. These latter steps are tedious and time-consuming and often produce particles that are irregular in size and shape, resulting in a heterogeneous mixture of imprinted nanoparticles. This kind of particles when they are packed in columns for use as stationary phases in chromatography can cause back-pressure problems. Also, grinding and sieving can cause a substantial loss of polymer and therefore the polymerization yield is drastically reduced [65]. Most of the known MIPs have been prepared by bulk polymerization, despite these obvious drawbacks, [11]. Figure 5 shows the electronic micrograph obtained for a polymer synthesized by bulk polymerization. The average particle size of this polymer was 0.3 μm. Polymers were analysed by electron scanning microscopy (SEM).

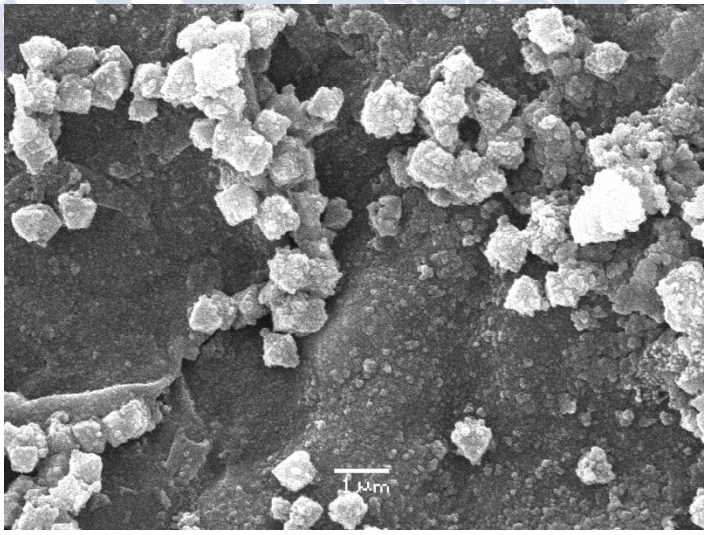


Figure 5. Scanning electron microscopy (SEM) micrograph of particles of molecularly imprinted polymers synthesized using bulk polymerization (authors' unpublished material)

To overcome common drawbacks of bulk polymerization, alternative methods have been developed, enabling the preparation of novel MIP formats, such as MIP beads, membranes, surface imprinted devices, molecularly imprinted monolayers and coatings of different materials [66]. New methods proposed include precipitation polymerization, suspension polymerization, stage swelling polymerization, monolayer polymerization and coating of different materials [49]. Regular beads are generally used to pack columns for

chromatography or SPE synthesized by polymerization in suspension and in emulsion, seed polymerization or precipitation polymerization. These techniques allow the formation of MIP particles with microspherical shapes of uniform sizes (see Figure 6) [67, 68]. In addition, their polymerization yields are quite high because the tedious grinding and sieving steps are avoided.

1.1.3.2.2 Precipitation polymerization

Precipitation polymerization is a relatively new technique used to obtain MIP spherical nanoparticles, first described in 1993 [69, 70]. This procedure is similar to bulk polymerization, but in this case a much more diluted reagent mixture is used working with a higher volume of solvent while maintaining the ratio template:monomer:cross-linker at 1:4:20. As a consequence, the growth of the polymer chains in formation are not capable of occupying the total volume of the solvent used, producing a dispersion of the spherical particles of the polymer within the dissolution [68, 71]. This avoids the need to break the container in which the polymerization takes place as in the case of bulk polymerization. Precipitation polymerization is a heterogeneous process since the working mixture is initially homogeneous but the formed polymer becomes insoluble upon initiation and precipitates. In general, microspheres and beads with a diameter of about 1 µm are obtained depending on the conditions [69]. In order to obtain larger particles, it is necessary to adjust the solubility parameters of the cross-linker with those of the solvents [72]. These criteria are usually met for copolymerization of divinylbenzene (DVB) in mixtures of acetonitrile and toluene [72]. Among the main advantages of this polymerization technique is, in addition to the aforementioned control of product morphology, the high polymerization performance as it is not necessary to subject the obtained polymer to the crushing and screening stages. In addition, it has been experimentally proven [73, 74] that particles obtained by precipitation have, compared to those obtained by bulk polymerization, a greater capacity and a more homogeneous distribution of the binding sites.

Several MIP studies have been published using this type of polymerization, such as the determination of fenuron in vegetable samples [75], benzimidazoles in water [76] or as a stationary phase for LC in the determination of thiabendazole in fruit [77]. They have also been developed for the detection of different veterinary drug residues in milk [8, 67, 78].

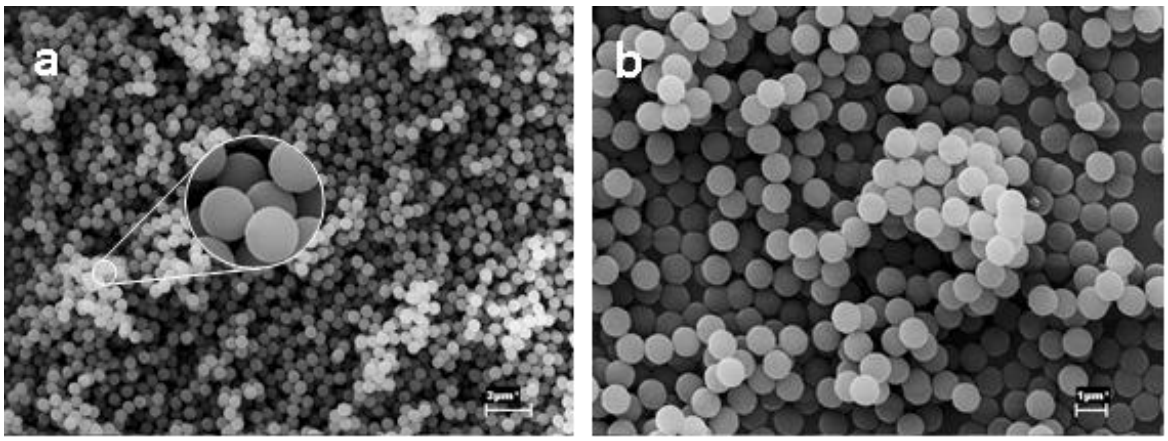


Figure 6. Scanning electron microscopy (SEM) micrographs of MIP (a) and NIP (b) spherical nano-particles obtained by precipitation polymerization. Reprinted with permission from Díaz-Bao, M. (2012) Copyright 2012, Springer-Verlag [67]

1.1.3.2.3Suspension polymerization

Suspension polymerization is a heterogeneous process described by Mayes and Mosbach in 1996, which allows the production of microspheres of a wide range of sizes, based on the use of a liquid perfluorocarbon as the dispersing phase [79]. In this procedure, the organic-based polymerization mixture is suspended as droplets in an excess of liquid phase under continuous mechanical agitation, and each droplet acts as a mini bulk reactor [80]. Due to the need for a polar (water) or expensive (perfluorocarbon fluid) suspension medium, this type of polymerisation is less frequent for MIP synthesis [1]. The use of an aqueous phase is much less frequent as it can seriously hinder the interaction between the analyte and the monomer. The agitation of the system is an important factor to control in this technique, because the size of the particles obtained depends on it. In addition to the monomer, initiator and solvent, chemical substances are added to help disperse the monomer in the continuous phase and keep the small droplets in suspension. The main advantage of this procedure is that it allows controlling the size of the particles (5-50µm) by varying the amount of stabilizing polymer [79] (polyvinylalchol or ionic liquid) or by controlling the agitation speed [81-83]. The beads that result from suspension polymerization are larger than those produced by precipitation (Figure 7).

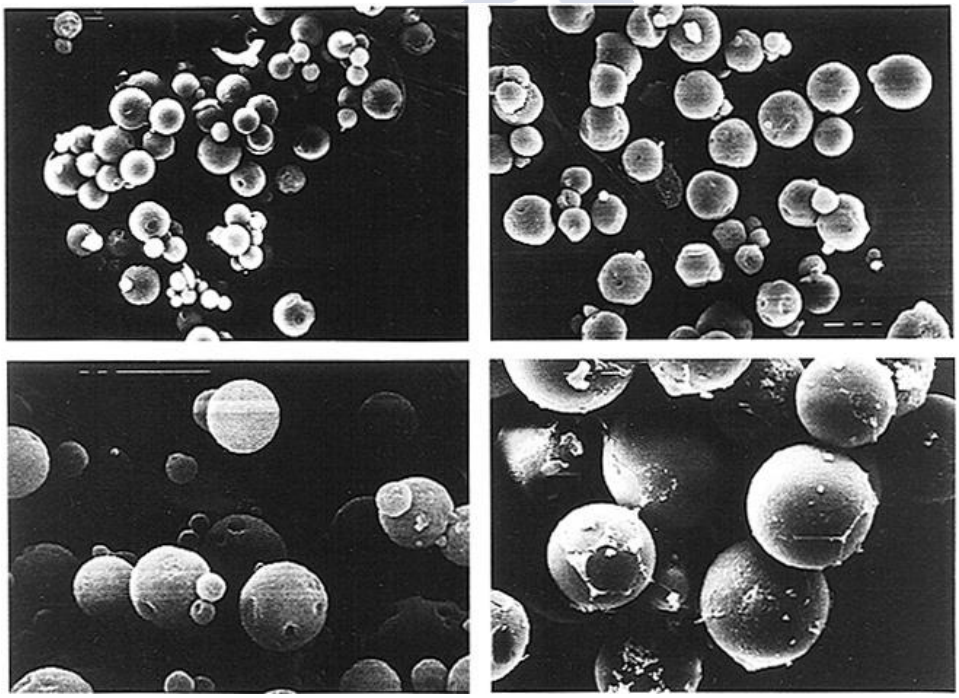


Figure 7. Scanning electron microscopy (SEM) micrographs of beads produced from suspension polymerization in perfluoro(methylcyclohexane). Modified with permission from Mayes and Mosbach (1996) Copyright 1996, American Chemical Society [79]

1.1.3.2.4 Two-step or multi-step swelling and polymerization

This type of polymerization allows producing spherical particles of uniform sizes, efficiently controlling size, distribution and shape and decreasing material loss. These particles have separation properties comparable to those prepared by bulk polymerization. However, they have much better column efficiency and peak shape in chromatographic applications. This polymerization method was initially described in 1994 and optimized in 2000 by the Hosoya group [84]. Two-step or multi-step swelling polymerization is based on

the synthesis of spherical latex particles in an aqueous medium, which swell by the addition of a solvent and are then impregnated with a prepolymerization mixture to synthesize a layer of polymer on the surface of the spheres. This method produces monodisperse particles of which the sizes range between 2 and 50 μm (Figure 8). These beads have separation properties comparable to those prepared by bulk polymerisation, but are more effective in chromatographic column applications. The difficulty of this method is to find a hydrophilic surface that does not modify the selectivity of the polymer and, on the other hand, the fact of using an aqueous medium makes non covalent interactions difficult, so it is necessary to work with other types of interactions [85]. However, even using these techniques, it is difficult to obtain both the desired particle characteristics (size, porosity, pore size, surface area) and the high affinity of the required binding sites [86]. As an alternative, different materials such as spherical silica particles were proposed as a support for the preparation of MIPs.

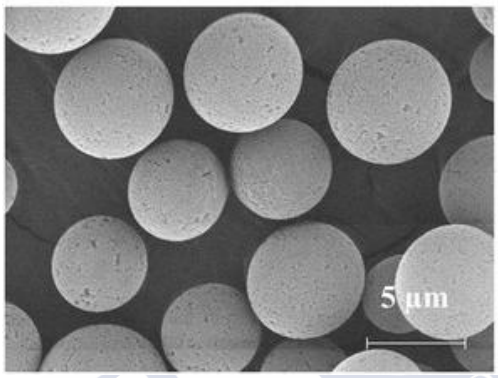


Figure 8. Scanning electron micrograph (SEM) of uniform-sized MIPs obtained by multi-step swelling and polymerization. Reprinted with permission from Nakamura *et al*. (2016) Copyright 2016, John Wiley and Sons [87]

1.1.3.2.5 Emulsion polymerization

Emulsion polymerization is a unique chemical process widely used to produce waterborne resins with various colloidal and physicochemical properties [88] and it is commonly used to prepare molecularly imprinted nanoparticles. In contrast to precipitation polymerization, emulsion polymerization approaches mix organic cross-linker and monomers with template and emulsify them in an aqueous phase containing a surfactant by vigorously stirring and/or sonication [89]. By applying a co-stabilizer such as sodium dodecyl sulphate (SDS) to the dispersed phase, the diffusion processes in the continuous phase are suppressed. The so-called mini-emulsion polymerization results in stable emulsions with a homogeneous droplet size [90]. Therefore, emulsion polymerisation implicates using liquid polymers or polymer solutions as disperse phase. Mini-emulsion polymerisation further applies stabilisers to generate particles in the dispersed phase with sizes around 50–1000 nm. Micro-emulsion polymerisation is defined as taking place in a dispersion made of water, oil and surfactant(s) [91]. Finally, the dispersion is purged by an inert gas and then polymerization begins. Haginaka *et al.* (2009) have used this methodology for the synthesis of imprinted beads using different templates [92] .This method is suitable for industrial applications, offers good particle size control resulting in small MIP nanoparticles with a narrow size distribution [93]. It also yields good binding site homogeneity and greater accessibility to binding sites as compared to bulk monoliths. On the other hand, for the elimination of surfactants it is necessary to use sophisticated washing procedures so it can be a tedious process [89].

1.1.3.2.6 Surface polymerization

Currently, in order to better control morphology and thus explore new applications, there is a growing interest in the search for alternative routes for the preparation of MIPs. There are polymerization methods which graft or coat thin films of MIP-phases on the surfaces of porous materials [92]. This type of imprinting techniques can be an alternative to difficulties in generating high affinity binding sites while simultaneously controlling porous properties, the morphology or other structural features. Moreover, the use of grafting and coating techniques allows decoupling of the imprinting step from the generation of a particular morphology [1]. It is common to refer to surface polymerization with the term "core-shell", although it should be noted that these are not only round particles as in the case of spheres, but also other types of supports. It is possible to use cores with specific properties, which would improve the performance of the formed system ranging from silica to magnetite [66]. Coating techniques consist of grafting a thin layer of imprinted polymer to a particular support (silica beads, electrodes, fibres) [1]. In the case of polymerization within pores of preformed silica beads, it simply consists of filling the pores with the polymerisation mixture and immediately after initiating the polymerization so it is a simple method [86]. One of the advantages of this technique is the lower consumption of reagents thus producing materials with minimal bleeding in the chromatography process [49].

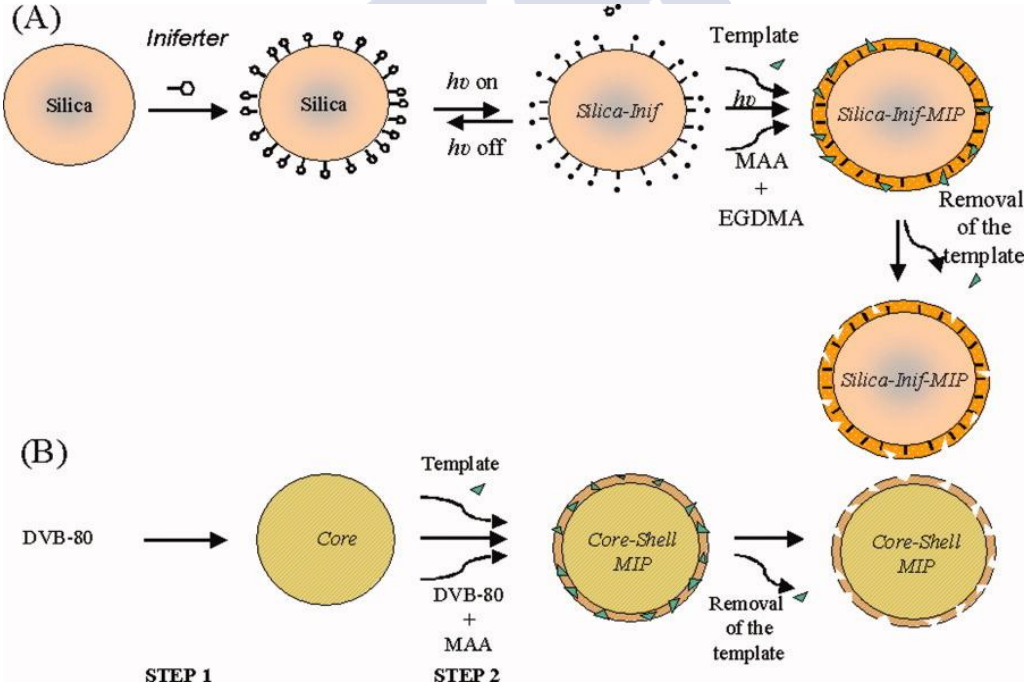


Figure 9. Schematic representation of the preparation of (A) thin layers of MIP via an iniferter-type initiator technique on spherical porous silica and (B) molecularly imprinted polymer microspheres with core-shell morphology prepared by precipitation polymerization in a two-step procedure. Reproduced from Barahona *et al*. (2010) Copyright 2010 Wiley Periodicals, Inc. [49]

In contrast, grafting technique consists of two variants which are described and represented with more detail in Figure 9. The first strategy ("grafting from"), involves the introduction of thin layers of MIP using an immobilized initiator on the particle surface (porous silica spheres or particles) [94]. The initiators may be azo [95] or iniferter-type [96].

Iniferters are asymmetrical radical initiators which, when fragmented, break down into two radicals, one capable of initiating polymerization and the other favouring recombination between radicals, resulting in the completion of polymerization. By their nature, iniferters are the only initiators capable of preventing polymerization within the solution, thus facilitating stricter control of the thickness of the polymer layer created and better mass transfer. With this type of starter, the growth of the polymer chains always occurs from the surface of the substrate, which allows for greater control of both the thickness of the layer and its density (Figure 9A).

In the second synthetic modality ("grafting to"), the immobilization of molecules with polymerizable bonds (monomers) is performed on the surface on which the polymerization is to be performed [94]. The polymerisation mixture (template, cross-linker, initiator and solvent) is then brought into contact with the modified surface and polymerisation takes place, resulting in the formation of a layer of MIP on the surface of the particles. This polymerization system has been used to obtain MIPs following both covalent and non-covalent models. However, this methodology presents a great difficulty when it comes to controlling the thickness of the polymer layer formed and, in addition, the maximum density of polymer formed is limited by steric and kinetic factors (Figure 9B). The following is a micrograph which shows the scanning electron of the bare silica support (Figure 10A) and the corresponding inif-MIP (Figure 10B). The successful grafting and moreover the individual integrity of the particles has been retained and no polymerization in solution was observed demonstrating the suitability of this methodology for the preparation of imprinted polymers.

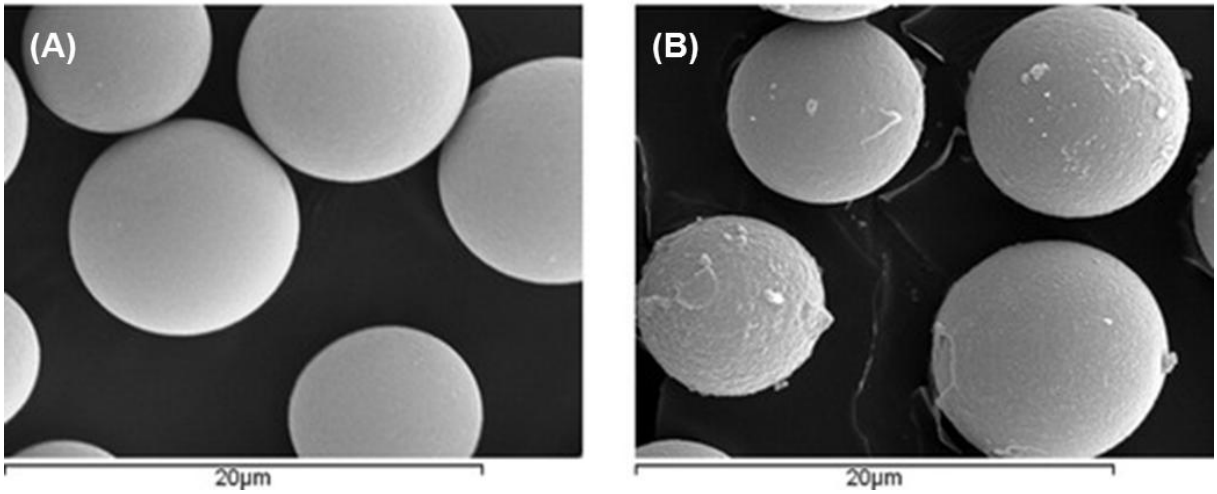


Figure 10. Micrograph of polymer coated silica spheres using the "grafting from" technique: (A) bare silica support and (B) inif-MIP beads. Modified with permission from Barahona *et al*. (2010) Copyright 2010 Wiley Periodicals, Inc. [49]

1.1.3.3 Combinations of MIPs with other materials

It is of great interest to search for new approaches that allow the modification of MIP and thus obtain materials adapted for new specific applications. More dedicated and less common applications of polymers have recently been found as a result of combining these imprinted sorbents with different materials such as silica, magnetite, metals, glass, carbon, polyetilene and other plastic supports and hybrid materials. Coatings, silane chemistry and/or surface-grafting approaches are some of the solutions developed to combine MIP with fibers,

nanoparticles, quantum dots, films or slides, nanotubes and/or beads of different materials [97-100], will be explained in more detail below.

1.1.3.3.1 Magnetic supports

At the same time to the developments achieved in molecularly imprinting, magnetic materials have been applied increasingly, alone or in combination with MIP. These materials contain small particles of ferromagnetic material such as magnetic iron oxide or magnetite (Fe_3O_4). Materials are only aggregate in the presence of a magnetic field, due to the small size of the magnetite particles. There is a wide range of methods to prepare magnetic particles. Solid magnetite or ferrofluids may be incorporated in suspension polymerization protocols or polymer beads may be "post-magnetized" by the precipitation of iron oxide from solution or inclusion of colloidal magnetite or ferrofluids [101]. In recent years, MIP sorbents have been combined with these different magnetic supports in order to achieve automated methods of extraction. These new combinations are known as magnetic molecularly imprinted polymers (MMIPs) (Figure 11). Generally, magnetic polymers are prepared by encapsulating inorganic magnetic particles with organic polymers.

The magnetic polymers and the analytes bound to them can be easily collected with the aid of an external magnetic field and without additional centrifugation or filtration (Figure 11).This possibility makes separations easier and faster than using traditional sorbents. Because of their large surface area, and unique physical and chemical properties, these MMIP have been widely applied in many fields such as cell separation, drug delivery and enzyme immobilization [102]. Core-shell magnetic MIPs (e.g., multiple Fe_3O_4 as core and SiO_2 as shell rather than a single Fe_3O_4 nanoparticle as supporter) have been designed not only to improve the magnetic performance but also to enhance the biocompatibility and stability of MMIP [103, 104]. A novel strategy to design well-organized, highly magnetic MIP particles was firstly proposed in 2009, in order to use them as optical sensing phases [105]. Recently, a research group reported its success in the design of well-defined nanospheres with a double-layer core-shell structure consisting of Fe_3O_4 nanoparticle core, an inner fluorescent layer and an outer layer of MIP [106]. With this regard, for the obtainment of MMIP, Fe_3O_4 nanoparticles are usually synthesized by a co-precipitation method.

