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Synthèse et caractérisation de polymères à empreintes moléculaires pour l'extraction sélective de pesticides organophosphorés dans les huiles végétales

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Soutenance prévue le 26 septembre 2017 devant le jury composé de :

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Avertissement au lecteur

Cette thèse est rédigée comme une succession de chapitres, dont certains sont construits sur la structure d'articles (publiés ou soumis) pouvant se lire indépendamment. Ceci peut donc engendrer des changements entre la langue française et anglaise ainsi que quelques redondances.

Tables de matières

Liste des publications et communications

Publications

S. Boulanouar, A. Combès, S. Mezzache, V. Pichon. **Synthesis and application of molecularly imprinted polymers for the selective extraction of organophosphorus pesticides from vegetable oils**. J. Chromatogr. A. 1513 (2017) 59–68. doi:10.1016/j.chroma.2017.07.067.

S. Boulanouar, S. Mezzache, A. Combès, V. Pichon, **Molecularly imprinted polymers for the determination of organophosphorus pesticides in complex samples,** Talanta. 176 (2018) 465–478. doi:10.1016/j.talanta.2017.08.067.

S. Boulanouar, A. Combès, S. Mezzache, V. Pichon. **Synthesis and characterization of molecularly imprinted silica for the selective extraction of organophosphorus pesticides from almond oil**. *Article à soumettre.*

S. Boulanouar, A. Combès, S. Mezzache, V. Pichon. **Reduction of matrix effect using molecularly imprinted silica applied to the extraction of organophosphorus pesticides from vegetable oils**. *Article à soumettre.*

Conférences présentées

Symposiums internationaux

S. Boulanouar, A. Combès, S. Mezzache, V. Pichon. **Synthesis and characterization of moleculary imprinted polymers for the selective extraction of organophosphorus compounds pesticides from vegetable oils**. 14th International Symposium on Hyphenated Techniques Chromatography and Separation Technology à Ghent, Belgique, 29 janvier 2016. Session jeunes.

S. Boulanouar, A. Combès, S. Mezzache, V. Pichon. **Development and application of molecularly imprinted polymers and molecularly imprinted silicas to the selective extraction of several organophosphorus pesticides from vegetable oils.** 19th International Symposium on Advances in Extraction Technologies à Saint Jacques de Compostelle, Espagne, 29 juin 2017. **Prix meilleur présentation orale.** Session jeunes.

Symposiums nationaux

S. Boulanouar, A. Combès, S. Mezzache, V. Pichon. **Synthesis and characterization of moleculary imprinted polymers for the selective extraction of organophosphorus compounds pesticides from vegetable oils.** Journées nationales de L'AFSEP à Orléans, 16 Juin, 2016. Session jeunes.

S. Boulanouar, A. Combès, S. Mezzache, V. Pichon. **Synthesis and characterization of moleculary imprinted polymers for the selective extraction of organophosphorus compounds pesticides from vegetable oils**. Journée du club jeune L'AFSEP à Rouen, 19 avril, 2016.

Communications par affiche

S. Boulanouar, A. Combès, S. Mezzache, Valérie Pichon. **Synthesis and characterization of moleculary imprinted polymers for the selective extraction of organophosphorus compounds pesticides from vegetable oils**. ISC 2016, 31st International Symposium on Chromatography, Cork, Irlande, 29 Août 2016.

S. Boulanouar, A. Combès, S. Mezzache, V. Pichon. **Synthesis and characterization of moleculary imprinted polymers for the selective extraction of organophosphorus compounds pesticides from vegetable oils**. 12ème Congrès Francophone sur les Sciences Séparatives et les Couplages de l'AFSEP à Paris, France, 29 Mars 2016.

Résumé

L'utilisation croissante de pesticides dans l'agriculture peut entraîner de graves risques pour la santé humaine. En effet, des résidus de pesticides peuvent être retrouvés à l'état de trace dans de nombreux produits de grande consommation. Leur identification et leur analyse quantitative à l'état de trace dans des échantillons complexes, tel que les huiles végétales, constituent un défi analytique majeur. Malgré le potentiel élevé de méthodes analytiques comme la chromatographie en phase liquide couplée à la spectrométrie de masse (LC-MS/MS), l'introduction d'une étape d'extraction et de purification des extraits avant l'analyse chromatographique s'avère nécessaire. Afin d'augmenter la sélectivité de cette étape de traitement de l'échantillon, la synthèse des supports à empreintes moléculaires générant un mécanisme de reconnaissance moléculaire a été envisagé. Cette étude se concentre sur une famille de pesticides, les organophosphorés (OP), qui présentent des disparités structurales importantes et une gamme de polarité assez large (log P compris entre 0,7 et 4,7). Deux approches ont été envisagées pour la synthèse de ces supports imprimés. La première approche consiste à réaliser une polymérisation par voie radicalaire en utilisant des monomères organiques dans des solvants organiques peu polaires pour obtenir des polymères à empreintes moléculaires (MIP). La seconde approche consiste à produire les supports par voie sol-gel via l'hydrolyse puis la condensation d'organosilanes dans un milieu polaire pour produire des silices imprimées (MIS). Pour les deux approches, différentes conditions de synthèse ont été criblées en utilisant différentes molécules empreintes, monomères et solvants. La sélectivité des polymères imprimés résultants a d'abord été évaluée en étudiant les profils de rétention des OP en milieu pur. Les interactions non-spécifiques ont été évaluées en étudiant parallèlement la rétention des OP sur des supports non-imprimés (NIP/NIS) qui ont été synthétisés dans les mêmes conditions que les MIP/MIS mais sans introduire la molécule empreinte. Il est apparu que les supports MIP/MIS présentaient une complémentarité en termes d'extraction sélective des OP visés : les OP les plus polaires ont été extraits sélectivement par le MIS alors que les OP modérément polaires ont été extraits sélectivement par le MIP. La capacité de ces supports a été évaluée et se révèle adaptée à l'analyse des OP à l'état de traces dans des huiles végétales. Après avoir vérifié la répétabilité de la procédure d'extraction et des synthèses, les performances de ces supports ont été étudiées dans des milieux réels.

Pour cela, ils ont été appliqués à l'extraction sélective des OP de différentes huiles végétales (huile d'amande, d'olive et de tournesol) conduisant à des résultats similaires pour les trois huiles. Enfin, leur potentiel en termes de capacité à éliminer les composés interférents provenant de la matrice s'est révélé être supérieur à ceux de la méthode conventionnelle qui utilise une extraction sur phase solide sur C18. Les limites de quantification obtenues sont inférieures aux teneurs maximales en résidus (LMR) de pesticides établis par le règlement 396/2005 de l'Union Européenne pour ces composés dans ces huiles.

Abstract

The increasing use of pesticides in agriculture causes serious health risks to humans. These pesticides may possibly be found in vegetable oils used as cosmetic ingredients. Their identification and reliable quantitative analysis at trace levels constitute a challenge for the safe use of such oils despite the high potential of analytical methods such as liquid chromatography coupled to mass spectrometry (LC-MS/MS). Their determination at low concentration levels in complex oil samples requires an extraction and a purification step. In order to increase the selectivity of the sample treatment step, the synthesis of imprinted sorbents can be considered. This study focusses on a group of pesticides, the organophosphorus (OPs) that present some structural disparity and belong to a wide range of polarity (log P values between 0.7 and 4.7). To produce imprinted sorbents, a first approach of synthesis consists in the radical polymerization of organic monomers in moderately polar organic solvents to obtain molecularly imprinted polymers (MIPs). The second one, the Sol-Gel approach, consists in the hydrolysis and then condensation of organosilanes in a polar medium to produce molecularly imprinted silicas (MIS). For both approaches, different conditions of synthesis were screened using different template molecules, monomers and solvents. The selectivity of the resulting imprinting polymers was first evaluated by studying the extraction profiles of OPs in pure media on MIP and MIS. The non-specific interactions were estimated by studying in parallel the retention of OPs on non-imprinted polymers synthesized in the same conditions as imprinted sorbents but in the absence of the template molecule. Both sorbents MIP/MIS present a complementarity in terms of selective extraction of the target OPs: polar OPs were extracted selectively using the MIS while moderately polar OPs were selectively extracted by the MIP. The capacity of these supports was evaluated and was consistent with the analysis of OPs at trace levels in real oil samples. After studying the repeatability of the extraction procedure and of the reliability of the syntheses, the performances of these supports were studied in real media. For this, MIP/MIS were applied to the selective extraction of OPs from different vegetable oils (almond, olive and sunflower oil) and similar results were obtained for the three different oils. Their potential in terms of ability to remove matrix interfering compounds were higher than those of the conventional method based on the use of C18 silica. The estimated limits of quantifications were lower than the Maximum Residue

Levels (MRLs) established by EU Regulation 396/2005 for these compounds in oils.

Liste des abréviations

ABAH: 2,2′-Azobis (2-amidino propane) hydrochloride

AA: Acetic acid

AA: Acrylic acid

AAM: Acrylamide

ABA: M-aminobenzoic acid

AC: Ammonium acetate

AChE: Enzyme acetylcholinesterase

ACN: Acetonitrile

AIBN: Azo-N,N'-bis-isobutyronitrile

APTES: Aminopropyl triethoxysilane

BMA: Butylmethacrylate

BPO: Benzoyl peroxide

$CHCl_3$: Chlorofom

CLE: Chlorpyriphos-ethyl

CLM: Chlorpyriphos-methyl

DCM: Dichloromethane

DCV: Dichlorvos

DEP: Diethyl(3-methyl ureido)(phenyl)methylphosphonate

DETP: Diethylthiophosphate

DIZ: Diazinon

DLLME: Dispersive Liquid-liquid Microextraction

DMF: Dimethylformamide

DMPTABA: 4-(dimethoxy phosphorothioylamino)butanoic acid

DMSO: Dimethylsulfoxide

DMT: Dimethoate

dSPE: dispersive solid phase extraction

DVB: Divinylbenzene

D4DNP: Diethyl(4-nitrobenzyl)phosphonate

ECD: Electron capture detection

EGDMA: Ehyleneglycoldimethacrylate

EMA: Ethyl methacrylate

EMR: Enhanced matrix removal-Lipid

EtOH: Ethanol

FA: Formic acid

FEM: Fenamiphos

FEN: Fenthion

FNT: Fenitrothion

FPD: Flame photometric detector

FSN: Fenthion sulfone

FSX: Fenthion sulfoxide

FTD: Flame thermionic detector

GA: Gallic acid

GC-MS: Gas chromatography-mass spectrometry

GDMA: Glycerol dimethacrylate

GMA: Glycidilmethacrylate

GPC: Gel permeation chromatography

HS-SPME: Headspace solid-phase microextraction

HEMA: 2-hydroxyethyl methacrylate

IA: Itaconic acid

LC-DAD: Liquid chromatography-diode array detector

LC-MS: Liquid chromatography-mass spectrometry

LLE: Liquid-liquid extraction

LODs: Limits of quantification

LOQs: Limits of quantification

MAA: Methacrylic acid

MAE: Microwave-assisted Extraction

MAL: Malathion

MBAA: N,N'-Methylenebisacrylamide

MCP: Monocrotophos

MeOH: Methanol

MIP: Molecularly imprinted polymer

MIS: Molecularly imprinted silica

MMA: Methyl methacrylate

MRLs: Maximum residue limits

MSPD: Matrix Solid Phase Dispersion

MTH: Methidathion

MWCNT: Multiwall carbon nanotube

NH_4OH: Ammonium hydroxide

γ-MAPS: γ-Methacryloxypropyl trimethoxysilane

NIS: Non-imprinted silica

NP: Nanoparticles

NPD: Nitrogen/phosphorus detector

NIP: Non-imprinted polymer

OH-TSO: Hydroxy terminated silicone oil

OPPs: Organophosphorus pesticides

OPs: Organophosphorus

PDMS: Poly(dimethylsiloxane)

PD: o-Phenylenediamine

PE: Parathion ethyl

PEG: Polyethylene glycol

PEI: Polyethyleneimine

PIM: Pyrimiphos methyl

PMHS: Poly (methylhydrosiloxane)

PSA: Primary secondary amine

PTMOS: Phenyltrimethoxysilane

QD: Quantum dots

QuEChERs: Quick, Easy, Cheap, Effective, Rugged, and Safe

RSD: relative standard deviation

SPE: Solid Phase Extraction

SPME: Solid Phase Microextraction

TED: Tetraethyl thiuram disulfide

TEOS: Tetraethyl orthosilicate

TFA: Trifluoroacetic acid

TFMAA: Trifluoromethylacrylic acid

THF: Tetrahydrofuran

TRIM: Trimethylol propane trimethacrylate

4-VP: 4-vinylpyridine

Introduction générale

Les propriétés des huiles végétales sont connues depuis l'antiquité, elles nourrissent, protègent et hydratent la peau et sont donc très utilisées dans l'industrie cosmétique. Cependant, des résidus de substances chimiques nuisibles à l'homme, tels que des pesticides, peuvent être présents dans ces huiles. En effet, ces substances sont de plus en plus utilisées pour augmenter la production agricole. Les principales familles de pesticides sont les organochlorés, les carbamates, les pyréthroïdes, les triazines et les organophosphorés. Dans cette étude, nous allons exclusivement nous intéresser à l'analyse des organophosphorés (OP) qui sont connus pour être des molécules neurotoxiques inhibitrices de l'acétylcholinestérase (AChE), une enzyme vitale pour le système nerveux. La reduction du taux sanguin de l'AChE déclenche l'accumulation de l'acétylcholine. Cela provoque des effets neurotoxiques tels que la paralysie neuromusculaire.

L'analyse des pesticides organophosphorés à l'état de trace dans des matrices telles que les huiles nécessite des méthodes de traitement de l'échantillon performantes compte tenu de la grande complexité des échantillons et des très faibles teneurs en contaminants à quantifier avant leur analyse généralement réalisée par LC-MS/MS ou GC-MS/MS. L'extraction sur phase solide (SPE) est la technique d'extraction de choix pour ce type d'échantillon. Divers supports d'extraction sont utilisés mais, compte tenu des mécanismes de rétention mis en jeu, généralement basés sur la polarité des molécules, ils peuvent entraîner des co-extractions de composés interférents. Pour relever ce challenge analytique il pourrait donc être intéressant de développer des supports sélectifs, des polymères à empreintes moléculaires, qui grâce à un mécanisme de reconnaissance structurale doivent permettre d'extraire sélectivement les OP sans co-extraire d'autres composés permettant ainsi une analyse quantitative plus fiable de ceux-ci.

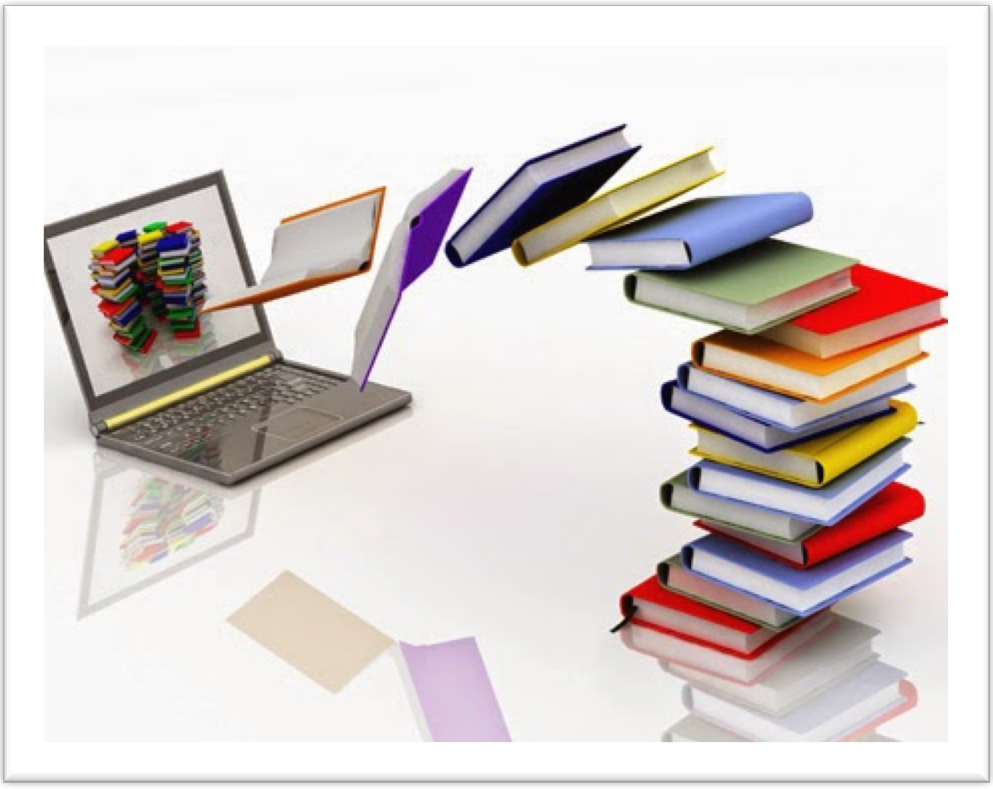
Ce travail de thèse a donc porté sur la synthèse et la caractérisation de polymères à empreintes moléculaires pour l'extraction sélective des organophosphorés identifiés comme prioritaires par rapport à leur présence dans les huiles végétales. Cependant, la synthèse de ce type de supports est un véritable challenge en raison des disparités

structurales importantes des OP et parce qu'ils appartiennent à une gamme de polarité assez large.

Ce manuscrit se divise en deux parties. Une partie bibliographique composée de deux chapitres et une partie expérimentale composée de trois chapitres sous forme d'articles. Dans la partie bibliographique, le premier chapitre décrit les techniques d'extractions les plus utilisées pour les pesticides OP dans les huiles végétales. Le second chapitre se présente sous la forme d'une revue, soumise à *Talanta,* qui décrit la synthèse et la caractérisation de supports imprimés sélectifs des OP qui ont été appliqués comme support d'extraction dans différentes méthodes et comme élément de reconnaissance dans des capteurs. Cette revue met notamment en évidence que peu de travaux ont porté sur le développement de ces supports pour aider à l'analyse des OP dans les huiles, ce qui constitue l'objectif du travail expérimental mené dans cette thèse. Ainsi, concernant la partie expérimentale, le premier chapitre décrit la synthèse, la caractérisation de polymères à empreintes moléculaires, appelés MIP, obtenus par voie radicalaire et leur application à l'extraction sélective d'OP ciblés dans différents huiles végétales. Cette approche n'ayant pas permis d'obtenir un support capable de piéger l'ensemble des OP ciblés, nous a porté sur la synthèse et la caractérisation de polymères à empreintes moléculaires obtenu par une approche Sol-Gel, appelé MIS, permettant d'extraire d'autres OP et qui ont fait l'objet d'études décrites dans les deux chapitres suivants.

Ces trois chapitres expérimentaux sont présentés sous forme d'articles. Le premier a été accepté et sera publié prochainement dans la revue *Journal of Chromatography A*. Les deux autres sont en cours de soumission pour acceptation par le comité scientifique du groupe L'Oréal pour être ensuite soumis à des journaux de rang A. Ainsi, ce manuscrit est majoritairement rédigé en anglais, sauf le résumé, l'introduction générale, les transitions entre les chapitres et la conclusion, et ce, à la demande de l'école doctorale.

PART I: BIBLIOGRAPHIC STUDY



PART I

Chapter I: Presence of organophosphorus pesticides in vegetable oils

I.1.Pesticides, generalities

According to International Union of Pure and Applied Chemistry (IUPAC): pesticides are substances or mixture of substances intended to control, to prevent or to dispose of animal and/or plant pests [1]. More than 4 million of tons of pesticides are used worldwide annually [2] and especially, over 140 000 tons in the European Union alone with the aim of increasing agricultural yields as well as limiting the transmission of diseases to humans through insects or rodents [3]. Thus, the systematic overuse of pesticides made them an essential factor of industrial agriculture however the accumulation of residues of pesticides in food is particularly dangerous even at trace levels. Hence, the quality of the products is altered and potentially unsafe for human consumption. Pesticides are some of the most toxic, yet environmentally stable and mobile substances. In general pesticides can be classified depending on:

- Their biological activity and/or the targeted pest species [1]. The main group of pesticides are herbicides, fungicides and insecticides. Further distinction is possible between acaricides, nematicides, and rodenticides [4].
- Their chemical composition. They are organized according to the chemical nature of the active ingredients such as the insecticides carbamates, organophosphorus, organochlorines, pyrethrum-derived pyrethroids, neonicotinoid or the herbicides triazines and ureas (see Figure I.1-1). These pesticides together with the fungicides such as phthalimides, triazoles, imidazoles or sulfamides are the most applied pesticides in crops [5].

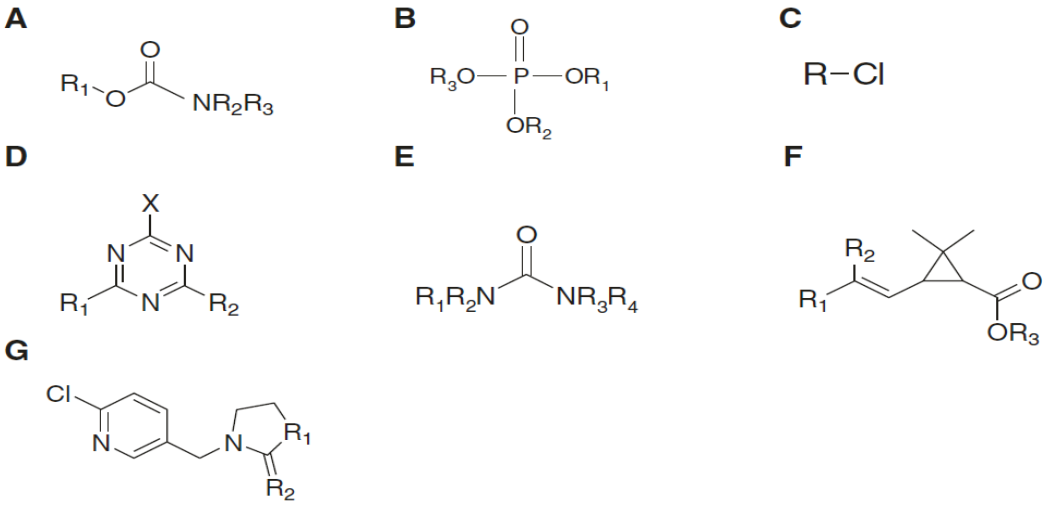


Figure I.1-1.Generic chemical structures of pesticides: A, carbamate pesticides; B, organophosphorus pesticides; C, organochlorine pesticides; D, triazines (X= halogen); E, urea analogs; F, pyrethrum-derived pyrethroids (R_1, R_2= H, halogen or other functions); and neonicotinoid [4].

- Their application according to the area of use, agricultural, domestic or directly on humans or animals [4].

According to data from European Union Pesticides Action Network, 350 different pesticides were detected in food produced in the EU in 2008. More than 5% of tested products contained pesticides at levels exceeding the EU's maximum permitted level. In 2012, the European Food Safety Authority (EFSA)[6], related data from EU Member States, Norway and Iceland, reported that among the 794 analyzed samples of olive oil 175 of samples contained one or several pesticides in measurable concentrations. In total, 26 different pesticides were detected. The most frequently found pesticides was an organophosphorus, chlorpyrifos and an herbicide, terbuthylazine, detected respectively in 14.1 and 12.0% of the samples. In Spain, other pesticides were detected at a concentration levels lower than the maximum residue limits such as terbuthylazine in four samples, the organochlorine, endosulfan, in one sample, the fungicide, famoxadone in one sample and the organophosphorus fenthion in three samples. The EFSA reported that the quantity of used pesticides was multiplied by two fold in almost 10 years. More than 774 different pesticides were found in the analyzed food products.

Hence it is important to control food samples such as vegetable oils. Indeed, with a world production of 177 million tons each year and because they are highly consumed, they are a way of contamination for people. Nevertheless, with developing technology and increasing health awareness, people pay close attention to chemical contaminants during oilseed plantation, refining, storage and consumption.

I.2.Vegetable oils

Vegetable oils are mainly constituted of triacylglycerols (95–98%) and complex mixtures of a wide range of minor chemicals (2–5%) and are an important source of human nutrition [7]. Their beneficial properties for the health is based on their wealth in saturated and unsaturated fatty acids, antioxidants, and other fat-soluble vitamin [8]. They are not only used in the food or pharmaceutical industry or cooking, they are also used in cosmetics industry, because they have been known since antiquity by nourishing, protecting and moisturizing the skin [9]. Most of vegetable oils are obtained from beans or seeds which furnish an oil and a protein-rich meal. Seed extraction is achieved by pressing and/or by solvent extraction. Oils such as palm and olive are

pressed out of the soft fruit (endosperm). The oil content recovered after the extraction of the seeds variate depending of the nature of the seed and it represents 58% of seeds weight for almond [10], 37% for olive [11], 50% for sunflower [12], 18% for soybean, 38.6% for rapeseed, 40.3% for groundnut, 15.1% for cottonseed or 62.4% for coconut [13].