To achieve MMIP, magnetic materials may be modified by silanization with tetraethyl orthosilicate (TEOS) and 3-methacryloxypropyl trimethoxysilane (MPTS) before imprinting, in order to promote the coating process of the polymer on the magnetic support [107-110]. A silica coating on the surface of magnetic nanoparticles by sol–gel process (Fe_3O_4@ SiO_2 nanoparticles) make them compatible with MIP and provide them with a silica-like surface easily modified with various groups for coating purposes, for instance subsequent modifications with, for instance, MPTS (Fe_3O_4@ SiO_2@MPTS nanoparticles) or 3-aminopropyl trimethoxysilane (Fe_3O_4@ SiO_2@APTMS nanoparticles) [109, 111]. Besides, the resulted silica shell provides hydrophilic surface which prevents the oxidation. Alternatively, oleic acid-modified Fe_3O_4 nanoparticles have also been used to obtain magnetic MIP [102, 112]. Oleic acid may be easily combined with DVB and styrene polymeric matrixes, which can be obtained easily and cheaply, as it provides adequate hydrophobic shell on the magnetic surface, obtaining stable and magnetic polymers [102]. It is worth mentioning the use of polyvinylpyrrolidone (PVP) as stabilizer dispersant agent in many of these approaches [102, 113]. Other option for obtaining magnetic imprinted materials is the use of magnetic nanocrystals, coated with a thin layer of MIP. In this case, the combination is successfully achieved using a salt chemistry with the iniferter method [101].

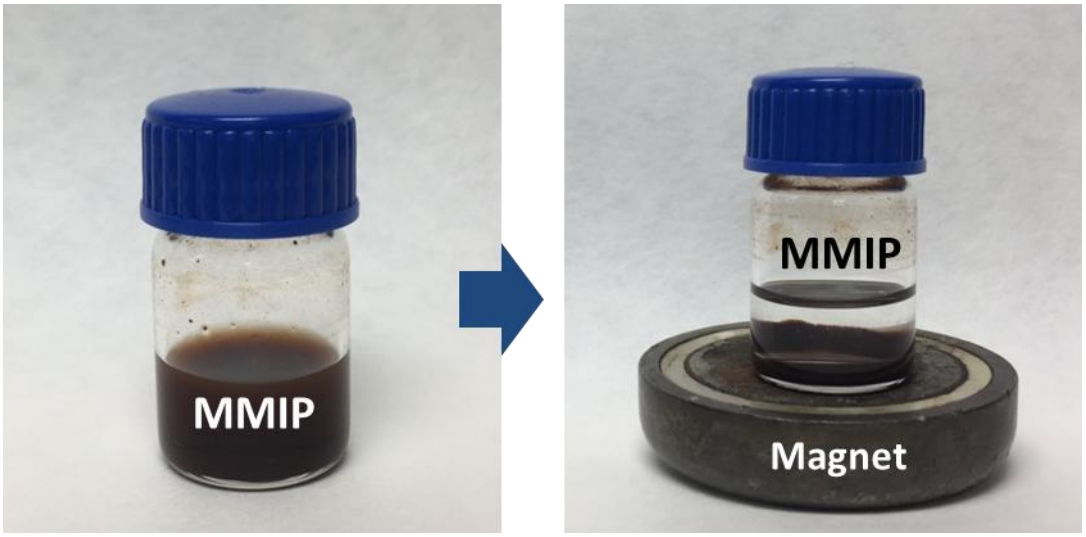


Figure 11. Example of magnetic molecularly imprinted polymer (MMIP) particles in solution that can be collected with the aid of an external magnet

1.1.3.3.2 Silica

Silica is another material used often as physical support in molecularly imprinting technology. Silica particles containing surface-bound free radical initiators have been used as supports for the grafting of thin films of molecularly imprinted polymers [100]. By confining the initiating radicals to the support surface a higher density of grafted polymeric chains can be achieved [94, 114]. In the absence of chain transfer, the chain will grow mainly from the surface of the support with minimal polymerization occurring in solution. The silica supports can be modified covalently with glycidoxypropyltrimethoxysilane (GPS) or (3-aminopropyl)triethoxysilane (APTS) and the azoinitiator 4,4‘-azobis(4-cyanopentanoic acid) (ACPA) (Si-GPS or Si-APS) or non-covalently with the diamidine azoinitiator 2,2‘-azobis(N,N‘-dimethyleneisobutyramidine) (ADIA). The chromatographic properties of these materials depend on the thickness of the polymeric layer on the silica surface, the solvent, the pore diameter, the cross-linker and the composition of the mobile phase, amongst others. A thin film of MIP can be grafted from preformed spherical porous silica particles using an immobilized iniferter-type initiator (inif-MIP), and as such this methodology has been reported (Figures 9 and 10) [115, 116]. The procedure involves coupling of an iniferter onto the silica, an initiator for free radical polymerization which is able to both initiate and terminate the polymerization process. In this manner, polymerization occurs only via the active radical immobilized onto the porous silica surface, and polymerization in solution is avoided [49].

The immobilization of methacryloxypropyltrimethoxysilane (MPTS) onto silica surface can also provide polymerizable groups for their copolymerization with monomers in solution. MPTS covalently attached to the surface of Stöber silica particles would result in SiO2-MPTS particles. This method has been recenlty used to prepare methacrylate-silica particles from MPTS precursor and tetraethylorthosilicate (TEOS) using a sol–gel process. Then, the silica particles covered with methacrylate groups can be used to prepare two types of molecular imprinted polymers, i.e. SiO2MAA@MIPs and SiO2MA@MIPs [100]. Apart from nanoparticles, polymerization can be performed on the surface of a silica fiber or nanotube. The preparation of imprinted fibers is performed by silanization step of the surface of silica

fibers which are subsequently immersed in fresh polymerization solution and repeatedly coated until the desired thickness is reached [104, 117-119].

1.1.3.3.3 Glass

The combination of MIP with glass supports is also possible using different approaches as it has been already demonstrated in some studies. To achieve successful MIP coating on glass surfaces, the uniform deposition of silane layer upon glass is the technique of choice. As a prerequisite for subsequent silanization, glass needs to be carefully cleaned, usually applying acidic and/or basic solutions [120]. After rinsing thoroughly with deionized water and dry it under N_2 stream, the glass is ready for silane modifications. Up to date, one of the most frequent silanization reagents for glass supports has been 3-mercaptopropyltrimethoxy silane (MPTS) [121, 122]. Glass may be used in the form of different formats and devices, such as glass-covered magnetic stir-bars, *e.g.* the one shown in Figure 12, or even glass slides. The effective coating of glass-covered stir-bars has been recently reported, using two types of coating procedures: physical and chemical coating [122]. In the first option, the authors simply used an epoxy adhesive to immobilize the MIP particles, previously synthesized by precipitation polymerization, over a previously conditioned glass stir bar, *e.g.* etched with fluorocarbon etchant to provide a rough surface. The silanization of the surface was not necessary in this case. In the second technique, the etched glass surface was submitted to a silanization step before immersing it within the pre-polimerization mixture. Polymerization was performed using an oven, resulting in a MIP-coated stir bar with the shape and the size of the container in which the procedure takes place. The authors concluded that the chemical coating is a better approach when desiring to combine MIP and glass supports [122].

Figure 12. Magnetic molecularly imprinted stir-bar (MMISB) developed coating a glass-covered magnet; the screw cap was placed below the stir-bar to illustrate its size. Reproduced from MDPI under CC-BY 4.0 [123]

Besides stir bars, other glass devices or supports have been used to obtain novel MIP-based detection methods. For instance, the combination of colloidal-crystal templating and a molecular imprinting technique resulted in a sensor platform that allowed the efficient detection of the analyte in aqueous solution [124]. The photonic polymer hydrogels are constructed in three steps: preparation of the colloidal-crystal arrays by vertical deposition of silica on glass slides, polymerization of pre-ordered complex of template and functional monomers in the interspaces of the crystal and removal of template and silica particles form the imprinted matrix (see Figure 13 for an example).

Finally, to overcome some of the limitations of traditional MIP methodologies such as template bleeding and high binding-site heterogeneity, the possible synthesis of MIP glass-

based nanoparticles in a solid-phase approach has been reported. Canfarotta *et al.* (2016) described an elegant method that relies on the covalent immobilization of the template onto the surface of glass beads. The obtained MIPs are virtually free of template and have demonstrated high affinity for the target molecule [125]. To create these MIP, the surface of the glass beads is activated by NaOH and amino groups are then introduced to the surface by MPTS. Moreover, the reusability of the glass beads-template complex for more than one MIP batch is also an advantage that allows minimizing the amount of template required for the synthesis [126].

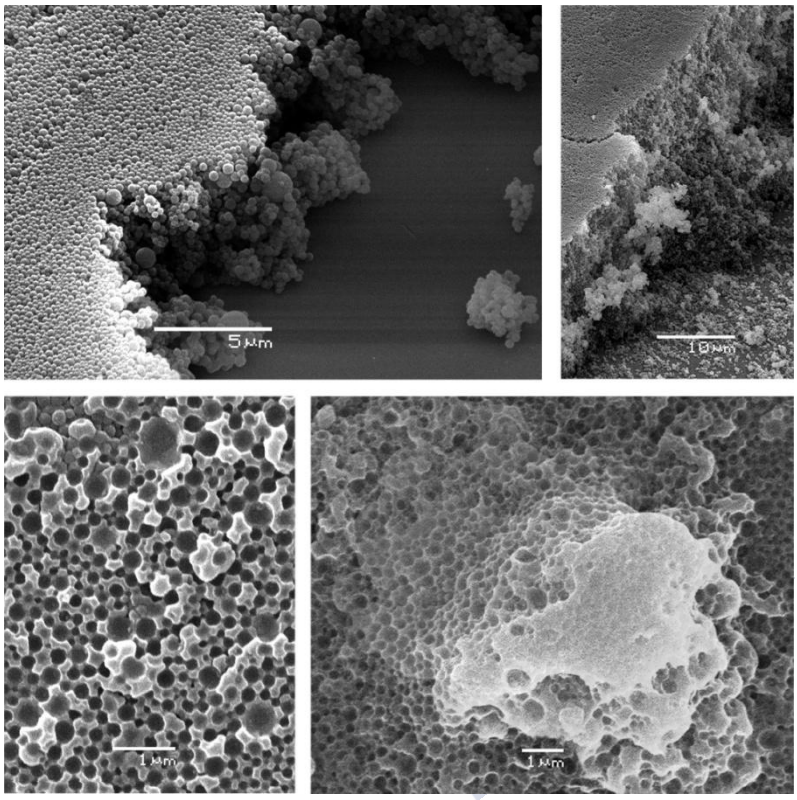


Figure 13. SEM images of the macroporous structure obtained using colloidal-crystal templating and molecularly imprinted, before (upper part) and after (lower part) removal of silica particles and template. Reprinted with permission from Díaz-Bao, M. *et al.* (2018) Copyright 2018 by Taylor and Francis [29]

1.1.3.3.4 Other materials

Materials other than magnetite, silica and glass, may also be combined with MIP for their application in analytical chemistry. Coatings and surface-grafting-to approaches are also a general form of merging the polymer and its support, which is presented in different forms such as fibers and nanotubes, beads (e.g. quantum dots or gold nanoparticles), or films, for achieving hybrid materials [127, 128].

Carbon nanotubes (CNT) are classified into multi-walled carbon nanotubes (MWNT) and single-walled carbon nanotubes (SWNT). The first ones are considered the ideal nanotube support for MIP because of high strength, stability under acidic conditions, lack of swelling and large surface areas. Molecularly imprinting technology has been applied to the surface of MWNT through the functionalization of the tube surface, using covalent or non-covalent methods. Besides, various kinds of nanoparticles may be incorporated on MWNT, modifying the properties of the obtained CNT@MIP complex [98]. Apart from silica beads, "core

particles" can be prepared by precipitation of DVB in acetonitrile and used as seed particles in the synthesis of MIP shells by copolymerization of functional and cross-linker monomers in an adequate porogenic environment [49]. Finally, the combination of quantum dots encoded microbeads with molecularly imprinted polymers has been recently reported, forming a core-shell material with multiplexing and selective recognition abilities [99].

Tips, membranes or stainless steel columns, amongst others, can be considered alternative options to classical MIP approaches, being their use much less frequent than the rest of materials [129-133]. However they present some advantages such as miniaturization of procedures, and/or simplicity of preparation. For instance, MIP monolith can be prepared in a micropipette tip, simply by in situ polymerization and with no need of chemical activation of the tip. The monolithic tip can be easily connected to a syringe to perform MISPE procedure. Monoliths synthetized inside a stainless steel column may be used as on-line clean-up procedures or even as chromatographic columns. Finally, the recent combination of MIPs with liquid membranes circumvents the traditional lack of recognition associated to MIPs in aqueous media thus opening new areas of application [134].

1.2 Food Safety: Control of Residues and Contaminants

Nowadays, food safety is one of the major concerns of society due to the health alarms generated in recent years. Among these alarms, some of the most relevant ones in EU should be highlighted, such as the case of "mad cow disease" or bovine spongiform encephalopathy (BSE), contamination of chickens with dioxins in Belgium, the removal of infant formulas manufactured in France associated with an outbreak of Salmonellosis in children in the same country, among others. The most recent alert (23th August 2019) in Spain has been caused by a *Listeria monocytogenes* outbreak linked to meat products that poisoned hundreds of people so far [135].

These crises are some examples that reflect the increasing demand from society for information on the presence of contaminants or residues of products that are harmful, toxic or hazardous to health, which may be present in food. A proof of this interest is the concern, both on the part of official organisms and the food industry, to control the presence of these substances in the food chain, "from farm to table". At this point, it is necessary to analyse the content of certain compounds in food for a number of reasons. Firstly, due to the intentional use of pesticides in agricultural holdings food contamination is possible, and secondly, the use of veterinary drugs in farms may lead to residues in food of animal origin. Such products may remain in food in the form of residues after application and their determination is therefore necessary. On the other hand, it is necessary to analyse the levels at which other substances that have not been intentionally used by humans are found, as in the previous case. These other substances are contaminants, which are present in the environment or in the material in contact with food, having reached this point via different routes and whose presence also poses a significant risk to human health.

All parties involved in the food sector, such as farmers and veterinarians, are responsible for both the quality and safety of food on the market, as they need to ensure which substances can be used safely in agricultural production. Thus, regulators must adopt food control measures but always taking into account international trade obligations. In the event of non-compliance, inspectors and laboratory technicians must provide evidence and know what can be enforced. On the other hand, judges decide on sanctions in case of illegal use of pharmacologically active substances. Finally, the consumer values the products on the market [136].

Due to the potential presence of high-risk substances in food, there is a need to develop new analytical methods that allow rapid and efficient sample preparation together with the reliable determination of the compounds of interest. Chemical hazards are a major cause of food contamination associated with foodborne outbreaks [137]. Food contaminants typically include environmental contaminants, food processing contaminants, adulterants and non-approved food additives, and migrants from packaging materials [138]. There is a wide variety of organic compounds found in foods as a consequence not only of their permitted use but also due to natural processes or human action. Organic compounds that appear in food due to their use during production or storage (pesticides, veterinary drugs) are classified as residues, while compounds that have been generated naturally during food production, storage or processing (mycotoxins, marine biotoxins) or by anthropogenic activities (dioxins, dioxin-like polychlorinated biphenyls) are referred to as contaminants [139].

1.2.1 Veterinary drugs residues in food

Veterinary drugs or veterinary medicinal products are used as prophylactic agents and to treat animal diseases. There is a wide variety of substances for this purpose such as antibiotics, anticoccidials, anthelmintics, sedatives, and steroidal as well as non-steroidal hormones. Apart from their desired effects, the incorrect or abusive use of these substances (e.g., with a withdrawal period that is too short or using growth-promoting hormones to accelerate animal-meat production) can also exhibit adverse properties [140]. Given this situation and in order to protect the health of consumers, it is essential to evaluate the safety of these substances, taking into account the toxicological risks, environmental contamination and the undesirable pharmacological and/or microbiological effects of their possible residues. This assessment of the safety of the product, together with its quality and efficacy, will enable the veterinary medicinal product to be authorised or not. Although, the use of these active substances benefits productivity, it is also accompanied by the risk associated with residues of veterinary drugs remaining in animal tissues at the time of slaughter [141].

Improvements in animal management and feeding, together with the rational use of veterinary drugs, have greatly contributed to achieving high productivity rates on most farms worldwide. The use of veterinary drugs in food-producing animals can generate residues in edible products such as milk, meat, honey and eggs so its irrational administration may pose a hazard to the health of the consumer [142]. Residues of veterinary drug are *"pharmacologically active substances (whether active substances, excipients or degradation products) as well as their metabolites which remain in foodstuffs obtained from animals to which the veterinary drug in question has been administered"* as defined by European Union (EU) and the Centre for Veterinary Medicine, an agency under the Food and Drug Administration (FDA/CVM) in the USA [142]. There are also veterinary drugs such as antimicrobials, anabolic hormonal compounds, thyreostatics, corticosteroids and β-agonists that through various metabolic mechanisms have the ability to improve animal production. Because of this, they have been used in livestock farming as growth promoters for many years [143]. However, in most European countries their use as growth promoters has been banned as a measure to protect consumers [144-146, 146]; therefore the use of alternative products is necessary. For prevention of risks it is necessary to use vet substances in a responsible way, using them only if necessary in the right dose and moment, and always administered respecting periods established for edible products under the indications of a veterinarian [143]. Veterinary drugs usually accumulate in the liver or kidney rather than other tissues. It has been noted that different residues levels can be found in different tissue positions such as site and route of administration [147]. Pesticides are similar to residues of veterinary drugs in

that they can present a risk to the individual by remaining in the meat and causing the transfer of antibiotic resistance and even inducing the risk of allergies [12].

The European Union requires that food products such as milk, eggs and meat, obtained from animals exposed to chemicals or treated with veterinary medicinal products used during animal husbandry do not contain residues at a level that may represent a hazard to the health of consumers. With the appearance in 1990 of Regulation 2377/90/EC those substances were classified into four Annexes and the use of veterinary medicinal products was regulated by defining a procedure for establishing maximum residue limits (MRLs) for veterinary products in foodstuffs of animal origin. MRLs are the maximum concentration of a residue of a given substance that may legally be present in food as a result of the use of that substance in the animal [148, 149].

In 2010, with the publication of Commission Regulation (EU) 37/2010, all pharmacologically active substances were included in a single Annex [142]. Two separate tables were established, one of them for allowed substances and the second table for substances that must not be used in food producing animals or in biocidal products for use in animal husbandry [142, 143]. Thus, Codex Alimentarius Commission (CAC) together with the European Union and other regulatory authorities around the world have established and implemented MRLs to ensure the limited presence of antibiotic residues in food of animal origin in addition to restricting the use of banned veterinary drugs [142, 150]. The Joint FAO/WHO Expert Committee on Food Additives and Contaminants (JECFA), at its 40th Session of the CAC, updated the MRLs for veterinary drug residues in foods, due to concerns about the presence of antibiotic residues in food of animal origin [150]. Similar regulations are found in countries such as the USA, where the Food and Drug Administration regulates veterinary drugs through the Center for Veterinary Medicine (CVM) at FDA website [151]. Although similar trends exist in countries such as Canada, China, Japan, Argentina, Australia or Brazil, there are important differences in the authorised substances among countries.

1.2.1.1 General classification of veterinary drugs

Veterinary medicinal products can be classified not only according to their therapeutic purpose but also according to other criteria, such as chemical properties, structure, mode of action or also the existence or otherwise of authorisation for their administration to food-producing animals all over the world. According to Regal *et al.*, (2018) and from the point of view of food residues, the following classification has selected as represented in Table 1.

Antimicrobial substances are the broadest and most varied group of all the existing groups of veterinary medicinal products as well as the most used in husbandry worldwide. There are hundreds of antimicrobial drugs, most of which belong to a few major classes (β-lactams, sulphonamides, quinolones and fluoroquinolones, tetracyclines, nitrofurans, aminoglycosides lincosamides, phenicols, nitroimidazols, ionophores…) [152]. The group of antibiotics includes antimicrobials/antibacterials both produced and derived from microorganisms, however it does not include substances that are synthetic or semi-synthetic or those obtained from animals or plants. Antimicrobials are substances produced both naturally and synthetically and are used to kill or inhibit microorganisms (bacteria, fungi or viruses, parasites in particular protozoa) [145]. Coccidiostats and histomonostats substances are used in the case of protozoa infections and also belong to the group of antimicrobials. There group of β-lactams antibiotics (BLAs) constitute one of the most widely used antimicrobial drugs in veterinary medicine especially to treat and prevent bacterial infections (urinary and mammary gland, respiratory or skin infections) of dairy cattle [153].

Tetracyclines, sulphonamides and fluoroquinolones are groups of synthetic antibacterial agents widely used in veterinary practice for the prevention and treatment of infections in food-producing animals due to their broad spectrum of activity, easy over the counter availability and low cost [154, 155]. Once antibacterial medications are given to animals to treat an infection or disease, if the use of them is improper (i.e. use as feed additives to promote growth), it can give rise to residues in edible tissues or derived products such as milk. It means have adverse effects on consumer health including bacterial resistance to these drugs in humans, and also problems in the dairy industry. To avoid risks to consumer health, the EU has defined safe maximum residue limits (MRLs) in Commission Regulation (EU) No 37/2010 [142]. In analytical chemistry, within the food field, the group of antibacterial substances is one of the most important [143]. Thus, recent research predicts a large increase in antimicrobial consumption by 2030 in EU, in comparison to 2010, and nearly double in India, Russia, Brazil, China and South Africa [156]. There are many other veterinary drugs besides antimicrobials that have also been authorized for administration in animal production. These include drugs that act on the nervous system (tranquilizers, sedatives, β-agonists), non-steroidal anti-inflammatory drugs (NSAIDs), and corticosteroids or glucocorticoids that do not stimulate growth (dexamethasone, betametaxone, methylprednisolone) [142].

Table 1. Veterinary medicinal products classified according to the existence of authorization for their use to food-producing animals in the European Union (EU). Information gathered from Regal *et al*. 2018 [143]

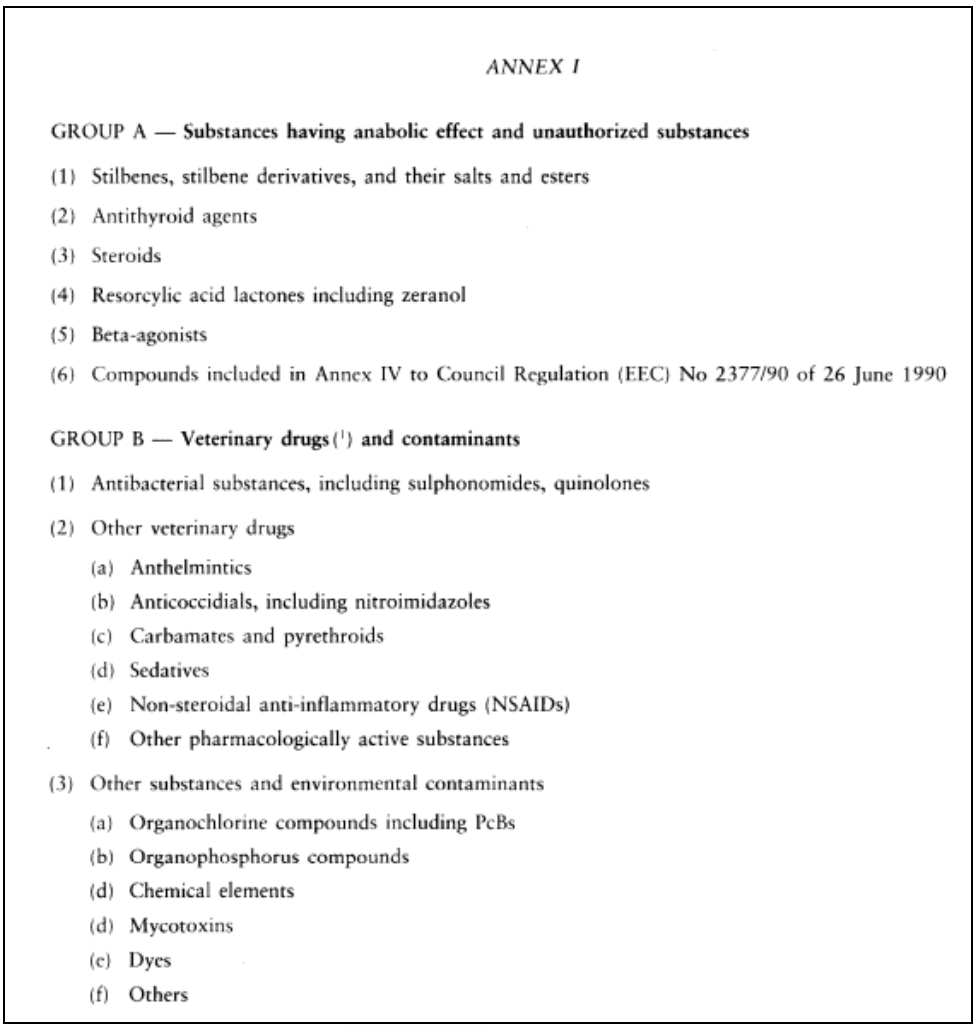
Authorized	Non-authorized	Natural presence/Others
	Antimicrobials (as growth promoters)	
	Corticosteroids/Glucocorticoids (as growth promoters)	
	ß-agonists	
Antimicrobials	Steroids (androgens, estrogens, gestagens)	Natural hormones
Corticosteroids/Glucocorticoids	Stilbenes, stilbene derivatives, and their salts and esters	Mycotoxins
Antiparasitic agents	Antithyroid agents	Prohormones
Sedatives, tranquilizers, anesthetics	Resorcycilic acid lactones	Selective-androgen receptor modulators
ß-agonists	Recombinant bovine somatotropin	Selective-estrogen receptor modulators
Non-steroidal anti-inflammatory	Chloramphenicol, chloroform, chlorpromazine, colchicine, dapsone, dimetridazole, metronidazole, nitrofurans (including furazolidone) and ronidazole	
	Synthetic dyes (malachite green)	

On the other hand, some substances whatever their level in food, pose a clear danger to consumer safety and cannot be used in veterinary practice in stock farming (non-authorized substances in Table 1). Council Directive 96/23/EC included all these veterinary medicinal products together in a single group called group A (Figure 16), containing banned substances according to Directive 96/22/EC, compounds with no MRL, growth promoters and drugs abused in animal fattening [144]. Growth promoters include substances which have a hormonal action (steroid hormones, resorcylic acid lactones, stilbenes), β-agonists and thyreostats (thiouracils and mercaptoimidazole analogues), all capable of enhancing growth rate [145]. Strictly speaking, thyerostats cannot be considered as growth promoters because their effect is not anabolic. Despite being banned in the EU, some of these compounds are authorised in the United States. On the other hand, in the case of corticosteroids, these are steroid hormones that are used as antipyretics, anti-allergens and anti-inflammatories in both human and veterinary medicine [67]. Corticosteroids are authorized for therapeutic treatments in food producing animals provided that waiting times between treatment and slaughter are respected [143]. Accordingly, maximum residue limits (MRLs) have been established for authorized corticosteroids in matrices such as milk, liver, muscle, kidney and fat [142]. Such drugs can also be used illegally as growth promoting agents, synergistically combined with other substances such as anabolic agents or β-agonists.

In addition to thyreostatic, β-agonists and corticosteroids, compounds such as androgenic, estrogenic and gestagenic (EGAs) can be used alone or in combination with growth promoting cocktails with low concentrations of different substances making it harder to detect. Chloramphenicol (CAP), chloroform, chlorpromazine, dapsone, dimetridazole, colchicine, nitrofurans (including furazolidone), metronidazole and ronidazole are veterinary drugs with no MRL because a safe residue limit could not be established. In particular, chloramphenicol (CAP) is a broad-spectrum bacteriostatic antibiotic which has been subject of study, commonly used in veterinary medicine for the treatment of mastitis in cattle due to its highly efficient and low-cost activity. Due to detection of toxic effects in humans such as Grey syndrome, bone marrow suppression, and fatal aplastic anaemia, the use of CAP in foodstuffs has been banned in EU since 1994 and no maximum residue limit (MRL) has been established in animal-derived foods. To reach a harmonized analytical performance of methods monitoring CAP, the European Commission defined a minimum required performance limit (MRPL) for CAP at 0.3 $\mu g.kg^{-1}$ [78].

Finally, the use of synthetic dyes with pharmacological activity to reduce parasitic, microbial and fungal diseases in aquaculture such as malachite green is not allowed in the EU. These substances are classified into triarylmethanes, xanthenes, phenothiazines, acridines and azoic compounds. Although their use is not permitted in EU they are still used illegally due to their high efficiency, availability and relatively low cost [143]. It is necessary to take into account that some natural compounds such as zearalenone can be found in animal feed due to contamination of cereals with fungi. Its presence can be considered natural and inavoidable, although they belong to the group of prohibited compounds (resorcyclic acid lactones). Due to the hormonal variability of animals, it is practically impossible to establish legal thresholds for the control of any exogenous administration of natural hormones to animals [143]. Selective androgen receptor modulators (SARMs) have less androgenic side-effects and they constitute a novel class of androgen-receptor ligands with anabolic properties. It cannot be discarded their potential misuse in animals although they have been already banned by World Anti-Doping Agency (WADA) [158]. Similarly, the group of anti-estrogenic substances includes selective estrogen receptor modulators (SERMs), but also anti-estrogens

andaromatase inhibitors. Their illegal use for fattening purposes poses a risk to animal husbandry. In addition, the following are prohibited in human sports doping [159].



ANNEX I

GROUP A — **Substances having anabolic effect and unauthorized substances**

(1) Stilbenes, stilbene derivatives, and their salts and esters

(2) Antithyroid agents

(3) Steroids

(4) Resorcylic acid lactones including zeranol

(5) Beta-agonists

(6) Compounds included in Annex IV to Council Regulation (EEC) No 2377/90 of 26 June 1990

GROUP B — **Veterinary drugs**(1) **and contaminants**

(1) Antibacterial substances, including sulphonomides, quinolones

(2) Other veterinary drugs

- (a) Anthelmintics
- (b) Anticoccidials, including nitroimidazoles
- (c) Carbamates and pyrethroids
- (d) Sedatives
- (e) Non-steroidal anti-inflammatory drugs (NSAIDs)
- (f) Other pharmacologically active substances

(3) Other substances and environmental contaminants

- (a) Organochlorine compounds including PcBs
- (b) Organophosphorus compounds
- (d) Chemical elements
- (d) Mycotoxins
- (e) Dyes
- (f) Others

Figure 14. Classification of groups of substances A and B according to the Directive 96/23/EC (EC 96) [157]

1.2.1.2 Analytical methods for veterinary drugs residue control

The large amount of food available to the consumer can give an idea of the enormous amount of analysis that is required. Moreover, the low concentrations at which veterinary drug residues are found in edible matrices make indispensable the need for highly sensitive analytical methods necessary for their detection, identification and quantification [160]. The development, implementation and validation of suitable analytical methods are indispensable to put legislation into practice and to have appropriate means for effective veterinary drug residue control. These methods may be subdivided into screening methods and confirmatory methods, both of them have their specific demands, which should be kept in mind when selecting an appropriate analytical technique [140]. Screening methods are analytical methods used for detecting the presence of a substance or a group of substances at the level of interest. This type of methods can be qualitative or semiquantitative and they are designed to sieve a large number of samples and to avoid false compliant results [160]. Examples of screening methods for veterinary drugs residues are enzyme-linked immunosorbent assays or ELISA

[161, 162], microbiological inhibitor tests [163], fluorescence-linked immunosorbent assays [164] or receptor tests [165]. Obtaining precise results is of great importance because false-negative or false-positive results bring great consequences for the consumer as well as for the producer [140].

On the other hand, confirmatory methods are used to provide complete or additional information which allows identifying a substance as well as quantifying it, if required. Unlike screening methods, confirmatory alternatives are mainly based in chromatography coupled to different detectors such as UV or mass spectrometers (MS) [166-169]. In the case of banned substances, the main objective is the identification of substances in as many matrices as possible, at the lowest possible concentration (zero tolerance). Although for substances with an MRL, methods for their quantitative determination in food matrices must be developed, for prohibited substances the development of qualitative methods is necessary, followed by (semi-)quantitative methods [160]. The MRL is linked to the level of the active substance which remains in the animal tissue at the end of medical treatment, and also to the amount of this particular food which is consumed by the population en a daily basis. Therefore, a MRL is set for each relevant food product such as milk, eggs, meat… for each relevant species [136, 142]. Moreover, for the detection of substances for which no permitted limit (MRL) had been introduced, minimum required performance limits (MRPL) are set [170]. In order to ensure the quality and comparability of the results obtained from the various analyses in the official laboratories [171], Commission Decision 2002/657/EC implementing Council Directive 96/23/EC laid down criteria and rules on how analyses are to be carried out and the interpretation of the results obtained. Moreover, there are concepts that must be established during the validation of an analytical method, such as decision limit (CCα) and detection capability (CCβ), terms introduced according to 2002/657/EC as quality parameters [140].

In the field of separation techniques, attempts have been made to improve separation speed and power. This has been reflected in a number of changes such as the move from thin layer chromatography (TLC), method used for the qualitative detection of banned substances such as thyrostats and certain anabolics of the time [160], to high performance liquid chromatography (HPLC), the introduction of ultra-high-pressure liquid chromatography (UHPLC) as well as the two dimensional liquid chromatography (2DLC). Nowadays, techniques such as HPLC and UHPLC with triple quadrupole tandem mass spectrometry (MS/MS) are frequently used and effective for confirmatory analysis [169, 172]. Although first HPLC instruments were not very robust, later ultraviolet (UV) detectors were introduced and nowadays this technique still often used for the detection for the detection of macrocyclic lactons after derivatization, amongst others [173]. In the past, for the detection of veterinary drugs, postcolumn derivatization and UV detection were often used and new devices such as GC-MS were introduced on the market. Thus, GC-MS methods were published for the analysis of different analites such as thryreostats [174], β-agonists [175] or CAP [176], among others. Despite all these applications, GC-MS methods have one important disadvantage and it is the often time-consuming preliminary derivatization steps. It was here that the LC-MS technique was introduced but however, it was not until the late 1990s that it became the obligatory standard equipment for residues. A review on the use of LC-MS in food safety was published by Malik *et al.* in 2010, where a large number of methods were described to detected different substances [177]. With the evolution of the analysis of veterinary drug residues, sophisticated equipment such as high-resolution mass spectrometry (HRMS) was required, which allows reliable identification in complex matrices due to its accuracy and mass resolution. The practically unlimited number of substances that can be analysed

simultaneously and the possibility of performing MS analysis and identifying new biomarkers, among others, are other advantages of this technique. Although it also has disadvantages such as high investment costs in addition to its reduced sensitivity when compared to the low-resolution mass spectrometry (LRMS) [160]. Last but not least, there is another important technology (GC-C-IRMS) that allows the distinction of endogenous steroids among their synthetic analogues [178].

The use of HPLC multi-residue method is a way to improve the cost-effectiveness and the speed by maximizing the number of analytes that can be determined by a single method, i.e. within one analytical run is capable of analysing multiple compounds, often belonging to different classes of residues [179]. To determine in a single run, the presence of residues of multiple veterinary drugs, multi-residue methods save solvents, samples, time and analytical costs and include a large number of compounds. Both the term multi-residues and multi-class are often used interchangeably, but it is necessary to take into account they are not and should not be considered synonyms [143]. Several examples for the simultaneous determination of more than 100 veterinary drugs along with their metabolites using LC-MS/MS in multiclass/residue methods have been described for different matrices such as bovine, porcine, and chicken muscle/meat/kidney [180, 181], milk [182, 183], honey [184, 185] and eggs [186]. There are many multi-class and multi-residue methods recently presented to contaminants, mycotoxins and others [186-190]. Although traditional methods are still used for the analysis of a single residue or a class of residues, more modern methods such as multiclass/multi-residue are predominant. This is due to the fact that, they are more cost-effective and time-effective. Moreover, they are selective and highly sensible (very low LODs and LOQs), and also accurate for identification and confirmation [168, 169, 172, 187, 191-193]. For the analysis of multiple residues of veterinary drugs and growth promoters the classical analytical techniques employed range frequently from liquid chromatography coupled to triple quadrupole mass spectrometers (HPLC-MS/MS), towards untargeted full scan mass spectrometry utilizing UHPLC and HRMS [194]. Recently published methods demonstrate very powerful technologies for both screening and confirmatory analysis of multiple residues of veterinary drugs in food, such as time-of-flight (TOF) and Orbitrap technologies [195, 196]. Figure 15 shows the most common analytical workflows in a graphical overview.

The selective, reproducible and accurate detection of several analytes at low concentrations in different matrices requires a careful optimization of the analytical method. In this context, sample preparation step is still the most laborious step in the analytical procedure, mainly in the case of multi-analyte analysis [197]. The extraction and cleaning of samples prior to instrumental analysis has evolved over time. As a general rule, the results of the instrumental technique are strongly correlated with the efficiency of the cleaning [198]. A through overview of the different aspects involved in sample preparation is collected in the Kinsella review [199]. Moreover, a recently published review by Mainero Rocca *et al.* [168] compiles some of the most common techniques like solid-phase extraction (SPE) or QuEChERS (quick, easy, cheap, effective, rugged and safe) method, both currently available for extraction of veterinary drugs and sample clean-up. Solvent extractions have also proven to be very simple and effective tools in the laboratory so they continue to be very popular in sample preparation in residue analysis. This type of extractions together with modern equipment such as LC and MS has proven their effectiveness in the simultaneous determination of hundreds of compounds [200, 201]. There is a rather novel extraction and clean-up technique which enables a supported liquid-liquid extraction (SOSLE) with a

relative polar organic acceptor phase such as acetonitrile and it is based on high salt concentration in the aqueous donor phase [143]. The concept of salting-out in combination with SOSLE technique was proposed by Kaufmann *et al.* in 2014 [202]. This technique resulted of equal or superior cleanliness and with higher average recoveries in milk extracts, than those obtained with SPE or QuEChERS. Its robustness in multi-residue analysis for matrices such as muscle and eggs has been reaffirmed in recent researches [203].

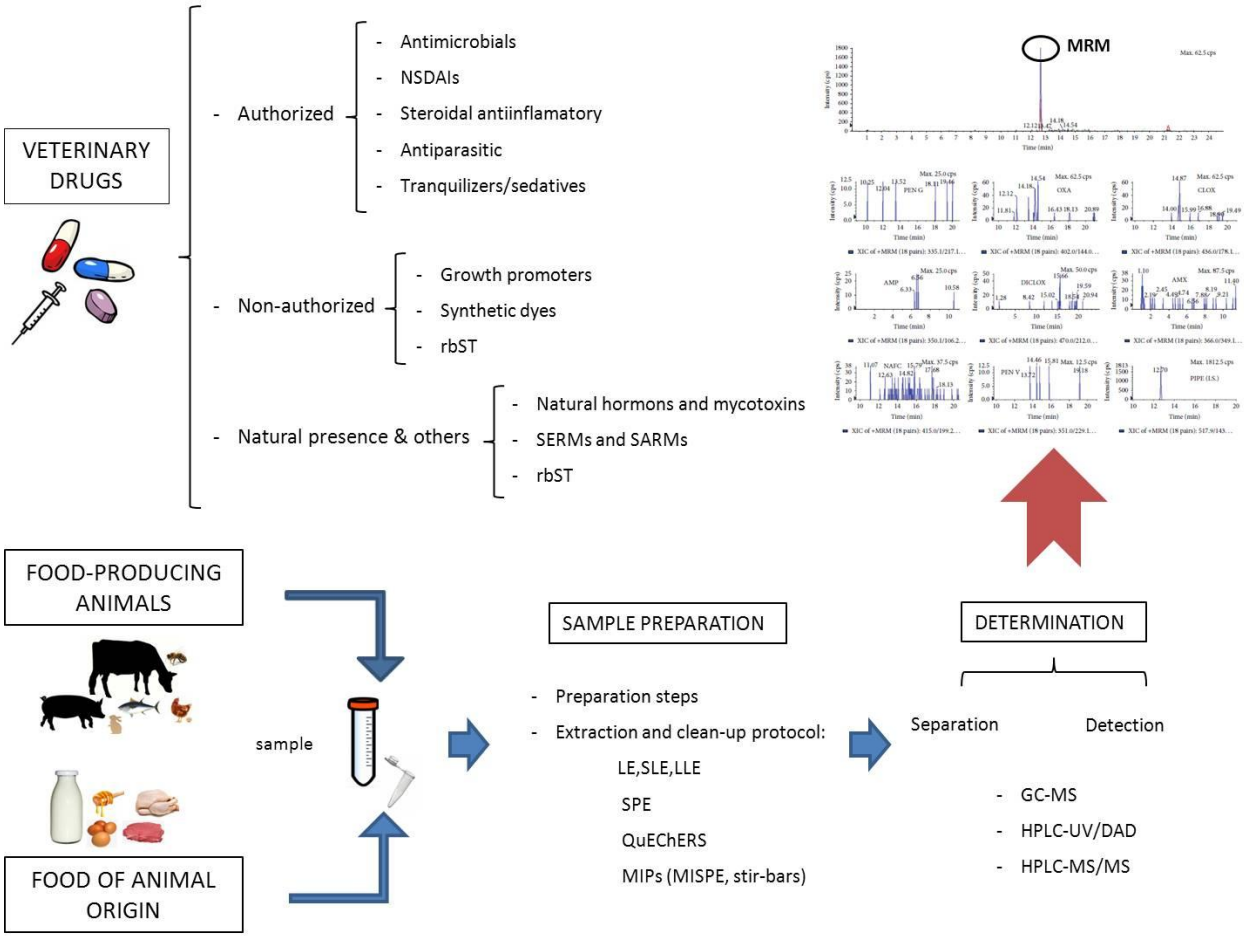


Figure 15. Analytical techniques most commonly used for the confirmation of residues of veterinary products in food (author's unpublished material)

Home-made extraction columns such as silica gel or solvent extraction columns were used for cleaning samples evolving with the SPE technique and immunoaffinity chromatography (IAC). It was at the end of 1990s when molecular imprinted polymers (MIPs) took over the job of sample preparation [198]. Nowadays, the SPE method is routinely used for the pre-treatment of food samples as it is easily automated and low cost [204]. However, the main drawback of the available SPE cartridges are the lack of selectivity of the sorbents and the low recoveries achieved. To overcome the limitations of common solid sorbents, molecularly imprinted polymers (MIPs) appeared as selective sorbents for SPE applications [44]. Molecularly imprinted solid-phase extraction (MISPE), a SPE method which uses MIPs as sorbent, provided selective extraction of different antibiotics from biological samples such as urine, milk among others [205, 206], and also in environmental

samples [118, 207]. In addition, new formats have emerged for the extraction of multi-analytes in food, such as molecularly imprinted instance stir-bars, monoliths and/or on-line clean-up columns, with a clear tendency towards miniaturization [208, 209]. Yet, some articles have been recently published using new techniques based on older principles [202, 210, 211].

1.2.2 Contaminants in food

Although food contamination dates back 8.000 years, globalization, the growth of agribusinesses and the consequent environmental pollution have helped to make the problem increasingly acute across the planet [212, 213]. The high concentration of chemicals present in the edibles is a matter of serious concern which poses serious health risks. Contamination of food occurs for multiple reasons including the natural presence of contaminants in the environment itself or their introduction by human action artificially [214]. Even though, these chemicals in food are regulated by the authorities prescribing minimum limits that are safe for human consumption yet measures still need to be taken to curb food contamination entirely. So, it is necessary to inspected and measured for the presence of chemical contaminants [12]. Codex Alimentarius defines a contaminant as follows: "any substance not intentionally added to food, which is present in such food as a result of the production (including operations carried out in crop husbandry, animal husbandry and veterinary medicine), manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food or as a result of environmental contamination. The term does not include insect fragments, rodent hairs and other extraneous matter". This definition of a contaminant implicitly includes naturally occurring toxicants including toxic metabolites of certain microfungi that are not intentionally added to food and feed (mycotoxins). Moreover, toxins that are produced by algae and that may be accumulated in edible aquatic organisms such as shellfish (phycotoxins) are also included [150].

Food contaminants usually include environmental contaminants, unapproved adulterants and food additives, and migrants from packaging materials. Environmental contaminants are impurities that appear naturally in soil, water or air or they are not introduced by human action. The direct sunlight that speeds deterioration of food and packaging even, adsorption of unwanted off-odor, are included in the group contaminating factors [12]. Moreover, excessive accumulation of heavy metals in agricultural soils through wastewater irrigation, may not only result in soil contamination entering into the food chain to infect the raw sources of food, but also lead to elevated heavy metal uptake by crops, and thus affect food quality and safety [215]. On the other hand, undesirable compounds formed in processes such as baking, roasting, canning, heating, fermentation or hydrolysis are contaminants in food processing [12]. Bacteria, viruses and parasites that occur naturally on raw food surfaces can also cause contamination as well as sewage, external surfaces, soil, live animals or the internal organs of meat animals [12]. Food contamination in the food production and processing is summarized in Figure 16. Depending on the evaluation of the problems that can cause this type of substances and the possible solutions that can be carried out, it could be necessary to establish maximums levels (MLs) of contaminants or other measures to control contamination of food and feed. MLs shall only be set for food in which the contaminant may be found in amounts that are significant for the total exposure of the consumer, taking into consideration the Policy of the Codex Committee on Contaminants in Foods for Exposure Assessment of Contaminants and Toxins in Foods or Food Groups [150].