Some oils are used for food without refining processes, such as virgin olive oil. For other oils, it is necessary to apply refining process in order to minimize undesirable materials such as phospholipids, monoacylglycerols, free acids, trace metals, sulfur components or pesticides. This process may also remove minor components with positive properties, like antioxidants and vitamins such as carotenes or tocopherols [13]. However, the residues of undesirable compounds like pesticides can still be present. It is estimated that more than 1000 active compounds have been applied to plant/corps protection in the past [14]. These highly lipophilic and stable pesticides can be easily bio-accumulated in oilseeds and hence will be co-extracted into the oils during the extraction process. Oils such as soybean, sunflower, olive or rapeseed oils are the most used consumed vegetable oils in the world and different pesticides are applied to increase their production. The organophosphorus pesticides such as dichlorvos, methyl parathion, chlorpyriphos, diazinon, fenitrothion or malathion are the principal group of compounds used to protect plants [3]. Therefore, governments and international organizations have established maximum residue limits of this pesticides in vegetable oils.

I.2.1. Regulation of organophosphorus in vegetable oils

Generally, the EU Regulation No 396/2005 sets maximum residue levels (MRLs) of pesticides that are legally tolerated in food or feed. As general default, a MRLs of 10 µg/kg are applied when a pesticide is not specifically mentioned. These limits established for pesticides can be found in the MRL database of the European Commission website.

The french Institute specialized in fats and oils (ITERG) have established a list of the most detected pesticides in vegetable oils according to their recent studies. For this work, this list was used to select the studied OPs. These compounds reported in the Table I.2-1 were present in several vegetable oils. However, in this study we have

focused only on three vegetable oils (olive, almond and sunflower) that can be used as raw material to elaborate cosmetic products. Hence in the Table I.2-1 we summarize the update (MRLs) established originally by the EU regulation No. 396/2005 in oil seeds.

Table I.2-1. Update MRLs of OPs in olive, almond and sunflower seeds established originally by the EU Regulation No. 396/2005.

OPs	Olive seed	Almond seed	Sunflower seed	Update MRLs
Dimethoate (DMT)	3000	10	10	Regulation (EU) No 2017/1135
Dichlorvos (DCV)	10	10	10	Regulation (EC) No 839/2008
Fenthion sulfoxide (FSX)	10	20	20	Regulation (EU) No 310/2011
Fenthion sulfone (FSN)	10	20	20	Regulation (EU) No 310/2011
Methidathion (MTH)	20	50	50	Regulation (EU) No 310/2011
Malathion (MAL)	20	20	20	Regulation (EU) No 2015/399
Diazinon (DIZ)	20	50	20	Regulation (EU) No 834/2013
Fenthion (FEN)	10	20	20	Regulation (EU) No 310/2011
Chlorpyriphos-ethyl (CLE)	50	50	50	Regulation (EU) No 2016/60
Pirimiphos-methyl (PIM)	10	10	50	Regulation (EU) No 2016/53
Chlorpyriphos-methyl (CLE)	50	50	50	Regulation (EC) No 836/2008
Fenitrothion (FNT)	20	20	20	Regulation (EU) No 899/2012

Nevertheless, for processed products such as vegetable oils, the MRL are not yet established. Hence FIEDOL, the EU vegetable oil and protein meal industry association, positioned itself in 2007 on the application of the European MRLs to processed products. In fact, the MRLs for pesticides in processed products should be derived from the MRLs for raw products, considering the concentration or dilution caused by the refining process. In the oil extraction process, the concentration/dilution factors depend on the type of processing. Moreover, the solubility of a given pesticide in water or in fat and/or in the solvents used for oil extraction have an impact on the concentration of the pesticide in the processed products. To establish the processing factors accurately it

would take a long time because there are more than 1000 pesticides and around 20 different types of crude oils that are of economic interest for the oil industry. It is possible to estimate the maximum residue levels in crude oils based on the physico/chemical properties of the pesticides and on the oil content of the raw materials. One of the criterias that can be used to predict the fate of a given pesticide during oil extraction is its polarity. Indeed, pesticides with high solubility in fat or in the extraction solvents may concentrate in crude oil. In this case the MRL for crude oil will be obtained by multiplying the MRL for seeds by the corresponding processing factor. For example, when the partition coeficient (log P) of a pesticide exceeds 3, the pesticide is considered as fat-soluble. Hence the estimated MRLs in crude oil will be calculated by taking a count only the concentration or the dilution done in the treatment of the seeds.

OPs were found in different vegetable oils (olive, sunflower or rapeseeds oils) as reported in Table I.2-2 showing the concentration ranging from 5 to 730 µg/kg. Generally the samples that were analyzed in Europe were lower than the MRLs, except in two cases [15,16]. In these samples, the concentration of dimethoate and fenthion in olive oil were higher than the MRLs.

Table I.2-2. Detected quantity of OPs in vegetable oils.

Samples	OPs	Detected quantity µg/kg	Localization	year	Ref.
7 olive oil extra virgin samples	Chlorpyrifos (4 samples)	5-26(< MRLs)	Almeria markets-Spain	2016	[5]
	Chlorpyrifos-methyl (1 sample)	In one sample: 21(<MRLs)			
	Phosmet (1 sample)	156(< MRLs)			
2 olive oil refined samples	-	-			
3 sunflower oils samples	-	-			
20 rapeseed oils samples	Diazinon (10 samples)	< LOQs	China	2012	[17]
79 olive oil samples	Fenthion (13 samples)	90-730 (>MRLs)	Sicilia, Apulia -Italy	2004	[15]

Samples	OPs	Detected quantity µg/kg	Localization	year	Ref.
65 virgin olive oil samples	Azinphos-ethyl, chlorpyrifos-methyl, diazinon in 4 samples, dimethoate in samples 29, fenthion, formothion, methidathion, parathion and parathion-methyl in 18 samples	30-120 (< MRLs) Dimethoate(>MRLs)	Campania, Italy	1999/ 2000	[16]
Sunflower and rapeseed oils	Dichlorvos, malathion and pirimiphos-methyl	100-250(< MRLs)	France	2006	[18]

I.2.2.Organophosphorus pesticides

Historically, organophosphorus have largely been used as pesticides and as nerve agents [1]. The first organophosphorus were synthesized in the 19th century, but they only started to be widely used in 1930s. The German chemist Gerhard Schrader synthesized many commercial OPs such as parathion that is still used as pesticide in crop production. At the beginning of the Second World War, the development of OPs switched to highly toxic compounds employed as nerve agents, e.g. sarin, soman and tabun. After this, the syntheses of OPs were oriented towards the development of less toxic compounds that could be used as pesticides. Moreover, this usage increased rapidly in the 70's, when the application of organochlorine pesticides, such as dichlorodiphenyltrichloroethane (DDT), was prohibited because of their toxicity on the nervous system of vertebrates and their long-life persistence in the environment. However, the OP pesticides are also neurotoxic. Indeed, they inhibit the activity of acetylcholinesterase (AChE), causing the accumulation of excessive acetylcholine in the synaptic cleft. This leads to neurotoxic effects such as neuromuscular paralysis throughout the entire body and in some cases to death [1,2].

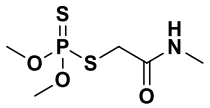
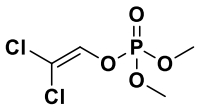
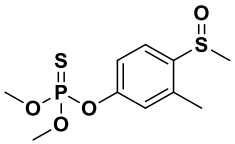
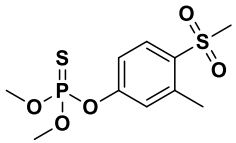
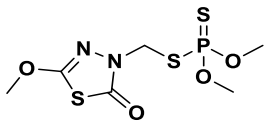
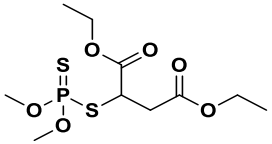
People are continually exposed to low OP concentrations by ingestion, inhalation, or skin contact. Long-term epidemiologic studies reveal the linkage on OPs to higher risk of cancer development [2].

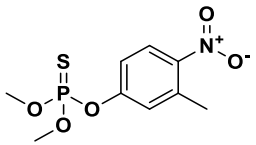
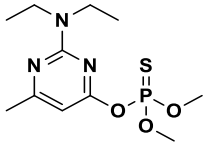
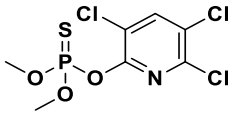
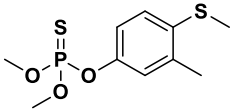
I.2.3.Physical and chemical properties

OP compounds are usually esters, amides, or thiol derivatives of phosphoric, phosphonic, phosphinic or thiophosphoric acids with two organic and additional side chains such as cyanide, thiocyanate and phenoxy group [19]. The general chemical structure of an organophosphorus comprises a central phosphorus atom (P) and the characteristic phosphoric (P=O) or thiophosphoric (P=S) bond.

Depending on their specific additional side chain groups the mechanism of action is different. For example, extreme toxicity is associated with those compounds in which this side group is a strongly electronegative such as halide, cyanide, or thiocyanate tabun, sarin or soman) [19]. While, in the case of the pesticides, the OPs are less toxic since this group is less reactive. In the Table I.2-3, structures, molecular weight and hydrophobicity of studied OP pesticides presenting a broad range of polarity and a large structural variety are reported. Therefore, their extraction at low levels of concentration in oil matrices is a challenging task since they belong to a broad range of polarity.

Table I.2-3. Physico-chemical properties of the OPs.

Common name	IUPAC name	Molecular formula	Structure	Molecular weight (g/mol)	Log of P [20]/[21]/ [22]
Dimethoate	2-dimethoxyphosphinothioylthio-*N*-methylacetamide $C_5H_{12}NO_3PS_2$	$C_5H_{12}NO_3PS_2$		229.3	0.7/0.8/ 0.7
Dichlorvos	2,2-dichloroethenyl dimethyl phosphate	$C_4H_7Cl_2O_4P$		221	1.9/1.47/1.9
Fenthion sulfoxide	*O,O*-dimethyl *O*-4-methylsulfinyl-m-tolyl phosphorothioate	$C_{10}H_{15}O_4PS_2$		294.3	ND/ND/1.92
Fenthion sulfone	O,O-Dimethyl O-3-methyl-4-(methylsulfonyl)phenyl phosphorothioate	$C_{10}H_{15}O_5PS_2$		310.3	ND/ND/2.25
Methidathion	3-dimethoxyphosphinothioylthiomethyl-5-methoxy-1,3,4-thiadiazol-2(3H)-one	$C_6H_{11}N_2O_4PS_3$		302.3	2.2/2.29/2.57
Malathion	diethyl (dimethoxyphosphinothioylthio)succinate	$C_{10}H_{19}O_6PS_2$		330.3	2.74/2.4/ 2.75

Common name	IUPAC name	Molecular formula	Structure	Molecular weight (g/mol)	Log of P [20]/[21]/ [22]
Fenitrothion	O,O-dimethyl O-4-nitro-m-tolyl phosphorothioate	$C_9H_{12}NO_5PS$		277.2	3.43/3.12/3.32
Diazinon	O,O-diethyl O-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate	$C_{12}H_{21}N_2O_3PS$, 30583-38		304	3.3/3.8/3.69
Pirimiphos-methyl	4-dimethoxyphosphinothioyloxy-N,N-diethyl-6-methylpyrimidin-2-amine	$C_{11}H_{20}N_3O_3PS$		305.1	4.2/ND/3.9
Chlorpyriphos-methyl	O,O-dimethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate	$C_7H_7Cl_3NO_3PS$		322.5	4.2/ND/4.07
Fenthion	O,O-dimethyl O-4-methylthio-m-tolyl phosphorothioate	$C_{10}H_{15}O_3PS_2$		278.3	4.84/4.09/4.84
Chlorpyriphos-ethyl	O,O-dimethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate	$C_9H_{11}Cl_3NO_3PS$		350	4.7/4.96/4.7

ND: non determined

I.3.Extraction techniques of organophosphorus from vegetable oils

A wide range of OPs are used legally for seeds protection and their residue content in vegetable oils must be accurately monitored for safe consumption. These matrices contain a high level of triglycerides and the possible presence of lipophilic analytes at low concentration [23] which requires complicated sample treatment procedures before chromatographic analysis. Indeed, it is a crucial step in the analytical procedure since even a small residual amount of lipids can damage LC columns or cause signal suppression during MS detection. It is then necessary to simplify the matrix by removing interfering compounds in order to improve detection of pesticide residues and to achieve the lowest limits of detection and quantification [4]. Basically, a sample treatment procedures is required prior to analysis by gas chromatography (GC) or high performance liquid chromatography (HPLC) determination and follows these basic steps:

- The food sample is homogenized or blended to obtain a uniform matrix.
- The pesticide residue will be extracted from the matrix with solvents.
- A cleanup step is used to remove interfering matrix components to decrease the matrix effect during chromatographic analysis.
- The eluent is concentrated and re-constitute in a solvent which is compatible with the GC or HPLC analytical conditions.

The analysis of OPs were carried out by gas chromatography (GC) coupled to different detectors such as flame thermionic detector (FTD)[24], nitrogen/phosphorus detector (NPD) [16,25], flame photometric detector (FPD) [15,26,27] or the more specific mass spectrometry (MS)[28–30] in selected ion monitoring (SIM) mode or in tandem MS/MS [5,23,27,31–33] or by liquid chromatography generally coupled to MS/MS detection [17,28,34–36] with an advantageous features for the analyses of polar pesticides in olive oil. Accordingly prior to the separation and detection, the most widely used techniques to extract OPs in vegetable oils are: liquid-liquid extraction (LLE) [5,15,23,25–30,33–37], solid-phase extraction (SPE) [16,25,38], solid phase microextraction (SPME)[24], matrix solid phase dispersion (MSPD) [26,28,36] or dispersive solid phase extraction (dSPE) [5,23,29,30,33–35] that is usually applied in QuEChERs methods. Other extraction techniques such lower temperature precipitation [17,27], gel permeation chromatography (GPC) [31] or microwave-assisted extraction

(MAE)[38] were also applied. The applications of these different methods of separation and detection and of extractions of OPs from vegetable oils are summarized in Table I.4-1.

I.3.1.Liquid-liquid Extraction (LLE)

Liquid-liquid extraction (LLE) is based on the relative solubility of an analyte in two immiscible phases and is defined by the equilibrium distribution/partition coefficient. LLE is traditionally one of the most common methods of extraction, particularly for organic compounds from aqueous matrices. Typically, a separating funnel is used and the two immiscible phases are mixed by shaking and then allowed to separate. To avoid emulsions, in some cases, a salt may be added and centrifugation can be used if necessary [39].

LLE is largely applied to extract OPs from vegetable oils. The most often used solvent in LLE partitioning are acetonitrile or a mixture of acetonitrile and hexane. It has be used without no subsequent clean up steps to extract and analyze some OPs directly from olive oils [15]. In this case, the resulting limit of quantification was relatively high (between 3 and 15 μg/kg), Table I.4-1. However, the extracts obtained after LLE contain a significant amount of residual fat that could interfere with the analysis. Nowadays, most of the methods described for the analyses of OPs in vegetable oils include a subsequent clean-up step. LLE had been used to extract OPs in vegetable oils combined with MSPD [26,28,36], dSPE [5,23,29,30,33–35], GPC [31], that allow the separation of the low molecular mass pesticides from higher molecular mass fat constituents of the oils, such as triglycerides, SPE [25] or with lower temperature precipitation [30,36]. The last extraction technique consists of a precipitation of the fatty component of the oils at lower temperature and generally ACN is used as extraction solvent. However when is applied without any supplementary extraction step, as is shown in Table I.4-1, the recoveries present high RSD (between 15 and 27%) due to the matrix effects [17,27].

Recently the need to reduce solvent usage has led to microextraction techniques [40], such as dispersive liquid–liquid microextraction (DLLME). This technique was emerged in 2006 and was described by Rezaee et al.[41]. It had shown high recovery and enrichment factors in comparison with classic LLE. In the DLLME technique, a mixture of an organic solvent as the extractant and a disperser solvent is rapidly injected into an aqueous sample so that the turbulence produced causes the formation of fine

droplets, which are dispersed through the aqueous sample. The emulsified droplets have a great interstitial area and, consequently, the equilibrium is reached rapidly and the extraction is almost instantaneous [42]. Recently this technique was used to extract 3 OPs from several vegetable oils (olive, flaxseed, walnut and coconut) by using as extractant magnetic water prior to the analysis by GC-MS/MS. This procedure led to recoveries included between 78 and 138% and to limits of quantification between 0.7 and 1.27µg/kg [32]. Even if the recoveries were higher than 100%, which is probably due to the matrix effects, this method of extraction coupled with a more specific detector (MS-MS) allowed an important decreased of the limits of quantification as compared to classical LLE [15].

I.3.2.Solid phase extraction (SPE)

As it requires a lower volume of solvent than LLE and it imply simple manipulations which are less time consuming and that could be automatized, solid phase extraction (SPE) was developed in 1970 as an alternative approach to LLE for separation, purification, pre-concentration and solvent exchange of analytes. SPE can be used directly as an extraction technique for liquid matrices, or as a cleanup steps for solvent extracts. A SPE method consists in four successive steps, as illustrated in Figure I.3-1. First, the solid sorbent should be conditioned using an appropriate solvent. Typically, for reversed phase sorbent, methanol is frequently used, followed by water or an aqueous buffer whose pH and ionic strength are similar to that of the sample. The second step is the percolation of the sample through the sorbent (in this step the analytes are retained on the sorbent). The third step consist in the washing of the sorbent with an appropriate solvent, to eliminate matrix components which have been retained without displacing the analytes. The final step is the elution of the analytes of interest by an appropriate solvent that allows to recover the analyte of interest without removing the retained matrix component [39]. In the SPE different sorbents can be used (e.g., florisil, alumina, aminopropyl, graphitized carbon black or silica gel). SPE has been used without any additional step to extract 18 OPs from olive using classical sorbents such as silica gel and C18 silica [16], the obtained recoveries were over 100% with RSD until 16%. However when this technique was combined with an additional extraction step such LLE [25] or with matrix accelerated extraction MAE [38] used also to extract

OP from olive oil, the obtained recoveries and the RSD were lower with also lower LOQs by using the same detector (NPD). Hence, the combination of several extraction steps allow better recovery yields and cleaner extracts thanks to the reduction of the matrix effects. Different SPE methodologies such as, SPME [24], MSPD [26,28,36] or dSPE [5,23,29,30,33–35]) were used to extract several OPs from vegetable oils and the performances of these techniques are described below.

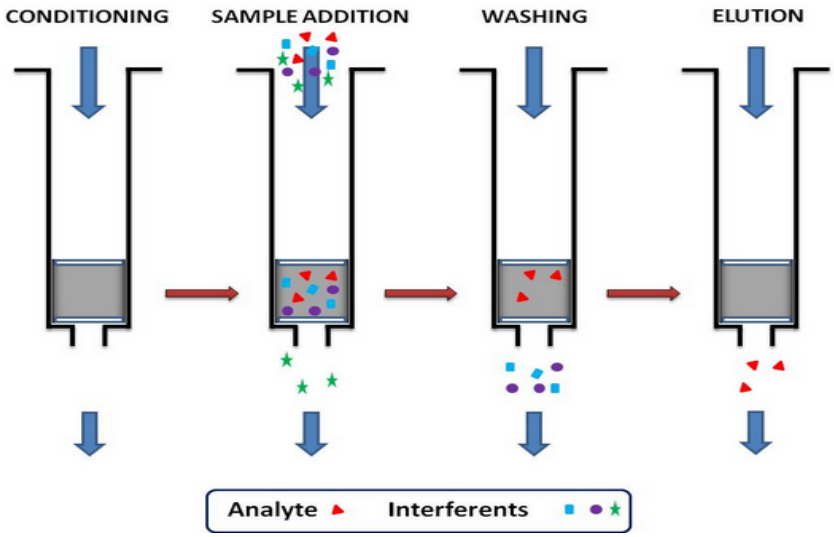


Figure I.3-1. Solid phase extraction procedure based on conditioning, sample addition, washing and elution [39].

I.3.3.Dispersive Solid Phase Extraction (dSPE)

The dispersive solid phase extraction (dSPE) was applied in QuEChERS "Quick, Easy, Cheap, Rugged and Safe'' method. This method was described by Anastassiades et al. [43] and it have been applied originally for pesticide multiresidues analysis in fruits and vegetables. Recently, it has been extended to determine multiresidues pesticides such as organophosphorus, organoclorines, carbamates, triazines and pyrethrois in vegetable oils [5,23,29,30,33–35]. QuEChERS is based on liquid-liquid partitioning with generally acetonitrile or a mixture of acetonitrile and hexane using salts such as NaCl or Mg_2SO_4 followed by a clean-up step with dSPE. The sorbents used are generally C18 [23,35], primary secondary amine (PSA)[23,34,35] or graphitized carbon black (GCB)[23,34,35]. However other sorbent such as florisil [30] or multiwalled carbon nano tubes and alumina as adsorbents [29] have been also used. Using QuEChERS good recoveries (between 70 and 120%, Table I.4-1) are obtained, however, the high content of lipids and fatty acids of oil matrices still affecting the recoveries (some recovery

higher than 100%) since these type of compounds can co-eluted during LC-MS/MS or GC-MS/MS analysis with pesticides. Therefore the addition of an extra step such as freezing step after LLE, was applied successfully to minimize the co-extract fat contents [5,30]. The combination of several sorbents such as C18, PSA and GCB have also been used and allow the decreasing of the LOQ for the targeted OPs [35]. Nevertheless the recoveries were drastically reduced, indeed some analytes were not recovered since GCB absorbs some interferents such primarily chlorophyll but also some OPs. Hence when this sorbent was removed, higher recoveries were obtained meanwhile the matrix effects were higher, indicating that the effectiveness of the clean-up step should be sacrificed to obtain adequate recoveries. PSA sorbent was tested alone in this experience giving high matrix effects and more variability on the results.

More selective sorbents were used to reduce matrix effects such as magnetic mesoporous ZrO_2 microspheres (m-$ZrO_2Fe_3O_4$) and n-octadecylphosphonic acid modified magnetic microspheres (Fe_3O_4-OPA) since ZrO_2 has an amphoteric characteristic and its surface possess a large amount of Lewis acid sites, which makes it a good adsorbent for Lewis bases such as fatty acids and glycerides [33].

Other works, usually use a matrix matched standards calibration methods for the good quantification of the pesticides in vegetable oils when the matrix effects is observed as was described in the work of He et al. [23].

Although the matrix effects were observed with the method of QuEChERS, good recoveries (between 70 and 120%) and enough sensitivity (globally the LOQ were below the MRL for OPs) were obtained for OPs pesticides at trace levels in different oil matrices.

I.3.4.Matrix Solid Phase Dispersion (MSPD)

Matrix solid phase dispersion (MSPD) was introduced in 1989 by Barker and was used in food, environmental or biological matrices [44]. In this technique, a liquid, semi-solid or solid sample is placed in a glass or agate mortar containing a sorbent material such as silica, alumina or C18 [39]. After blending, this material is packed into a small column, where the analytes are eluted by a relatively small volume of a suitable eluting solvent. This step can be accomplished together with a “co-column” clean-up, to achieve a further degree of matrix removal. The co-column material (florisil, GCB or silica, as example) is packed into the bottom of the same column of the sorbent, cleaning the

sample as it elutes from the MSPD sorbent-matrix mixture (Figure I.3-2). Therefore, MSPD enables the development of extraction and clean up steps [28].

This extraction technique was applied to extract OPs and other pesticides from olive oil and olives [28], palm oil [36] or cameilla oil [26] in association with GC-MS [28], LC-MS/MS [36] and GC with FPD detection [26]. For all of these work, MSPD was used after a first extraction step by LLE to achieve low LOQs between 1.5 and 5µg/kg [36] when LC-MS/MS was used, however higher LOQs were obtained by GC-MS (10 - 60 µg/kg) and GC-FPD (44 -222 µg/kg). The difference of LOQs was due to the performances of the detector. Concerning the recoveries, they were similar, between 71 and 115%, but the RSD values were higher (almost 19 %) when cameilla oil was used [26]. Probably because in that work they have used less clean up steps compared to the other works where additional clean up by using florisil or GCB sorbent or also it could be because of the different nature of this oil compared to others: palm and olive oil.

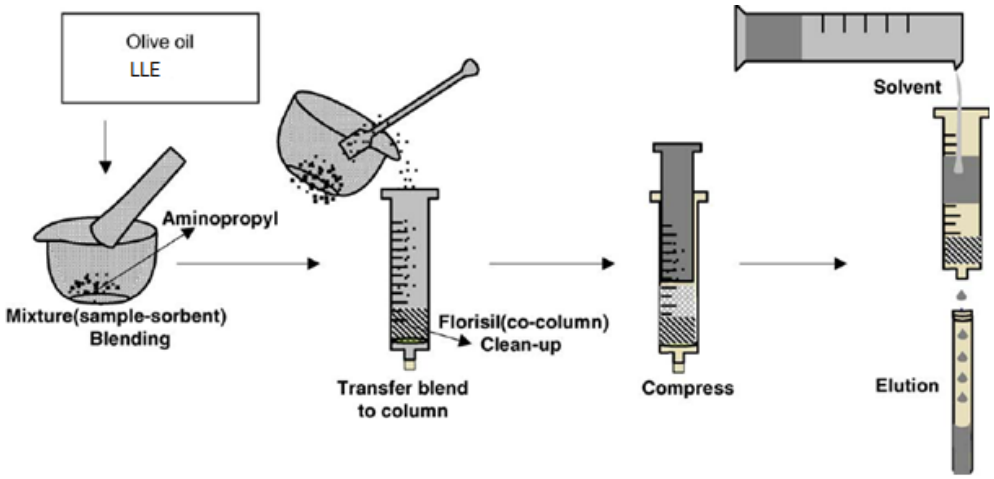


Figure I.3-2. Schematic representation of the MSPD extraction procedure applied to olive oil [28].

I.3.5.Solid Phase Microextraction (SPME)

Solid Phase Microextraction (SPME) is a relatively recent technique, it was introduced in the early 1990s by Pawliszyn and coworkers. This technique used a fused-silica fiber that is coated on the outside with an appropriate stationary phase [39]. The SPME process is composed of two basic steps: (i) partitioning of analytes between the extraction phase and the sample matrix and (ii) desorption of concentrated extracts into an analytical instrument [45]. In SPME, the extraction of the target analytes from the sample matrix to the fiber happens either directly, with the coated fiber immersed in the

liquid sample (direct SPME), or in the headspace SPME (HS-SPME), in this case, the coated fiber is suspended above the sample, as presented in the Figure I.3-3 [46]. HS-SPME reduce the matrix effects and the interferences that were present in liquid samples compared to the direct SPME [47].

C. Tsoutsi et al. have used HS-SPME to extract 9 OPs from olive oil with good recoveries between 80-106%[24]. They also compared the efficiency of different type of fibers (carboxen and poly(dimethylsiloxane)) with different film thickness. The results showed that PDMS fiber with a thickness of 100 µm was the most suitable fiber for the analysis of OPs in olive oils.

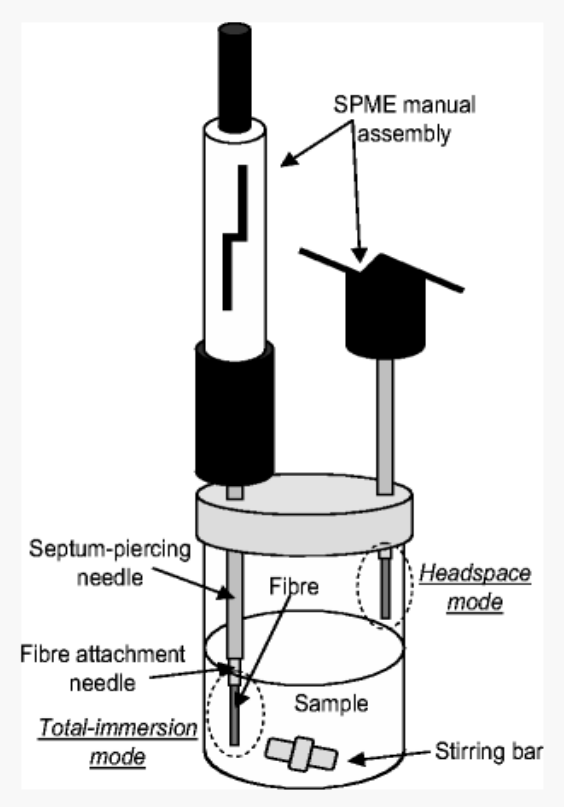


Figure I.3-3. SPME procedure for total-immersion and headspace sampling according to Nerin et al. [46].

I.4.Conclusions

The development of sample treatment procedures for the determination of OPs residues in oil samples with a high fat content is a demanding task, since even small amounts of co-extracted fat must be removed to keep the chromatographic system in working order and to allow the sensitive quantification and reliable detection of OPs at trace levels in such matrices. For this reason, clean-up steps had to be included in the extraction procedures. Different sample handling strategies that we discussed above

(LLE, GCP, SPE, dSPE, MAE, SPME, MSPD or low temperature precipitation) can circumvent the main problems associated with this kind of matrix and permit the development of multi-residue methods when combined with selective and sensitive analytical methods such GC-MS/MS and/or LC-MS/MS. However, the use of these sample treatments necessitate many manipulations of the sample, they are time and solvent consuming. In addition, they suffered of a lack of specificity. Indeed, even if they decrease the matrix effects, the different results exposed above showed that the removal of the matrix component is not total and that it remains interfering compounds in the samples after this step of sample treatment. In order to circumvent this problem a more specific step of sample treatment could be developed by using a sorbent based on the molecular recognition mechanism and named molecularly imprinted polymers.

Table I.4-1. Principal extraction procedures for the determination of OPs in vegetable oils

Sample	Pesticides	Sample preparation technique	Separation technique	Recovery (%)	RSD (%)	LOQ in sample (µg/kg)	Ref.
Olives and olive oil	13 pesticides including OPs	LLE (ACN/petroleum eter)+ MSPD (aminopropyl) + clean up (florisil)	GC-MS and LC-MS/MS	85-115	<10	(LC–MS/MS) < 5 (GC–MS) 10 – 60	[28]
Palm oil	7 Pesticides including OPs	LLE (ACN) + low temperature precipitation + MSPD (PSA)+ clean up (GCB) + sonication	LC-MS/MS	73-91	<14	1.5-5	[36]
Camellia oil	15 OPs	LLE (ACN/H_20)+ MSPD (aminopropyl)	GC-FPD	71–104	<19	44-200	[26]
Olive oil	5 OPs	LLE (ACN)	GC-FPD	78–97	<10	10-50	[15]
Olive oil	26 pesticides, including OPs	LLE (n-hexane/ ACN) + GPC	GC-MS/MS	83- 100	<6	0.3-3.6	[31]
Vegetable oils: olive, flaxseed, colleseed, walnut and coconut oil	3 OPs	DLLME (magnetic water)	GC-MS/MS	78-138	<7.5	0.7-1.27	[32]
Virgin olive oil	18 OPs	Two SPE (silica gel + C18 silica): percolation (hexane) and elution (ACN)	GC-NPD	82–110	<16	10-66	[16]
Olive oil	35 pesticides including OPs	LLE (n-hexane/ ACN) + SPE (ENVI-Carb)	CG-NPD/GC-ECD	70.9 - 107.4	<12	CG-NPD (1.6-47.8) GC-ECD (2.6-43.3)	[25]

Sample	Pesticides	Sample preparation technique	Separation technique	Recovery (%)	RSD (%)	LOQ in sample (µg/kg)	Ref.
Olive oil	9 OPs	MAE (ACN/dichloromethane) + SPE (ENVI-Carb) elution in dichloromethane	CG-NPD	62- 99	<11	7 - 20	[38]
Olive oil	9 OPs + 4 metabolites	HS-SPME (PDMS fiber)	GC–FTD	80-106	<10	< 33	[24]
Soybean oil, peanut oil, sesame oil	14 OPs	Low temperature extraction (ACN)	GC-FPD	> 50	<15	8-18	[27]
Rapeseed, rapeseed oil, and rapeseed meal	27 pesticides, including OPs	Low temperature extraction (ACN or acidified ACN)	LC-MS/MS	70–118	<27	0.3 -18	[17]
Soybean oil	95 pesticides including OPs	QuEChERs: LLE (ACN/n-hexane) + low temperature extraction + dSPE (florsil)	GC-MS	80-114	<14	4-30	[30]
Vegetable oils: olive, peanut, soybean, sesame, colza blend oils, flaxseed and perilla seed	225 pesticides including OPs	QuEChERs: LLE(ACN/H_2O) + dSPE (PSA +C18)	GC-MS/MS	70–120	<20	5-50	[23]

Sample	Pesticides	Sample preparation technique	Separation technique	Recovery (%)	RSD (%)	LOQ in sample (µg/kg)	Ref.
Peanut oil	9 OPs	QuEChERs: LLE (ACN) + dSPE (multiwalled carbon nano tubes + alumina)	GC-MS	86-114	<8.5	2.2-5.3	[29]
Edible oils and other food matrices	OPs and carbamates	QuEChERs: LLE (ACN) + dSPE (PSA or PSA/C18 or PSA/CGB) all used depending of the fat of the extract.	LC-MS/MS	70-120	20	10	[34]
Vegetable oils: Olive, sunflower, palm, rapeseed oil	41 pesticides including OPs	QuEChERs: LLE (ACN)+ dSPE : (A) PSA+CGB +C18 (B) PSA+C18 (C) PSA applied on 4 oils	LC-MS/MS	(B) 70-120> C>A	>20	(A)1-5 (B)10-50 (C)10-50	[35]
Peanuts, rapeseed, soybean and sesame oils	52 pesticides including OPs	QuEChERs: LLE (ACN)+ dSPE : magnetic mesoporous ZrO_2 microspheres (m-$ZrO_2Fe_3O_4$) +n-octadecylphosphonic acid modified magnetic microspheres (Fe_3O_4-OPA)	GC-MS/MS	69.1-120.0	<15	0.1–4.1	[33]
Soybean, sunflower and extra-virgin olive oil	213 pesticides including OPs	QuEChERs : LLE (ACN) + low temperature + dSPE (EMR-lipid)	GC-MS/MS	70-120	1-15	10	[5]

ACN: acetonitrile; dSPE: dispersive solid phase extraction; ECD: electron capture detection; EMR: enhanced matrix removal-Lipid, FTD: flame thermionic detector; FPD: flame photometric detector; GPC: gel permeation chromatography; GC: gas chromatography; HS-SPME: headspace solid-phase microextraction; LLE: liquid–liquid extraction; MAE: microwave-assisted liquid-liquid extraction; LOQs: limits of quantification; LODs: limits of quantification; MRLs: maximum residue limits; NPD: nitrogen/phosphorus detector; LC: liquid chromatography; MSPD: matrix solid-phase dispersion; SPE: solid phase extraction; PDMS: poly(dimethylsiloxane); PSA: primary secondary amine; QuEChERs : quick, easy, cheap, effective, rugged, and safe RSD: relative standard deviation.

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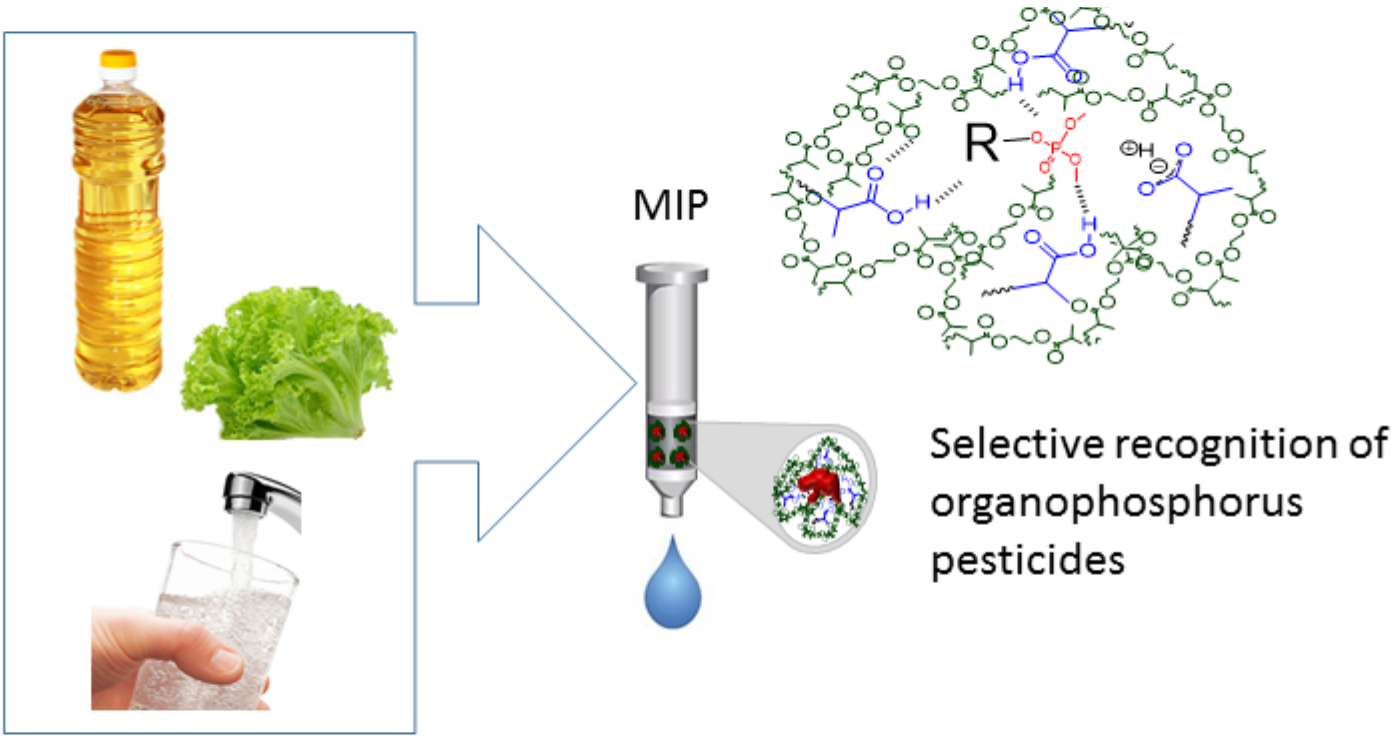
Chapter II: Molecularly imprinted polymers for the determination of organophosphorus pesticides in complex samples



II.Review

This review was submitted in July 2017. All the statistics were stopped at this date.

Molecularly imprinted polymers for the determination of organophosphorus pesticides in complex samples

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II.1.Abstract

Organophosphorus compounds constitute an important class of pesticides whose the toxicity of which arises from the inhibition of the acetylcholinesterase enzyme. They exhibit a wide range of physico-chemical properties, thus rendering their determination in complex oil samples particularly difficult. To facilitate their analysis at the trace level in various samples (environmental waters, soils, vegetables…), molecularly imprinted polymers (MIPs) that are synthetic polymers possessing specific cavities designed for a target molecule have been prepared. Often called synthetic antibodies, MIP can replace antibodies in different application fields. Indeed, as immunosorbents, MIPs can be used as selective sorbents for the solid phase extraction of target analytes from complex matrices. Their synthesis, characterization and use as selective sorbent for the selective recognition of organophosphorus pesticides have been already largely described and are summarized in this review.

Keywords: organophosphorus pesticides; molecularly imprinted polymer; solid-phase extraction; sensors; trace analysis.

II.2.Introduction

The increasing use of pesticides for agricultural purposes cause serious risks to the human and animal health. Organophosphorus pesticides (OPPs) are among the most used pesticides. As mentioned in a recent review related to their analysis in fruit and vegetables, they are found mutagenic, carcinogenic, cytotoxic, genotoxic, teratogenic and immunotoxic [1]. Their determination, at very low concentration levels in environmental samples and foodstuff, constitutes a real analytical challenge. Indeed, OPPs exhibit a wide range of physico-chemical properties thus explaining the possibility to analyze some of them either by gas chromatography (GC) for the most volatile compounds or by liquid chromatography (LC) for the most polar ones. For their analysis through GC, different types of detectors have been used including some specific detectors such as flame photometric detector (GC-FPD) or nitrogen phosphorus detector (NPD) and mass spectrometers for their identification capabilities [1,2]. These recent years, OPPs analysis through LC have been carried out in association with mass spectrometry (LC-MS) with regard to its higher sensitivity and identification capabilities, as compared to UV detection [1–3]. However, despite the advances in the development of such highly sensitive analytical instruments including high resolution mass spectrometry that can be associated with different ionization sources, a pre-treatment is usually necessary in order to extract and isolate the analytes of interest from complex samples before their determination [2].

The analysis of pesticide residues, including OPPs in environmental samples (waters, soils, sediments…), foodstuffs and biological fluids has been often reviewed, showing that numerous extraction methods have been developed for the treatment of solid and liquid samples those last ten years. Some of these reviews have focused on the development of various methods for the treatment of a given type of samples such as water samples [4], foodstuffs [5,6], such as fruits and vegetables [7], fatty vegetable matrices [8], foods of animal origin [9], olive and olive oil [10], baby-food [11] and honey using various techniques [12]. Others have reported the potential of a method or a group of close methods for the treatment of various types of samples such as solid-phase based extraction method for food and environmental samples [13], stir bar sorptive extraction (SBSE) for fruits and vegetables [14], single drop liquid extraction (SDLE) for waters and fruit juices [15] or liquid-phase micro-extraction for water samples including SDLE and dispersive liquid-liquid extraction [16], matrix solid phase dispersion (MSPD) for

foodstuffs such as vegetables [17] or food from animal origin [17,18].

Despite the use of these efficient extraction and clean-up methods, matrix components are unavoidably present in final extracts thus causing a risk of matrix effect during GC or LC determination [19,20]. The effects caused by these matrix components can be reduced by improving the chromatographic resolution as can be achieved using multidimensional chromatography or by improving the selectivity during the sample treatment.

Selectivity, during sample pretreatment, can be obtained by using sorbent able to retain compounds by a molecular recognition mechanism. For this, it comes therefore possible to use immunoaffinity supports (*i.e*. immunosorbents, ISs) based on the use of specific antibodies that target a molecule of interest. The high selectivity and affinity of the antigen-antibody interactions allows a selective clean-up to being reached with high enrichment factors as already demonstrated for numerous pesticides in complex samples [21] including OPPs from water samples [22]. Other selective supports, called oligo-sorbents, have been recently proposed using aptamers immobilized onto a solid support. Aptamers are oligonucleotides with a specific sequence able to bind a given molecule with the same affinity as antibodies. Aptamers were recently successfully applied to the selective extraction of different target analytes from biological fluids and food samples [23,24]. A DNA sequence was previously described for the recognition of OPPs but not applied yet to their extraction from real samples [25]. Once the sequence is available, developing an oligosorbent is less expensive than an IS. However, despite their high potential, a limited number of sequences is, to date, available. This molecular recognition mechanism can also be exploited using molecularly imprinted polymers (MIP) that are synthetic polymeric materials possessing specific cavities designed for a template molecule. MIPs are often called synthetic antibodies in comparison with IS. They offer some advantages including easy, cheap and rapid preparation and high thermal and chemical stability [26]. The use of MIPs as selective sorbents for solid-phase extraction (SPE) is recent. It was initially proposed by Sellergren *et al.* in 1994 for extracting pentamidine present at low concentration in urine [27]. Since this first application, numerous MIPs were developed for the selective extraction of target analytes from complex samples [28–31]. Because of their high selectivity, they have been also already successfully used in several other fields such as sensors [32–34], bioassays [35,36] and enantiomeric separation [37].

Their synthesis, characterization and use as selective sorbent for the selective recognition of OPPs have been already largely described and mainly developed to be integrated in sensors or used in solid phase extraction. Figure II.2-1a gives an idea of illustrates the proportions of the application of MIPs for the determination of OPPs in these different fields. As shown by Figure II.2-1b, this field of research is very active since more than 70% of the papers have been published those last five years.

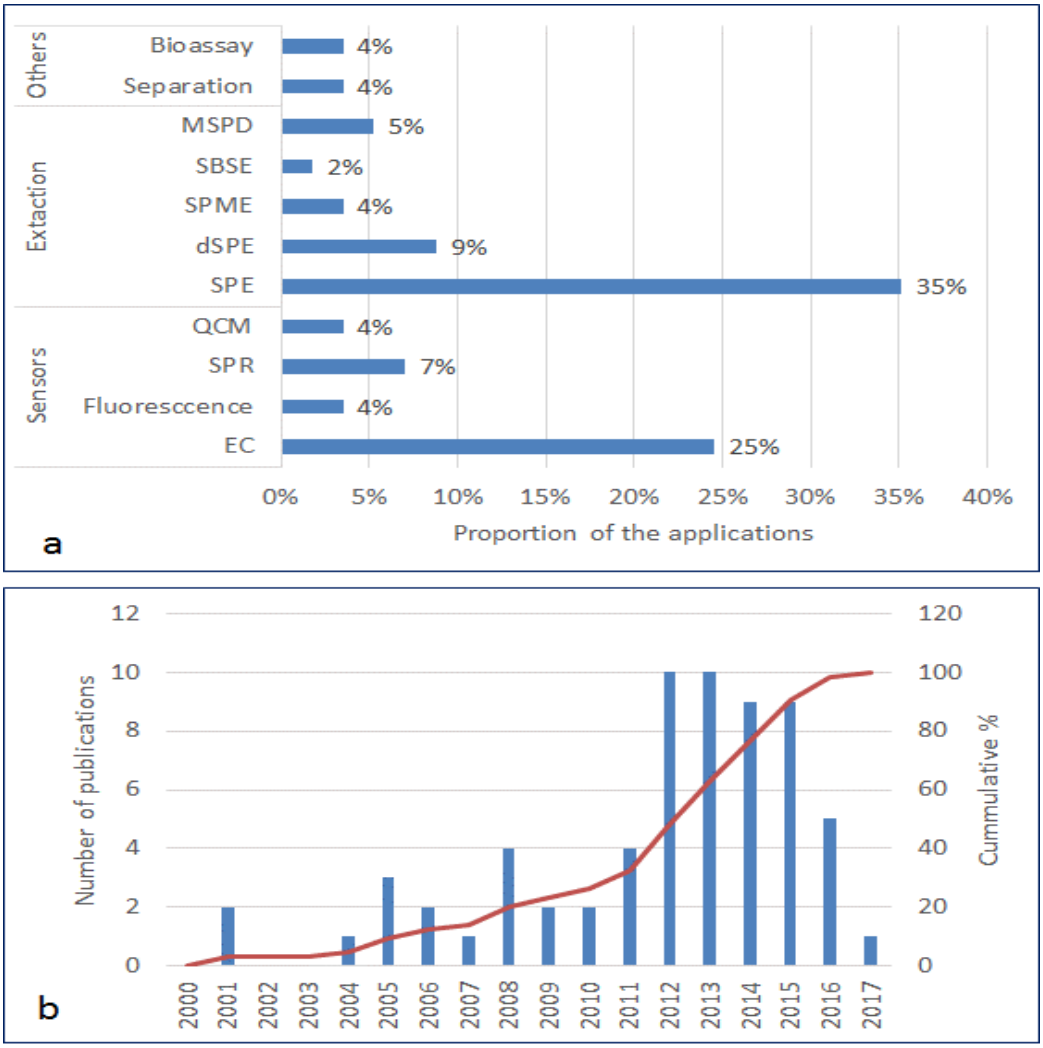

Figure II.2-1. Percentages of use of MIPs in the different fields such as sensors of different types e.g. piezoelectric (using using quartz cristal microbalance (QCM) or surface plasmon resonance (SPR)), optical (i.e. fluorescence) or electrochemical (EC)), as extraction sorbent in conventional SPE, in dispersive mode applied to liquid samples (dSPE) or solid samples (matrix solid-phase dispersion, MSPD), in micro-solid-phase extraction (SPME), in stir-bar solid-phase extraction (SBSE) or as stationary phase for separation purposes or in bioassays (a) and cumulative percentages (red curve) of publications related to the development of MIPs for dedicated to OPPs (b).

Therefore, this review focuses on the presentation of polymerization methods used to produce MIPs for OPPs, their characterization in pure media and their

performance as SPE sorbents or as selective tools of sensors for the determination of OPPs in real samples.

II.3.Synthesis of MIPs

In the common approach, the synthesis of molecularly imprinted polymers (MIPs) involves first the solution complexation of a template molecule with functional monomers, through non-covalent bonds, followed by polymerization of these monomers around the template with the help of a cross-linker in the presence of an initiator. The choice of the chemical reagents making the MIP must be judicious in order to really create specific cavities designed for the template molecule. For these reasons, a monomer is chosen to develop strong interactions with the target analyte, *i.e*. an OPP or a structural analog acting as the template, in a porogen solvent. By the presence of a cross-linker, the polymerization takes place around the template. The template molecule is then removed, producing a polymer with binding sites complementary to the template in size, shape and position of the functional group. The conditions of synthesis of MIPs for OPPs (polymerization mode, reagents used), as reported in the literature, are summarized in Table II.3-1.