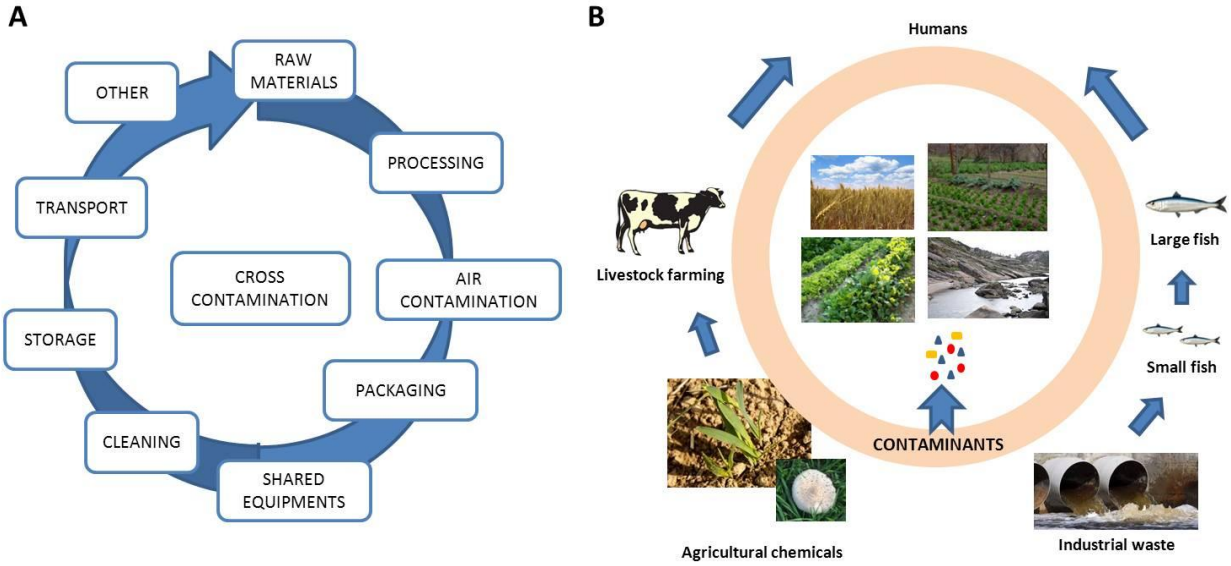


Figure 16. Pathways of food contamination: A) contamination in the food production and processing, B) contamination due to environmental influences. Adapted from Rather, I.A. (2017) Frontiers Pharmacology, under CCBY [12]

1.2.2.1 Mycotoxins in food

Among the most relevant types of contaminants that may be present in food, special emphasis will be placed on the case of mycotoxins. Fungi are considered ubiquitous microorganisms found in nature, whose spores are able to travel through different countries and continents all over the planet [216]. Mycotoxins are natural substances with low-molecular-weight, very diverse in terms of structure and abilities which are produced by a wide variety of different species filamentous fungi such as *Aspergillus*, *Penicillum* and *Fusarium* as secondary metabolites. They are toxic microorganisms that enter the food chain through contaminated crops, especially cereals. Their presence is strictly linked to environmental and storage conditions [217]. Generally, temperature and moisture content are the parameters that define the probability of a food being invaded by fungi even water activity or the relative vapour pressure have an influence on growth rate of these microorganisms. Hence, keeping foods dry or drying is an effective decision to prevent fungal growth and subsequent synthesis of mycotoxins [218]. In general, production is maximum between 24 ºC and 28 ºC, corresponding to tropical ambient temperatures. In refrigeration (as would happen in the case of moulds that proliferate, for example, on cheese), not only the fungal growth would be lower, but also the proportional production of mycotoxins [219]. The situation with this type of substance is becoming increasingly worrying because several of these mycotoxins remain stable throughout food processing and can therefore reach the final products [217].

There is a very wide variety of mycotoxins that can affect human health and livestock, depending on the fungus that produces them, and the type of food. Some mycotoxins are formed mainly in the field (during cultivation), others during harvest and others during storage (or in several stages at the same time). Once present in the food, it can no longer be decontaminated, resisting the processes of drying, grinding and processing. In addition, due to their thermal stability, they do not usually disappear during cooking. These mycotoxins enter the food chain normally through contaminated cereals which are destined for food and feed [219]. In addition, the same mycotoxin can be produced by different species of fungi just as

the same fungus can produce different mycotoxins. The Western diet is very varied so individuals are exposed to a wide range of mycotoxins. On the other hand, third-world nations are not safer despite having relatively consistent diets, as they depend on cereal crops grown and processed under laxer regulation and prone to show significantly higher levels of contamination [216].

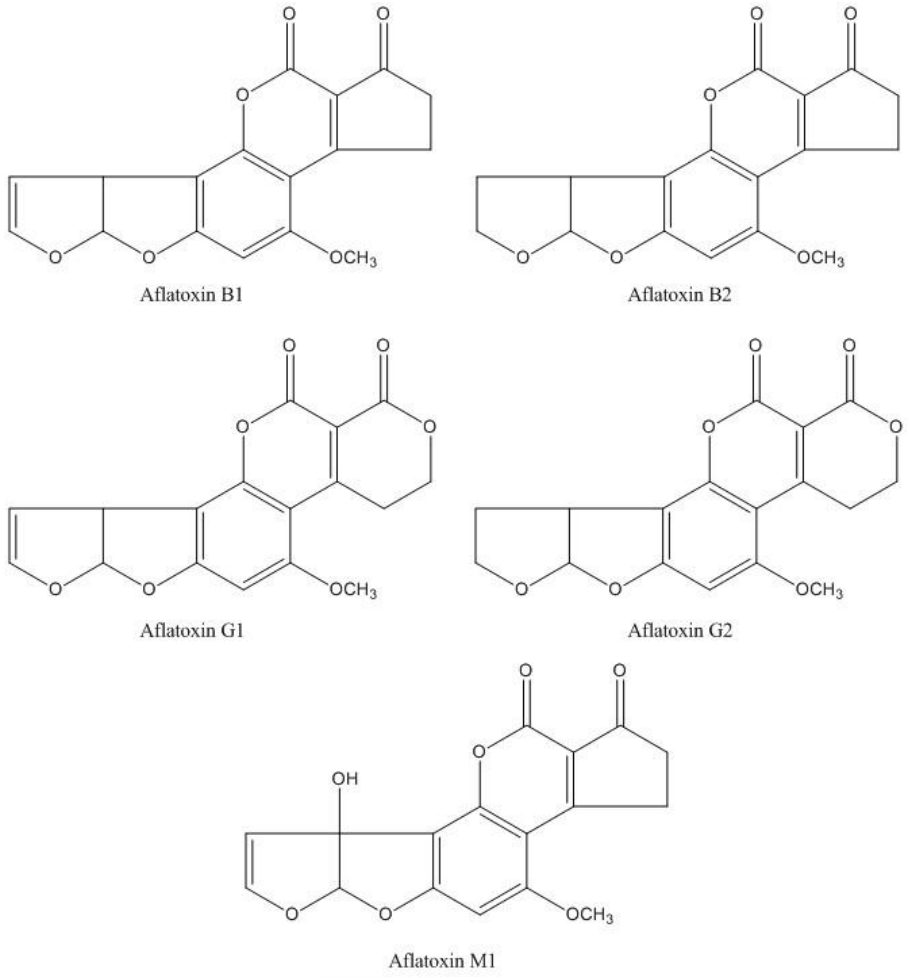


Figure 17. Structures of common aflatoxins (AFs)

One of the most important classes of mycotoxins in terms of their presence and toxicity are aflatoxins (AFs) [217]. These difuranocoumarin derivatives are produced by some *Aspergillus* species, namely *A. parasiticus* and *A. flavus* [220], or even by the rarely *A. nomius* [221]. Although more than twenty AFs have been identified, only some of them, aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) are classified as human's carcinogens [221]. Furthermore, aflatoxin M1 (AFM1), which is a major metabolite of AFB1 in humans and animals, can be present in milk obtained from animals fed with feed contaminated with aflatoxin B1. Interestingly, aflatoxin M_1 is also an important metabolite in humans and its analysis in breast milk may serve to assess the "internal exposure" to aflatoxins [218]. The chemical structures of the most common AFs are shown in Figure 17. The exposure to these substances can cause acute toxic, chronic (mutagenic, carcinogenic, teratogenic and immunosuppressive) and even death in consumers because they act as tumour initiators and promoters. Infants and children may be exposed to AFs through various special commercial products for them, such as cereal-based foodstuffs,

formulas, or even through their mothers' milk [217]. On the other hand, Patulin (PAT) is an important mycotoxin produced by over 30 genera of mold such as *Penicillium*, *Aspergillus*, and *Byssochlamys*. In particular, *Penicillium expansum* is recognized as the main source of PAT and it has been commonly associated with apple rot [222]. Although, PAT is mainly detected in apples and derived products, is known to invade silage, cereals, berries, fruits, vegetables, bread and meat products [218]. Patulin structure is shown in Figure 18. These molds grow easily in damaged fruit or in derived products such as juices, if storage conditions are deficient. Some of the most serious health effects of PAT ingestion in humans are convulsions, agitation, edema, intestinal ulceration, inflammation and vomiting.

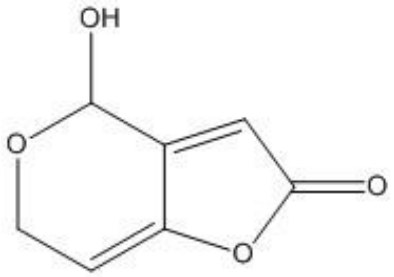


Figure 18. Structure of patulin (PAT)

In the literature over the years, several outbreaks of mycotoxicosis have been reported starting with "turkey disease X" from aflatoxins in 1963. Many mycotoxicosis have been associated with the consumption of AFs, and the incidence of liver cancer in Africa has been proven in epidemiologic studies to be highly correlated with aflatoxin intake, among others [218]. Both humans and animals are at risk of exposure to mycotoxins and this public health problem is longstanding. There are alternate routes such as dermal inhalation and absorption of toxinogenic molds containing mycotoxins although the ingestion is its main route of expousure [223]. According to FAO, one third of all foodstuffs produced for world's population are lost from field to consumer (nearly 1.3 billion metric tons each year) and 25% of the crops in the world are damaged by mould or fungal growth highlighting their economic impact [223]. The toxicity of these molecules has led to the set-up of strict regulations in many countries for their control in food and feed, and the consequent establishment of official legislation. The maximum levels of AFB1 for processed cereal-based foods and baby foods for infants and young children have set by European Commission at 0.1 $\mu g.kg^{-1}$. The limit of AFM_1 in milk and infant formulas and follow-on formulas, including infant milk and follow-on milk, was set at 0.05 $\mu g.kg^{-1}$ and 0.025 $\mu g.kg^{-1}$, respectively [224]. In the case of PAT, the European Commission Regulation (EC) 1881/2006 established the maximum content of 10 $\mu g.kg^{-1}$ in infant fruit juices, 25 $\mu g.kg^{-1}$ in fruit-derived products and 50 $\mu g.kg^{-1}$ for fruit juices for adults [225]. To minimize the exposure to mycotoxins is necessary to take steps such as the use of sensitive, selective and effective analytical methods. The demand for a fast, simultaneous and accurate determination of multiple mycotoxins together with the heterogeneity of food matrices creates enormous challenges for routine analysis [226].

1.2.2.2 Analytical methods for mycotoxins determination in food

The heterogeneous distribution of fungi in agricultural products, the complexity of food matrices and the diversity of analyte structures, make it impossible to use only one technique for the determination of all toxins. Increasing the speed of analysis and the applicability to a wider range of matrices are very important factors to take into account when developing a method for mycotoxins [226]. Normally, analytical methods for mycotoxins detection are

divided in two different groups: fast-screening methods and the more specific confirmatory methods. The first type is mainly based on immunochemical reactions occurring in lateral flow devices or enzyme-linked immunosorbent assays (ELISAs), and are available for almost all relevant mycotoxins [218]. Although screening methods are less precise and sensitive than confirmatory methods, such as HPLC-MS; they allow for the analysis of a large number of samples. ELISA is a widely used technique based on specific antibodies which offer the advantages such as ease of operation, sensitivity, speed, and high sample throughput despite of high matrix dependence [226, 227]. Fluorescence polarization immunoassays have been developed for mycotoxins such as DON, ZON, and AFs [228] and are commercially available. Furthermore, rapid disposable membrane-based assay tests have been developed in multiple formats, for example test strips [229], flow-through tests [230], and dip sticks [231]. In addition, many rapid screening tests and a number of new techniques are rapidly emerging [228, 232, 233]. Screening results must be confirmed by chromatographic methods to obtain an accurate analyte content of positive samples [226]. Advances have come in the areas of sample purification techniques and in separation science with the development of HPLC and associated detectors [234]. High-performance liquid chromatography (HPLC) coupled to diode array (DAD), fluorescence (FLD), single mass spectrometry (MS) or tandem mass spectrometry (MS/MS) detectors and gas chromatography (GC) coupled with electron capture (ECD), flame ionisation (FID), or MS detection are classical analytical methods used for mycotoxins analysis [226]. However, HPLC with fluorescence or mass spectrometric detection is the most popular technique for determining AFs [235].

In this context, to isolate the compounds from milk and/or cereals solid-phase extraction (SPE), clean-up with organic solvents or immunoaffinity columns (IAC) have been frequently applied [217, 220, 236, 237]. Guidelines for mycotoxins safety precautions are given by the Association of Official Analytical Chemists (AOAC). Thus, AOAC International has adopted official methods which are predominantly based on IAC clean-up, prior to LC with fluorescence detection [217]. Immunoaffinity columns (IAC) in combination with LC–MS/MS have numerous advantages, for instance, low detection limits, separation of a wide range of mycotoxins with different physic-chemical properties and high selectivity. In addition, this type of immunoaffinity columns has been used in tandem to achieve multiple mycotoxin extractions at levels close to the EU limits for adult and infant foods [236]. However, there is now growing interest in the development of reliable, simple and cheap detection systems for the analysis of mycotoxins in infant foods [236, 238-240]. Biosensors are one of the most advanced recent techniques which focus on features such as simplicity, easy-to-use, relatively fast and easy portability. The receptors developed for mycotoxin detection are biomolecules (antibodies, DNA and enzymes) and synthetic chemicals (MIP, aptamers, mimotopes etc) [241]. At this point, the use of MIPs is an easy, affordable, cheap, and highly versatile extractive alternative [217]. MIPs have become very popular and promising materials which are used to extract different analytes that are present in food [1, 8]. Moreover, these molecules in combination with magnetic supports, the so-called magnetic molecularly imprinted polymers (MMIP), allow the laboratories to obtain automated methods in which the steps of classical extractions are performed with the aid of an external magnetic field [217]. Sometimes analytes are extracted from the matrix in the polymer coating immobilized in a glass tube with a magnetic core [122]. In addition to selectivity, the combination of magnetic properties with the advantages of MIP provides the ability to separate in a single step [108]. In the case of complex samples, the combination of MIPs with stir bar sorptive extraction technique (SBSE) can be especially useful and bring huge benefits to sample preparation strategies [242]. A rapid and affordable extraction method has been

developed to isolate five different aflatoxins that may be present in foods using molecularly imprinted stir-bars [217]. Although the authorities have taken appropriate measures to minimise exposure to mycotoxins further analitycal action is needed to reduce health risks.

1.3 Applications of Molecularly Imprinted Polymers (MIPs) in Food Safety

Food safety is a scientific discipline comprising a number of routine analyses and controls that should be followed to avoid the presence of potential health hazards for humans in food. With this regard, safety considerations include the origins of food, food labelling, hygiene, additives, contaminants, residues and pathogens, amongst others. Food contamination refers to the presence in food of harmful chemicals and microorganisms, and it can be a result of a fraudulent practice or non-intentional and unavoidable. In the particular case of chemicals, there is an increasing demand for innovative analytical methods to control their presence in food. The use of powerful and modern mass spectrometers coupled to chromatographic separations allows the reliable identification and quantification of hazardous substances that represent a risk for the consumers. However, in almost all cases, food samples require specific preparative solutions prior being analysed using instrumental techniques. Also, chromatographic separations of closely related compounds are not always achieved in straightforward manner. Determining a wide range of compounds in different types of matrices/food complicates the procedure and calls for highly specific, easy-to-use and selective options. Molecularly imprinted polymers (MIPs) have been used effectively as active sorbents in both preparative options and chromatographic separations. Current trends in their use in food safety and potential directions in this field are outlined in this section.

As mentioned in the preceding section 1.1., molecular imprinting technology (MIT) represented an advance in the field of chemical, environmental, pharmacological and food analysis, as well as in the field of public health, given that molecular imprinting polymers (MIPs) have a high physical and chemical stability, as well as a high capacity for recognition by the analyte-template with which they were synthesized. Specific MIPs have been prepared for proteins, sugars, vitamins as well as environmental contaminants such as pesticides, pharmaceuticals and veterinary products [44]. MIPs were first used in solid phase extraction as effective chromatographic phases for the separation of isomers and structurally related molecules, as well as selective adsorbents in sample preparation stages. Some of the applications in which the use of this type of polymer is most commonly used are described below.

1.3.1 Use of MIPs in solid-phase extractive solutions (MISPE)

Nowadays, within the analytical procedure, the most laborious stage is still the preparation of the sample, since there are several factors that affect it, such as the nature of the matrix and the properties of the analyte. The pre-concentration of the analyte as well as the elimination of interfering compounds are two main objectives of sample pre-treatment [1]. If the analyte is present in low concentration, it is necessary to concentrate it so that it can be detected later by analytical techniques. In addition, in the case of a complex sample of similar compounds, washing steps are necessary. With this regard, solid-phase extraction (SPE) is a frequently used technique for sample pre-treatment because it has low-cost and it is easy to automate. A wide variety of sorbents exist in the market, but their main drawback is usually their lack of selectivity for the target analytes. Molecularly imprinting polymers (MIPs) are commonly used as a selective material in SPE processes for the selective cleaning of samples. Solid phase extraction using molecular imprinting polymers is called MISPE (molecularly imprinted solid phase extraction) and has two basic modes: off-line and on-line extraction.

The first example of MISPE was described in 1994 by the Sellergren group that used a selective pentamidine MIP for the pre-concentration and continuous cleaning of enriched urine samples [243].The use of MIPs in SPE extractions is the most important application due to the high selectivity of MIPs compared to other conventional adsorbents such as modified silica (C18), polystyrene-divinylbenzene or ion exchange resins. Normally used in off-line mode, this mode is more useful and effective for detection of several analytes for which the MIPs are able to recognize several structural analogues, for example a group of antibiotics [8]. Because MIPs are synthesized specifically for a particular analyte or group of structurally related compounds, stronger combinations of solvents can be used to obtain cleaner final extracts from the cartridges. On the other hand, the on-line mode allows a direct loading of the sample, washing of possible interferences, elution of the analytes and subsequent detection of the analytes by directly connecting the MISPE column to a detector. Due to the high selectivity of the MIPs, in one step by directly coupling a MISPE column in-line with the detection system, the extraction process, enrichment, separation and detection of the analytes can be achieved [126, 208, 209, 244]. For these on-line applications, MIPs monoliths are usually preferred over particles.

Thus, the potential advantages of MISPE are clearly evident and include selectivity and reproducibility, along with fast, easy and low-cost synthesis. This makes the cleaning efficiency of the samples higher, similar to the efficiency that would be obtained if immunosorbents were used. The procedure is very similar to the chromatographic one, it consists of packing a small quantity ~ 50 mg of the imprinted polymer, inside a SPE cartridge, where later the analyte of interest will be retained, extracting it in a small volume of suitable solvent mixture [245]. The MIP cartridge shall be subjected to the stages common to the SPE processes, including pre-conditioning of the polymer, loading of the sample onto the polymer, selective fixation of the analyte in the polymer cavities, washing of the matrix compounds non-specifically retained in the polymer and elution of the analytes with the appropriate solvent. The choice of the most suitable solvent for each of these stages should be made based not only on the solubility of the analytes in the different solvents used in each of the stages, but also on the type of interaction involved in the polymer-analyte bond and in the solvent used during the polymerization process. Figure 19 shows different MISPE extraction cartridges synthesized in the laboratory. To carry out the evaluation of the polymer, in parallel with the synthesis of MIPs, another polymer identical to this one is prepared, with the only difference that it does not contain template and is called non-imprinted polymer (NIP). If loading, washing and elution solvents in both polymers are compared, the formation or not of the bonding sites can be checked. The steps involved in developing a new extraction protocol in SPE based on MIPs are as follows: synthesis of the material, testing for its recognition properties, and development of an extraction protocol and validation of the protocol.

Some of the most common chemicals found in food for human consumption are antimicrobial residues. These analytes are mainly present in products of animal origin as a result of legal veterinary treatment or fraudulent use. A large number of very useful and effective MISPE protocols have been proposed for the analysis of antimicrobial agents in food, demonstrating their great potential and usefulness for isolating these compounds [8, 78, 246, 247]. In addition to antimicrobial agents, there are other chemicals that can contaminate food such as mycotoxins, environmental contaminants such as pesticides, herbicides and organic contaminants, veterinary drugs other than antimicrobials and food adulterants such as melanin. Several studies have demonstrated the selectivity of MIPs in SPE processes for these hazardous chemicals [10, 67, 76, 118, 133, 248, 249]. Therefore, it is demonstrated that

molecular imprinting technology is useful for the successful extraction of such compounds in food matrices. For environmental contaminants and pollutants, molecular imprinting sorbents are most often applied to aqueous samples (surfaces or waste water) or for the purification of extracts resulting from the treatment of solid samples (soil or sediment) [250]. However, the presence of pesticides and other agrochemicals can also be extended to crops, vegetables and fruits, in which case the MISPE can also provide an appropriate solution for the analysis of their residues [10].

Figure 19. MISPE cartridges prepared in the laboratory for different analytes using 50 mg of imprinted polymer

Unlike classical SPE sorbents, MISPE are more selective and allow the isolation of similar analytes within the same sample. Immunosorbents offer a high selectivity for solid phase extraction applications, however, the obtaining of antibodies is expensive and long lasting, and they need to be immobilized in adequate supports to preserve their binding capacity. MIPs are a good alternative to immunosorbents, since the cavities obtained after removing the template offer free binding sites, which are complementary in shape, size and functionality. The specificity of MIPs as receptors in some cases is comparable to monoclonal antibodies [251-254].

1.3.1.1 Optimization of the MISPE process

As in the case of any solid phase extraction; MISPE protocols comprise the followings common steps: polymer conditioning, sample loading, washing to eliminate possible interference and elution of the analyte. Before loading the sample, a pre-conditioning stage of the cartridge is required to remove any moisture that may be present in the sorbent packaging, as loading in organic solvent would reduce its efficiency. Washing the cartridge with the future elution solvent would remove any type of interference that may co-elute with the analytes in the final stage. Finally, a last conditioning using the same solvent as for the sample loading stage will facilitate the transfer of the material [255-258]. Once the MISPE cartridges have been conditioned, the sample is loaded. To choose the solvent, it is necessary to take into account not only the solubility of the analytes in the different solvents for each stage, but also the type of interaction involved in the polymer-analyte bond and in the porogen used during the polymerization process. For example, polymers prepared in toluene have a higher recognition of the analyte when the sample is loaded in toluene than if it is loaded in another solvent such as acetonitrile. A study conducted by Spivak's *et al.* in 1997 found that in the extraction processes on a polymer prepared in acetonitrile, recoveries were higher if the analyte was loaded in acetonitrile than in a more apolar solvent such as chloroform [259]. In subsequent studies this trend was confirmed so that it can generally be said that the most

suitable solvent for the loading stage of the sample will be the one that favours the interaction between the analytes and the polymer, the one in which polymerisation has been carried out, *e.g.* porogen [260]. According to the above theoretical foundations, it is concluded that all solvents with low polarity are suitable for the loading stage, provided that the analyte is soluble in them. In this way the retention of the analyte will be greater when the apolarity of the solvent increases, since the formation of hydrogen bonds with the monomer will be favoured. Generally, synthesis processes are carried out in non-polar media such as chloroform or toluene, since polar solvents such as methanol or water inhibit the formation of the template-monomer complex and the beginning of the polymerization process. For this reason, the direct addition of samples containing a high percentage of water, such as environmental water or biological fluids, is inappropriate because it will not produce molecular recognition or will be practically non-existent. However, when the analytes of interest are capable of non-specific interaction with the polymer matrix, subsequent binding to specific sites is possible if a suitable solvent is added after the loading process.

The next stage in MISPE protocols is washing aimed at retaining the compounds of interest and at the same time to break down non-specific interactions with the polymer matrix and thus eliminate possible interferences from the matrix. At this stage, very polar solvents should not be used as specific interactions MIP-analyte may be broken down. To optimize this stage, the recovery percentages obtained in the MIP are compared with those obtained with the corresponding non-imprinted polymer (NIP). As it is a polymer with no specific voids, the bond that will occur when loading any type of compound will always be non-specific. In this way, a study of different recoveries with different solvents and volumes is carried out for both MIP and NIP, in order to demonstrate the existence of specific interactions between the analytes of interest and the polymer itself, ruling out the option that the recoveries may be due to non-specific interactions. The amount of solvent used for the removal of non-specific interactions depends on the strength of the interactions and the solvent used. While in some cases it is possible to eliminate these interactions with 1 or 2 ml of the solvent used in the load, in other cases it is necessary to use higher volumes of a more polar solvent or solvent mixture. In general, this mixture of solvents usually consists of the solvent used in the charge and different proportions of another solvent of a higher polarity [60, 260].

Elution is the final stage of the MISPE process and polar solvents, protics or a mixture of both are generally used. In some cases small percentages of weak acids have also been used in order to break specific hydrogen bridge interactions between the compounds under study and the polymer. It is important to mention that abrupt changes in the polarity of the solvents used can cause loss of selectivity in the polymer as there are contractions and swelling of the polymer, distorting the specific voids, so it is necessary to make an elution using solvents with sequential polarity [55]. When analyzing the elutions, the presence of remains of template used to synthesize the MIP (template "bleeding") can be a drawback of MISPE at trace levels. This is due to the fact that the washing process used to remove the template has not been completely effective. Because of this, false recoveries can be obtained during the first analyses. To avoid this, it is possible to use another compound similar in structure and properties to the target compound (dummy template) for MIP synthesis but it is not always possible.

In Figure 20, the four steps involved in a MISPE process are shown. This includes pre-conditioning of the polymer with the solvent in which the sample is to be loaded onto the polymer and selective fixation of the analytes in the cavities of the polymer itself, washing of

the non-specific interfering compounds retained in the polymer and elution of the analytes with the appropriate solvent.

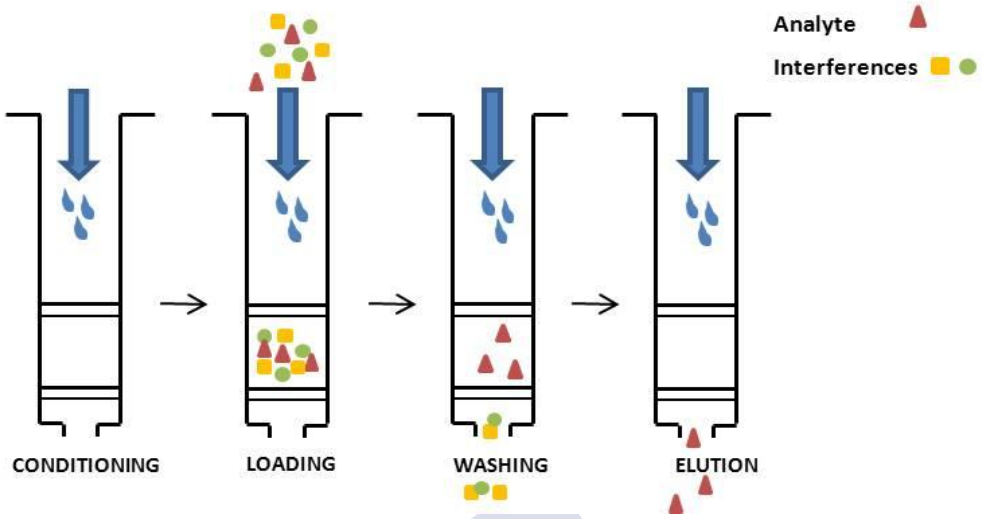


Figure 20. Schematic representation of MISPE process (authors´unpublished material)

1.3.2 Use of MIPs in chromatographic separations

The use of molecular imprinting polymers (MIPs) as stationary phases for chromatography is one of its main applications. MIPs have been used as column fill materials for effective chromatographic separations for the separation of isomers and structurally related molecules [49, 77, 118]. As previously mentioned, by using precipitation polymerization, the two-stage swelling method or the silica particles for polymer synthesis, small spheres are obtained that are optimal for this function. Some studies have already demonstrated the selectivity of MIPs prepared using precipitation as filling materials to column for liquid chromatographic separations of hazardous chemicals that may be present in food, such as antibiotics and fungicides [77, 118, 261]. In addition, MIPs synthesized within the pores of silica particles result in spherical beads with a homogeneous and appropriate morphology; therefore, they present optimal characteristics to be used as stationary phases in liquid chromatography. These molecular imprinted polymer phases allow the direct injection of complex samples with analytes (herbicides) that are not only separated from each other but also from compounds that interfere with the matrix [86]. In this regard, the good yield of the spheres obtained from the core-shell technology and/or by iniferter type starter techniques was demonstrated in a study with fungicides in fruit and vegetable extracts [49]. This type of material can also be used in the development of on-line SPE methods.

The use of MIPs as stationary phases in HPLC, poses a number of problems that have considerably limited their application. Among these problems are the heterogeneity of the cavities generated during polymer synthesis, the need for spherical polymer particles with a homogeneous distribution of sizes and the low speed at which mass transfer processes to the cavities take place. By optimizing the conditions for polymer synthesis, some of these problems have been solved. Thus, for example, a covalent or semi-covalent synthesis method can be used, since this way a more homogeneous distribution of voids in the polymer matrix can be achieved. Polymerization on silica particles solves the problems of heterogeneity in particle size and mass transfer, as they may or may not dissolve after synthesis of the MIP [85]. Monolithic columns shows higher permeability and lower flow resistance than conventional liquid chromatography columns, providing high performance, resolution and

separation with high performance in short run times. This type of columns is earning interest as exceptional substitutes for conventional particle-packed columns [129]. Monoliths, as one of the methods of preparing MIPs, combine the advantage of monolithic columns with molecular imprinting technology. Monoliths, in addition to allowing sample concentration, also facilitate the selective separation of analytes of interest into actual samples, which is crucial for the quantitative determination of analytes in complex samples. Monolithic columns have been applied to the analysis of residues, contaminants and food constituents, which demonstrates their effectiveness and success for this type of control [133, 209, 262-265].

1.3.3 Use of MIPs in the development of sensors

In recent years, numerous sensors have been developed based on the use of molecular imprinting polymers (MIPs) for the selective detection of different analytes, including pesticides and other contaminants as well as those of clinical or biochemical interest such as some drugs. The high specificity and selectivity of this type of materials is an excellent alternative to the use of biomolecules, generally used as a means of selective recognition in the manufacture of sensors. The integration of MIPs in sensors can be done by polymerization in situ, using photochemical or thermal initiation, or by inserting it into a surface by means of a chemical initiator or by UV radiation. The latter system allows the surface of an electrode to be modified with very thin sheets of specific polymers. Electro-polymerization can also be carried out on the surface of a transduction platform [62]. In the context of food safety, sensors have been developed for the determination of antibiotic residues in foods, such as those of the fluoroquinolone family in water and milk, at concentrations lower than the MRLs established by the European Union for these substances, using a fluorimetric sensor based on an MIP [266]. They have also been applied for determination of chloramphenicol and/or tetracyclines in complex samples such as honey and milk [267, 268].

1.3.4 Use of MIPs in catalysis processes

In biochemical processes, enzymes are selectively bonded, stabilizing the transition state of a given reaction. The limitation in their applications lies in the low stability in organic solvents as well as the limitations of work at temperatures or extreme pH values. For this reason we are currently looking for synthetic substances that imitate their behaviour, so MIPs are adjusted to these needs due to their high selectivity and resistance to be used in conditions of high temperature, extreme pH and with different organic solvents. Today, MIPs have already been used as catalysts in various chemical reactions imitating the behaviour of enzymes [44]. A structural analogue of the transition or intermediate state of a chemical reaction was used as a mould molecule. In this way, this polymer would have the capacity to incorporate and stabilize this state of transition or intermediate of the reaction in the polymeric matrix, showing a catalytic effect similar to that of enzymes [269].

1.3.5 Novel formats, mini-devices and magnetic alternatives

MIP formats have ranged from the traditional MISPE columns to disks, micro-columns, fibers and capillaries, membranes, stir-bars, magnetic beads or even needle and micropipette tips [119]. With regard to magnetic MIP and their application in food safety, they have been one of the most popular solutions for the detection of many different analytes in the last years. Recently, a research group reported its success in the design of well-defined nanospheres with a double-layer core-shell structure consisting of Fe_3O_4 nanoparticle core, an inner fluorescent layer and an outer layer of MIP, for simultaneous separation and recognition of estrogenic disrupting chemicals [106]. A similar approach was reported for the selective enrichment of endocrine disrupting chemicals in water and milk samples [109]. Magnetic MIP have also

been employed for the detection of malachite green residue, a parasiticide and antibacterial agent used in freshwater aquaria, in fish samples [112]. An additional example is the development of magnetic dummy molecularly imprinted nanoparticles based on functionalized silica and used as an efficient sorbent for the determination of acrylamide in potato [111]. This kind of polymers can also be useful for determining important natural food components such as vitamins and nutrients. A good example is the application of magnetic molecularly imprinted polymers for selective determination of resveratrol in wine [270].

A sensitive and selective electrochemical sensor for metronidazole, an antibiotic and antiprotozoal agent, was developed by attaching a core–shell metronidazole-magnetic molecularly imprinted polymer to the surface of magnetic glassy carbon electrode, in order to prepare an electrochemical sensor. The imprinted sensor exhibited high recognition ability and affinity for the analysis of metronidazole in milk samples and honey samples [271]. Silica particles containing surface-bound free radical initiators have been used also as supports for the grafting of thin films of molecularly imprinted polymers, demonstrating its success for separation of patulin, a mytoxin that can be present in fruit as a consequence of fungal contamination [100, 114]. Another attractive alternative to particle-based MIP are imprinted monoliths, which are easy to obtain and possess also great potential for the preparation and clean-up of complex food samples. In-situ polymerization inside appropriate supports has allowed the development of several micro-extraction formats, such as needle and pipette tip-based extractions [129]. A pipette tip-based molecularly imprinted polymer monolith microextraction method was developed for the selective extraction of difenoconazole, a triazole fungicide, in tap water and grape juice [132]. Monoliths synthesized inside capillary columns have enabled the on-line isolation of aflatoxins [133]. Novel selective solid phase extraction formats include MIP grafted to stir-bars or to porous polyethylene frits for selective extraction of herbicides in food and environmental samples [49, 118].

The imprinting of bacteria and viruses, ultimately considered as extremely large molecules, is a very recent application of these MIP. Literature brings several examples of successful imprinting microorganisms with different structures [254, 272, 273]. In these strategies, the virus/bacteria surface is imprinted for instance on silica nanoparticles as a core material or in the form of micro-gel granules. The application area certainly has the potential to be extended to cover other classes of cells.

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2 OBJECTIVES

Food security as a global concern has been the subject of study of many research projects worldwide. However, the complexity of food matrices, as well as the presence of traces of different substances, requires highly specific detection methods in addition to efficient sample cleaning and concentration procedures. Nowadays, sample preparation is still the bottleneck stage of the analytical process, due to factors such as the nature of the matrix and the physico-chemical properties and concentration of the analytes of interest. In spite of the advantages and disadvantages of different alternatives, the simplicity and quickness of solid phase extraction (SPE) have made it the most used technique for environmental and food samples, providing relatively high recovery percentages. This technique is based on the retention on a solid adsorbent of the desired compounds dissolved in a liquid sample or extract. Over time, the selectivity of these solid sorbents have gained importance and conventional materials have been improved with the introduction of functional groups, enhancing the efficiency, and robustness of methods and contributing to lower detection limits of the analytical procedure. In this context, imprinting technology has emerged, which consists of the synthesis of stable polymers with properties of selective molecular recognition towards the analytes of interest. Molecular imprinted polymers (MIPs) are synthetic and porous materials with a predetermined affinity for an analyte or group of compounds similar in structure and functionality. These materials are obtained by polymerisation in presence of a target molecule, known as template, which generates cavities with molecular memory in these synthetic materials. In this way, the materials obtained can be used to capture the molecule of interest when they come into contact with it again, and thus selectively extract it from the analytical matrix.

Based on the above, the objectives of this Doctoral Thesis were established as follows.

Objective 1: Review of the state of the art of MIPs in food safety

In view of the potential of MIPs in analytical chemistry and due to the large number of factors that come into play in their design and polymerisation, it became essential to carry out a broad and detailed search of the different materials and, polymerisation techniques used to guarantee the suitability of MIPs in their subsequent applications in food analysis. This objective was divided in two tasks:

1.1 Latest applications of MIPs in the development of methodologies, such as clean-up and chromatography to monitor the presence of residues of veterinary products in food

1.2 Advances and improvements in the use of innovative materials and formats based on MIPs for the analysis of residues and contaminants in food

Objective 2: Design and application of MIPs in SPE format for veterinary drugs analysis in food

Design and application of MIPs to be used as classic solid-phase sorbents (MISPE) for selective extraction of residues of different veterinary drugs in food, using milk as an example of analytical matrix. This objective was divided in three tasks:

2.1 Design and synthesis of MIPs, and optimization of MISPE protocol for corticosteroids extraction

2.2 Design and synthesis of MIPs, and optimization of MISPE protocol for amphenicols extraction

2.3 Design and synthesis of MIPs, and optimization of MISPE protocol for penicillins extraction

Objective 3: Design and application of MIP-based innovative solutions for contaminants analysis in food

Design and application of MIPs combined with magnetic supports to be used for selective extraction of contaminants in food, using milk, cereals and fruit as examples of analytical matrix. This objective was divided in two tasks:

3.1 Design, synthesis and optimization of magnetic molecularly imprinted stir-bars (MMIP-SB) for extraction of aflatoxins

3.2 Design, synthesis and optimization of magnetic molecularly imprinted stir-bars (MMIP-SB) for extraction of patulin

Public funding:

The present research project of Doctoral Thesis was developed within the framework of the following official research project financed by regional and national public funds: "Desarrollo y aplicación de nuevos modelos de reconocimiento molecular en el control de antibióticos y corticosteroides en alimentos y medioambiente" (INCITE09 261 380 PR, Xunta de Galicia); "Desarrollo de polímeros de huella molecular (MIP) para la detección simultánea de antibióticos y corticosteroides por LC-MS/MS en productos de origen animal (leche y productos lácteos)" (AGL2009-14707, Ministerio de Ciencia e Innovación); "Desarrollo de polímeros de impronta (molecularly imprinted polymers) para su aplicación en el campo alimentario (MIPFOOD)" (IPT-060000-2010-14, Ministerio de Ciencia e Innovación) and "Desarrollo de nuevas metodologías para la extracción y purificación de analitos basadas en el uso de polímeros de impronta molecular acoplados a sólidos magnéticos para el control alimentario" (EM 2012/153, Xunta de Galicia).

The Doctoral Thesis belongs to the research developed by the national research network "MICOFOOD" (Red de Excelencia sobre las micotoxinas y hongos toxigénicos y de sus procesos de descontaminación, http://micofood.es/).

3 RESULTS

This Doctoral Thesis reports on the results entitled "Design and application of molecularly imprinted polymers (MIPs) for detection of veterinary residues and contaminants in food". Eight scientific contributions have been published and are presented below, following the three objectives initially established:

- ✓ Results of the objective 1: Review of the state of the art of MIPs in food safety (2 review articles, 1 encyclopaedia chapter).
- ✓ Results of the objective 2: Design and application of MIPs in SPE format for the determination of corticosteroids, amphenicols and penicillins in milk (3 original articles).
- ✓ Results of the objective 3: Design and application of MIPs in innovative solutions for mycotoxins analysis in baby products (based on milk and cereals) and apple (2 original articles).

3.1 REVIEW ON THE STATE OF THE ART OF MIPS APPLICATIONS IN FOOD SAFETY

An extensive review of the different materials, polymerisation techniques, formats and applications of MIPs in food safety has been performed. The potential of this kind of polymers in analytical chemistry becomes evident as well as the large number of factors that should be taken into consideration in their design. Thus, this first objective was achieved in two review article and one encyclopaedia chapter, presented below.

3.1.1 Latest applications of MIPs in the development of methodologies, such as clean-up and chromatography to monitor the presence of residues of veterinary products in food

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

Regal, P., Díaz-Bao, M., Barreiro, R., Cepeda, A., & Fente, C. (2012). Application of molecularly imprinted polymers in food analysis: clean-up and chromatographic improvements. *Central European Journal of Chemistry*, 10(3), 766-784. DOI: 10.2478/s11532-012-0016-3

https://link.springer.com/article/10.2478/s11532-012-0016-3

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.

3.1.2 Advances and improvements in the use of innovative materials and formats for the analysis of residues and contaminants in food

3.1.2.1 Recent advances and uses of monolithic columns for the analysis of residues and contaminants in food

Díaz-Bao, M., Barreiro, R., Miranda, J., Cepeda, A., & Regal, P. (2015). Recent advances and uses of monolithic columns for the analysis of residues and contaminants in food. *Chromatography*, 2(1), 79-95. DOI: 10.3390/chromatography2010079

https://www.mdpi.com/2227-9075/2/1/79

Abstract

Monolithic columns are gaining interest as excellent substitutes to conventional particle-packed columns. These columns show higher permeability and lower flow resistance than conventional liquid chromatography columns, providing high-throughput performance, resolution and separation in short run times. Monoliths possess also great potential for the clean-up and preparation of complex mixtures. In situ polymerization inside appropriate supports allows the development of several microextraction formats, such as in-tube solid-phase and pipette tip-based extractions. These techniques using porous monoliths offer several advantages, including miniaturization and on-line coupling with analytical instruments. Additionally, monoliths are ideal support media for imprinting template-specific sites, resulting in the so-called molecularly-imprinted monoliths, with ultra-high selectivity. In this review, time-saving LC columns and preparative applications applied to the analysis of residues and contaminants in food in 2010–2014 are described, focusing on recent improvements in design and with emphasis in automated on-line systems and innovative materials and formats.

3.1.2.2 Smart Polymers: Molecularly Imprinted Polymers

Díaz-Bao, M., Barreiro, R., Cepeda, A., & Regal, P. (2018). Smart Polymers: Molecularly Imprinted Polymers. *Encyclopedia of Polymer Applications*, First Edition, 2426-2440. Taylor & Francis. DOI: 10.1201/9781351019422-120054090

https://www.taylorfrancis.com/books/9781351019422

Abstract

Assuring food safety has historically been the purpose of thousands of research projects all over the world, promoted and supported by government authorities. Dangerous substances in food may include natural toxicants, contaminants, and chemicals and drugs deliberately used to increase the food supply, among others. Analytical methods are crucial to detect and quantify hazardous substances and assure and achieve this way the required safety and quality of food products. In this context, molecularly imprinted polymers (MIPs) have offered a broad range of versatile options to support and complement existing analytical techniques. These "smart" polymers are designed as artificial antibodies, capable of specifically rebinding the template used during their synthesis, or analogue compounds. The target analyte is usually surrounded by a biological matrix that complicates its determination, but these MIPs facilitate enormously the process thanks to their ability to rebind the template within the imprinted sites and isolate it. Several polymerization techniques are available for the analyst, as well as different possible hybridization of MIPs with other materials and supports (magnetite, quantum dots, silica, glass, carbon nanotubes, and so on), all of this in order to obtain the desired device, with the desired characteristics and behavior. In this sense, solid-phase extraction is one of the most popular applications studied for these imprinted materials, while more recent applications include small-sized devices, monoliths, sensors, stationary phases for liquid chromatography, and magnetic sorbents, among others. MIPs have widely demonstrated their suitability as supports to extract residues and contaminants in food, and the field is still growing.

3.2 DESIGN AND APPLICATION OF MIPS IN SPE FORMAT FOR THE DETERMINATION OF CORTICOSTEROIDS, AMPHENICOLS AND PENICILLINS IN MILK

The design and application of MIPs to be used as classic solid-phase sorbent for selective extraction (MISPE) of different veterinary residues in food was achieved using milk as an example of analytical matrix. Thus, the results of objective 2 were reported in three original articles, presented below.

3.2.1 Design and synthesis of MIPs, and optimization of MISPE protocol for corticosteroids extraction

3.2.1.1 Evaluation of molecularly imprinted polymers for the simultaneous SPE of six corticosteroids in milk

Díaz-Bao, M., Barreiro, R., Regal, P., Cepeda, A., & Fente, C. (2012). Evaluation of molecularly imprinted polymers for the simultaneous SPE of six corticosteroids in milk. *Chromatographia*, 75(5-6), 223-231. DOI: 10.1007/s10337-012-2182-z

https://link.springer.com/article/10.1007/s10337-012-2182-z

Abstract

A molecularly imprinted solid-phase extraction procedure was optimized for the simultaneous extraction of six corticosteroids commonly employed in animal husbandry. Two different molecularly imprinted polymers (MIPs) were synthetized by precipitation polymerization, using dexamethasone (DM) and flumethasone (FLU) as template molecules. Methacrylic acid was used as functional monomer and divinylbenzene as cross-linker, and the selected porogen was a solution of acetonitrile and toluene (3:1). After removal of the template, both molecularly imprinted and non-imprinted polymers were placed into glass cartridges to perform solid-phase extraction experiments. Loading, washing and elution steps were optimized for each MIP in order to test the suitability of the selected template molecules DM and FLU to provide selective polymeric sites for corticosteroids. Recovery measurements were performed by HPLC–MS/MS. The maximum recovery for all corticosteroids was achieved with DMbased MIP when loading with toluene and washing with 5% acetonitrile in toluene, and eluting with 1% acetic acid in methanol. When cleaning-up milk samples spiked with corticosteroids, the recovery of DM-based polymer decreased. However, the efficiency of FLU-based polymer clearly improved even when a strong matrix effect was observed. Additionally, two frequently used commercial solid-phase extraction (SPE) cartridges were compared with imprinted polymers. The commercial SPE cartridges gave good recoveries only for specific analytes while MIPs provided good recoveries for all analyzed corticosteroids.