Table II.3-1. Conditions of synthesis of MIPs for the recognition of OPPs. Underlined reagents correspond to reagents that were finally selected in the studies.

Template	Synthesis	M/CL	Solvent	Initiation	Ref.
Acephate	Bulk	MAA/ EGDMA	$CHCl_3$: H_20 (8:3)	AIBN/58°C	[38]
	Pickering emulsion	MAA /EGDMA	$CHCl_3$		[39]
	Surface grafting on Au	MAA/ TRIM	CH_3CN	ABAH/ UV	[40]
Chlorpyrifos	Polymerization on silica NPs	MAA/ EGDMA	CH_3CN / Toluene 3/1	BPO/50 to 85°C	[41]
	Polymerization on silica particles	AA/ EGDMA	CH_3CN	AIBN	[42]
	Bulk	MAA/ EGDMA	CH_3CN / MeOH 2/1	AIBN /60°C	[43]
	Polymerization on QD	AAM/ EGDMA	EtOH	AIBN /60°C	[44]
	Electropolymerization on NP	PD	-	-	[45]
	Polymerization on NP	dopamine	-	-	[46]
Chlorpyrifos methyl, diazinon	Polymerization on a fiber	vinylbenzoate + Europium/styrene+DVB	Water/ MeOH	AIBN /60°C	[47]
D4DNP	Precipitation	imidazole + Co+ /DVB	CH_3CN	AIBN /60°C	[48]

Template	Synthesis	M/CL	Solvent	Initiation	Ref.
DEP	Precipitation	MAA, IA, AAM /EGDMA	$CHCl_3$	60°C	[49]
DETP	Bulk	4-VP/ EGDMA	CH_3CN	AIBN /65°C	[50]
Diazinon	Bulk	MAA/ EGDMA	$CHCl_3$	AIBN /60°C	[51]
	Dispersion	MAA+HEMA /EGDMA	MeOH / CH_3CN 1/4	AIBN /60°C	[52]
	Precipitation and suspension	MAA/ EGDMA	$CHCl_3$	AIBN /60°C	[53]
	Sol-gel coating	PEG /TEOS, PMHS	Toluene		[54]
Dichlorvos	Bulk	MAA / TRIM	CH_3CN	AIBN	[55]
Dimethoate	Bulk	BMA, MMA, EMA / EGDMA	THF	AIBN /60°C	[56]
	Membrane coating	MAA,AA/ polyacrylonitrile/ N-methylpyrrolidone	-	-	[57]
	Living radical	MAA/ EGDMA	CH_3CN	AIBN, TED /UV	[58]
	Precipitation	MMA, MAA, AAM/ EGDMA	CH_3CN	AIBN /20°C	[59]

Template	Synthesis	M/CL	Solvent	Initiation	Ref.
DMPTABA	Bulk	AAM/ EGDMA	CH_3CN	AIBN /60°C	[60,61]
	Film produced at the surface of 96-well plate	MAA/ EGDMA	-	AIBN	[62]
	Sol-gel	APTES/ TEOS	-	ammonia	[63]
Fenitrothion	Bulk	MAA/ EGDMA	CH_2Cl_2	AIBN /65°C	[64]
Fenthion	Bulk	AAM/ EGDMA	DMF	AIBN	[65]
Isocarbophos	Electropolymerization	PD/GA/ABA	-	-	[66]
Malathion	Precipitation	MAA/ EGDMA MAA+GMA /EGDMA	CH_3CN / $CHCl_3$ $CHCl_3$	AIBN /70°C	[67] [68]
Methamidophos	Bulk	MAA/ EGDMA	CH_2Cl_2	AIBN /58°C	[69,70]
Methidation	Bulk	MBAA ,IA, MAA, TFMAA/ EGDMA	DMF	AIBN /80°C	[71]

Template	Synthesis	M/CL	Solvent	Initiation	Ref.
Monocrotophos	Bulk	MAA/ EGDMA	CH_2Cl_2	AIBN /58°C	[72–74]
			-	-	[75]
			$CHCl_3$	AIBN /58°C	[76]
	nylon membrane	MAA, AA, AAM /EGDMA	Toluene, CH_3CN, CH_2Cl_2	AIBN /65°C	[77]
	Phase inversion/Nylon-6	Nylon-6	-	-	[78]
	Precipitation	MAA/ EGDMA	Toluene	AIBN /60°C	[79]
O,O-dimethyl thiophosphoryl chloride (dummy)	Bulk	MAA/ EGDMA	$CHCl_3$	AIBN /58°C	[80]
Omethoate, dimethoate, monocrotophos	Bulk	IA, MAA, TFMAA/ EGDMA	DMF	AIBN /80°C	[81]
Parathion	Bulk	MAA/ DVB	EtOH	AIBN /60°C	[82]
	silica gel grafting	PEI /EGDMA	basic solution		[83]
	Precipitation	MAA/ EGDMA	$CHCl_3$	AIBN /60°C	[84]
	Sol-gel film (spin-coating)	p-tert-butylcalix[6]arene /PMHS, TEOS, OH-TSO -1,4-crown-4	CH_2Cl_2/ EtOH	TFA	[85]

Template	Synthesis	M/CL	Solvent	Initiation	Ref.
Parathion or paraoxon	Sol-gel film (spin-coating)	PTMOS, APTES/ TEOS	EtOH	HCl	[86]
Parathion methyl	Electropolymerization	aminothiophenol	-	-	[87]
	Electropolymerization	quercetin, resorcinol, dodecanethiol	-	-	[88]
	Electropolymerization	phenol	-	-	[89]
	Precipitation	MAA /EGDMA	MeOH	AIBN /60°C	[90]
	Polymerization on NP	MAA :4-VP/ EGDMA	$CHCl_3$	AIBN	[91]
	Sol-gel	calix[4]arene /PMHS, TEOS, OH-TSO	CH_2Cl_2	TFA	[92]
Parathion methyl /parathion/paraoxon	Polymerization on MWCNTs	AAM/ EGDMA	CH_3CN / Toluene/ DMF (15/2/3)	AIBN /70°C	[93]
Profenofos	Precipitation and spin-coating/in situ self assembly on Au electrode	MAA/ EGDMA or TRIM	CH_3CN / DMSO	AIBN, ABAH /60°C	[94]
	Coating of Ag film	MAA/ TRIM	DMSO		[95]
	Polymerization on Au chip	MAA/ TRIM	DMSO	ABAH /60°C	[96]

Template	Synthesis	M/CL	Solvent	Initiation	Ref.
Tolchlofos-methyl (dummy)	Dispersion polymerization with γ-MAPS activated silica NP	MAA/ EGDMA	CH_3CN	AIBN /60°C	[98]
Trichlorfon	Bulk	MAA/ EGDMA	$CHCl_3$	AIBN /60°C	[99]
	In-situ polymerization into a capillary	MAA /γ-MAPS	MeOH / toluene 3/2	AIBN /50°C	[100]
	Bulk	MAA/EGDMA	$CHCl_3$	AIBN/58°C	[101]
Trichlorfon + monocrotophos	Bulk	MAA/ EGDMA	$CHCl_3$	AIBN /58°C	[102]

4-VP: 4-vinylpyridine, AA: acrylic acid ; AAM: acrylamide; ABA: m-aminobenzoic acid ; ABAH: 2,2′-Azobis (2-amidino propane) hydrochloride; AIBN: 2,2-Azobis-(2-methylpropionitrile); APTES: aminopropyl triethoxysilane; BMA: butylmethacrylate ; $CHCl_3$: chlorofom; CH_2Cl_2: dichloromethane; CH_3CN: acetonitrile; CL : cross-linker ; BPO : benzoyl peroxide ; D4DNP: diethyl(4-nitrobenzyl)phosphonate ; DMPTABA: 4-(dimethoxy phosphorothioylamino)butanoic acid; DCM: dichloromethane ; DEP: diethyl(3-methyl ureido)(phenyl)methylphosphonate ; DETP: diethylthiophosphate; DMF: dimethylformamide; DMSO: dimethylsulfoxide ; DVB: divinylbenzene ; EGDMA; ethyleneglycoldimethacrylate; EMA: ethyl methacrylate; EtOH : ethanol ; GA : gallic acid ; GDMA: glycerol dimethacrylate; GMA: glycidilmethacrylate; HEMA: 2-hydroxyethyl methacrylate ; IA: itaconic acid; M: monomer ; MAA: methacrylic acid; MBAA: N,N'-Methylenebisacrylamide MMA: methyl methacrylate ; MeOH: methanol; MWCNT: multiwall carbon nanotube NP: nanoparticles ; OH-TSO: hydroxy terminated silicone oil ; PEG: polyethylene glycol; PEI: polyethyleneimine ; PMHS: poly (methylhydrosiloxane); PTMOS: phenyltrimethoxysilane; QD: quantum dots ; TED: tetraethyl thiuram disulfide; TEOS: tetraethyl orthosilicate ; TFA: trifluoroacetic acid ; TFMAA: trifluoromethylacrylic acid ; THF: tetrahydrofuran; TRIM: trimethylol propane trimethacrylate ; γ-MAPS: γ-methacryloxypropyl trimethoxysilane ; PD: o-phenylenediam

As shown in this table, MIPs were prepared by radical polymerization of organic acrylate of acrylic based monomers. In most of the cases, a conventional bulk polymerization is achieved and gives rise to a monolith that as to be ground and sieved to obtain particles that present an heterogeneous size distribution. This procedure is easy to achieve but it is time-consuming and its yields are less than < 50%, mainly explained by the loss of MIP, as fine particles removed during a sedimentation step. In order to obtain more regular and homogeneous beads or microspheres, MIPs can also be prepared by precipitation polymerization that results from an increased amount of porogen or by more sophisticated methods such as suspension polymerization or multi-step swelling or surface-grafting. It was also proposed to develop MIPs by the hydrolysis and the condensation of organo-silanes around the template, thereby thus giving rise to a hybrid sol-gel material. This synthesis achieved in aqueous media presents the advantages to facilitate the dissolution of polar templates.

As shown by data reported in Table II.3-1, more than 20 different OPPs were used as template molecule, the most frequently reported OPP templates being chlorpyriphos, parathion, parathion methyl, dimethoate and monocrotophos. The structure of the main studied OPPs and their log P values are reported in Annexe I (Figure 1).

The use of a structural analog has been proposed to prepare MIP for other chemicals to decrease the cost of the material when the target molecule is expensive as it is can be the case for some toxins but not for OPPs. It is also a way to circumvent the risk of residual template leaking from the polymer that may cause erroneous results when applying the MIP to trace analysis. Indeed, the complete removal of the template from the MIP after its synthesis can be difficult to achieve and necessitates extensive washing steps. The use of this molecule, named “dummy molecule”, that can be distinguished from the target analysis during its determination in real samples, particularly by chromatographic methods when the MIP is used as extraction sorbent, constitutes an easy way to limit the risk caused by this leaking. The dummy molecule must resemble the target analyte in terms of shape, size and functionalities to obtain cavities that are able to bind the target analyte. This dummy approach was reported by different groups to produced OPP MIPs [48,49,60,63,80], including the use of a metabolite of OPP, *i.e.* DETP [50].

As for other molecules, the most common approach used for the development of

OPP MIP for extraction purposes, consists of a non-covalent imprinting. To exploit the non-covalent polar functions of the OPP during the polymerization reaction, polar organic monomers such as MAA, AAM, 4-VP and IA were selected. For the same reasons, the solvent of polymerization was a weakly polar and aprotic solvent such dichloromethane, chloroform and acetonitrile.

As mentioned by some authors, the selection of the monomer(s) and the template/monomer(s) ratio can be carried out by studying the changes in UV spectra of the template when adding increasing concentration of the monomer in the solvent selected for the synthesis [67,69,77]. Other spectroscopic methods were used such as NMR to highlight the presence of hydrogen bonds between the template and MAA [39,67,69,72,73] and FT-IR to highlight the interactions between OPP and OH-group of MAA [39,46,55,61,70,76,80,91,97,98,102]. This method was also used to control the template removal by comparing MIP spectrum before and after its washing [64,93,99], this control being most of the time ensured by analyzing the template amount in the washing solution by UV detection or by chromatographic analysis.

Computational design was also proposed to simulate monomer-template interactions and then to select the best monomer, *i.e.* the monomer that gives the highest interaction energy with the template [39,56,65,67] or to elucidate the best template/monomer ratio [79]. It was reported that results obtained using this approach, despite its high potential, must be confirmed by retention measurements (HPLC, SPE profiles) [56,81] or binding experiments [71] thus implying the synthesis of several MIPs with a selection of templates and/or monomers selected by the simulation.

II.4.MIP characterization

The potential of a MIP is related to the occurrence of selective cavities that promote a high interaction with the target OPP. In most of the works, a non-imprinted polymer (NIP) is synthesized in the same conditions as the MIP but without in the absence of the template. This control polymer, that does not possess any cavities, is studied in parallel during the MIP characterization.

The first evaluation of the synthesized MIP may consist in characterizing by SEM the surface of MIP/NIP [39,54,77,78,97,102], the shape and size of particles [50,58,59,79,98]. BET adsorption method can also be used to measure and compare the

porosity of MIP/NIP [64].

Binding tests that consist in introducing a given amount of MIP/NIP in the presence of a known amount of an OPP and then measuring, after a fixed time, the amount retained by the MIP and the NIP was used to select the best monomer [49,59,82], the template/monomer ratio [49,51,59]. In these cases, the solvent used is very close or similar to the solvent used for the synthesis of the polymers. The adsorption isotherm resulting from these binding experiments also allows, by using different models (Langmuir, Freundlich, Langmuir-Freundlich), to define number of binding sites and their affinity towards the template molecules [80,93] and, in some cases, towards structural analogs to being defined [55,60,64,69,70,76,79,82,86,90,99].

Binding experiments were also carried out in a pure solvent, very similar to the nature of the sample matrix, such as aqueous buffer or pure water or acetonitrile for the further analysis of OPPs in aqueous or acetonitrile vegetable extracts, respectively [50,51,76,80,93,99]. These binding experiments were also carried out in heptane, the chosen solvent to dilute oil samples [58]. This approach allows a better evaluation of the retention potential and of the selectivity that can be expected in real samples to being better assessed. In the same way, by testing different solvents by binding experiments, the solvent that favors the highest selectivity can then be used to dilute the sample or sample extracts [65,78]. On the opposite, the solvent that gives the lowest affinity can be chosen as eluting medium in SPE procedure [78].

The effect of the nature of solvents on the retention properties was also studied by HPLC measurements. Indeed, this method was used to evaluate the solvent that favors the retention [56,72] and to evaluate the selectivity towards different analogs [56].

II.5.MIP for selective extraction

As already demonstrated by numerous reviews related to the development of MIPs for the selective extraction of compounds [28,103–106], this field of application is very active and represents more than half of the developments of MIPs for OPPs as shown by Figure II.2-1a. Since the pioneer work of Sellergren in 1994 [27] who developed and used a MIP as SPE sorbent for the selective extraction of pentamidine from urine, different extraction devices are now envisaged. Indeed, despite the large use

of MIP as solid-phase extraction sorbent (MIP-SPE), after its packing into disposable cartridges to carry out exhaustive extraction, beads of MIP were dispersed in liquid samples and solid sample to develop selective dispersive SPE (dSPE) or selective matrix solid-phase extraction (MSPD) methods, respectively. Other non-exhaustive extraction methods such as solid-phase micro-extraction (SPME) or stir-bar sorptive extraction (SBSE) have been developed for the selective trapping of OPPs from various types of samples [31,103,104]. These different developments are summarized in Table II.5-1.

Table II.5-1. MIPs involved in extraction methods. Underlined compounds correspond to are those studied in real samples, compounds in bold are those whose selectivity was proven by a comparative study on NIP as control SPE sorbent (not only by binding experiments).

Use	Template	Synthesis	Monomer / CL/ Porogen	Matrix	Studied compounds	Ref.
SPE	chlorpyrifos	Dispersion/ silica particles	AA / EGDMA /ACN	spinage	chlorpyriphos, methyl-parathion, parathion	[42]
	DETP	Bulk	4-VP /EGDMA /ACN	urine	DETP, DEDTP	[50]
	diazinon	Bulk	MAA /EGDMA /$CHCl_3$	cucumber	**diazinon**	[51]
	dichlorvos	Bulk	MAA/TRIM /ACN	water, vegetables	dichlorvos, phoxim, chlorpyriphos	[55]
	dimethoate	Living radical polymerization	MAA/ EGDMA/ACN	olive oil	**dimethoate,** omethoate, malathion, fenthion methidation	[58]
	DMPTABA	Bulk	AAM /EGDMA/ ACN	cucumber	dimethoate, isocarbophos, methyl-parathion	[61]
	fenitrothion	Bulk	MAA/EGDMA /CH_2Cl_2	tomato	**fenitrothion**	[64]

SPE	fenthion	Bulk	AAM/ EGDMA /DMF	olive oil	**fenthion, fenthion sulfoxide**, dimethoate, methidathion, malathion	[65]
	malathion	Precipitation	MAA /EGDMA ACN : $CHCl_3$ 1 :1	tap water, soil, cabbage	malathion, malaoxon, profenofos, triazophos	[67]
			MAA: GMA /EGDMA $CHCl_3$	honey	malathion, ethophos, phorate, terbuphos, dimethoate, fenamiphos	[68]
	methamidophos	Bulk	MAA/EGDMA /CH_2Cl_2	surface water, soil	**methamidophos**, acephate, monocrotophos, phosphamidon	[70]
	methidation	Bulk	MBAA/ EGDMA / DMF	olive oil	**methidation**, dimethoate, malathion, fenthion	[71]
	monocrotophos	Bulk	MAA/EGDMA /CH_2Cl_2	river and tap water, soil	monocrotophos, mevinphos, phosphamidon, omethoate	[73]
			MAA /EGDMA/ $CHCl_3$	rape, cauliflower, leek	monocrotophos	[76]

		Polymerization/ nylon membrane	MAA/ EGDMA/ Toluene	pure water	**monocrotophos**, mevinphos, phosphamidon, omethoate	[77]
	O,O-dimethyl thiophosphoryl chloride	Bulk	MAA/ EGDMA/ $CHCl_3$	water, vegetables	dichlorvos, methamidophos, acephate, folimat, monocrotophos, methyl-parathion, phosphamidon, malathion	[80]
	omethoate	Bulk	IA / EGDMA/ DMF	olive oil	**omethoate, dimethoate**, methidation, monocrotophos, malathion, fenthion	[81]
	quinalphos	Bulk	MAA / EGDMA / ACN	fruit	**diazinon, quinalphos, chlorpyriphos**	[97]
	trichlorfon	Bulk	MAA /EGDMA/ $CHCl_3$	vegetables	trichlorfon, omethoate, acephate	[99]
	trichlorfon + monocrotophos	Bulk	MAA /EGDMA/ $CHCl_3$	leek	trichlorfon, monocrotophos, methamidophos, acephate	[102]
dSPE	acephate	Pickering emulsion / SiO_2 NP	MAA /EGDMA/ $CHCl_3$	waters	acephate, methamidophos, isocarbophos and malathion.	[39]

	chlorpyrifos	Dispersion/ silica NPs	MAA /EGDMA /ACN : Toluene 3 :1	green vegetable	chlorpyriphos, profenofos, ltriazophos, phoxim	[41]
	diazinon	dispersion / Fe_3O_4 NP (magnetic)	MAA: HEMA /EGDMA / MeOH: ACN 1:4	soil, cucumber	diazinon	[52]
	dimethoate	Precipitation	MAA / EGDMA / ACN	cucumber	dimethoate, methamisophos, carbaryl	[59]
	methyl-parathion	polymerization / Fe_3O_4 NP (magnetic)	MAA: VP /EGDMA/ CHCl3	soil	methyl-parathion, malathion, methamidophos	[91]
MSPD	DMPTABA	Bulk	AAM / EGDMA / ACN	apple, pear	trichlorfon, malathion, acephate, methamidophos, omethoate, dimethoate, phosphamidon, monocrotophos, methyl parathion	[60]
	monocrotophos	Bulk	MAA/EGDMA /CH_2Cl_2	soil	monocrotophos, fenitrothion, parathion, fenthion, phoxim	[74]
	tolchlofos-methyl (dummy)	dispersion / γ-MAPS activated silica NP	MAA / EGDMA / ACN	carrot, yacon	tolclophos methyl, methyl-parathion, chlorpyrifos, phoxim, iprobenphos	[98]

SBSE	monocrotophos	Phase inversion/ Nylon-6	Nylon-6	soil	monocrotophos	[78]
SPME	chlorpyriphos	Bulk	MAA/EGDMA /ACN:MeOH 2 :1	apple, grapes	chlorpyriphos, diazinon, malathion, parathion	[43]
	diazinon	Sol-gel coating	PEG /TEOS, PMHS /toluene	water, vegetable	methyl-parathion, diazinon, pirimiphos-methyl, isocarbophos	[54]
	methyl-parathion	Sol-gel	calix [4]arene/PMHS, TEOS, OH-TSO/CH_2Cl_2	fruits	methyl-parathion, parathion, fenitrothion, fonofos, fenthion	[92]

II.5.1.MIP-SPE of OPPs

As shown by the conditions of synthesis reported in Table II.5-1 , MIPs for SPE were mainly prepared by bulk polymerization. The resulting monolith was ground to obtain 25-50 µm particles that were packed between two frits in disposable cartridges and applied as conventional SPE sorbent (C18 silica, polymers…) to the extraction of OPPs from real samples.

Except in one case for which three OPPs were studied as template before eventually choosing omethoate as template [81], the reported works described the use of a unique OPP to prepare a MIP for this molecule and then for its selective extraction from real samples.

In more than 75% of the reported studies, MAA was used as monomer without any preliminary studies related to the selection of this monomer. The computational screening of monomers was only reported by Bakas *et al.* [65,71,81] that allows them to select a unique monomer that presents the highest interaction energy with the template. In one of these studies, several MIPs were synthesized using the several selected monomers (IA, MAA, TFMAA) and SPE was carried out to definitively select IA, since leading to a MIP that provides the highest retention and the best selectivity for omethoate [81].

In most of the cases, the presence of specific cavities was proven by binding experiments in a pure solvent spiked with increasing amounts of the target molecule. These experiments allow the affinity of the binding sites of the MIP to being compared with those of the NIP and then to evaluate the presence of specific cavities in the MIP. This approach was also used by Zhu et al. to evaluate the best monomer and solvent among three to produce cavities of high affinity for monocrotophos [77].

If binding experiments can also be used to determine the affinity of the MIP towards other OPPs [67,77], the ability of a MIP to trap several OPPs has been mainly done by measuring extraction recoveries on MIP and on NIP after the application of a SPE procedure previously optimized by studying the target compound alone. In a SPE procedure, different parameters can be studied such as (i) the nature of the percolated solution that must favor the retention, (ii) the composition of the washing solution that constitutes a key parameter for differentiating to differentiate the MIP from and the NIP and (iii) the nature and the volume of the elution solution to recover the target analyte. This is particularly well illustrated with the results reported by Bakas et al. [65] and

related to the selective extraction of fenthion and four other OPPs from olive oil using a MIP produced with fenthion as template and AAM as monomer in dimethylformamide. After studying the retention of fenthion on MIP/NIP in different solvents, heptane was selected for its ability to favor the retention of this compound on MIP and different solvents were further tested as washing solvent to select the one that allows the retentions of between MIP and NIP to being differentiated. As shown by results reported in Figure II.5-1, the use of dichloromethane allows fenthion to being partially removed from the NIP (40%) during the washing step (Figure II.5-1a) while maintaining the retention on the MIP (Figure II.5-1b). To improve the selectivity of the procedure, an increasing amount of acetonitrile was added in dichloromethane. This study showed that the use of 5% acetonitrile in dichloromethane (v/v) allows 98% fenthion to being removed from the NIP (Figure II.5-1c) while maintaining its retention on the MIP (Figure II.5-1d).

This procedure optimized with fenthion was applied to four other OPPs and results, reported in Table II.5-2, showed that it was possible to extract both fenthion and fenthion sulfoxide with recovery rates above higher than 93% and with a high selectivity, these compounds being not retained on the NIP. In return, the three other OPPs were not retained by the MIP.