3.2.2 Design and synthesis of MIPs, and optimization of MISPE protocol for amphenicols extraction

3.2.2.1 Development of a HPLC-MS/MS confirmatory method for the simultaneous determination of amphenicols in baby formulas using molecularly imprinted polymers

Barreiro, R., Díaz-Bao, M., Regal, P., Miranda, J. M., & Cepeda, A. (2013). Development of a HPLC-MS/MS confirmatory method for the simultaneous determination of amphenicols in baby formulas using molecularly imprinted polymers. *Analytical Methods*, 5(16), 3970-3976. DOI: 10.1039/c39ay40374b

https://pubs.rsc.org/en/content/articlelanding/2013/ay/c3ay40374b#!divAbstract

Abstract

Chloramphenicol (CAP) is a broad-spectrum bacteriostatic antibiotic commonly used in veterinary medicine. However, toxic effects in humans such as Grey syndrome, bone marrow suppression, and fatal aplastic anaemia have been described. As a consequence, the use of CAP in foodstuffs has been banned within the European Union since 1994 and no maximum residue limit (MRL) has been established in animal-derived foods. On the other hand, thiamphenicol (TAP) and florfenicol (FLP) are allowed but different MRLs have been set in foodstuffs of animal origin. In this work, precipitation polymerisation has been used and different MIP sorbents were tested and optimized for the solid-phase extraction (MISPE) of a group of three, structurally related amphenicols in milk powder. Recoveries were calculated using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) working in negative mode. The proposed confirmatory method was validated according to the Commission Decision 2002/657/EC, resulting in CCα ranging from 0.06 to 10.5 ng mL−1 and reaching the required working levels. The applicability of thesepolymers for the extraction of amphenicols in spiked samples of baby formulas has been tested.

3.2.3 Design and synthesis of MIPs, and optimization of MISPE protocol for penicillins extraction

3.2.3.1 Fast HPLC-MS/MS method for determining penicillin antibiotics in infant formulas using molecularly imprinted solid-phase extraction

Díaz-Bao, M., Barreiro, R., Miranda, J. M., Cepeda, A., & Regal, P. (2015). Fast HPLC-MS/MS method for determining penicillin antibiotics in infant formulas using molecularly imprinted solid-phase extraction. *Journal of Analytical Methods in Chemistry*. DOI: 10.1155/2015/959675

https://www.hindawi.com/journals/jamc/2015/959675/

Abstract

The dairy cattle may suffer from different infections relatively often, but the inflammation of the mammary gland is very important to the farmer. These infections are frequently treated with penicillin antimicrobial drugs. However, their use may result in the presence of residues in animal products, such as milk powder and/or infant formulas, and it represents a potential risk for consumers. To monitor this, the EU has defined safe maximum residue limits (MRLs) through Commission Regulation (EU) number 37/2010. Although LC-MS is a trustful option for confirmation and quantification of antibiotics, the analysis of real samples with complex matrices frequently implies previous clean-up steps. In this work, precipitation polymerization has been used and different molecularly imprinted polymer (MIP) sorbents were tested and optimized for the fast and simultaneous solid-phase extraction (MISPE) of eight common penicillins (ampicillin, amoxicillin, oxacillin, penicillin G, penicillin V, cloxacillin, dicloxacillin, and nafcillin).The extracts were analyzed using liquid chromatography coupled to tandem mass spectrometry (LCMS/MS) and the applicability of these polymers as sorbents for the extraction of penicillins at MRL levels in milk powder (infant formulas) was proved. The limits of detection and quantification were below the legal tolerances, except for LOQ for oxacillin and cloxacillin.

3.3 DESIGN AND APPLICATION OF MIPS IN INNOVATIVE SOLUTIONS FOR CONTAMINANTS (MYCOTOXINS) ANALYSIS IN BABY PRODUCTS AND FRUIT

The design and application of MIPs combined with magnetic supports to be used for selective extraction of contaminants in food, was achieved using baby products (based on milk and cereals), and apple as examples of analytical matrix. Thus, the results of objective 3 were reported in two original articles, presented below

3.3.1 Design, synthesis and optimization of magnetic molecularly imprinted stir-bars (MMIP-SB) for extraction of aflatoxins

3.3.1.1 A facile method for the fabrication of magnetic molecularly imprinted stir-bars: A practical example with aflatoxins in baby foods

Díaz-Bao, M., Regal, P., Barreiro, R., Fente, C. A., & Cepeda, A. (2016). A facile method for the fabrication of magnetic molecularly imprinted stir-bars: A practical example with aflatoxins in baby foods. *Journal of Chromatography A*, 1471, 51-59. DOI: 10.1016/j.chroma.2016.10.022

https://www.ncbi.nlm.nih.gov/pubmed/27751523

Abstract

A fast and facile method for the fabrication of magnetic molecularly imprinted stir-bars (MMIP-SB) hasbeen developed, using a combination of imprinting technology and magnetite. Magnetite was preparedin the laboratory from the raw and embedded into molecularly imprinted polymers through a process ofbulk polymerization. This novel design was applied to the analysis of aflatoxins, one of the most impor-tant groups of mycotoxins in terms of occurrence and toxicity. In the context of food safety, molecularlyimprinted polymers are a promising tool to achieve selective and accessible methods of extraction fordifferent residues and contaminants. Considering the toxicity of aflatoxins, a dummy template was pre-ferred for the synthesis of the imprinted polymers. A rapid and affordable extraction method for isolatingfive different aflatoxins that may be present in food was developed. The MMIP-SB was used as a conven-tional stir-bar and combined with high performance liquid chromatography and mass spectrometry forthe determination of aflatoxin M1in milk powder (infant formulas) and aflatoxins B1, B2, G1 and G2 in cereal-based baby foods. The results showed an average recovery of 60%, 43, 40, 44 and 39%, respectively,and RSD below 10%. These in-house prepared stir-bars featured good stirring and extraction performance,and recognition abilities, offering a good alternative to more complicated.

3.3.2 Design, synthesis and optimization of magnetic molecularly imprinted stir-bars (MMIP-SB) for extraction of patulin

3.3.2.1 Design of a Molecularly Imprinted Stir-Bar for Isolation of Patulin in Apple and LC-MS/MS Detection

Regal, P., Díaz-Bao, M., Barreiro, R., Fente, C., & Cepeda, A. (2017). Design of a Molecularly Imprinted Stir-Bar for Isolation of Patulin in Apple and LC-MS/MS Detection. *Separations*, 4(2), 11. DOI:10.3390/separations4020011

https://www.mdpi.com/2297-8739/4/2/11

Abstract

Mycotoxins are a very diverse group of natural products produced as secondary metabolites by fungi. Patulin is produced by mold species normally related to vegetable-based products and fruit, mainly apple. Its ingestion may result in agitation, convulsions, edema, intestinal ulceration, inflammation, vomiting, and even immune, neurological or gastrointestinal disorders. For this reason, the European Commission Regulation (EC) 1881/2006 established a maximum content for patulin of 10 ppb in infant fruit juice, 50 ppb for fruit juice for adults and 25 ppb in fruit-derived products. In this work, a rapid and selective method based on magnetic molecularly imprinted stir-bar (MMISB) extraction has been developed for the isolation of patulin, using 2-oxindole as a dummy template. The final extraction protocol consisted of simply pouring in, stirring and pouring out samples and solvents from a beaker with the MMISB acting inside. The magnetic device provided satisfactory recoveries of patulin (60–70%) in apple samples. The successful MMISB approach has been combined with high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) to determine patulin.

4 GENERAL DISCUSSION

Over the years, the interest in the detection of a wide range of natural and synthetic substances in food has made it necessary the development of different analytical methods to use in laboratories around the world and ensure food safety. These methods are crucial for detection and quantification of substances that compromise food quality. To further prevent the presence of residues of veterinary drugs and contaminants and thus protect the health of consumers, official surveillance plans have been implemented using validated analytical methods. Moreover, improvements in different fields such as molecular biology, immunology or computer technology, among others, have facilitated the development of faster, more sensitive and more convenient alternatives in food analysis. The ideal analytical method should combine cost-effective one-step isolation, pre-concentration and quantitative determination of analytes. It is necessary to note that each additional step in the analytical procedure increases the probability of analyte losses, sample contamination and analytical error [217]. Due to the complexity of food matrices, the direct detection of analytes in real samples is often difficult. In order to achieve analytical success, it is usually necessary to implement effective clean-up protocols, which allow possible interferences to be removed and the analytes to be subsequently extracted from the matrix. Moreover, good chromatographic separation methods are also necessary to distinguish closely related analytes.

There are several procedures for preparing samples of different matrices prior to the detection process. These are new techniques introduced over the years to analyse foodstuff that improve the extraction process by LC-MS/MS or GC-MS/MS among others. To date, solid-phase extraction (SPE) is one of the most frequently used preparative techniques due to its low-cost and easy automation. It can be coupled to both liquid and gas chromatography and there is a wide variety of SPE commercial cartridges used in the case of complex matrices. This pre-concentration technique has almost totally replaced the traditional liquid-liquid extraction (LLE), and some of its related branches are currently gaining more popularity as they are magnetic SPE (MSPE), dispersive SPE (d-SPE), stir-bar sorptive extraction (SBSE) and solid-phase microextraction (SPME) [274]. In this context, molecular imprinting technology (MIT) emerges, providing a wide range of versatile options to support and complement existing analytical techniques. The use of molecularly imprinted polymers (MIPs) facilitates the clean-up process and in turn the extraction process thanks to their high degree of selectivity. Moreover, nowadays their use in solid-phase extraction, so-called molecularly imprinted solid-phase extraction (MISPE), is by far the most advanced technical application of MIPs [11]. Molecularly imprinted polymers (MIPs) are designed as artificial antibodies, capable of specifically rebinding the template used during their synthesis, or analogue compounds. To obtain the desired polymer, with the desired behaviour and characteristics, different polymerization techniques are available for the synthesis, as well as different possible hybridizations of MIPs with other materials and supports such as magnetite, silica, or glass. Recent applications studied for these imprinted materials in food analysis

include monoliths, sensors, and magnetic sorbents among others. In the case of monolithic columns, these have been gaining interest also as excellent substitutes for conventional columns filled with particles due to their higher permeability and lower flow resistance in comparison to conventional liquid chromatography columns, which provides high resolution and separation performance in short run times [129]. MIPs have widely demonstrated their suitability as supports to extract residues and contaminants in food, and the field is still growing. The superiority of MIPs as selective SPE sorbents (MISPE) has been extensively demonstrated in the last decade for analysis of various contaminants and of residues in food [9, 250].

In this research project of Doctoral Thesis an extensive review of the state-of-the-art in MIP science has been and compiled [1, 29, 129], including information on polymerization techniques and approaches, monomers, templates and/or existing formats. As a matter of fact, different approaches have been also tested and applied for MIP synthesis and design, in an attempt of combining modern solutions such as magnetic devices but also taking advantage of well-established classical formats such as MISPE. The developed imprinted materials were successfully implemented in the laboratory for extraction of various groups of veterinary drugs and contaminants in food. On the one hand, MISPE techniques were developed for veterinary drugs analysis in dairy products [8, 67, 78], and on the other hand, new magnetic materials with stirring abilities were created for extraction of contaminants in cereal-based and milk-based baby foods and in fruit [123, 217].

4.1 Advances and applications of MIPs in Food Safety

In the present Thesis, the design and application of various MIPs for the detection of veterinary drugs and contaminants in food has been carried out. Initially, based on the first objective established, an exhaustive and updated bibliographic review of the different scientific publications existing in the field was achieved. As a result of this search, two reviews and a chapter of an encyclopaedia have been published. The first review explains in detail the different materials, polymerization techniques used to guarantee the suitability of this kind of molecularly imprinted polymers and also, it describes the most important applications of this technology in the development of new methodologies to detect the presence of veterinary drug residues in food products [1]. The second review shows the different applications of monolithic chromatographic columns for LC that allow time-saving chromatographic methods when processing samples for the analysis of residues or contaminants in food matrices. Moreover, it explains the different improvements in design, new materials and innovative formats such as molecularly imprinted monoliths which are finding their way into separation science and into solid-phase micro-extraction devices, improving the selectivity and resolution of analytical methods [129]. Finally, a chapter has been published in an encyclopaedia (Encyclopedia of Polymer Applications by Taylor & Francis, edition 2018), including different concepts on these smart polymers and their applications in food safety, ranging from design, existing techniques and polymerization approaches, combination with new materials and different applications in food analysis [29]. The information gathered in these three bibliographic compilations provided a solid foundation for the accomplishment of the other two objectives of this Thesis, which resulted in five experimental peer-reviewed articles, accepted and published [8, 67, 78, 123, 217].

From the three works, it can be stated that MIPs have widely demonstrated their suitability as supports to extract residues and contaminants in food, and the field is still growing. Similar perceptions have been highlighted in recent reviews and chapters,

sufficiently showing the high selectivity of this "smart" polymers, which are able to distinguish similar molecules within the same group that may be present simultaneously in food matrices [274-283]. The results obtained here are framed within the official research projects INCITE09 261 380 PR and EM 2012/153 from Xunta de Galicia (Spain), and AGL2009-14707 from the Spanish Ministry of Science and Innovation.

4.1.1 MIP evolution in the field of food analytical chemistry

The successful performance of MIPs as selective sorbents in solid-phase extractions of contaminants and residues in food has been thoroughly demonstrated, both in the shape of monoliths obtained by bulk polymerization or as particles [1, 129]. Bulk polymerization is still the most frequently used polymerisation method yet additional novel techniques as precipitation polymerization are replacing it rapidly, in particular aiming at obtaining micro-particles of controlled size and morphology and eliminating the steps of crushing and sieving. The extensive use of MAA as functional monomer in MIP synthesis is justified by its capability to interact with almost any template [29]. As far as the cross-linker is concerned, EGDMA and TRIM are the two most popular alternatives, while DVB has been specifically preferred for precipitation polymerization [1, 29].

MISPE is by far the most frequently reported way of application of these smart polymers in food analysis, both off-line and on-line. Unlike off-line, the direct connection of MISPE columns to an analytical system provides automation and speed to analytical methods. Apart from particle-based MISPE cartridges, imprinted monoliths possess also great potential for the clean-up and preparation of complex food samples. The process to obtain monoliths is simple and adds the possibility of *in situ* polymerization inside appropriate supports as capillaries or pippete tips. Animal matrices such as meat, kidney, liver, honey, eggs or milk have been the target of hundreds of MIP-based preparative methods, usually combined with LC-MS detection [8, 67, 78]. These techniques have been implemented to determine the presence of group A and group B substances as included in Directive 96/23/EC [157].

Novel formats have emerged in this last decade, including MIP-based extractive disks, micro-columns, fibers and capillaries, LC-columns, membranes, stir bars, magnetic beads, or even needle and micropipette tips, among others [1, 123, 217]. The previous alternatives often require inclusion of different materials (glass, silica, magnetite, carbon nanotubes…) to enhance MIP performance and increase their capabilities, allowing method miniaturization, automation, stirring abilities and magnetic properties. It is worth mentioning that MISPE commercial cartridges are already in the market for off-line clean-up of several veterinary drugs and contaminants, including for instance beta-agonists, steroids, chloramphenicol, fluoroquinolones and quinolones [1].

4.1.2 MIPs as stationary phases in LC columns: monoliths and particles

Molecularly imprinted monoliths are finding their way into SPE but also in the separation field as stationary phases for LC [8, 67, 78]. Despite the fact that monolithic columns were firstly used in gas chromatography many years ago, their application in liquid chromatography has been scarce to date. In LC, particle-based phases are usually preferred over monoliths, being porous silica microspheres the most popular option. In this sense, MIPs synthesized within the pores of spherical silica offer an interesting alternative as they add the recognition capability of the imprinted polymer [29, 118, 129], becoming the compounds not only separated from each other but also from matrix-interfering compounds. Core–shell technology and iniferter-type initiator techniques can be used in the development of MIP particles for stationary phases in LC.

Despite all the aforementioned, monoliths are excellent alternatives to this conventional particle-packed columns, providing higher permeability and lower flow resistance. Monolithic columns are made of a single piece of molecularly imprinted porous polymer, where the material entirely fills the column minimizing the inter-particle space typical of particle-packed LC columns [29]. In this sense, successful methods have been published reporting both development and application of MIP monoliths as phases for liquid chromatographic separation of residues present in food, such as [77, 86, 284].

4.2 POLYMERISATION MIXTURES AND OPTIMIZATION MISPE APPROACHES IN VETERINARY DRUG ANALYSIS

The selection of the substances of interest targeted in the development of objective 2 has been made on the basis of their frequency of use in the field of livestock, and on the basis of the European Directive 96/22/EC and 37/2010 [142, 144]. Also, the selection criteria for analytes and matrices was related to the objectives established in the official research projects AGL2009-14707 and IPT-060000-2010-14, from the Spanish Ministry of Science and Innovation. In this context, MIPs specifically designed for three groups of substances (corticosteroids, amphenicols and penicillins) have been synthesized and applied to milk and milk product samples.

One of the groups of veterinary drugs selected was the group of corticosteroids. These substances are prescribed for a wide range of conditions in both human and veterinary medicine. These treatments are intended to reduce inflammation, also they are anti-allergy and antipyretic [285]. In food producing animals, this type of molecules are authorized for therapeutic treatments as long as they respect withdrawal times established for each case. Thus, maximum residue limits (MRLs) have been established for authorised corticosteroids in different matrices such as milk, muscle, liver, kidney and fat [142]. However, their use as growth promoters is totally prohibited in the European Union [145]. In the literature, a wide range of methods have been developed to the identification of this group of substances in food. The most frequently used method to quantitatively determine corticosteroids in complex matrices has been liquid chromatography coupled to mass spectrometry [199, 286-288]. In the present Thesis, a molecularly imprinted solid-phase extraction (MISPE) procedure has been developed and optimized to allow the simultaneous extraction of six corticosteroids commonly employed in animal husbandry [67]. A library of cortisol-imprinted polymers was prepared by Baggiani *et al.* (2010) combining 10 different functional monomers, 7 cross-linkers and 5 porogen solvents, and testing their selectivity towards various synthetic and natural corticosteroids [289]. Their experiments showed that different combinations of functional monomers, cross-linkers and porogen imprinted polymers with very different selectivity patterns, highlighting the importance of a careful optimization of the pre-polymerization mixture. One of the objectives in this Doctoral Thesis was testing the suitability of two different corticosteroids, dexamethasone (DM) and flumethasone (FLU) for being used as template molecule in the design of MIPs for solid-phase extraction of corticosteroids in milk. The optimized MISPE protocol was combined with liquid chromatography coupled to tandem mass spectrometry for analyte determination in milk. It is important to notice that only one method for synthetic corticosteroids determination using MIPs was found in the literature, for dexamethasone extraction in skincare products [290], highlighting the need to develop novel multi-analyte methods for food.

The next group of substances of interest was the amphenicols group. This type of substances was selected because of the great importance of chloramphenicol (CAP), since it is

a broad-spectrum bacteriostatic antibiotic commonly used in medicine. The use of this substance has been totally banned in food producing animals within European Union due to its serious effects in humans such as Grey syndrome, fatal aplastic anaemia and bone narrow. In the case of the rest of amphenicols, thiamphenicol (TAP) and florfenicol (FLP), they are permitted in cattle but with different MRLs established for food products of animal origin [142]. For their determination, laborious methods are often applied combining SPE or QuEChERs with LC-MS/MS [291-293]. Also, molecularly imprinted technology has been previously applied to the extraction of different components of this antimicrobial group in milk, in different formats such as polymer monolith micro-extraction [294], MISPE cartridges [295, 296], MIP-modified electrodes [297] and on-line MISPE microcolumns [298]. However, these studies have been focused in CAP, using analogues as template for MIP synthesis [295] or even commercial MISPE cartridges [296]. Also, a recent study of Liu *et al.* published in 2018 used a magnetic MIP to detect TAP in milk samples with satisfactory results [299]. On the basis of their importance as potential residues in food, in this Thesis a molecularly imprinted solid-phase extraction (MISPE) procedure has been developed and optimized, testing the suitability of CAP as template molecule in the design of MIPs for the simultaneous recognition of the three amphenicols [78]. The optimized MISPE protocol was applied to milk powder and combined with liquid chromatography coupled to tandem mass spectrometry for analyte determination.

Finally, penicillins group was selected due to its importance for the treatment of food-producing animals, in particular, dairy cattle [300]. Penicillins are antimicrobial agents belonging to the β-lactam family, used against various microorganisms and exert their activity inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls [8]. The incorrect and frequent use of these antibiotics may result in the presence of residues in edible tissues or derived products such as milk [301, 302]. To avoid health risks for the consumer, MRLs of these compounds in milk have been established through Commission Regulation (EU) number 37/2010 [142]. Although there are recent studies demonstrating that LC-MS is a trustful method for confirmation and quantification [303, 304], a recent review of Mohnsenzadeh et al. highlights the role of MIPs as effective sorbents for separation, clean-up, pre-concentration and analysis of different antibiotics in milk [278]. A few MIP alternatives have been published to isolate penicillins from meat or milk [305] or tap water [128], using amoxicillin and benzylpenicillin as template molecules, respectively. However, the aforementioned studies developed MIP alternatives for single-compound extractions. In this doctoral Thesis, a molecularly imprinted solid-phase protocol (MISPE) has been developed and optimized for simultaneous extraction of eight penicillin antibiotics, testing the suitability of various compounds as template molecule and various cross-linkers and porogens [8]. The MISPE protocol was applied to milk powder spiked with the eight antibiotics at the level of interest (MRLs), and analytes were measured using liquid chromatography coupled to tandem mass spectrometry.

4.2.1 Templates, functional monomers, cross-linkers, porogenic solvents and initiators

There are a large number of experimental variables influencing the design and design of MIP such as the nature and amount of template, the wide variety of functional monomers and cross-linkers available, solvents and initiators, as well as the method of initiation and duration of polymerisation [306]. All these mentioned parameters have to be assessed since they can immensely influence the final morphology, properties, and performance of the polymers in terms of affinity, selectivity, loading, capacity, etc., their proper selection will ensure that

polymers with the appropriate properties are obtained for a particular application [260]. A summary table is presented below showing the combinations that have allowed the highest performance to be achieved for the different groups of substances analysed (corticosteroids, amphenicols and penicillins). In addition, it is possible to check the polymerization process used and the optimized conditions of the final MISPE process applied to real samples.