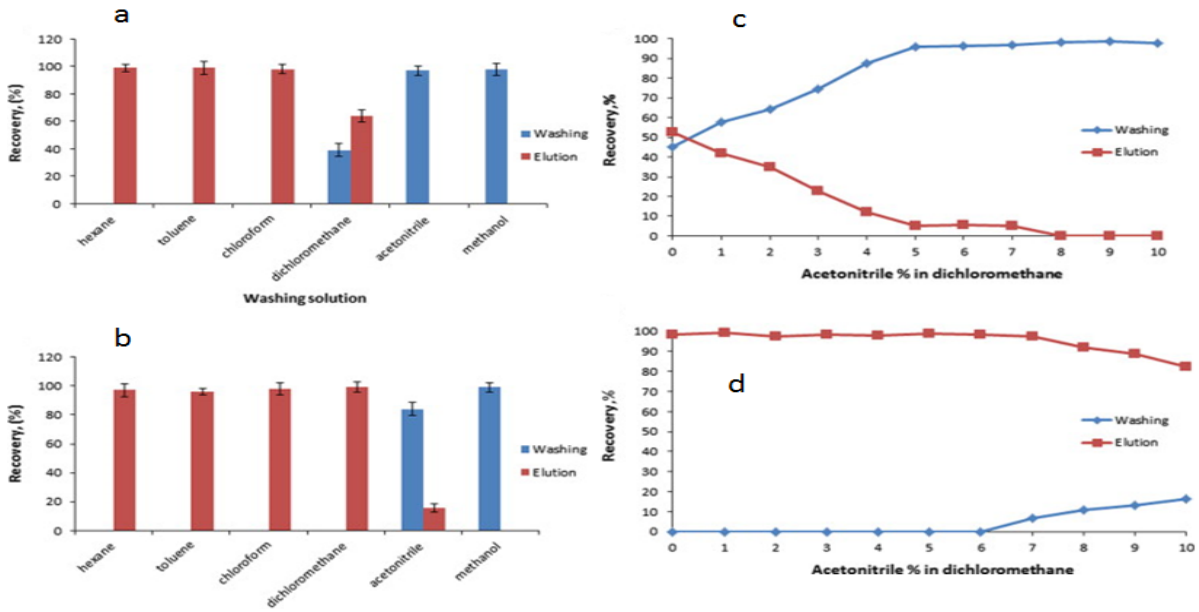

Figure II.5-1. Recovery of fenthion in the washing (blue) and elution (red) fractions after loading 1 mL of 1 mg L^{-1} pesticide on NIP (a,c) and MIP cartridges (b,d). Washing step: 2 mL of the solvents (a,b) or with different % of acetonitrile in dichloromethane (c,d); elution step: 1 mL of methanol/2% TFA [65].

This low ability of the MIP to recognize a large number of OPPs was reported by numerous groups after this optimization with the MIP and the NIP in pure media [58,65,70,73,81]. This can be explained by the fact that the phosphate group of OPPs is substituted by very different chemical groups as shown by the structure reported in Annexe I (Figure 1). In this case, the recognition of fenthion sulfoxide was certainly favored by the fact that it comprises, as fenthion, an aromatic group unlike the three other studied compounds. The effect of the structural similarity on the ability of a MIP to recognize selectively three OPPs was demonstrated by Sanagi *et al*. who developed a MIP using quinalfos as template. This MIP was able to selectively extract quinalfos but also diazinon and chlorpyriphos, *i.e.* three molecules that comprise an aromatic ring with one or two linked nitrogen atoms [97].

Table II.5-2. Recovery rates (%) of 5 OPPs loaded as 5mL aliquots of 1 mg L^{-1} solution onto acrylamide-based MIP and corresponding NIP. The calculations are based on triplicates; the RSD values are below 5% [65].

Analytes	MIP		NIP	
	Washing	Elution	Washing	Elution
Fenthion	4 ± 3.2	97 ± 4.1	95 ± 4.8	4 ± 2.1
Dimethoate	98 ± 3.7	nd	97 ± 3.2	nd
Fenthion-sulfoxide	nd	93 ± 3.3	95 ± 4.4	nd
Methidathion	96 ± 3.5	6 ± 3.6	95 ± 3.6	nd
Malathion	97 ± 5	nd	98 ± 4.2	nd

nd : not detectable.

Some authors reported the ability of the MIP to extract up to six OPPs with high recovery rates but the NIP was not used in parallel to the MIP when optimizing the SPE procedure [42,50,61,68,76,80]. In these conditions, the retention was certainly favored by the solvent selected as percolating medium but the real selectivity, believed to being brought by the cavities of the MIP, was not really proven.

To ensure the simultaneous trapping of two OPPs, *i.e.* monocrotophos and trichlorfon, from vegetable extracts with a high efficiency, two tailor-made MIPs were prepared using each target as template [76]. These MIPs were prepared by applying very similar conditions of synthesis to ensure the possibility to develop a unique extraction procedure based on the same chemical interactions. The authors just adapted

the amount of each MIP to be introduced in the cartridge to ensure a high recovery of extraction for both molecules. To reach the same objective, a MIP was prepared using both molecules, monocrotophos and trichlorfon, as templates for the synthesis of a unique MIP [102].

A SPE sorbent specific to two different groups was also proposed by preparing a dual-layer cartridge containing both a MIP prepared with dimethoate as template (in synthesis conditions previously reported by Martins et al. [58]) and a MIP prepared using terbuthylazine as template for the simultaneous determination of both targets in olive oil samples [107].

As illustrated by studies reported in Table II.5-1, MIPs were applied to the selective extraction of OPPs from different matrices such as water samples, aqueous or hydro-organic extracts of vegetables or of soil. They were also applied to the selective extraction of olive oil by Bakas et al. that mentioned the necessity to dilute the oil sample in hexane to favor the retention of the target analytes while decreasing matrix effects [65,71,81]. To demonstrate the potential of the MIP in terms of selectivity, chromatograms resulting from the use of the MIP were compared to chromatograms resulting from liquid-liquid extraction or an extraction on a conventional C18 silica sorbent [70,73,81,102]. In all cases, the chromatograms showed that the MIP removed compounds that co-extracted with OPPs when using non-selective approaches. Two studies also compared the recovery rate on MIP and NIP applied to the same sample: the higher recovery rates obtained on MIP than on NIP confirm the real contribution of the specific cavities in the extraction process of the target analyte of fruit and oil samples [71,97].

II.5.2.Other extraction methods

Instead of introducing MIP particles between frits in a cartridge, MIP particles can be dispersed in a liquid sample to be put in contact with the target analytes for a suitable time. To obtain well defined particles, authors proposed to replace the polymerization in bulk by precipitation polymerization [59], polymerization on silica particles [39,41] or on iron (II,III) oxide (Fe_3O_4) magnetic nanoparticles [52,91], the magnetic properties of the MIP particles greatly facilitating the development of extraction procedure as recently reviewed [108]. For the extraction of OPPs with this non-exhaustive dSPE method, MIP beads were incubated for 20 min and 2 hours in the

sample to favor the binding of the target analytes. After this time that often corresponds to the equilibrium time required for the binding of the target, the beads are separated from the sample by centrifugation [59], by filtration on a membrane [39,41] or by a simple magnetic field when magnetic nanoparticles are used [52,91]. Beads are then put in contact with a washing solvent to improve the selectivity. The desorption of the target is further ensured by introducing the beads in a solvent that disrupts the MIP-target compounds interactions during an incubation time that must be again optimized.

This dispersive method was applied to the extraction of OPPs from soil [52,91] and vegetable extracts [41,52,59] or water samples [39]. As an example, Figure II.5-2 shows the application of a MIP-dSPE to the extraction of chlorpyriphos from different vegetables [41]. For this, a MIP was polymerized at the surface of silica nanoparticles (NPs) and 20 mg of these NPs were introduced, dispersed in 5 mL of a chloroform extract of vegetable samples. After incubating for 30 min, NPs were recovered by a filtration through a 0.22 µm membrane to be washed with chloroform. Desorption of chlorpyriphos was achieved in 1h in acidified methanol. Despite this time-consuming procedure, a very high selectivity was obtained as illustrated by the comparison of the chromatograms corresponding to the use of MIP NPs to the direct injection of the same extract. The selectivity is also demonstrated by the absence of peak of chlorpyriphos when using non-imprinted (NPs).

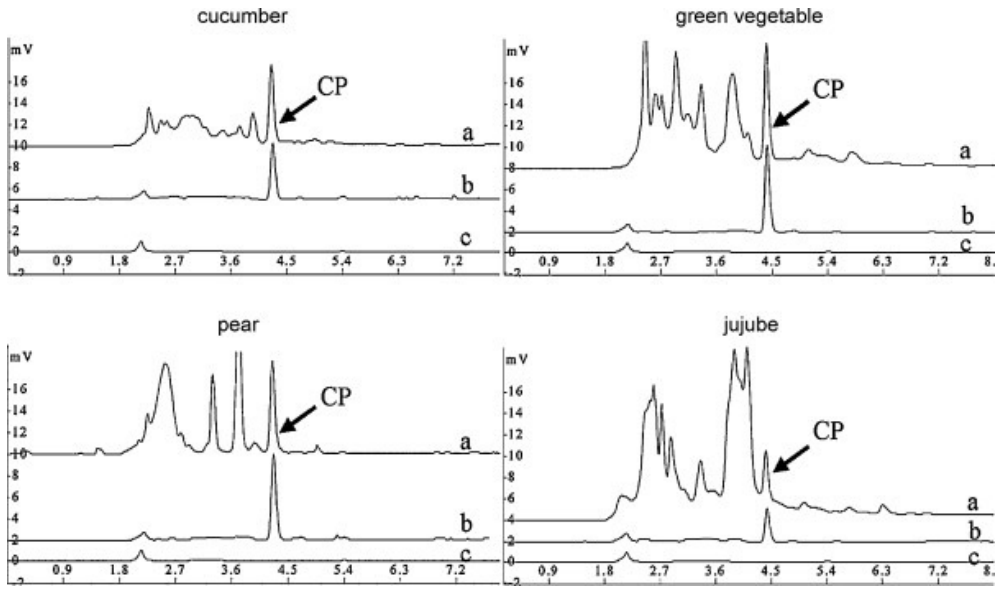

Figure II.5-2. HPLC chromatograms of (a) spiked sample solution containing 1 µg mL^{-1} chlorpyriphos (CP), (b) spiked sample solution extracted with CP-imprinted nanoparticles, and (c) spiked sample solution extracted with non-imprinted nanoparticles [41].

In a similar manner, MIP particles can be dispersed directly in solid matrices to develop a selective matrix solid-phase dispersion (MSPD) method [60,74,98], the MIP replacing conventional sorbent such as silica, bonded silica (C18), Florisil... In this method, forces are applied to the sample by mechanical blending with the sorbent to produce complete sample disruption and the interactions of the sample matrix with this sorbent. The blended material is then transferred and packed into a cartridge suitable for conducting sequential elution with solvents [109]. This approach was used for the extraction of nine OPPs from fruit samples using a MIP produced by dummy approach using 4-(dimethoxyphosphorothioylamino) butanoic acid as template. In their work, authors focused more on finding conditions that favor the recoveries for the nine compounds than on the real selectivity brought by the MIP, the NIP being not studied in parallel in MSPD experiments [60]. To shorten the extraction time and solvent consumption, it was also proposed to combine MSPD with accelerated solvent extraction by submitting the blended material, introduced in a stainless-steel extraction cell, to a pressurized hot organic solvent for the desorption of the target analyte [74].

Other non-exhaustive but nonetheless quantitative methods also based on the equilibrium of the target analyte(s) between small amount of sorbent and the sample such as micro-solid phase extraction (SPME) [43,54,92] or stir-bar sorptive extraction (SBSE) using a MIP as sorbent were proposed for the extraction of OPPs [78].

For SBSE, the stir bar was coated by an imprinted Nylon-6 film imprinted with monocrotophos prepared by phase inversion method [78]. For SPME, the fiber coating was achieved by immersing an activated silica fiber in a sol-gel solution [54,92], the thickness of the MIP layer being defined by the immersion time. A MIP fiber was also prepared by introducing the polymerization mixture in a capillary as a mold [43]. One of this MIP fiber was prepared by sol-gel approach using methyl parathion as template calixarene as functional monomer. As shown Figure II.5-3 the three home-made fibers (MIP, NIP and a blank fiber (synthesized without introducing calixarene)) displayed much better extraction ability than those of the commercial fibers. The positive effect of calixarene on the recognition properties was also proven by the better performance of MIP and NIP-coated fibers compared to blank fiber. This was explained by the contribution of π–π interactions, hydrophobic interactions and inclusion interactions provided by calixarene to enhance the affinity between the fiber and the studied OPPs [92].

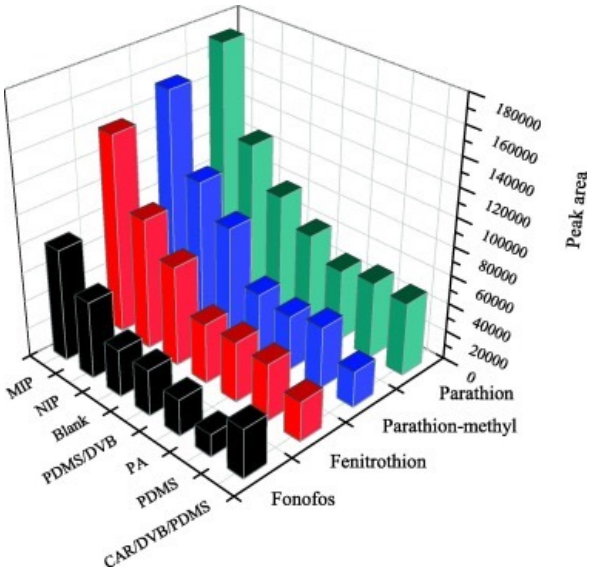

Figure II.5-3. Extraction capability of the prepared fibers and the commercial fibers (polydimethylsiloxane (PDMS), polyacrylate (PA), PDMS/DVB and Carbowax (CAR)/DVB/PDMS) in spiked water samples [92].

This fiber was applied to the extraction of OPPs from different fruits by diluting 2 g of fruit with 2 ml of water and by extracting the OPPs by introducing the fiber in the head-space of the vial. After 30 minutes, the fiber was introduced in the injector of the gas chromatograph for the thermal-desorption of the OPPs at 250°C. The selectivity brought by the MIP is illustrated by the chromatograms reported on Figure II.5-4 that show that the MIP fiber presents higher extraction capabilities than the NIP fiber when applied to pineapple sample [92]. To ensure that the temperature applied for the thermal-desorption of the OPPs from the fiber will not damage the fibers, some authors made thermo-gravimetric analysis of the synthesized polymer showing that a methacrylic MIP supports a temperature up to 400°C [43] while a MIP prepared by sol-gel was thermostable up to 350°C [54].

Among the studied parameters that affect the recovery rate on the fibers, there are the extraction time, extraction temperature [43,92], pH of the sample for methacrylate based MIP [43] and the salt content of the sample [92].

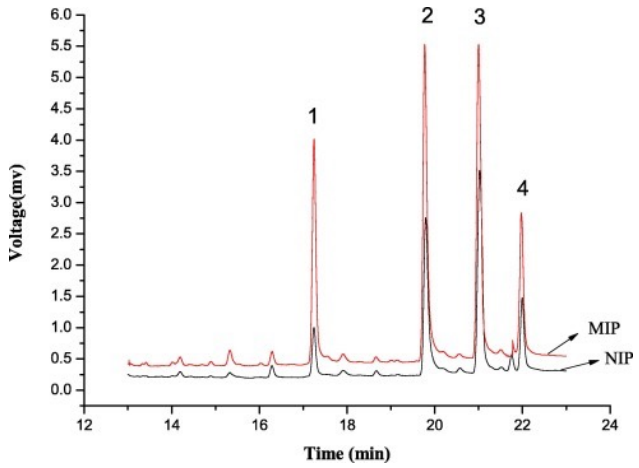

Figure II.5-4. HS-SPME/GC chromatograms of the spiked pineapple samples. Peaks and spiking levels: ***1****, fonofos, 10 µg kg⁻¹;* ***2****, parathion-methyl, 100 µg kg⁻¹;* ***3****, fenitrothion, 60 µg kg⁻¹;* ***4****, parathion, 30 µg kg⁻¹[92.]*

II.6.MIP used as sensors

The development of sensors to detect OPPs constitutes an important field of research as reported by Hassani *et al.* in a review dedicated to the development of biosensors for these pesticides in the environment [110]. This recent review only focused on biological tools as sensing elements of recognition (antibodies, aptamers, enzymes, cells). However, Figure II.2-1 shows that the use of MIPs as recognition elements for the development of sensors represents one third of the applications of MIPs for OPPs. Moreover, as recently reviewed, the introduction of MIP in the development of sensors constitutes todays a very active research field for a broad range of target molecules [111–114]. For this field of application, the major attracting feature of MIPs is their high stability in real media: they can operate in acid or alkaline conditions, at high temperature in aqueous or organic media… Different types of sensors were developed for OPPs such as piezoelectric sensors (QCM, SPR), optical sensors (fluorescence) and electrochemical sensors that are the most reported sensors as illustrated by the works summarized in Table II.6-1.

Table II.6-1. MIP used as recognition element in sensors for OPPs

Type of sensor	Measured signal or technique	Template	Mode of polymerization	Medium	T	Linear range	LOD	Ref.
QCM	frequency shift	parathion or paraoxon	dip-coating of a sol-gel film on QCM crystal	gas phase	10 min	-	-	[86]
	frequency shift	profenfos	in-situ self-assembly of MIP film on gold electrode	real water (after LLE)		10^{-8} to 10^{-5} mg mL^{-1}	0.2 µg L^{-1}	[94]
SPR	wavelength shift	acephate	ultra-thin film anchored on Au slide glass	apple and cole (aqueous extracts)		0.5 to 8 pM	1.14 10^{-13} M (apple), 4.29 10^{-14} M (cole)	[40]
	angle shift	chlorpyriphos	MIP film on Fe_3O_4 NP surface	apple (ACN extract)	> 12h	0.001-10 µM	0.76 nM	[46]
	angle shift	profenofos	ultra-thin film anchored on the surface of an Au-chip	tap water (after LLE)	30 min	0.001 to 0.1 µg mL^{-1}	3.6 10^{-4} µg mL^{-1}	[96]
	wavelength shift		dip-coating of a MIP film on Ag film supported by an optical fiber	real waters		10^{-4} to 10^{-1} µg L^{-1}	2.5 10^{-6} µg L^{-1} (PBS); 2 10^{-4} µg L^{-1} (drinking water); 2 10^{-2}µg L^{-1} (tap water)	[95]

Fluorescence	quenching of QD fluorescence	chlorpyriphos	MIP film on QD	river water	40 min	0.3 to 60 µM	50 nM	[44]
	effect on Eu^{3+} luminescence	chlorpyrifos methyl, diazinon	MIP film on a fiber optic probe	pure water	15 min	5-7 ppt to 100 ppm	250 ppb	[47]
Electro-chemical	SWV	diazinon	MIP NPs mixed with graphite powder	well water, apple	10 min	2.5nM to 0.1 µMol	$7.9\ 10^{-10}$ mol L^{-1}	[53]
	DPV, $Fe(CN)_6^{3-}$	isocarbophos	electropolymerization of a MIP film on GCE	ethanol extracts of vegetable diluted in water	5 min	75 nM to 50 µM	20 nM	[66]
	LSSV	Parathion methyl	electropolymerization of a MIP film on Au-NP modified MWCNT-GCE	ethanol extract of apple and cucumber (concentrated x15 and diluted x 50 in buffer), tap water	6 min		0.08 ng mL^{-1}	[87]
	CV, $Fe(CN)_6^{3-}$		electropolymerization of a MIP film on Au electrode modified by nitrogen-doped graphene sheet	river water		0.1 to 10 µg mL^{-1}	0.01 µg mL^{-1}	[89]
	Impedance /$Fe(CN)_6^{3-}$		electropolymerization of a MIP film on the surface on Au-NP	tap, river, rain waters	280 s	70 nM to 1 µM	34 nM	[88]

	DPV		film formed with a mix of MIP beads (precipitation), ionic liquid and graphene oxide at the surface of GCE	cabbage and apple peel extract diluted in PBS (x10)	150 s	0.01 to 7 µM	6nM	[90]
	DPV	parathion	spin-coating of sol-gel film on CGE	phosphate buffer	20 min	5nM to 0.1 mM	≈ 1 nM	[85]
	SWV		MIP particles (obtained by precipitation) incorporated in a CPE	tap water and cabbage sample	10 min	1.7 to 900 nM	0.5 nM	[84]
	CV		MIP particles obtained by silica gel grafting immobilized on GCE using chitosan	cucumber, cabbage (aqueous extracts)	50 min	0.015 to 15 mg kg^{-1}	3 µg kg^{-1}	[83]
			coating of MIP particles on GCE with DPH	pear juice (diluted in buffer)	210 s	0.1 µM to 10 µM	54nM	[82]
	CV	parathion or paraoxon	spin-coating of a sol-gel film on activated GCE	phosphate buffer	-	-	-	[86]
	DPV	methyl parathion/ parathion/ paraoxon	polymerization of a film at the surface of vinylized MWCNTs	pear, cucumber (extract diluted in a buffer)	3-5 min	0.2 µM to 10 µM	60 nM	[93]

	DPV, $Fe(CN)_6^{3-}$	DMPTAB	electrodeposition of a film of MIP of GCE coated by Fe_3O_4-MWCNT	kidney bean, cucumber (buffer extract)	20 min	10^{-4} to 10^{-1}0M (acephate); 10^{-5} to 10^{-11}M (trichlorphon)	9-70 pM	[63]
Photoelect rochemical	photocurrent measurements	chlorpyriphos	electropolymerization of a film on Au-NP-TiO2-NT	vegetable (hexane extraction, methanol addition, dilution x 1000 with buffer)	15 min	0.05 to 10 µM	0.96 nM	[45]

DPV: differential pulse voltammetry; GCE: glassy carbon electrode; CPE: carbon paste electrode; CV: cyclic voltammetry; DHP: dihexadecyl hydrogen phosphate; LSS: linear stripping sweep voltammetr ; NP: nanoparticles; NT: nanotubes, QD: quantum dots; SW: Square wave voltammetry

One case excepted, the molecule chosen as template for sensor developments was the target molecule that has to be detected in samples when applying the sensor [40,42,44–47,53,66,82,83,85–90,93–96]. In a unique case, DMPTABA was used as template to develop a sensor able to determine the presence of two compounds, acephate and trichlorfon by the same sensor [63].

The development of MIPs for SPE mainly consists in the preparation of MIP as particles (mainly by grinding a monolith obtained by bulk polymerization or by precipitation polymerization). Concerning sensors, in 80% of the cases, MIPs were prepared as a film at the surface of activated NPs, of QDs, of chips, of fibers or on electrodes depending on the type of developed sensors. This film was produced by different methods such as dip-coating, spin-coating, electropolymerization… Nevertheless, MIP particles were mainly involved in electrochemical sensors through immobilizing them on the electrode with the help of a binder. Recently, Gao et al. compared the potential of a sensor developed using MIP particles spin-coated on a QCM electrode or a thin film of MIP produced by *in-situ* self-assembly at the surface of a gold electrode [94]. They showed that the film-based sensor revealed better performances. Indeed, the interface adhesion between the MIP particles and the transducer surface can be poor and the response time can be extremely long due to a because of low mass transfer. Recently, an electrochemical sensor was developed by mixing MIP particles with graphite powder to prepare carbon paste electrode, showing that nanoparticles were better suited than micro particles to develop a highly sensitive electrochemical sensor [53].

Whatever the types of sensor, the film thickness controls the performance of the final sensor. It has been shown that, in the development of electrochemical sensors, a too thick layer gives rise to insulation phenomenon [82]. For these types of sensor, the electropolymerization gains in popularity, certainly owing to the easiest control of the thickness of the MIP film using this approach [114].

As regards QM sensors, the quantification of an OPP was done directly in gas phase [86] or in real water sample extracts after a previous liquid-liquid extraction [94]. For water sample extracts, the quantification was performed by measuring the frequency shift that can be directly correlated with the logarithm of the concentration of profenofos in the liquid sample. The MIP film being prepared at the surface of an electrode, cyclic voltammetry (CV) was first used to evaluate the presence of cavities by

comparing the imprinted film to a non-imprinted one [94].

As for SPR sensors, the quantification of the OPPs was achieved by measuring the angle or the wavelength shift caused by the bounding of the target OPP that is again proportional to the logarithm of the concentration of the target OPP. For a sensor developed for profenofos by dip-coating a MIP film on an optical fiber, a limit of quantification (LOQ) of 2 $x10^{-2}$ µg L^{-1} was reported for tap water [95]. To optimize the sensitivity of this sensor, the authors have studied the effect of the amount of template to be introduced during the preparation of the probe on the sensitivity of the sensors.

Regarding the optical sensors for OPPs, the quenching of QD fluorescence when the MIP layer binds chlorpyriphos [44] or the enhancement of the luminescence intensity of the europium-OPP complex [47] was used to quantify the bound amount of OPP.

Most of the electrochemical sensors were based on the measurement of the current resulting from the reduction of the nitro group of some OPPs such as parathion [82,83,85,90], parathion methyl [90,93] and diazinon [53], the measured current being proportional to the amount of OPPs trapped by the MIP. When OPPs cannot be reduced, the sensors were based on the reduction of hexaferrocyanate that is affected by the OPP binding on the MIP film that alters the electron transfer through the film [63,66,88,89]. With these electrochemical sensors, the measure of the signal can be achieved by different methods such as cyclic voltammetry (CV), linear stripping sweep voltammetry (LSSV), square wave voltammetry (SWV) and differential pulse voltammetry (DPV).

To enhance the selectivity, a washing step with water was introduced between the incubation of the sensors with the sample and the measurement of the amount of OPP bound to the MIP [66,85,88,93–95]. This washing was optimized by Alizadeh showing that-washing for 15 s in a water/acetonitrile (98/2) mixture allows the signal on the NIP to being decreased while maintaining the same signal level on the MIP thus improving the selectivity of the response [84].

The potential of sensors is given by the linearity range and the limit of detection that can be reached for pure sample and its selectivity towards the target OPP. Most of the developed sensors were selective towards the target OPP with a low ability to recognize other OPPs. An illustration of this selectivity is given by Figure II.6-1 that shows the signal obtained for profenofos and 4 other OPPs using an SPR sensor developed for profenofos and by comparing the signal obtained with the MIP-film versus

that with NIP-film [96].

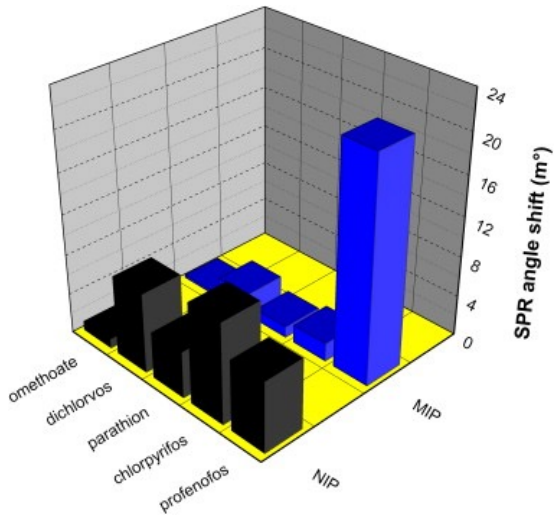

Figure II.6-1. Selectivity of the profenofos SPR-MIP sensor towards the target OPP and four structural analogs. Sample concentrations were 1 and 10 µg mL^{-1} for MIP and NIP, respectively [96].

Using the MIP-film, the responses of the analogs were significantly lower than those of profenofos. In return, the responses of the five compounds using the NIP-film are similar. These results indicate that the MIP-film has cavities that are complementary only to profenofos in shape, size, and function. The absence of cavities on the NIP film gives rise to non-specific absorption that is similar for the five studied compounds and of relatively low strength, the sample being tenfold more concentrated for experiments on NIP than on MIP.

This selectivity can be improved for electrochemical sensors based on the reduction of nitro group because the measured signal cannot be affected by compounds that cannot be reduced in the same conditions. Among the studied parameters to enhance the sensitivity and the selectivity of the sensor response, there is the pH value of the sample when MAA was used as monomer [40,53,82,86,94–96]. The incubation time is also generally studied to optimize the signal. As shown by incubation times reported in Table II.6-1, the latter varies from 3 min to 10 min for half of the applications but it can reach 12 h. These sensors were rarely applied directly to real samples. Only a few studies reported the direct application of the MIP sensors to real water samples with limits of detection (LODs) ranging 8-17 µg L^{-1} (34-50 nM) [44,88,89]. To reach low concentration level in water samples, a previous liquid-liquid

extraction step was introduced to concentrate and transfer the target OPP in an adapted buffer [94,96]. Regarding vegetables, OPPs were previously extracted by water or an aqueous buffer [40,42,63,85] or an organic solvent [45,46] that can be further diluted with water or an appropriate buffer [66,82,87,90,93].

The repeatability of the preparation of the sensor was demonstrated by preparing 5 different electrodes for a sensor developed for methyl parathion with a RSD value of only 6.4% on the signal. Moreover, this sensor showed the same performances after one-month storage [90]. For other electrochemical sensors developed for the same target but prepared by another approach (electropolymerization instead of precipitation polymerization), the stability was ensured for 10 days but a loss of 43% of the signal was observed after 1 month [87]. The repeatability of the synthesis of a sensor for isocarbophos prepared by electropolymerization was also demonstrated by 6 independent preparations and their stability was demonstrated over 30 days [66]. Similar results were obtained for sensors developed for acephate and trichlorfon [63] and for chlorpyriphos [45]. In addition to the study of the stability of the sensor during storage, some authors reported the possibility to re-use them more than 5 [95], 6 [96], 30 [85], 50 [45] up to 200 times [42].

II.7.Miscellaneous applications

Some MIPs were also prepared to be used as stationary phases in HPLC or in electrochromatography. Indeed, to obtain homogeneous particles, MIP particles were prepared by precipitation polymerization and packed in a 150 x 4.6 mm I.D. column to be applied to the separation of several OPPs [49]. The high efficiency that can be expected when using electrokinetic separation methods was exploited by Zhao et al who prepared organic-inorganic hybrid monolithic column by in-situ synthesis of the polymer in a 35 cm x 100 µm I.D. capillary. This capillary was applied to the analysis of trichlorfon in cucumber and cauliflower extracts by electrochromatography [100].

A MIP for trichlorfon was also prepared by bulk polymerization using MAA, EGDMA in chloroform and the resulting particles were used to replace antibodies in an immunoassay- capillary electrophoresis method [101]. As for a conventional immunoassay, an enzyme conjugate was prepared by linking the pesticide to horse radish peroxidase (HRP). A competition between the OPP and its conjugate for the MIP

takes place and after the removal of the supernatant, MIP particles were eluted to inject trichlorfon in capillary electrophoresis for its analysis. The ability of the MIP to recognize other OPPs was measured and low cross-reactivity values of 16% and 13.3% were obtained for monocrotophos and omethoate, respectively, in this competitive context. A LOD of 0.13 µg L^{-1} was obtained in pure media. This method was further applied to vegetable extracts.

II.8.Conclusions

This review demonstrates that the predetermined recognition ability of MIPs for a target OPP, their stability, relative ease and low cost preparation in under various formats (particles, membrane, film…) make them very attractive for being used as alternatives to biological entities such as antibodies for the development of extraction devices and sensors. Although most of the development of MIPs has been carried out in the biological and the clinical fields, their potential as selective tools in analytical techniques dedicated to the environmental domain and food survey is particularly well-illustrated by the numerous developments related to the analysis of OPPs.

Their use in extraction devices certainly remains the most active area but the increasing development in the field of sensors highlights the high potential of MIP for targeted analysis. Indeed, the large range of physico-chemical properties of OPPs renders difficult the design of adequate conditions of synthesis of a MIP able to trap a large number of molecules of this class of pesticides as it was demonstrated for other classes of pesticides such as triazine herbicides.

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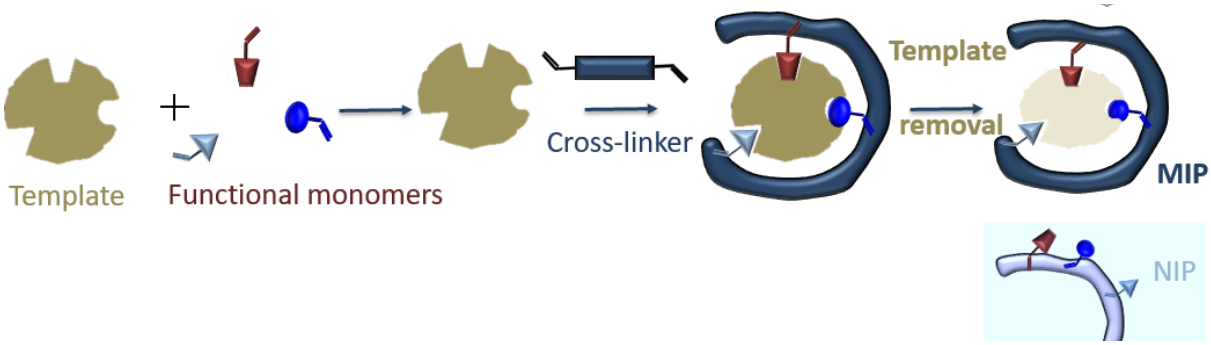
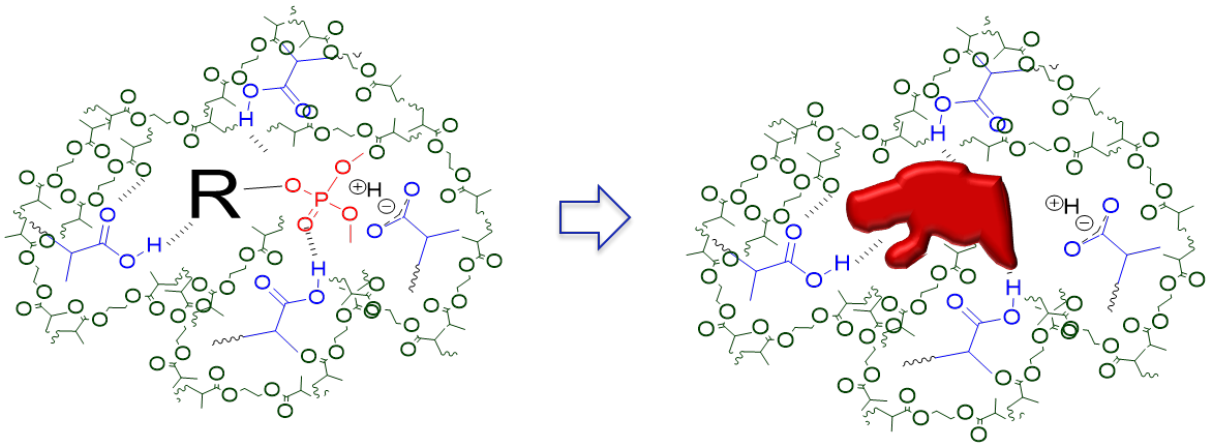


PART II: EXPERIMENTAL

Chapter III: Synthesis and application of molecularly imprinted polymers for the selective extraction of organophosphorus pesticides from vegetable oil



Outres leur utilisation dans l'alimentation, les huiles végétales peuvent également être utilisé comme matières premières pour l'élaboration de produits cosmétiques appliqués sur la peau. Des résidus de pesticides utilisés pour leur culture peuvent donc se retrouver dans ces huiles (notamment la famille des OP largement repondus). Par conséquent le règlement EU No 396/2005 a établi des limites maximales résiduelles (LMR) pour les graines (fixé par défaut à 10 µg/kg) à l'origine de la production de ces huiles. L'étude bibliographique a mis en évidence le potentiel des polymères imprimés pour l'extraction sélective des pesticides de la famille des organophosphorés (OP) de différents types d'échantillon aqueux. Cependant aucune étude n'a porté pour l'instant sur l'extraction sur des polymères imprimés de plus d'un composé organophosphoré à la fois dans les huiles végétales. Une première approche de synthèse de polymère imprimés utilisant une synthèse par voie radicalaire a donc été mise en œuvre pour piéger le plus grand nombre de composés organophosphorés à la fois dans les huiles végétales. Dans cette première partie expérimentale, nous allons introduire rapidement les résultats décrits dans l'article « Synthesis and application of molecularly imprinted polymers for the selective extraction of organophosphorus pesticides from vegetable oils » qui a été accepté le 19 juillet 2017 par « Journal of Chomatography A » et qui se trouve dans les pages suivantes mais également quelques expériences supplémentaires qui ont été réalisés mais qui n'ont pas été intégrés à cet article.

Les OP analysés présentant des disparités structurales importantes et appartenant à une gamme de polarité assez large (log P compris entre 0,7 et 4,7), une méthode de séparation en chromatographie en phase liquide couplé à une détection en UV spécifique des différents OP a donc été développée, pour permettre leur identification et leur quantification. Compte tenu des différences de polarité des OP étudiés, différentes colonnes non polaires : (atlantis C18 (150 x 2.1 mm, 3.5 µm, Waters), fused-core Zorbax Poroshell 120 EC-C18 (50 x 2.1 mm, 2.7 µm, Agilent), accucore RP-MS 120 EC-C18 (100 x 2.1 mm, 2.6 µm, Thermoscientific) et PFP Accucore (150 x 2,1 mm, 2,6 µm, ThermoScientific) ont été testées en utilisant différents gradients de phase mobile. La meilleure séparation (Annexe II (Figure 1)) a été obtenue en utilisant une colonne PFP Accucore (150 x 2,1 mm, 2,6 µm, ThermoScientific) avec un gradient linéaire en utilisant de l'eau (A) et ACN (B). Le gradient commence avec 8% de B pendant 2,5 min et augmente à 60% en 23,5 min, maintenu pendant 2 min, est retourné à la composition initiale en 2 minutes et laissez 2 min pour équilibrer le système. Le

temps de rétention, les longueurs d'onde, la gamme de linéarité de la droite d'étalonnage et les limites de quantification pour chacun des OP sont décrites dans l'Annexe III (Table 1) Les LOQ obtenus sont compatibles avec l'analyse des OP nécessaire à la caractérisation des différents MIP synthétisés en milieu pur.

Un criblage des conditions de synthèse (différentes molécules empreinte, monomères ou solvants) a été réalisé et à conduit à la synthèse de six polymères à empreintes moléculaires différents (MIP). Les performances de ces différents MIP ont été évaluées par extraction sur phase solide afin de déterminer le plus sélectif et celui capable de piéger le plus grand nombre d'OP possible. La sélectivité a été évaluée en étudiant en parallèle l'extraction des OP en milieux pur par les MIP et par les polymères non imprimés (NIP) correspondants (analyse des fractions issus de l'étape de percolation, de lavage et d'élution). L'analyse chromatographique de ces fractions issues de l'extraction sur MIP/NIP nécessite une étape d'évaporation préalable (solvant utilisé lors de l'extraction sur MIP/NIP incompatible et/ou insoluble avec la phase mobile). Des tests d'évaporation ont donc été réalisés avec des solutions standards, il n'a été observé aucune perte pour les différents OP sauf pour le dichlorvos (composé volatile avec une tension de vapeur très élevé par rapport aux autre pesticides, de 2700 mPa à 25° C) pour lequel une perte pouvant atteindre jusqu'à 15% pour cette seule étape d'évaporation a été observé. Ce composé a donc finalement été écarté de l'étude.

Le support MIP le plus prometteur a été obtenu en utilisant le monocrotophos, comme molécule empreinte, l'acide méthacrylique, en tant que monomère et le diméthacrylate d'éthylène glycol, comme agent réticulant et un ratio molaire de 1/4/20 respectivement pour ces trois éléments. Ce MIP a permis d'extraire sélectivement cinq OP modérément polaires : le methidathion, le malathion, le diazinon, le fenitrothion et le fenthion (log P compris entre 2,5 et 3,7) en milieu pur « hexane » (solvant choisis car il s'agit du solvant couramment utilisé pour extraire et/ou diluer les échantillons d'huiles). Ensuite après avoir montré la répétabilité de la procédure d'extraction sur le MIP en milieu pur, déterminé la capacité du support ainsi que la répétabilité de la procédure de synthèse du MIP, les performances de ce polymère ont été évaluées en milieu réel.

Au vu de la complexité de la matrice « huile » et du niveau de concentration recherché dans ces échantillons, une méthode de séparation et de quantification a été développée en LC-MS/MS pour les OP ciblés (détails dans la section Matériel et méthode

de la publication). Une première procédure d'extraction des OP réalisée sur trois huiles différentes (olive, tournesol et amande) sur le MIP a montré que le comportement du MIP est similaire pour les trois huiles testées. Néanmoins une baisse du rendement importante ayant été observée dans ces conditions, une optimisation de la procédure d'extraction (volume des étapes de lavage) a donc été réalisée sur l'une des huiles (l'huile d'amande). Cette optimisation a permis d'obtenir de la rétention et de la sélectivité pour trois OP (methidathion, malathion et diazinon) dans l'huile d'amande.

III.Article 1

Synthesis and application of molecularly imprinted polymers for the selective extraction of organophosphorus pesticides from vegetable oils

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III.1.Abstract

The increasing use of pesticides in agriculture causes environmental issues and possible serious health risks to humans and animals. Their determination at trace concentrations in vegetable oils constitutes a significant analytical challenge. Therefore, their analysis often requires both an extraction and a purification step prior to separation with liquid chromatography (LC) and mass spectrometry (MS) detection. This work aimed at developing sorbents that are able to selectively extract from vegetable oil samples several organophosphorus (OPs) pesticides presenting a wide range of physico-chemical properties. Therefore, different conditions were screened to

prepare molecularly imprinted polymers (MIPs) by a non-covalent approach. The selectivity of the resulting polymers was evaluated by studying the OPs retention in pure media on both MIPs and non-imprinted polymers (NIP) used as control. The most promising MIP sorbent was obtained using monocrotophos (MCP) as the template, methacrylic acid (MAA) as the monomer and ethylene glycol dimethacrylate (EGDMA) as the cross-linker with a molar ratio of 1/4/20 respectively. The repeatability of the extraction procedure and of the synthesis procedure was demonstrated in pure media. The capacity of this MIP was 1 mg/g for malathion. This MIP was also able to selectively extract three OPs from almond oil by applying the optimized SPE procedure. Recoveries were between 73 and 99% with SD values between 4 and 6% in this oil sample. The calculated LOQs (between 0.3 and 2 µg/kg) in almond seeds with a SD between 0.1 and 0.4 µg/kg were lower than the Maximum Residue Levels (MRLs) established for the corresponding compounds in almond seed.

Keywords: molecularly imprinted polymers; organophosphorus pesticides; solid phase extraction; vegetable oils; liquid chromatography; mass spectrometry.

III.2. Introduction

Vegetable oils occupy a large place among food products and their nutritional or health contribution does no need demonstration. Their constituents play a very important role in human health. In addition, their beneficial properties in cosmetics have been known since antiquity by nourishing, protecting and moisturizing the skin. However, pesticides used in agriculture may possibly be found in vegetable oils. The EU harmonization of the pesticides Maximum Residue Levels (MRLs) within Regulation 396/2005 has led to specific MRLs being set on raw materials (oil seeds and oil fruit), but not on processed products. A processing factor was proposed by FEDIOL (vegetable oil and protein meal industry association), to define the limits allowed in the processed products such as vegetable oils, fats and meals. To reach the MRLs values that are established at 10 µg/kg by EU for pesticides as general default for food or feed constitutes a significant analytical challenge for the safe use of such oils.

Organophosphorus (OPs) compounds constitute an important class of pesticides whose toxicity arises from the inhibition of the acetylcholinesterase

enzyme. They exhibit a wide range of physicochemical properties thus rendering their determination in complex oil samples particularly difficult. Their analysis often requires a previous extraction step using gel permeation [1] or a liquid-liquid extraction (LLE) step [2] that is nowadays usually followed by a purification step by dispersive solid-phase (dSPE) extraction, *i.e.* a global QuEChERS-based procedure adapted for fatty matrices [3–5]. Primary secondary amine (PSA), octadecylsilica (C18) and graphitized carbon black (GCB) are the three most commonly used sorbents for QuEChERS. However, their amount and their proportion when they are used in combination must be optimized to reach the most powerful clean-up effect without affecting the extraction recovery of the target analytes [5]. Indeed, it was recently shown that the addition of GCB to PSA/C18 was efficient for trapping oil components and their removal from the extract but also affects the extraction recovery for some compounds [3].

These drawbacks led to the recent development of molecularly imprinted polymers (MIPs). These synthetic polymeric materials possess specific cavities designed for a template molecule involving a retention mechanism based on molecular recognition. The MIPs have been already successfully used in several fields, such as sensors, organic synthesis and separation of enantiomers [6–9]. The first application of a MIP as SPE sorbent was carried out by Sellergren *et al.* in 1994 for extracting pentamidine present at low concentrations in urine [10]. The principle of selective extraction on a MIP is the same as for a conventional SPE sorbent. After a conditioning step, the sample is percolated through the MIP and a washing step removes the interfering compounds. The desorption of analytes is achieved by percolating a solvent able to develop interactions with the sorbent in order to desorb the analytes retained on the MIP. Several MIPs dedicated to the selective extraction of mycotoxins, drugs, pollutants or steroids are now commercially available.

The development of MIPs for the extraction of OPs has been largely reported these last years. MIPs were prepared as particles to be used in cartridges between two frits as SPE sorbent [11–26] or as dispersive sorbent for dSPE [27–31] and for matrix solid-phase dispersion (MSPD) [32–34] or as a thin film in solid-phase microextraction (SPME) [35–37] or in stir bar sorption extraction (SBSE) [38]. They were applied to the selective extraction of OPs from vegetable extracts (cucumber, lettuce, apple, pear…) and environmental samples such as waters and soil extracts.

In the common approach, the synthesis of MIPs involves first the complexation of a template molecule with functional monomers through non-covalent bonds in a porogenic solvent, followed by polymerization of these monomers around the template with the help of a cross-linker in the presence of an initiator. The choice of the chemical reagents used for the synthesis of the MIP must be judicious in order to really create specific cavities designed for the template molecule. In 85% of the reported works, MIP for OPs were produced in fixed conditions without optimizing the nature and the ratio of the reagents. The target OP was taken as template molecule, methacrylic acid as monomer, ethylene glycol dimethacrylate as cross-linker in a non protic solvent (mainly acetonitrile, dichloromethane and chloroform). The effect of the template was studied only once for the development of an MIP for dimethoate and its metabolite omethoate showing that the metabolite was better adapted for the trapping of both molecules [19]. A few studies described the synthesis of an MIP by varying the nature of the monomer [11,19,28,30,31] and/or the porogen [11,25,30,31] or the template/monomer ratio [24]. In some studies, the choice of the monomer for a given template resulted from studies by molecular modeling and computational design [17,19,23]. Once, the MIP synthesized, its selectivity was mainly evaluated by binding experiments or retention studies in pure media. These evaluations were achieved by comparing results using the MIP with results obtained using a non-imprinted polymer (NIP) that is prepared in the same conditions as MIP but in the absence of template. In most reported works, these studies were carried out using up to three OPs including the OP used as template. This comparison between MIP and NIP achieved in spiked pure media allows to put in evidence the presence of cavities in the MIP and is also useful to optimize the extraction procedure that must give rise to high extraction recovery on the MIP and low one on the NIP [8]. Except for one reported work [21], the conditions of extraction finalized in pure media were applied to real samples [11,17–20,22–27, 38] without a control of the selectivity by using the NIP or without re-optimization of the extraction conditions to circumvent matrix effects as already reported [8]. These matrix effects were well illustrated by Sanagi *et al.* who reported recoveries obtained in pure media and in real samples after applying the same extraction procedure on MIP and on NIP [21]. While recovery of extraction for quinalphos in pure media was 92.3% and 43.9% for MIP and NIP respectively, the recovery was 99% and 64.8%, respectively for a real sample, thus illustrating the effect of the matrix components that increase the retention on both

sorbents and induce consequently a loss of selectivity. At last, very few works reported the application of MIPs for the selective extraction of OPs from vegetable oils. These works were carried out by Bakas *et al.* who studied the extraction of methidathion [17], dimethoate [19] and fenthion [23] from olive oil samples.

The objectives of this work were to prepare a MIP able to extract from vegetable oil the maximum number of OPs that were selected by taking into account the risk of their occurrence in such samples. For this, different conditions of synthesis were screened by varying the nature of the template, of the monomer and of the porogenic solvent in order to find the conditions of synthesis of a MIP able to selectively trap the largest number of OPs from vegetable oils The MIP resulting in best selectivity for five OPs was studied more in detail by investigating its behavior towards ten OPs from pure media but also from vegetable oils whose content may affect the recoveries on the MIP. At last, to highlight the potential of the developed MIP, a comparison with results obtained while applying C18 silica to an almond oil extract was performed.

III.3. Materials and methods

III.3.1.Chemicals

HPLC-grade acetonitrile (ACN), methanol (MeOH), dichloromethane (DCM) and toluene were supplied by Carlo Erba (Val de Reuil, France). High purity water was dispensed by a Milli-Q purification system (Millipore, Saint Quentin en Yvelines, France).