Table 2. Pre-polymerization mixture and optimum conditions selected for the MISPE procedure of corticosteroids, amphenicols and penicillins in real dairy samples

MIP characteristics		CORTICOSTEROIDS	AMPHENICOLS	PENICILLINS
Pre-polymerization mixture	Template	FLU	CAP	NAFC
	Monomer	MAA		
	Cross-linker	DVB		EGDMA
	Porogen	ACN/TOL (3:1)		ACN
	Initiator	AIMN		
MISPE conditions	Loading	TOL		ACN
	Washing	TOL (5%ACN)		
	Elution	MeOH (1% HAc)		
Polymerization method		Precipitation		

FLU: Flumethasone, **CAP:** Chloramphenicol, **NAFC:** Nafcillin, **MAA:** Methacrylic acid, **DVB:** Divinylbenzene, **EGDMA:** Ethylene glycol dimethacrylate, **AIMN:** 2,2′-azobis-(2-methyl-butyronitril), **ACN:** Acetonitrile, **TOL:** Toluene, **MeOH:** Metanol, **HAc:** Acetic acid

A wide range of templates molecules have been successfully used to obtain MIPs for veterinary drugs extraction in animal matrices [1]. The selection of template molecules for the groups of corticosteroids, amphenicols and penicillins was made on the basis of the most important and structurally representative molecules of each group. The obtained results demonstrated that with appropriate MIP design and exhaustive template removal, it is possible to obtain polymers with no detectable bleeding and excellent extraction ability and selectivity for different veterinary drug groups [1, 8, 78]. For corticosteroids, dexamethasone (DM) and flumethasone (FLU) were tested as templates. Unfortunately, no methods have been reported for simultaneous extraction of synthetic and natural corticosteroids in food using MIPs. Only an article was found reporting the use of DM as template for MIP synthesis on magnetic nanoparticles, but it was applied to cosmetic samples [290]. In the present case, DM-based MIP provided the maximum recovery in solvent tests, but FLU-based polymer was more efficient in real milk samples [67]. The template selected for synthesizing a polymer to extract amphenicols was CAP, the most commonly used inside its group and the one with the lowest level of interest among them (MRPL). The very few polymers found in the literature designed for CAP extraction in milk used structural analogues in order to avoid possible CAP bleeding [295, 307], as it can be a serious problem during CAP determination [296, 308]. However, other authors demonstrated that the use of the target molecule as a template not necessarily leads to bleeding [1, 294, 295]. Actually, the work developed in this Thesis demonstrated that the use of CAP as template is feasible, and provides polymers capable of extracting the 3 components amphenicol family from milk [78]. On the other hand, in the study of penicillin antibiotics, oxacillin (OXA), amoxicillin (AMX) and nafcillin (NAFC)

were selected as template and thus, different polymeric sorbents were synthesized and optimized for solid phase. Urraca *et al.* 2016 used 2-biphenylylpenicillin, a surrogate or dummy template with a close resemblance to β-lactamase-resistant penicillins in terms of size, shape, hydrophobicity, and functionality, as template for the analysis of OXA, CLOX and DICLOX in milk samples [309]. Alternatively, amoxicillin and benzylpenicillin have also been reported as templates with no detectable bleeding in the application of the MIP, but for single analyte extraction [128, 305]. In the present Thesis, nafcillin was effective to obtain a MISPE protocol useful to extract eight compounds of the penicillin family in milk powder [8], with no detectable bleeding of the template.

In terms of the choice of an appropriate functional monomer, this step is considered of extreme importance in order to create highly specific cavities in the imprinted polymer [31] since they are responsible for binding interactions in the imprinted binding sites. As it can be seen in Table 2, methacrylic acid (MAA) was selected as functional monomer in the three studies developing MISPE alternatives for veterinary drug analysis included in this Doctoral Thesis. Up to now, MAA has been the most frequent functional monomer in non-covalent approaches, being its use restricted to those templates able to interact by hydrogen bonding. Moreover, all templates selected in the aforementioned studies (FLU, CAP and NAFC) posse functional groups in their structures as ketone and hydroxyl, which will theoretically interact with MAA by hydrogen bonding. In the literature several studies were reported showing the frequent use of MAA as functional monomer for different groups of substances. For instance, MAA has been recently used for developing MISPE cartridges for extraction of some penicillins in meat or milk [305, 310], or for amphenicol compounds in various matrices such as meat, fish or milk [307, 311]. In view of the results and literature evidence, MAA can be considered a very useful functional monomer for MIP synthesis for groups of veterinary substances chemically very different [278].

It is well known that the selectivity of any MIP is also greatly influenced by the type and amount of cross-linking agents used for synthesis [29]. Moreover, the type of cross-linker influences the final size and yield of MIP nanoparticles during the polymerisation process by precipitation [29, 40]. That is why different cross-linking agents have been tested in the different studies of veterinary drug of this Doctoral Thesis [8, 67, 78]. According to the literature, ethylene glycol dimethacrylate (EGDMA) and trimethylolpropane trimethacrylate (TRIM) are the two most commonly employed cross-linkers for MIP synthesis [260, 312]. However, TRIM usually results in polymers with more rigidity, structure order, and effective binding sites than EGDMA [29]. In Table 2, the selected combinations for MISPE of veterinary drugs are shown, being divinylbencene (DVB) the cross-linker selected for corticosteroids and amphenicols, and EGDMA for penicillins. The appropriate porogenic solvent was selected in accordance, being a mixture of acetonitrile with toluene (3:1) more suitable for DVB polymers, and pure acetonitrile for EGDMA polymers. When using DVB as cross-linker the combination of acetonitrile and toluene is required to obtain polymers with good performance and with the production of monodisperse, imprinted polymers beads with well-developed, permanent pore structures [197, 313, 314]. In the design and synthesis of MIP for amphenicols extraction, EGDMA has been tested too but it was impossible to obtain a complete dilution of CAP in two of the porogens tested (toluene, dichloromethane), which it is very convenient in any MIP synthesis prior to cross-linker addition. Consequently, EGDMA polymers for amphenicols could only be achieved using methanol or acetonitrile as porogens, in accordance to the literature [307]. Even when the synthesis of MIP for amphenicols has mainly involved EGDMA in combination with different functional acrylic

monomers [278, 307], in this research DVB-polymers provided higher recoveries of the 3 amphenicols extracted simultaneously. In particular case of penicillins, cross-linkers such as EGDMA and TRIM have been also reported for milk clean-up, including different solvents in the polymerization mixture [305, 310]. In the case of the 8 penicillins selected in this study, EGDMA polymerised in acetonitrile provided a selective polymer for their extraction in milk powder, in accordance with the literature [8, 305].

All the designed and synthetized polymers for the extraction of veterinary drugs compiled in the present Thesis were prepared by precipitation polymerisation method, since it allows the formation of spherical polymer particles with uniform sizes. Besides, it is one of the most attractive and reliable methods available for the routine production of imprinted polymer beads with desirable characteristics [72, 75]. Furthermore, MIPs obtained by this type of polymerisation usually have a quite high yield because grinding and sieving steps are not required [1]. In the studies of this Doctoral Thesis the performance of polymerisation reactions was evaluated calculating the final yield percentage in comparison to theatrical values. Ideally, a reaction should yield 100% but in real practice the amount of polymer produced during precipitation varies over a wide range, mainly due to the losses that occur during handling and in particular during the Soxhlet template removal. For example, the MIP synthesized for corticosteroids using DM as template and DVB as cross-linker yielded 94%, while using FLU as template the same monomers yielded 65% [67]. Curiously, the MIP selected to be implemented in real milk samples analysis was FLU-based MIP, even when the reaction seemed not so successful. Similarly, MIPs developed for amphenicols using DVB also yielded 65%, being the imprinting effect also more evident than in EGDMA-polymers, which yielded 100% [78]. Yoshimatsu *et al.* (2007) obtained also lower polymerisation yield using DVB as cross-linker, in comparison to TRIM polymers [40]. These results demonstrated that selectivity is not directly related to the amount of MIP produced during polymerisation, but it is related to the inner reactions carried out in the pore sites of the polymer. Moreover, precipitation polymerisation leads to a more homogenous binding site distribution in comparison to that present in polymers obtained by bulk polymerisation [75]. The polymer beads formed in this way are protected from aggregation during polymerisation by their cross linked surfaces and are completely surfactant free. Particles with diameters of a few micrometres (~5µm) can be readily accessed and are more appealing as far as routine MISPE and MIP-column LC applications are concerned [77]. Precipitation polymerisation has been used for similar SPE applications by other research groups, including determination of other antibiotics such as tetracyclines [315] in foodstuffs. Alternatively, suspension and bulk polymerisation have been also applied to obtain MISPE cartridges for extraction of penicillins [305, 310] and amphenicol compounds [311], respectively.

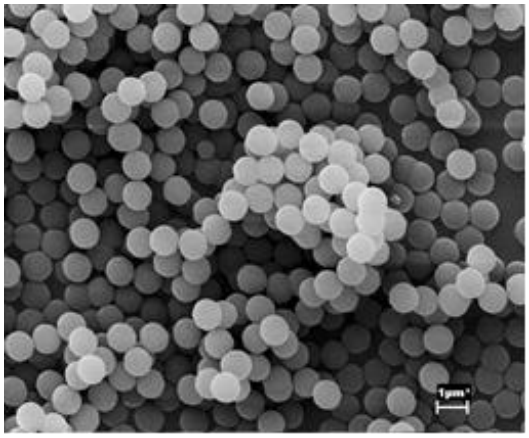

Figure 21. Spherical MIP particles obtained by precipitation polymerisation using MAA and DVB for corticosteroid extraction. Reprinted with permission from Díaz-Bao, M. (2012) Copyright 2012, Springer-Verlag [67]

Polymerisation process takes place thanks to a chemical initiator which depends on the method of initiation is to be used. In the three studies [8, 67, 78], 2,2′-azobis-(2-methyl-butyronitril) (AIMN) was used as initiator since it is one of the most commonly used initiators for precipitation polymerisation[115, 316]. All the components of pre-polymerisation mixture (template, functional monomer, cross-linker and initiator) are dissolved in porogenic solvent, which plays an important role in the formation of the porous structure of MIPs. Moreover, it is responsible for creating pores within the macroporous polymers [29]. The most common solvents used for MIP synthesis were tested in the three groups analysed such as acetonitrile, toluene, methanol and dichloromethane. Once the pre-polymerisation mixtures have been prepared, the procedure of polymerisation takes place into a controllable incubator equipped with a low-profile roller (Figure 22). After 24 hours at 60 ºC polymerising, the template is removed by Soxhlet extraction, following the protocol purposed by Cacho *et al.* (2003), which has proven to be adequate for the production of spheres suitable for the packaging of columns [60]. Accordingly, non-imprinted polymers (NIPs) were also prepared for each group of substances following the same procedure as for MIPs but without the addition of the template molecule. These NIPs have been used to demonstrate the existence of specific imprinting sites or cavities in MIP sorbents and discard unspecific interactions MIP-analyte.

Figure 22. Roller and incubator used for precipitation polymerisation

Once polymers have been obtained, cleaned and dried, 0.05 g were in-house packed into empty SPE glass cartridges of 6 mL between a Teflon (PTFE) frit at the bottom and 0.5 cm of folded filter paper (73 g/m^2) on the top of the columns. MISPE columns were connected to an SPE manifold connected to a vacuum pump.

4.2.2 Optimization of MISPE protocols and application to real food samples

Before their first use, MIP and NIP cartridges were always conditioned with acetonitrile, methanol and toluene. Several experiments were carried out loading different amounts of each analyte in different loading solvents (acetonitrile, ethyl acetate, toluene), and using several washing solutions (acetonitrile, methanol, toluene, toluene with different percentages of acetonitrile). Real samples usually have a high degree of complexity and the protocol needs to be optimised to be able to apply it later and in order to obtain quantitative recoveries of the analytes with a high degree of selectivity. In parallel, the same experiments were carried out on NIPs cartridges to prove the existence of template-specific imprinted sites into the MIPs. Additionally, once the MISPE protocol was optimized in solvent, a comparison was made between MIPs designed to extract corticosteroids and two types of commercial cartridges, i.e. OASIS HLB (Waters) and Strata-X (Phenomenex) [67]. In this study, the imprinted polymers provided more stable and balanced recoveries when extracting the six corticosteroids simultaneously, demonstrating the usefulness of the imprinting effect for extracting a group of structurally related molecules. Another advantage of MISPE cartridges is their reusability of the contrary to commercial alternatives, making them less costly in use. Further studies have demonstrated the superiority of MIPs in solid-phase extraction to extract residues present in food, in comparison to commercial counterparts [305, 315].

Concerning the loading solution, first experiments of optimisation were made using acetonitrile in the three studies [8, 67, 78]. In the case of corticosteroids and amphenicols MIPs, and also in their corresponding NIPs, no imprinting effect was observed and analytes of interest eluted during the loading step. However, in the case of penicillin antibiotics the use of acetonitrile as loading solvent was successful as the MIP selected for their extraction was synthesized in ACN. It has already been reported that most times recoveries are higher whenever the sample is loaded in the same solvent used as a porogen in the MIPs preparation [60, 67]. This may be the result of the porogenic memory of MIPs, so polymerisation solvents are usually the most suitable for the loading step. For this reason, in the case of corticosteroids and amphenicols, as it can be seen in Table 2, the solvent selected to load the sample was pure toluene instead of ACN. Optimization of the washing solvents is critical in MISPE as if the solvent is not selected with the appropriate polarity to maintain interactions between the analytes of interest and the polymer, elution can occur and therefore also loss of the analytes at this point. The washing step is generally achieved by the same solvent using during loading or with the addition of a small proportions of a more polar solvent, in order to limit the non-specific interactions of the target analytes with the external surface of the MIP [60, 67]. As it is shown in Table 2, toluene with 5% acetonitrile was selected as washing solvent for both corticosteroids and amphenicols MISPE protocols. It was assumed that a small percentage of acetonitrile in toluene would promote the imprinting effect, and this theory was correct in both MIP synthesized from DVB. The same was true for other MIPs synthesized using toluene as porogen, for which more evident imprinting effect was observed adding acetonitrile to the toluene used for washing [60]. In the case of penicillins, the optimized washing was performed with acetonitrile. These differences between MIPs have their origin in the cross-linkers used for their synthesis, being DVB and EGDMA, respectively. In the case of amphenicols and penicillins, pure methanol and acetonitrile were tested too. Methanol was

too aggressive for washing in both cases provoking analyte elution at this point, but acetonitrile was suitable for penicillins EGDMA-based MISPE [8, 78]. Methanol 1% acetic acid was selected as optimal solvent for elution the analytes in all cases, in accordance to similar studies [307, 317].

In most cases, after optimization of the MIP-based clean-up process, techniques such as protein precipitation, centrifugation, filtration, among others, are required prior to the application of real samples [211, 317, 318]. In the present research project, after MISPE optimization for veterinary drugs, samples of commercial milk and infant formulas were spiked at the level of interest (MRLs, Table 3) with a mixture of the selected analytes. This way, the usefulness of developed MIPs to extract them from milk or from milk powder was assessed, as well as the existence of possible matrix effect was verified. The real samples analysed were commercial milk in the study of corticosteroids, and milk powder (baby formulas) in the case of amphenicol and penicillin antibiotics. For both corticosteroids and amphenicols, samples were loaded in toluene based on the previous optimization of the MISPE protocol. In the case of corticosteroids, prior to MISPE, protein precipitation with ACN was required. After centrifugation, ACN was evaporated and redissolved in toluene (loading solvent). In the study of amphenicols, the analytical MISPE procedure included a prior protein precipitation with ethyl acetate, followed by evaporation and reconstitution in the loading solvent (toluene). Ethlyl acetate was chosen to extract amphenicols group prior to MISPE because several authors have reported its efficiency for removing these analytes from different matrices [295, 319-323]. Moreover, the direct loading (without evaporating) of ethlyl acetate supernatant into the MIP cartridge was tested too; in this case, recoveries of CAP and FLP decreased about 10%. In the case of corticosteroids, acetonitrile was used for protein precipitation, in accordance with other works on steroid analysis in dairy products [197, 307, 317]. However, in the case of BLAs (penicillins) the loading of the precipitated sample (supernatant) was direct thanks to the cross-linker used for the synthesis, EGDMA. This cross-linker resulted in the use of ACN as optimal loading solvent, simplifying the method and avoiding the evaporation step. Moreover, in milk powder samples the percentage of water was negligible, facilitating the direct loading of the extract.

MISPE extracts were analysed using HPLC-MS/MS methods, and in the case of amphenicols the selectivity and efficiency of the optimised procedure coupled to the sensitivity of the detector, lead to a validated confirmatory method [78]. This validated method can be used to control the presence of amphenicol residues in milk powder, including CAP, TAP and FLP. In Table 3, MRL or MRPL, MISPE recoveries in real samples, analytical matrix and validation parameters of the LC-MS method are presented for each compound. In real samples, recoveries ≥85% were obtained for all corticosteroids in spiked milk samples. No report on MIP use to extract these compounds from milk was found in the literature for comparison. For the amphenicols group, recoveries in milk powder samples using MISPE were within the range of 91-110%. In this sense, Zhao *et al.* (2017) reported similar recoveries for CAP with a polymer synthesized using a thiamphenicol as dummy template in combination with MAA and EGDMA in methanol [307]. However, Liu *et al.* (2018) developed a magnetic MIP for TAP extraction in milk, achieving recoveries of 85% using the same compound as template molecule [299]. Finally, recoveries in the range of 56-88% were obtained for penicillin antibiotics in milk powder. Van Royen *et al.* (2016) reported recoveries for benzylpenicillin in the range of 70-80% using MISPE cartridges prepared with MAA and TRIM in acetonitrile and the same compound as template molecule [324]. Curiously, very few

multi-analyte methods are available in the literature using MIPs for food analysis, as it has been confirmed in this Doctoral Thesis, particularly for the selected compounds.

Table 3. MRLs established by Commission Regulation (EU) in milk and MISPE performance in real samples

ANALYTES		MRLs (μg.kg^{-1})	NOTES	MISPE		VALIDATION
				Recovery (%)	Analytical matrix	LC-MS/MS
Corticosteroids	COR	-	Natural hormones	105	Milk	Non Validated
	HCOR			120		
	FLU			105		
	DM	0.3		85		
	PRED	6		150		
	MPRD	-	Not for use in animals from which milk is produced for human consumption	130		
Amphenicols	FLP			110	Milk powder (infant formulas)	Validated CCα CCβ
	TAP	50		91		
	CAP	-	Prohibited substance but MRPL has been established (0.3μg.kg^{-1})	105		
Penicillins	OXA	30		88		Validated LOD LOQ
	CLOX			82		
	DICLOX			63		
	NAFC			77		
	AMP	4		65		
	AMX			58		
	PEN G			56		
	PEN V	-	It was applied the Penicillin G limit, 4μg.kg^{-1}	70		

COR: Cortisone, **HCOR:** Hydrocortisone, **FLU:** Flumethasone, **DM:** Dexamethasone, **PRED:** Prednisolone, **MPRD:** Methylprednisolone, **FLP:** Florfenicol, **TAP:** Thiamphenicol, **OXA:** Oxacillin, **CAP:** Chloramphenicol, **CLOX:** Cloxacillin, **DICLOX:** Dicloxacillin, **NAFC:** Nafcillin, **AMP:** Ampicillin, **AMX:** Amoxicillin, **PEN G:** Penicillin G, **PEN V:** Penicillin V, **LOD:** Limit of detection, **LOQ:** Limit of quantification, **CCα:** Decision limit, **CCβ:** Detection capability

Commercial SPE cartridges are single-use alternatives, resulting in increased costs for laboratories. The durability of SPE cartridges prepared with molecularly imprinted polymers is one of their major strengths as alternatives for sample preparation. In this sense,

conservation is a key factor in MIP usage, since, theoretically, MISPE cartridges should be preserved from a high load of remaining matrix, in order to increase their durability and keep the recognition properties of the polymer unaltered [325]. For the maintenance and conservation of the cartridges it is recommended, after each use, to pass through the cartridge two volumes of solvents with different polarity and keep the polymer in the loading solvent upon next analysis. Alternatively, a two-step MISPE procedure for preserving MIPs properties was proposed by Tamayo *et al.* (2005), consisting of the inclusion of a previous clean-up step using a non-imprinted polymer before the MISPE procedure [325]. Also, the possibility of in-house preparing tailor made MIPs for specific purposes/analytes, expands the scope of action of laboratories without being totally dependent on commercial clean-up solutions.

4.3 MAGNETIC ALTERNATIVES: MOLECULARLY IMPRINTED STIRRING-BARS FOR MYCOTOXINS ANALYSIS IN FOOD

Veterinary drug residues aside, there is a possibility that certain contaminants may appear in food unintentionally, such as fungal toxins. The selection of the substances of interest targeted in the development of objective 3 has been made on the basis of their incidence in foodstuff and toxicity for humans. Also, the selection criteria for target mycotoxins and matrices was related to the objectives established in the official research project EM 2012/153, from Consellería de Cultura, Educación e Ordenación Universitaria (Xunta de Galicia, Spain). In this context, MIPs specifically designed for six mycotoxins (5 aflatoxins and patulin) have been synthesized in combination with magnetite and magnetic supports, and further applied to real samples. The formats used in the accomplishment of objective 3 are more innovative in comparison to classical MISPE cartridges developed in objective 2. In this context, fast and selective methods were developed for the fabrication of magnetic molecularly imprinted stir-bars (MMIP-SB) and applied to baby products (dairy and cereal-based) and apple samples [123, 217].

Mycotoxins are a widely diverse group of natural toxins produced by fungi as secondary metabolites. Aflatoxins (AFs) are one of the most important classes of mycotoxins in terms of occurrence and toxicity. This group of substances has been selected because they are toxic compounds that are introduced into the food chain through contaminated crops, mainly cereals. AFs presence depends mainly on environmental conditions, storage and even the inappropriate manufacturing process. Milk is one of the matrices in which aflatoxins (AFM1) can be found, obtained from animals fed with contaminated feed. Thus, children and infants may be exposed to AFs through different commercial products, such as formulas and cereal-based foodstuffs, or even through breast milk [238, 240, 326-328]. Juan *et al.* in 2014 indicated mycotoxins presence in a high variety of baby food samples [240]. This situation compromises newborns and infants health, and it calls for reliable and affordable methodologies. The exposure to these mycotoxins can cause significant health problems in humans and even death in some cases. For this reason, the European Commission Regulation (EC) 165/2010 sets maximum levels for AFB1 for processed cereal-based products and baby foods and AFM1 in milk and infant formulas [224]. Recent studies have determined the presence of mycotoxins and other contaminans in cereals by LC-MS/MS extracting them from the matrix using LLE or QuEChERS [329, 330]. Recently, Jayasinghe *et al.*, proposed the use of MIPs for µ-SPE, in combination to LC-MS/MS, for the determination of AFs in fish feed [331]. In the present Thesis, a combination of imprinting technology with magnetite is proposed, resulting in a fast and simple method for fabrication of MMIP-SB [217].