Certified reference material : dimethoate (DMT) 98%, fenthion sulfoxide (FSX) 99%, fenthion sulfone (FSN) 99%, methidathion (MTH) 98%, malathion (MAL) 99%, fenitrothion (FNT) 98%, diazinon (DIZ) 98%, pirimiphos methyl (PIM) 99.5%, fenthion (FEN)99% and chlorpyrifos-ethyl (CLE) 99.5% were supplied by Cluzeau Info Labo (Saint-Foy-La-Grande, France). Individual stock solutions from each OP were made at a concentration of 100 mg/L in ACN. A stock solution mixture containing 5 mg/L of each OP was prepared in ACN and stored at 4 °C until further use.

Parathion ethyl (PE), monocrotophos (MCP), fenamiphos (FEM), 2-trifluoromethyl acrylic acid (TFMA) 98%, acetonitrile anhydrous 99.8%, ammonium acetate for HPLC 99.0% (AC), n-hexane, methacrylic acid (MAA) and ethylene glycol dimethacrylate (EGDMA) were supplied by Sigma-Aldrich (Saint Quentin Fallavier, France). Washed EGDMA and MAA were distilled under vacuum in order to remove

inhibitors. Azo-N,N'-bis-isobutyronitrile (AIBN) was purchased from Acros Organics (Noisy-le-Grand, France). Acetic and formic acids (AA and FA respectively) were purchased from VWR (Fontenay-sous-Bois, France).

III.3.2.Apparatus and analytical conditions

The LC-MS/MS analyses were performed using a liquid chromatograph (UltiMate 3000®, Thermo Scientific, Illkirch, France) coupled with Triple Stage Quadrupole Mass Spectrometer (TSQ Quantum Access MAX, Thermo Scientific, Illkirch, France) equipped with a heated electrospray ionization source (HESI2). The chromatographic separation was performed on Accucore PFP column (150 x 2.1 mm, 2.6 µm, ThermoFisher Scientific, Villebon Courtaboeuf France) maintained at 32 °C with a column oven (Croco-cil, Interchim). Samples were analysed using linear gradient elution with water containing 0.1% (v/v) of FA and 4 mM of AC (A) and MeOH containing 0.1% (v/v) of FA and 4 mM of AC (B). The gradient started at 20% of B during 2.5 min and increased to 80% of B in 23.5 min, held for 2 min, and returned to initial composition within 2 min and let 2 min to equilibrate the system. The flow rate was set at 0.4 mL/min and the injection volume was 2 µL.

For the capacity study, the LC gradient was shorter. This new gradient started with an equilibration during 2 minutes with 20% of B and increased to 80% in 5 min, held for 3 min, and returned to initial composition within 2 min and let 2 min to equilibrate the system.

MS was operated in positive ion mode with MRM detection using an electrospray voltage of 3500 V and a skimmer offset of 5 V. Capillary and vaporizer temperatures were set at 280 °C and 295 °C respectively. Sheath gas pressure and auxiliary gas pressure were set respectively at 55 and 15 units. Nitrogen was used as nebulizer and desolvatation gas and argon as the collision gas at a pressure of 1.5 mTorr. For the optimization of the MS detection, each OP was infused at a concentration of 5 mg/L in the mixture A/B (50/50, v/v). The quantification of 10 the OPs was performed in MRM mode using the specific transitions FEN and FNT both gave a very low signal intensity during infusion. A second transition was used for confirmation purposes and to avoid false positive responses. The m/z, tube lens and collision energies values corresponding to quantitation and confirming ions were summarized in the Annexe IV (Table 2).

The LC-DAD analyses were performed using a liquid chromatograph (LC) Agilent

1200 series (Agilent Technology, Massy, France) system equipped with a binary pump, an auto sampler and a diode array detector (DAD) controlled by a Chemstation software. OPs were separated using the same column, flow rate and injection volume as for LC-MS/MS analysis. Samples were analysed using linear gradient elution with water (A) and ACN (B). The gradient started with 8% of B during 2.5 min and increased to 60% in 23.5 min, held for 2 min, returned to initial composition within 2 min and let 2 min to equilibrate the system. DMT, MTH, MAL were quantified at 210 nm, FSX at 240 nm, FSN at 230 nm, FNT at 270 nm, DIZ, PIM and FEN at 250 nm and CLE at 290 nm.

III.3.3. Synthesis of the MIPs

MIPs were synthesized as bulk using a non-covalent approach. Different combinations of templates; monomers and solvents were tested (Table III.3-1). A template/monomer/cross-linker molar ratio of 1/4/20 was used for all syntheses. Briefly 0.25 mmol of template and 1 mmol of monomer were dissolved in 1.4 mL of solvent in a glass tube (14 mm i.d.). Then, 5 mmol of the cross-linker (EDGMA) and 10 mg of the initiator AIBN, were added to the mixture and purged by nitrogen for 10 min. The tube was sealed and placed in a water bath at 60 °C for 24 h. A non-imprinted polymer was simultaneously prepared in the same conditions but without adding the template. Each obtained polymer was crushed, ground automatically in a mixer MIL MM 301 from Retsch® at 35 s^{-1} for 3 x 1 min and sieved in a vibratory sieve shaker from Retsch ® using amplitude of 15 mm/g for 5 min. The particles sizes between 25 and 36 µm were collected and a sedimentation with 4 x 5 mL of MeOH/water 80/20, v/v was performed to remove the thin particles and then dried 24 h at room temperature.

After that, between 25 and 35 mg of polymer were packed in a 1 mL disposable cartridge of propylene (Interchim) between two polyethylene frits (20 µm, Sigma-Aldrich). The polymer was washed with MeOH (approximately 10 mL) containing 10% of AA (v/v). The washing fractions were evaporated and suspended in MeOH, ACN and H_2O (40/10/50, v/v/v) for the MCP template, and in ACN for the other templates before injection in LC-UV. The polymers were washed until the template could no longer be detected in the washing fraction by LC-UV at 210 nm for MCP, 250 nm for DIZ and F, and 280 nm for PE. Then the cartridge was washed with 10 mL of MeOH to remove residual AA.

Table III.3-1. Conditions of the synthesis of six MIPs, using AIBN as initiator and a molar ratio template/monomer/cross-linker of 1/4/20. NIPs were synthetized in the same conditions without introducing the template. MAA: methacrylic acid, EGDMA: ethylene glycol dimethacrylate, TFMA: 2(trifluoromethyl) acrylic acid, DCM: dichloromethane, ACN: acetonitrile.

Sorbent	Template	Monomer	Cross-linker	Porogen
MIP 1	PE	MAA	EGDMA	DCM
MIP 2	MCP	MAA	EGDMA	DCM
MIP 3	F	MAA	EGDMA	DCM
MIP 4	DIZ	MAA	EGDMA	DCM
MIP 5	DIZ	MAA	EGDMA	ACN
MIP 6	DIZ	TFMA	EGDMA	ACN

III.3.4. SPE procedure applied in pure media

Different studies were carried out on the synthesized polymers to optimize the SPE procedure, as the selection of the percolation solvent or the washing solution. Before the percolation, the cartridges were conditioned with 4 mL of the used percolation solvent. Then 1 mL of percolated solvent (toluene, DCM, hexane or mix of hexane, DCM and ACN (70/29/1, v/v/v)) spiked with 1 mg/L of PE was passed through MIP/NIP 1 cartridges. To study the washing solvents, a spiked solution of hexane using six OPs at 1 mg/L was used as percolation solution on the 6 synthesized MIPs/NIPs. Three washing steps were included in an SPE procedure: 1 mL of hexane and DCM (80/20, v/v), 1 mL of hexane, DCM and ACN (80/18/2, v/v/v) and 1 mL of hexane, DCM and ACN (80/15/5, v/v/v). The second procedure applied to the six synthesized MIPs consisted in a single washing step with 1 mL of a mixture of hexane and DCM (95/5, v/v). After the washing step, the cartridge was dried by 5 mL of air. Finally, the OPs were eluted with 1 mL of ACN. Each fraction resulting from each step was evaporated to dryness by a nitrogen stream and was resuspended in 0.5 mL of ACN before injection in the LC-DAD system.

III.3.5. Extraction of OPs from the vegetable oils

III.3.5.1.Preliminary extraction procedure for the vegetable oils

Before the SPE procedure using MIP or NIP sorbents, an LLE was performed on oil samples. This LLE procedure was described by the ITERG (French Institute

specialized in fats and oils) and used before an SPE step using a C18 sorbent. LLE was carried out using 3 x 1 mL of a mixture of ACN and DCM (90/10, v/v) for 200 mg of oil. The obtained oil extract was evaporated to dryness under nitrogen stream and was spiked with 2.5 mg/kg of ten OPs in 1 mL of hexane and passed through the MIP 2 and NIP 2 cartridges. After a conditioning step with 4 mL of hexane, the oil extract was percolated and 1 mL of a mixture of hexane and DCM (95/5, v/v) was used for the washing step. Finally, the OPs were eluted in 1 mL of ACN. The elution fraction was directly injected in the LC-MS/MS and LC-UV systems. For the clean-up on C18, 12 mL of MeOH and 12 mL of ACN were passed through the cartridge for conditioning, then 3 mL of oil extract resulting from the LLE step were percolated, and 1.5 mL of MeOH was used for the elution step. The elution fraction was recollected and evaporated under nitrogen stream. Finally, the dry extract was suspended in ACN, before its analysis by LC-MS.

III.3.5.2.Optimized extraction procedure on MIP for the vegetable oils

Optimization of the SPE procedure was necessary to reach the MRLs established by the regulation (EC) No 396/2005 of the European Parliament and of the Council of 23 February 2005. LLE was carried out as in the previous section. The obtained oil extract was evaporated to dryness under nitrogen stream, diluted with 10 mL of hexane and was spiked with a low concentration of 100 µg/kg of three OPs (MTH, MAL, DIZ). After conditioning the MIP/NIP with 4 mL of hexane, 1 mL of the oil extract was percolated through MIP/NIP cartridges and different volumes of washing solution hexane and DCM (95/5, v/v) were tested: 0.4, 0.65, 0.8 and 1 mL. Finally, the OPs were eluted with 1 mL of ACN. The elution fraction was evaporated to dryness under nitrogen stream and suspended in 100 µL of ACN before injection in the LC-MS/MS system.

III.3.5.3. Study of the capacity

The study of the capacity of the MIP was performed using percolation solutions that contained different amounts (between 0.5 and 87 µg) of MAL in 1 mL of hexane through the MIP and NIP cartridges. Before percolation, the cartridges were conditioned with 4 mL of hexane. Then, for the washing step, 1 mL of hexane and DCM (95/5, v/v) was passed through the cartridge. Finally, the OPs were eluted with 1 mL of ACN. The elution fractions were diluted with ACN taking into account the linearity range of MAL

(10-250 µg/L) and were directly injected in LC-MS/MS using the specific transition (348→ 127) for the quantitation of MAL.

III.4. Results and discussions

III.4.1. Development of the LC-UV and LC-MS analyses

To ensure a good quantification of the OPs, the development of an analytical separation was necessary. Taking into account the hydrophobicity of the studied OPs (see Figure III.4-1), different non-polar columns were tested using different linear gradient modes. The first column, Atlantis C18 (150 x 2.1 mm, 3.5 µm, Waters), was not able to separate PE and FEN. Hence, a fused-core column Zorbax Poroshell 120 EC-C18 (50 x 2.1 mm, 2.7 µm, Agilent), was tested but a low resolution was obtained for DIZ and FEN. A third column, Accucore RP-MS 120 EC-C18 (100 x 2.1 mm, 2.6 µm, Thermoscientific) yielded a better resolution for DIZ and FEN, but the separation of MAL and FNT was not possible with this column. Finally, the best separation was obtained using an Accucore PFP column (150 x 2.1 mm, 2.6 µm, Thermoscientific). The LOQ values (defined as the concentration level that gives a signal to noise ratio S/N of 10) ranged from 30 to 300 µg/L depending on the OPs (Table III.4-1) using the LC-UV conditions described in Section III.3.2.

These values of LOQ allowed the OPs to being quantified and the performance of the MIPs to being evaluated in pure media. However, for the studies related to the application of the MIPs to oil samples, it was necessary to develop and to use the more sensitive LC-MS/MS method in MRM mode in order to decrease the LOQs (operating conditions described in Section III.3.2). The obtained LOQs are summarized in Table III.4-1, and range from 0.4 to 7 µg/L for the OPs in pure media, with the exception of FEN whose, the estimated LOQ was 1000 µg/L.

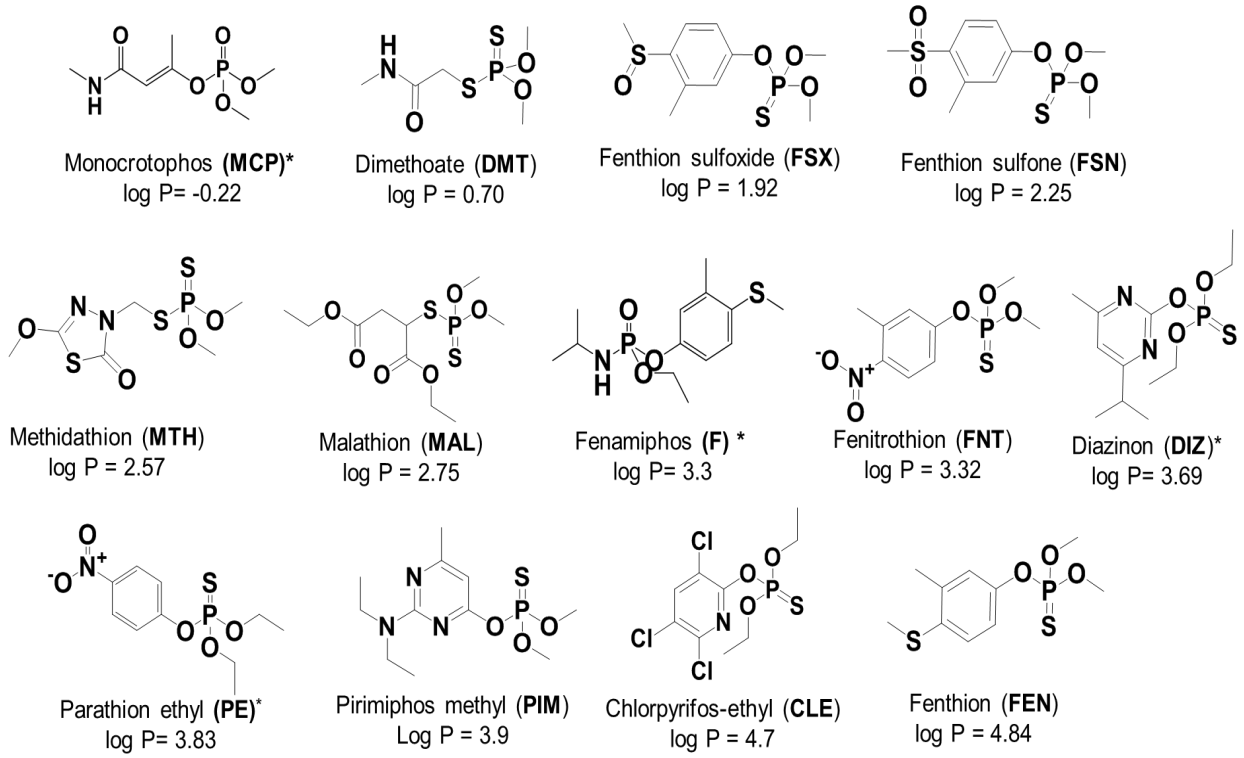

Figure III.4-1. Chemical structure and partition coefficient of ten OPs and of the templates. Log P values are issued from Pesticide Properties Database from University of Hertfordshire.*

Table III.4-1. Comparison of LODs (S/N= 3) and LOQs (S/N= 10) in µg/L obtained in LC-UV and LC- MS/MS and estimated by injecting OPs at 200 µg/L in LC-UV and at 5 µg/L in LC-MS (except for FEN, 1000 µg/L).

Compounds	LC-UV		LC-MS/MS	
(OPs)	LOD	LOQ	LOD	LOQ
DMT	50	160	0.6	2.2
FSX	20	70	0.1	0.4
FSN	2	10	2.1	6.9
MTH	50	170	0.3	0.9
MAL	90	300	0.2	0.8
DIZ	50	160	0.08	0.3
FNT	20	50	No signal	No signal
FEN	10	30	300	1000
PIM	20	60	0.2	0.8
CLE	30	90	0.4	1.3

III.4.2.Screening of the synthesis conditions

III.4.2.1.Choice of the MIP synthesis conditions

Several synthesis conditions were screened in order to determine which ones resulted for the largest number of OPs in the highest selectivity during the extraction procedure, *i.e* a low retention of OPs on the NIP and a strong retention on the MIP. The non-covalent approach was selected because it is the most common one used to prepare MIPs for SPE [40]. The synthesis of MIPs involves first the complexation of a template molecule with a functional monomer, through non-covalent bonds, followed by polymerization of this monomer around the template with the help of a cross-linker and in the presence of an initiator [41]. Finally, the template molecule is removed from the highly cross-linked polymer, thus leaving specific cavities complementary to the template in shape, size and functionality.

Target analytes were selected by taking into account the most frequently detected OPs in different vegetable oils by ITERG. As these OPs presented a large structural variety and a broad range of octanol-water partition coefficients (Figure III.4-1), different templates were used to prepare the MIPs (Table III.3-1). PE was selected for the MIP 1 synthesis because it is an analogue of FNT. MCP was used for the MIP 2 synthesis because it has a linear structure like DMT and MAL. F was selected for the MIP 3 synthesis because it presents similarities, namely the benzyl and the phosphate groups with FEN, FSX and FSN. Finally DIZ, although it is also a target OP, was used for the MIP 4 synthesis because it presents similar heterocycles and a thiophosphoric (P=S) group as CLE, PIM and MTH. The choice of the functional monomer also constitutes one of the most important factors governing the properties of MIPs. MAA was used for MIPs 1 to 5 and TFMA for MIP 6. These monomers were selected because the OPs include nitrogen, oxygen and sulfur atoms that can form electrostatic interactions with these acidic monomers or hydrogen bonds. In order to enhance this type of interactions, slightly polar and non-protic solvents, DCM (MIP 1 to 5) or ACN (MIP 5) were tested. To obtain a highly cross-linked structure, an excess of cross-linker, EGDMA, was added to the polymerization mixture. Once the six MIP/NIPs were synthesized, the optimization of the SPE procedure was necessary to evaluate the performance of these supports.

III.4.2.2.Choice of the percolation solvents

In order to select the solvent favoring the retention of OPs on the synthesized MIPs, a preliminary experiment was carried out by percolating different solvents spiked with one OP on one MIP only, MIP 1, the nature of the expected interaction between the OPs and the MIPs being similar, *i.e*. polar interactions. PE was selected for this experiment, as it is one of the selected templates. To favor the specific interactions between the monomer and the target compounds during the percolation step, solvents with low polarities (toluene, DCM, hexane and a mix of hexane, DCM and ACN (70/29/1, v/v/v)) spiked with 1 mg/L of PE were passed through MIP/NIP 1 cartridges. PE was not retained during the percolation step in toluene, and was weakly retained in DCM (50%) and in the mixture of hexane, DCM and ACN *(*70/29/1*,* v/v/v*)* (50%), whereas using hexane as percolation solvent, the retention was strong on MIP and on NIP. Therefore, hexane was selected as the solvent of percolation to evaluate the retention on other MIPs.

III.4.2.3.Comparison of the synthesized MIPs

To favor the selectivity brought by the MIPs, the washing step was optimized to decrease the retention on NIPs (that is caused by non-specific interactions at the surface of the polymer) while maintaining a high retention on the MIPs by specific interactions that should take place in their cavities. For this experience, and to limit data treatment, the retention of six OPs among the ten was studied on the six synthesized MIPs/NIPs (Table III.3-1). These OPs (FSX, MAL, DIZ, FNT, FEN and CLE) were selected according to their polarity, from one of the most to the less polar (FSX, FEN respectively) and by adding four other OPs of intermediate polarities in order to cover the whole range of polarity. The cartridges were conditioned first with 4 mL of hexane, then 1 mL of hexane spiked with 1 mg/L of six OPs was percolated on each MIP/NIP. Three successive washing steps were applied: 1 mL of hexane and DCM (80/20, v/v) (W1), 1 mL of hexane, DCM and ACN (80/18/2, v/v/v) (W2) and then 1 mL of hexane, DCM and ACN (80/15/5, v/v/v) (W3), the augmentation of the polarity of the mixture increasing its elution strength. As observed for PE, most of the six OPs were retained during the percolation step, but more than 70% were lost during the first washing step (W1) from the six MIPs/NIPs. However, CLE was not retained (loss during percolation step) on the six MIPs/NIPs because it was not able to develop strong polar interactions with the MIP.

To optimize the selectivity for the retained OPs, a washing solution of a lower elution strength was tested by introducing only 5% of DCM in hexane (1mL). The elution step was ensured with a more polar solvent, *i.e* acetonitrile. In these conditions, a strong retention of the studied compounds was obtained but without any selectivity for MIP 5 and MIP 6. Indeed, similar extraction profiles were obtained on MIP 5 and NIP 5 and on MIP 6 and NIP 6. Therefore, these two supports were removed from the study. For the four other MIPs/NIPs, the recovery of the five OPs in the elution fraction by applying this extraction procedure is reported on Figure III.4-2 . The comparison of MIPs and NIPs in these conditions shows that no selectivity (MIP 1 and MIP 3) or a low selectivity (MIP 2 and MIP 4) was obtained for FSX, which is the most polar of the studied compound. At this stage, an improvement in selectivity for all the MIPs could be expected for this strongly retained compound by increasing the elution strength of the washing. Nevertheless, MIP 4 presented a good retention for the five OPs but a lower selectivity than the three other MIPs for MAL and FEN. MIP 4 was then removed from the study. The three other MIPs were very similar in terms of retention and selectivity. However, MIP 1 appears less retentive than MIPs 2 and 3 (especially for DIZ and FEN). Finally, according to the retention and the selectivity observed for DIZ that was higher on MIP 2 than on MIP 3, MIP 2 was selected and named MIP for the rest of the study.

Additional studies were carried out on this MIP to improve the retention of these four compounds by changing the elution strength of the washing solution using 7% or 3% of DCM instead of 5%, but no improvement was observed in terms of selectivity or retention.

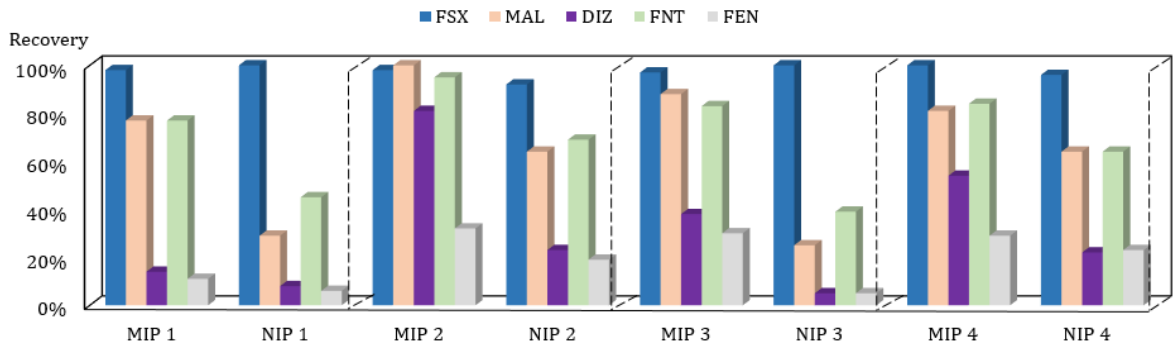

Figure III.4-2. Recovery of five OPs in the elution fraction obtained on four MIPs/NIPs by applying the screening extraction procedure including the percolation of 1 mL of hexane spiked with 1 mg/L of each OP, a washing with 1 mL of hexane/DCM 95/5 (v/v) and an elution with 1 mL of ACN. The average recovery (%) ± SD (n= 3) for MIP/NIP 2 and MIP/NIP 4 and the average recovery (%), (n= 2) for MIP 1 and MIP 3 are reported.

III.4.3.Potential of the MIPs towards OPs

To evaluate the potential of this MIP for the selective extraction of the ten OPs, the previously developed extraction procedure was further applied in triplicate to the ten OPs of interest (Figure III.4-3A). To confirm its potential, the same experiment was carried out on a MIP resulting from a second independent synthesis (Figure III.4-3B). The extraction profile represents recovery obtained in the percolation, washing and elution fractions on MIP and on NIP (Figure III.4-3A and B). The target OPs can be gathered together in three different groups according to their behavior on MIP/NIP. The MIP does not present any selectivity for the most polar OPs (DMT, FSX, FSN and DMT) because the retention was strong (up to the elution fraction) and was the same on MIP and NIP. Some selectivity was obtained for the non-polar OPs (PIM and CLE) because their extraction profiles on MIP and NIP were different but their retention was low since they were mainly recovered in the washing fraction. In return, a high retention and a satisfactory selectivity for moderately polar compounds was obtained. For example, the recovery of DIZ in the elution fraction was 81 ± 8% for the MIP and 23 ± 11% for the NIP. SD values between 2 and 13% (n= 3) also indicate the good repeatability of this MIP-SPE procedure.