On the other hand, patulin (PAT) is a toxin that appears frequently in fruits and derived products, such as apples and apple juice. Patulin can occur in these agricultural products of great importance in North-western Spain, and in particular in Galicia [332, 333]. For example, in the case of the apple, a very important part of the cider apple produced in Spain comes from Galicia, surpassing in the last campaign the 8 million Kg [334]. The climate and humidity characteristic of northwestern areas of Spain facilitate the growth of fungi in agricultural products [335, 336]. [336]Patulin is an important mycotoxin produced by species of fungi normally associated with plant products and fruits and its ingestion can cause agitation, convulsions, edema, intestinal ulceration, inflammation, vomiting and even immunological, neurological or gastrointestinal disorders. For this reason, the European Commission Regulation (EC) 1881/2006 established a maximum patulin content of 10 $\mu g.kg^{-1}$ in children fruit juices, 25 $\mu g.kg^{-1}$ in fruit products and 50 $\mu g.kg^{-1}$ in adult fruit juices. All the previous, resulted in an increasing demand of sensitive, selective and effective analytical methods for patulin determination. Nowadays, the official analytical method adopted by AOAC International is HPLC with UV detection, using clean-up with ethyl acetate and sodium carbonate. However, the diverse drawbacks of this method have originated interest in alternative options, such as LC methods coupled to mass spectrometry [222]. Also, this toxin is frequently included in multi-mycotoxin methods for apple and apple products, in which the extraction focus on the reduction or elimination of co-eluting matrix components, including solid phase extraction (SPE), inmunoaffinity separation (IAC) or liquid-liquid extraction (LLE) [337]. However, techniques such as QuEChERS are gaining more interest for multi-mycotoxin determinations [338] although they are not able to remove all the co-eluting matrix components. A recent study of Zhao *et al.* determined the presence of PAT from food samples using MISPE technique coupled with LC-MS/MS [339]. In the present Thesis, a combination of imprinting technology with a glass-covered magnetic stir-bar is proposed, resulting in a fast and simple method for fabrication of MMIP-SB for PAT determination in apple products [123].

4.3.1 Magnetic supports, templates, functional monomers, cross-linkers, porogenic solvents and initiators

Since alternative strategies to classic clean-up approach are demanded, new combinations of MIPs with different materials have emerged [29]. The combination of MIPs with magnetic supports is one of the most attractive and demanded alternatives, since it has allowed the laboratories to obtain automated methods in which the steps of classical extractions are performed with the aid of an external magnetic field.

In the present Thesis, the synthesis of magnetic molecularly imprinted stir-bars (MMIP-SB) for the extraction of aflatoxins was developed combining imprinting technology with magnetite [217]. Alternatively, the usefulness of glass-covered commercial magnets as physical supports for MIP has been demonstrated in the study of patulin [123]. In the literature, other options can be found to fabricate MMIP-SB, usually implying the use of commercial materials (stir-bar, fibers, glass) as supports [122, 340-344]. In the latter cases, surface-activation steps are necessary in order to firmly attach the MIP to the surface of the device. The MMIP-SB developed for PAT isolation was achieved by grafting a MIP onto a glass-covered magnetic stirring bar. A grafting protocol adapted from the study of Turiel and Martin-Esteban [122] was implemented. It is necessary to properly clean the surface of the glass and silanize it. Once the magnetic support is ready to use, the corresponding pre-polymerisation mixture is prepared and grafted to the activated (silylated) glass-surface. On the other hand, the MMIP-SB for AFs was developed using a combination of in-house

prepared pieces of magnetite and bulk MIP, polymerised inside a glass-vial lying down in the incubator. By simply breaking the vial, a stir-bar was released in the shape of a polymeric semi-cylinder filled with magnetite, i.e. with the aspect of a nuts "cake". The combination of magnetic properties with the advantages of MIP not only affords the selectivity for the target molecule but also provides the ability of one-step separation [108]. Figures 23 and 24 show the steps of fabrication of the MMIP-SB for aflatoxin extraction and the result, respectively.

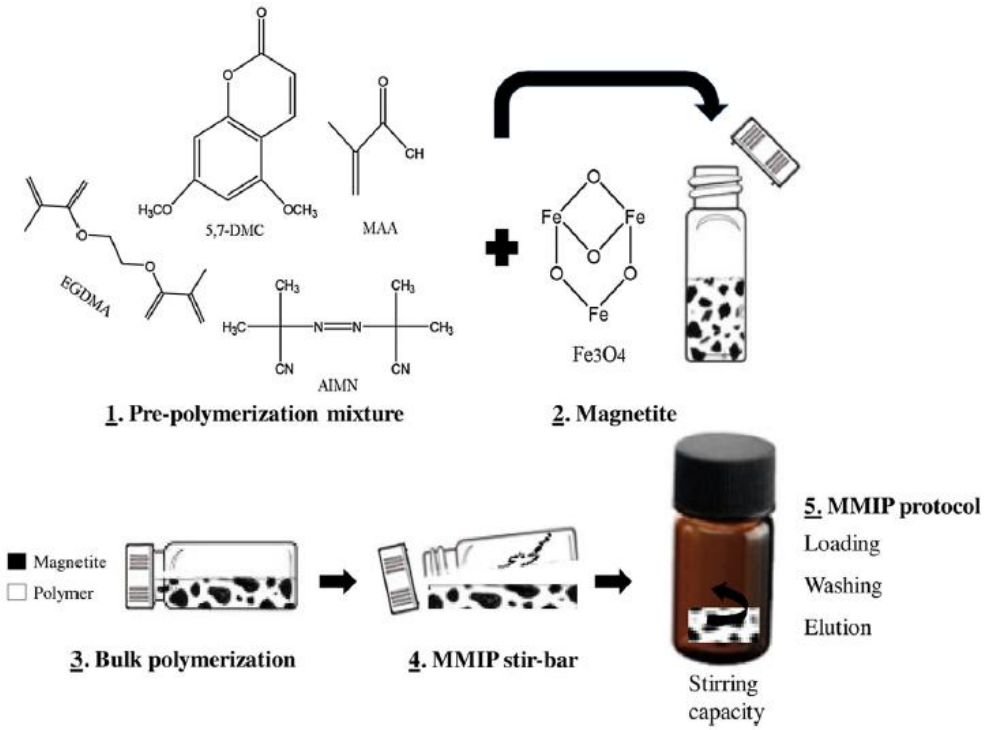

Figure 23. Steps of synthesis in the laboratory and application of the magnetic molecularly imprinted stir-bars. Reproduced from Díaz-Bao, M. *et al.* (2016), Elsevier B.V. All rights reserved [217]

Figure 24. Magnetic molecularly imprinted polymer (MMIP) and non-imprinted (MNIP) stir-bars developed for the extraction of aflatoxins. Reproduced from Díaz-Bao, M. *et al.* (2016) Elsevier B.V. All rights reserved [217]

Template bleeding is often outlined as the main risk of using the target molecule of MIP as template during synthesis and for this reason dummy templates were proposed [114]. However, sometimes the substances selected as templates for MIP synthesis have also high toxicity for humans. For this reason and to avoid their manipulation, as they are dangerous analytes for laboratory staff, a dummy template may also be preferred. In the design, synthesis

and optimization of MMIP-SB for extraction of AFs compiled in the present Thesis [217], the 5,7-dimethoxycoumarin (DMC) was used as template. This selection was made on the basis of previous MIP studies, as it is an structural analogue of AFs [133, 345-347]. In the case of PAT, a dummy template reported in the literature, 2-oxindole was also preferred [249]. Both 2-oxindole and DMC have not been classified as hazardous for humans and are much less expensive than AFs or PAT commercial standards [348]. Additionally, when it comes to very low detection levels as in the case of fungal toxins template bleeding can be an additional analytical problem and for this reason a dummy template is recommended.

Once dummy template molecule has been selected, different polymers were synthesized under different conditions, i.e., changing the porogenic solvents and the cross-linker employed, in order to achieve a robust and durable stir-bar. For PAT, the polymer coated on the surface of the stir-bar was prepared by a non-covalent approach, based on the formation of non-covalent interactions between the template and the functional monomer. MAA was used as functional monomer, based on its extended use for MIP synthesis and versatility [62]. In the case of AFs, the same non-covalent approach was applied, using MAA. This is a choice also supported by scientific literature for the detection of these compounds using MIPs [100, 345, 347]. Moreover, two different cross-linkers were tested (EGDMA and DVB) in acetonitrile and in toluene with different percentages of methanol. For AFs stir-bar the pre-polymerisation mixture was combined with magnetite, which had been prepared in the laboratory from the raw and through a process of bulk polymerisation it was embedded into MIPs. Conversely, for PAT stir-bar the pre-polymerisation mixture was grafted on the surface of the activated/silylated stir-bar by bulk polymerisation. This type of polymerisation was preferred in both cases because it does not require sophisticated instrumentation and the reaction conditions can be easily controlled [349]. AIMN at 2% wt. relative to monomers was used to initiate bulk polymerisation in both studies. To obtain the corresponding non-imprinted stir-bar, the same protocol was followed but without the addition of template. Once polymerisation had finalised, the template molecule was removed by Soxhlet extraction with methanol and acetic acid.

Once the polymerisation finalised, stirring properties were demonstrated in the obtained semi-cylindrical polymer for AFs extraction. It was observed that the choice of cross-linker for the synthesis of the polymer is a key point since many of the characteristics of the monolith/bar obtained such as colour, hardness, porosity, stiffness, strength, etc., will depend on it. DVB-polymers were fragile and had a plastic and yellowish appearance, with low porosity, not being possible to obtain a strong and durable polymer. On the contrary, the results were satisfactory using EGDMA since the polymers were clearly whiter and more resistant to scratch and pressing than those obtained with DVB, showing more durability under compressive strength conditions [217]. These characteristics were enhanced when toluene (combined with 10% methanol) was used as a porogen instead of acetonitrile. These results are in agreement with the work developed by Turiel and Martín-Esteban in 2012, in which case a mixture of toluene/methanol 80:20 (v/v) was used as porogen to synthesised a stir-bar for selective extraction of thiabendazole in fruits [122]. Several published studies have been found using EGDMA as a cross-linker for the determination of aflatoxins [133, 274, 350]. In the case of Szumski *et al.* (2014) developed a polymeric capillary column, for them they used pure toluene as porogenic solvent instead of combinations of solvents. Non-polar organic solvents are usually preferred in non-covalent imprinting processes, as they promote the monomer-template interactions and result in most effective rebinding [133]. As in the previous case, for PAT stir-bar EGDMA was selected as cross-linker as the polymers were

stronger than those obtained with DVB, showing more resistance to compressive strength and even more when using toluene with 10% methanol [123]. In Table 4, the selected components for MMIP-SB are shown, along with the optimised MMIP solid-phase extraction (MMISPE) protocol performed with the corresponding stir-bar.

Table 4. Pre-polymerisation mixture, magnetic components and optimum conditions selected for the MMIP-SB extraction procedure of aflatoxins and patulin in real dairy and cereal-based baby food samples and in apple

Design of MMIP-SB		AFLATOXINS	PATULIN
MIP components	Dummy Template	DMC	2-oxindole
	Monomer	MAA	
	Cross-linker	EGDMA	
	Porogen	TOL/MeOH (90:10)	
	Initiator	AIMN	
	Magnetic support	Magnetite	Glass-covered magnet
MISPE conditions	Loading	H_2O	
	Washing	H_2O	
	Elution	MeOH (25% HAc)	
Polymerisation method		Bulk	

DMC: 5,7-dimethoxycoumarin, **MAA:** Methacrylic acid, **EGDMA:** Ethylene glycol dimethacrylate, **AIMN:** 2,2′-azobis-(2-methyl-butyronitril), **TOL:** Toluene, **MeOH:** Metanol, **HAc:** Acetic acid

Other alternatives are available for fabrication of molecularly imprinted stir-bars, as compiled in the article by Díaz-Bao et al. in 2016 [217]. For example, glass-covered magnets can also be combined with MIPs using a physical coating approach, preparing MIP particles beforehand and using epoxy adhesives [122]. Other authors have reported the use of silylated glass capillaries coated with MIP and with magnetic cores [340, 342] or Teflon-coated disks with miniature magnetic stir-bars to obtain rotating disk extraction device [341]. The major advantages of the procedure presented in the aflatoxins study are its simplicity, using bulk polymerisation, and also the easy preparation of magnetite and the fact that no high chemical skills are required to obtain the MMIP-SB. Alternatively, the protocol of fabrication presented in the patulin study adds some complexity due to the silanization step, but it is also relatively easy to perform.

4.3.2 Optimization of MMIP-SB protocols and application to real samples

To optimize the extraction protocol for mycotoxins using the obtained stir-bars, it was necessary to carry out several experiments, using different solvents and/or loading times in order to seek the highest analytes recoveries. Before application to real samples, optimisation experiments were performed using the dummy template as analyte of interest. In this sense, the final conditions of the optimised MMIP solid-phase extraction (MMISPE) protocol are presented in Table 4.

To obtain the maximum recoveries of AFs it was necessary a minimum loading time of 45 min, and for PAT 30 minutes. These loading times may seem tedious but it must be taken into account the fact that automatic stirring is performed. Also, a prudent time was expected to be necessary as it is often difficult to reach the active pores within the bulk polymer matrix of this kind of stir-bars [122]. After having tried different solvents and combinations it was demonstrated that the use of water as loading and washing solvent was optimal for both MMIP-SB (Table 4), providing better results than those obtained using the porogen of the synthesis. Water was also preferred because of its green aptitudes and its lack of toxicity [351]. After optimizing the elution solvent and time, it was proved that 25% of acetic acid in methanol was efficient to obtain the maximum AFs and PAT recoveries with the stir-bars after 45 and 30 minutes of stirring, respectively. The use of a relatively high percentage of acid was expected due to monomeric polymers usually require stronger conditions to liberate the analyte than other easily-accessible polymers such as nanoparticles [217]. Moreover, the existence of template-specific imprinted sites in the MMIP-SBs was demonstrated by lower recoveries in the corresponding non-imprinted stir-bars.

Once the MMIP-SB protocol was optimized for AFs extraction (Table 4), it was applied to spiked samples of milk powder, (baby formulas) and cereal-based baby food [217]. There is a recent study which indicated mycotoxins presence in a high variety of baby food samples [240]. This situation compromises infants' health and calls for creation of reliable and affordable methodologies. The magnetic solid-phase extraction process was used in combination with a previous step with aqueous formic acid (1%) to avoid the saturation of the stir-bar during the analysis eliminating fat and/or cream, as previously recommended [352]. Then, two extractions with chloroform were performed [353, 354] and solvent was evaporated under nitrogen stream to subsequently redissolve the extract in water for loading. In the case of cereal-based baby food, the same preparative protocol was used. Once MMISPE was performed and elution was obtained, it was evaporated, redissolved in mobile phase and filtered for injection in the HPLC-MS/MS system. The levels tested for AFM1 were above the corresponding maximum concentrations permitted in the European Union. For cereals and all products derived from cereals, including processed cereal products, the European Commission has established a maximum level of 4µg.kg^{-1} for the sum of aflatoxins B1, B2, G1 and G2. The stir-bar was tested at 1µg.kg^{-1} in baby formula for AFM1 and at 4 µg.kg^{-1} in cereal-based baby food for B1, B2, G1 and G2. Upon MMISPE optimisation, it was demonstrated that DMC can be considered an adequate dummy template to develop MIPs for these five AFs, as it can see in the Table 5, satisfactory recoveries of AFM1 in baby formulas and sum of AF B1, B2, G1 and G2 in cereal-based food were obtained.

Several methods for the analysis of AFM_1 in breast milk and baby formulas have been reported [240, 327]. However, the protocols for milk or formulas samples usually imply the use of IAC clean-up or multiple liquid-liquid extractions and filtration using larger amounts of sample. Also, for cereal-based baby food methods are available in the literature [238, 240, 355-357]. Usually, these options require multi-step preparations, including IAC columns, various solvent extractions, filtration and/or classical SPE cartridges. In this sense, MMIP-SB offers an affordable alternative for routine analysis.

Table 5. MRLs established by Commission Regulation (EU) No 165/2010 for certain contaminants in foodstuffs as regards AFs and Commission Regulation (EU) No 1881/2006 for PAT, recoveries obtained for each matrix using the molecularly imprinted stir-bars (MMIP-SB)

ANALYTES	MRLs (μg.kg^{-1})		MMIP-SB		VALIDATION
			Recovery (%)	Matrix	LC-MS/MS
Aflatoxins	M1	0.025	60	Infant formulas	Non Validated
		0.05		*Milk	
	B1	0.1	43	Cereal-based baby food	
	Sum of B1, B2, G1, G2	4	39-44		
Patulin	50		60-70	Apple	Validated LOD LOQ

* Raw milk, heat-treated milk and milk for the manufacture of milk-based products. **LOD:** Limit of detection, **LOQ:** Limit of quantification

The efficiency of the obtained MMIP-SB for PAT was evaluated in fresh apple samples [123], as depicted in Figure 25. As in the case of AFs, the extraction protocol consisted simply of pouring in, stirring and pouring out solvents from a baker with the aid of an external magnet. Apple samples were spiked with water containing PAT at level of interest (Table 5) and the supernatant was transferred to a baker with the MMIP-SB, requiring a loading time of 30 min under stirring [217]. As can be seen in the Table 5, satisfactory recoveries of PAT were obtained using this molecularly imprinted glass-covered stir-bar. These results are in accordance with those obtained by other authors such as Wang *et al.* (2017), who used a graphene-based magnetic material to extract PAT from apple juice with recoveries in the range of 69–84% [358]. Similar recoveries (>77%) were obtained by Lucci *et al.* applying commercial MISPE columns for detection of PAT in apple products [359].

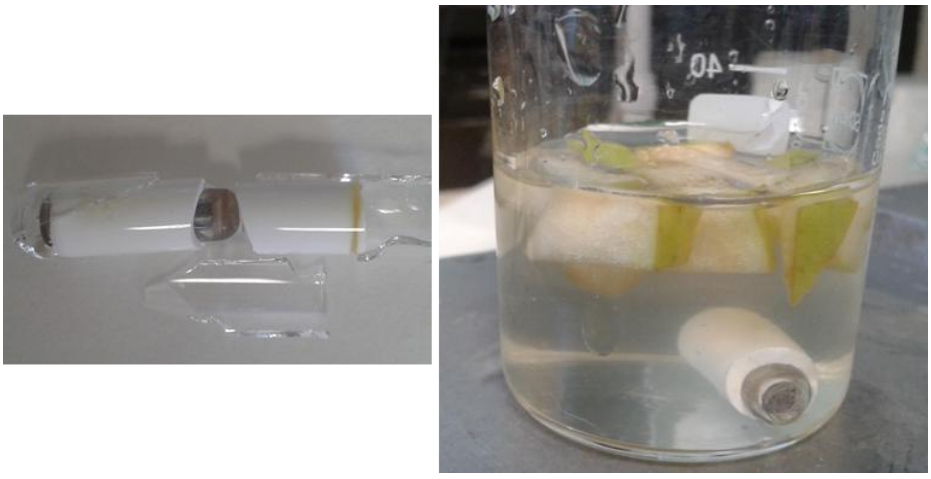

Figure 25. Magnetic molecularly imprinted stir-bar (MMIP-SB) developed for the extraction of patulin in apple

One of the most outstanding advantages of these stir-bars is their imperceptible deterioration after multiple sample extractions and several hours of stirring, indicating that the MMIP-SB developed here are strong and stable. Between analyses of different samples, MMIP stir-bars must be kept wet, immersed in methanol, for long-term storage, since they break down if getting dry. This fact is attributed to the degree of cross-linking achieved [122]. Imprinted polymers, especially the ones cross-linked with DVB, are stable over a long time and can be reused [360]. Thanks to these simple extraction protocols for AFs and PAT, consisting on pouring in, stirring and pouring out solvents from a beaker, with the aid of an external magnet, laboratory safety is improved since handling and processing of samples that may contain mycotoxins are mostly automatic. Additionally, this method based on the use of MMIP-SB, permits to comply with almost all the principles of green analytical chemistry described by Gałuszka *et al.* (2013), avoiding large volumes of waste and toxic reagents, minimizing sample treatment and sample size, allowing the use of automated methods and also increasing the safety of the operator by reducing sample handling to the minimum [351].

5 CONCLUSIONS

On the basis of the eight articles (2 review articles, 1 encyclopaedia chapter and 5 original research articles) collected in this Doctoral Thesis, the most remarkable conclusions are presented below:

1. Molecularly imprinted polymers (MIPs) are highly selective synthetic polymers for a given analyte and/or a group of structurally related compounds, making these materials ideal for use in extraction procedures for the detection of veterinary drug residues and contaminants in foodstuff.
2. Molecularly imprinted solid-phase extraction (MISPE) is the most frequent application of MIPs to residue (veterinary drugs) analysis in food, while new applications such as small-sized devices, sensors, monoliths and magnetic sorbents, among others, are in full development.
3. Bulk polymerisation is a simple straightforward technique for MIP synthesis, while precipitation polymerisation provides a good alternative to overcome its drawbacks as it yields micro-spherical particles of uniform size and shape and avoids tedious grinding and sieving steps.
4. Precipitation polymerization allows obtaining spherical particles of controlled morphologies and about 1µm of diameter, highly suitable for MISPE applications.
5. Methacrylic acid (MAA) is an excellent functional monomer candidate for MIP synthesis, since it is capable of interacting with chemically very different analytes, including corticosteroids, amphenicols, penicillins, aflatoxins and patulin.
6. Ethylene glycol dimethacrylate (EGDMA) and trimethylolpropane trimethacrylate (TRIM) are the two most popular cross-linkers in MIP synthesis, while divinylbenzene (DVB) has been specifically preferred for precipitation polymerization; the possibility of using one or another depends on the template and porogen used.
7. The type of cross-linker and the porogen have strong influence in the final size and yield of the MIP nanoparticles in the precipitation polymerisation process; when precipitating DVB, mixtures of acetonitrile and toluene are preferred as porogen.
8. Dummy templates are useful to synthesize selective MIPs for food analysis, eliminating the risk of template bleeding and as alternatives in hazardous and/or expensive analytes such as mycotoxins.
9. Dummy templates provide polymers with lower recovery capabilities and poorer cross-selectivity for structurally related compounds than those prepared using the analyte as template, as it was observed in the MIP-based protocols developed for mycotoxins and veterinary drugs, respectively.

10. During MISPE optimization, the selection of loading, washing and elution solvents must be made on the basis of the porogens used for MIP synthesis in order to promote imprinting interactions and fully exploit the selectivity of these sorbents, in particular when a group of structurally related analytes is to be extracted simultaneously.
11. The washing solvents are critical in MISPE, as they should maintain interactions between the analytes of interest and the polymer and simultaneously remove interfering compounds; the washing step is generally achieved by the same solvent used during loading (porogenic solvent or one of similar polarity) or with the addition of a small proportion of a more polar solvent.
12. MIP obtained by precipitation polymerisation can be implemented in the laboratory (MISPE) to analyse milk and milk products with satisfactory recoveries according to the maximum residue limits legally established for corticosteroids, amphenicols and penicillins, all in combination with HPLC-MS/MS.
13. In the design of magnetic molecularly imprinted polymer stir-bars (MMIP-SB), EGDMA provides stronger and more resistant coatings to cope with stirring conditions.
14. In the design of MMIP-SB, both in-house prepared magnetite and commercial glass-covered magnets (stir-bars) are suitable supports to combine with MIP coatings for mycotoxins isolation.
15. MMIP-SBs minimize sample manipulation and enabling method automation (magnetic stirring), as these clean-up protocols simply consist on pouring in, stirring and pouring out solvents from beaker, with the aid of an external magnet.
16. MMIP-SBs fabricated in the laboratory using dummy templates can be implemented to analyse baby products (cereal-based) and apple with satisfactory recoveries according to the maximum residue limits legally established for aflatoxins (sum of B1, B2, G1, G2) and patulin, respectively, in combination with HPLC-MS/MS.

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