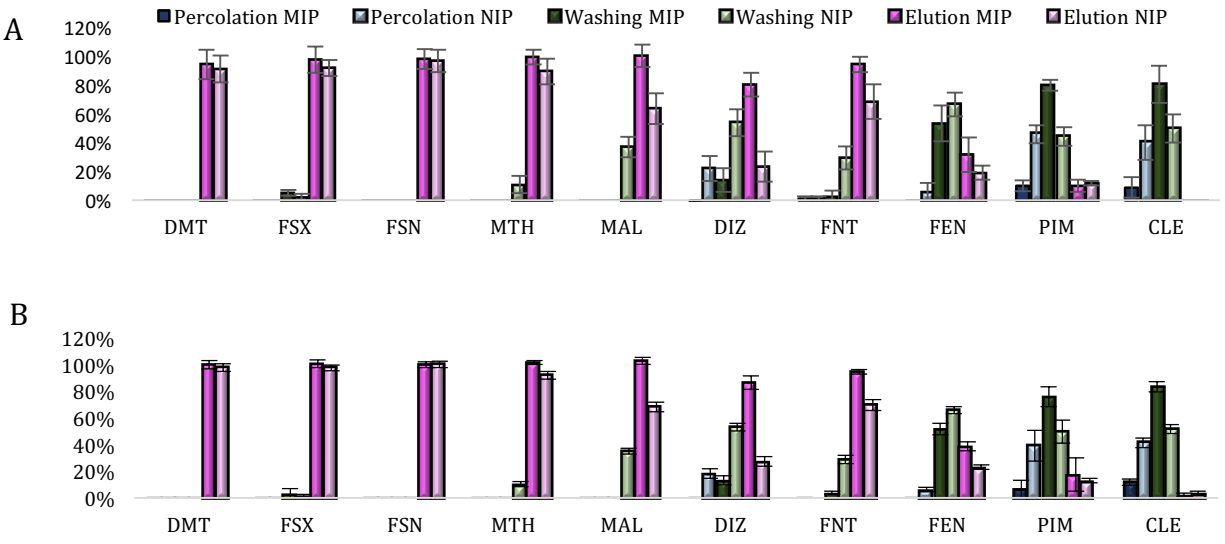

Figure III.4-3. Extraction profiles obtained when percolating the ten OPs (A) on MIP/NIP (n= 3 assays) and (B) on two MIPs/NIPs synthesized independently using the same condition of synthesis (n= 3 assays on each synthesis of MIP, n=6). The extraction procedure was the same as in Figure III.4-2.

In addition, the extraction profiles obtained for the two syntheses were similar as demonstrated by results reported on Figure III.4-3B that corresponds to the average

extraction profiles observed with the use of the MIPs/NIPs resulting from two different syntheses (extraction in triplicates on both MIP/NIP, n= 6). Indeed, SD values of the recovery were between 3 and 12%. Moreover, the analysis of variance, ANOVA test, (α= 5%) demonstrated that there was not a significant variation between recoveries obtained on the MIPs resulting from the two syntheses. These last two observations show the good repeatability of the extraction procedure and the reliability of the synthesis.

III.4.4. Study of the capacity of the MIP in pure media

The capacity of the MIP, which corresponds to the maximum amount of a compound that can be retained by the imprinted polymer with a constant recovery, was studied. This parameter is linked to the number of specific cavities that are available for the trapping of the target compounds. Therefore, the determination of the capacity was performed using MAL, which presents a selective behaviour on this polymer as shown in pure media (Figure III.4-3): recoveries of 100% with a SD of 8% on the MIP and of 64% with a SD of 11% on the NIP. To determine this capacity, samples of hexane were spiked with increasing amounts of MAL and percolated on MIP and on NIP and the extraction procedure described on Figure III.4-2 was applied to each sample. The amounts of MAL in the elution fraction of the MIP were plotted as a function of the percolated amounts. The resulting curve reported on Figure III.4-4 presents two different parts. For the lowest percolated amounts of MAL, the trend is linear, meaning that there is a constant recovery of extraction for this range of percolated amounts. The slope of this linear part corresponds to a recovery of 113%. This value was very close to the recoveries previously obtained for MAL using the same extraction procedure (Figure III.4-3A). For higher amounts of MAL loaded on the MIP, the curve reaches a plateau. The recovery decreases since the capacity of the cartridge was overloaded. Considering the point where the two parts of the curve intercept as the maximum amount of MAL retained on the MIP with constant recovery, the capacity can be estimated at about 32 µg of MAL for 32 mg of MIP, which corresponds to a capacity of about 1 mg/g or to 3.31 µmol/g of MIP. Over this value, quantitative analyses are not reliable since there is a decrease in the recovery extraction. This capacity value was in good agreement with the capacity values reported in the literature namely, between 0.37 µmol/g and 40 µmol/g [17,42,43].

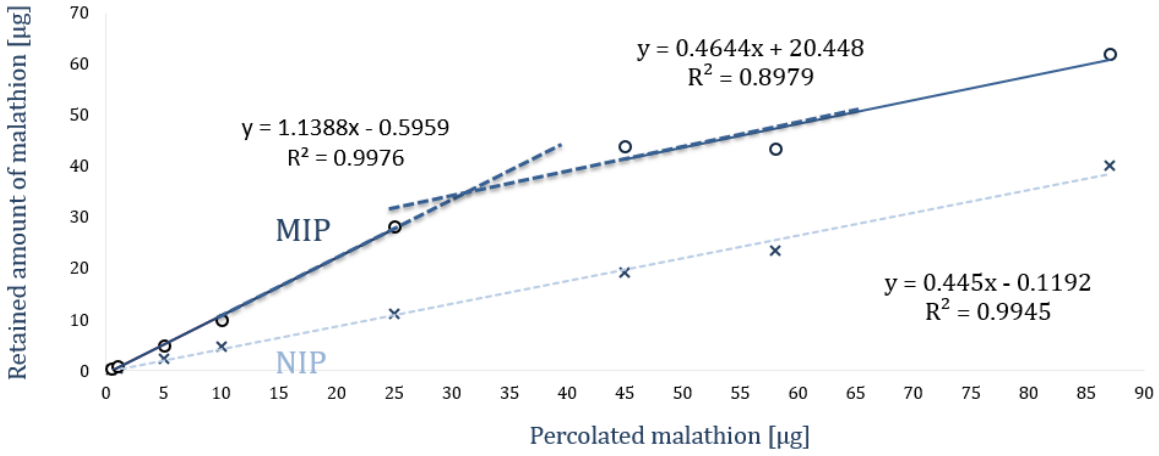

Figure III.4-4. Calibration curves obtained by plotting the amount of malathion recovered in the elution fraction of the MIP and the corresponding NIP after the percolation of different amounts of malathion spiked in 1 mL hexane. The extraction procedure was the same as in Figure III.4-2.

III.4.5.Extraction of OPs from different oils

III.4.5.1.Preliminary study of the repeatability of the extraction procedure in different vegetable oils

In order to evaluate the potential of the MIP for the extraction of OPs from real media, three vegetable oils (sunflower, almond and olive oils) were spiked at 2.5 mg/kg with the nine OPs. The analysis was carried out with LC-UV for FNT and with LC-MS for the other OPs. Despite the selectivity obtained for FEN in pure media, it was not considered in this study because its LOQ in MS or in UV were too high for its determination at this spiking level in the fractions resulting from SPE on MIP**.** Figure III.4-5 shows the recoveries of the nine OPs in the elution fraction for the three oils. Recoveries obtained in pure media were also reported on this figure. The results obtained for oil samples confirmed the results on pure media: the MIP was not selective towards the most polar OPs (DMT, FSX and FSN) with this extraction procedure. Moreover, a matrix effect causes a decreased of recovery for all the compounds, this recovery being lower for spiked oil samples than for spiked pure media. Therefore, the less polar compounds that were only slightly retained in pure media were weakly or no more retained on the MIP/NIP.

For the moderately polar OPs, MTH, MAL, DIZ and FNT, the retention was lower (especially for DIZ) than in pure media because of the matrix effect. However, the selectivity of the extraction on MIP was maintained, even slightly improved as shown by

the highest difference between recoveries on MIP and NIP. Indeed, MIP becomes more selective towards MTH in oil samples than in pure media. This can be explained by the fact that the matrix components may greatly weaken the non-specific interactions as compared with the specific ones. To improve the recoveries for these OPs, the optimization of the SPE procedure appears to be necessary.

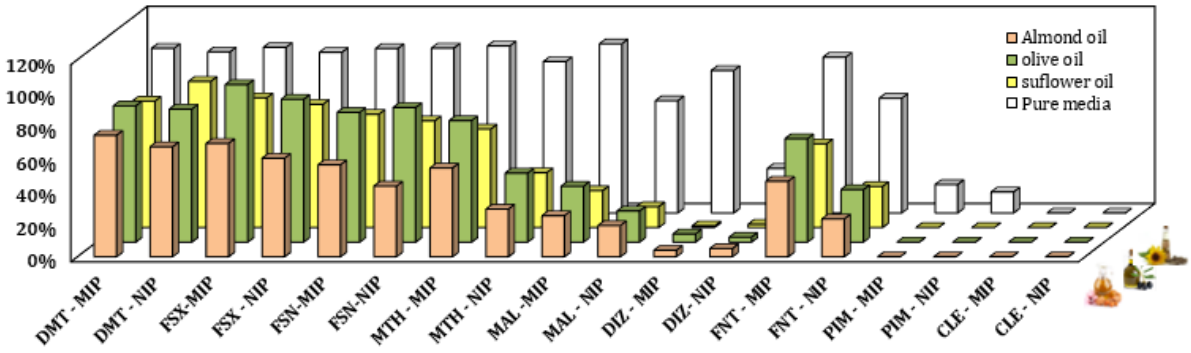

Figure III.4-5. Recovery obtained on MIP/NIP after applying the extraction procedure on different types of vegetable oils (almond, olive and sunflower) spiked at 2.5 mg/kg with nine OPs. Extraction conditions: see part III 3.5.1. Recovery obtained in pure medium (spiked hexane) correspond to those already reported in Figure III.4-3 A.

III.4.6.Optimization of the SPE procedure using almond oil

As the extraction profile (Figure III.4-5) seems not to be affected by the nature of the oil, being similar for the three types of oils, this optimization was only carried out for almond oil samples. Despite the selectivity obtained for the four moderately polar compounds, this part of the study only focusses on the three OPs that can be analyzed by LC-MS. The MRLs values were taken as reference to set the spiking level of OPs in the almond oil. Nevertheless, the MRLs for pesticides in processed products like crude oils (and refined oils) are not specifically set in the EU legislation. To compare the LOQs of pesticides in crude oils with the MRLs of pesticides in seeds, a processing factor proposed by FEDIOL (the vegetable oil and protein meal industry association) was applied. This processing factor is calculated taking a count the oil content and the hydrophobicity of the OPs at the same time. As an example, the proposed processing factor for hydrophobic pesticides (log P= 3) in nut seed with 54% in oil content was 2.5. For this case, the average oil content of almond oil is 58% [44], thus the estimate processing factor for this oil was 2.6. This proposal value, was used to estimate the LOQs of OPs in almond seed (see Table III.4-2). The almond extract was spiked at 100 µg/kg oil, instead of 2.5 mg/kg, and diluted 10 times to limit the matrix effect during the

extraction procedure on MIP/NIP as proposed by Barkas *et al*. for olive oil [17]. In addition, different volumes of washing solution hexane and DCM (95/5, v/v) were tested: 0.4, 0.65, 0.8 and 1 mL.

The washing with 0.65 mL presented a good compromise in terms of recovery and selectivity. Indeed, for 0.8 and 1 mL the extraction recovery decreases particularly for MAL and DIZ. In return, if a washing with 0.4 mL gives rise to the same recoveries as for 0.65 mL, this higher volume was preferred because it must allow the removal of a higher amount of matrix components than the smaller one. Recoveries in the elution fraction obtained by applying the dilution of the extract and this washing volume to almond oil extract are reported in Table III.4-2. Recoveries of extraction were corrected for MAL by taking into account the low amount of this compound (4 µg/kg) detected in the blank oil sample. The SD values were between 4 and 6% (n= 3). Those values are comparable to those obtained in pure media (between 5 and 8%). The selectivity is highlighted by the higher recoveries obtained on MIP (between 73 and 99%) than on NIP (between 34 and 75%).

Table III.4-2. Recovery obtained in the elution fraction using almond oil spiked with 100 µg/kg of the three OPs after LLE and SPE clean-up using MIP/NIP or C18. LOQs correspond to S/N= 10.

OPs	MRLs[a] in almond seed (µg/kg)	Sorbent	Recovery (%)	LOQ in almond oil (µg/kg)	Processing factor [b]	Estimated LOQs [c] in almond seed (µg/kg)	Matrix effect (%)
MTH	50	MIP	99 ± 6	2 ± 1	2.6	0.8 ± 0.4	7 ± 3
		NIP	75 ± 13				
		C18	106 ± 1				21 ± 6
MAL	20	MIP	73 ± 4	5 ± 1	2.6	2 ± 0.4	17 ± 8
		NIP	42 ± 5				
		C18	115 ± 7				34 ± 13
DIZ	50	MIP	81 ± 6	0.8 ± 0.3	2.6	0.3 ± 0.1	11 ± 3
		NIP	34 ± 8				
		C18	134 ± 9				35 ± 8

a: MRLs according to EU regulation N° 396/2005; b: processing factor from FEDIOL (vegetable oil and protein meal industry association); c: estimated LOQs according to FEDIOL processing factor.

These results were also compared with those obtained by using conventional C18 silica sorbents after the same LLE step (Table III.4-2). For this, the same spiked level was used for the three OPs in almond oil as the objective was also to compare the matrix effect in similar conditions. The recovery for the three OPs was over 100% using C18 silica, which indicates that the results obtained using C18 silica could be affected by a

matrix effect. The contribution of matrix effect in the quantification of OPs was then evaluated for both sorbents. After applying the whole extraction procedure to a non-spiked oil sample (LLE and SPE on MIP or on C18), the elution fraction was spiked with three OPs. This extract was injected in LC-MS, and the obtained signals were compared to those of a standard solution in pure media at same concentration level. The results indicate that the contribution of matrix effects using C18 silica was higher than when using the MIP. As an example, for MTH, the matrix effect was 21% on C18 and 7% on MIP. Therefore, the comparison of the two extraction procedures performed on MIP and C18 indicates that the use of MIP as a selective sorbent limits the matrix effect that occurs when using a conventional sorbent by a factor two to three. This can be explained by a more efficient removal of matrix components from the MIP than from C18 silica. This can also be illustrated by comparing the LC-UV (210 nm) chromatograms resulting from the analysis of the elution fraction after using C18 or MIP (Figure III.4-6). The chromatogram corresponding to C18 contains more peaks of interfering compounds than those obtained from MIPs. The MIP allowing a larger part of interfering compounds to being removed thus improving the reliability of LC-UV and LC-MS analyses.

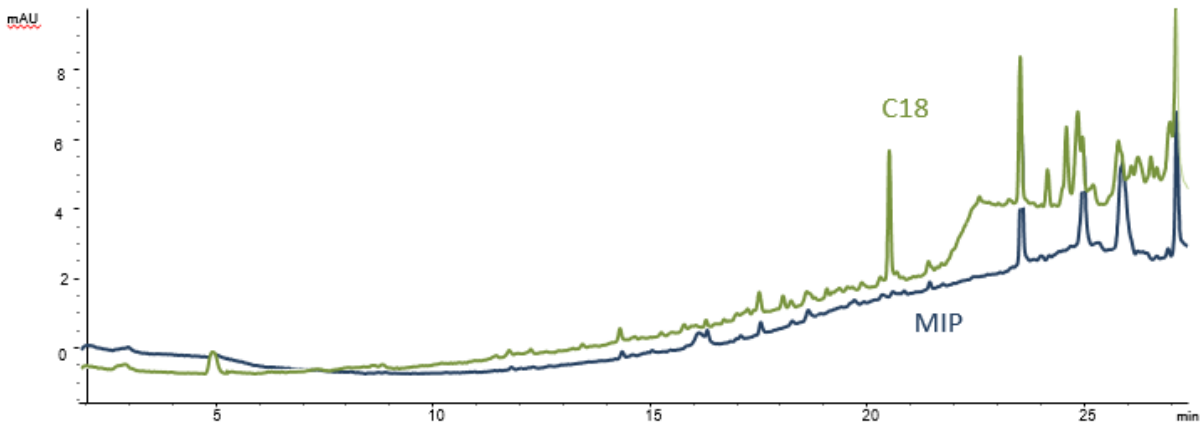

Figure III.4-6. LC-UV chromatograms (210 nm) of elution fraction of almond oil extract spiked at 100 µg/kg with eight OPs using C18 or MIP after LLE. The extraction procedure was described in the part III.3.5.2 (washing volume of 0.65 mL).

Annexe V (Figure 2) corresponds to the LC-MS analysis in (MRM mode) of the elution fraction from the MIP for an almond oil sample spiked at 100 µg/kg. The calculated LOQs for the three target OPs (MTH, MAL and DIZ) are reported in Table III.4-2 and range from 0.3 to 2 µg/kg in almond seed. These results mean that this analytical method allows the determination of concentration levels of OPs lower than their MRLs (20 to 50 µg/kg). Moreover, it is important to mention than these results

obtained for vegetable oils (comparison of oils, optimization of washing conditions, studies of matrix effect) were achieved on the same MIP without observing any decrease in its performance, thus highlighting its good chemical stability and its reusability for more than 100 experiments.

III.5. Conclusions

Different conditions of synthesis were screened to determine those that allow the synthesis of a MIP able to selectively extract OPs that belong to a very broad range of molecular structures and log P values (between 0.7 and 4.7). Among the six synthesized MIPs, one of them was able to selectively trap five OPs (MTH, MAL, DIZ, FNT and FEN).

After studying the repeatability of the optimized SPE procedure and of the reliability of the MIP synthesis in pure media, the performances of this polymer were evaluated in real media. The retention of OPs on the MIP was similar using three different oils (olive, sunflower and almond oils). Therefore, a rapid optimization of the SPE procedure on almond oil was achieved and allowed us to obtain recoveries for three OPs (MTH, MAL and DIZ) between 73 and 99% using the MIP and of only 34 to 75% using the NIP. The MIP also allows the matrix effects to being reduced by a factor of two to three: the matrix effects were between 7 and 11% using the MIP and between 21 and 35% using the C18 silica sorbent for a sample of almond oil spiked at 100 µg/kg. The LOQs obtained for almond seeds (between 0.3 and 2 µg/kg, estimated taking a count the LOQs of spiked almond oil), were lower than the MRLs (between 20 and 50 µg/kg) established for the almond seeds.

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The processing factor (PF) of almond oil is 1.7 instead of 2.6. This PF was calculated taking account the average of oil content of almond oil (58%).

PF =1/0.58= 1.7.

Therefore, the LOQs of almond that depends of this PF will be sligthly modified (1.2 ± 0.6, 2.9 ± 0.6, 0.5± 0.2 µg/kg for MTH, MAL and DIZ, respectively).

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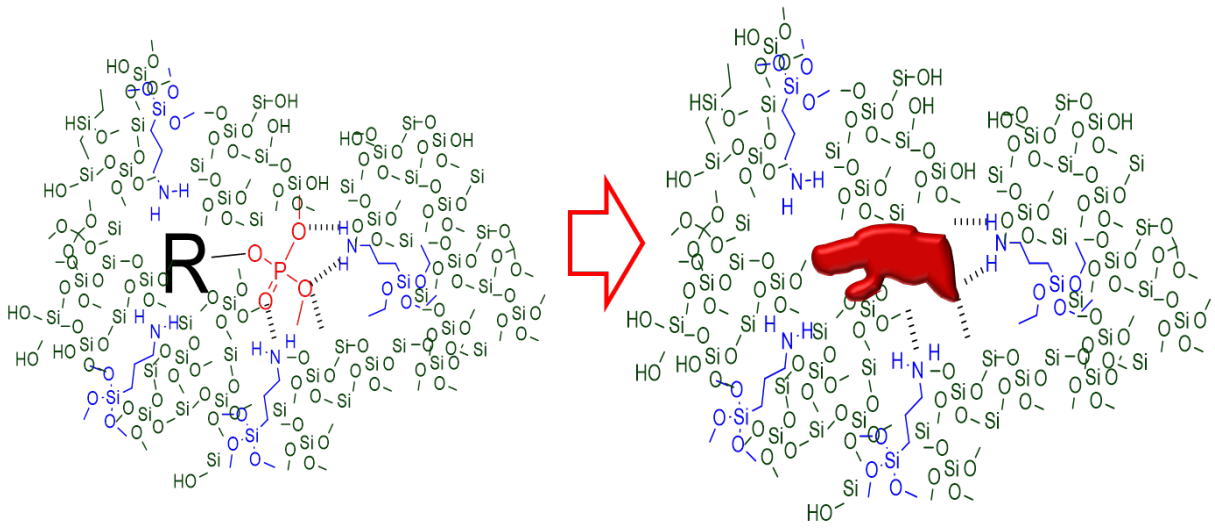
Chapter IV: Synthesis and characterization of molecularly imprinted silica for the selective extraction of organophosphorus pesticides from almond oil



Dans le chapitre précédant nous avons synthétisé un MIP, répétable en termes de procédure d'extraction et de synthèse. Ce MIP piège sélectivement trois OP présentant une polarité modérée dans trois huiles végétales différentes, avec des taux de récupérations similaires par exemple environ 62% pour le dimethoate. Cependant, l'objectif de ce travail de thèse étant de piéger le plus grand nombre possible d'OP à la fois, une approche de synthèse alternative de polymères imprimés (voie sol-gel), a été envisagée dans le chapitre II. Cette approche consiste à utiliser des organosilanes qui par hydrolyse puis condensation autour d'une molécule empreinte conduisent également à la formation des cavités complémentaire en taille, forme et groupement fonctionnel de la molécule empreinte. Tout comme pour les MIP, différentes conditions de synthèse ont été criblées afin d'identifier celle conduisant à un support imprimé à base de silice (MIS) capable d'extraire sélectivement le maximum d'OP en milieu pur. Le MIS sélectionné a été obtenu en utilisant le monocrotophos comme molécule empreinte, le 3-aminopropyl triéthoxysilane comme monomère et le tetraethyl orthosilicate comme agent réticulant avec un ratio molaire 1/4/20. Ce support a permis de piéger sélectivement six OP (dimethoate, fenthion sulfoxide, fenthion sulfone, methidathion, malathion et diazinon) en milieux pur, dont les trois composés les plus polaires (dimethoate, fenthion sulfoxide, fenthion sulfone), ayant des log de P compris entre 0,7 et 2,2, avec des taux de récupération élevés (73 and 99 %). Dans un premier temps, il a été démontré que la procédure d'extraction et de synthèse sur ce support étaient répétables et que la capacité du support était suffisante pour permettre l'extraction des OP présent à des concentrations très élevés dans les échantillons réels.

Les performances de ce support ont donc ensuite été évaluées dans l'huile d'amande pour deux composés, le DMT et le FSX, qui peuvent être analysé par LC-MS/MS. Une ré-optimisation de la procédure SPE a été réalisée pour améliorer la rétention et la sélectivité en milieu réel. Des rendements de récupération de 100 et 114% dans la fraction d'élution du MIS ont été obtenu pour respectivement le FSX et le DMT. La LOQ calculée pour ces OP, en tenant compte du facteur de transformation (lié à la concentration des composés durant le processus d'extraction et de raffinage permettant de passer de la graine à l'huile correspondante), dans les graines d'amandes était plus de 10 fois inférieure aux LMR établis par la Commission européenne. Par conséquent, ce MIS montre un potentiel élevé pour extraire sélectivement deux OP présent à l'état de trace dans l'huile d'amande.

Finalement nous avons pu constater que les supports MIP et MIS utilisés comme support d'extraction après une étape préalable d'extraction liquide/liquide de l'huile d'amande pour diminuer les effets de matrice, ont présenté une complémentarité en termes d'extraction sélective des OP visés. En effet, les OP le plus polaires ont été extraits de l'huile d'amande sélectivement par le MIS (DMT, FSX) alors que les OP modérément polaires (MTH, MAL et DIZ) ont été extraits sélectivement par le MIP.

IV.Article 2

Synthesis and characterization of molecularly imprinted silica for the selective extraction of organophosphorus pesticides from almond oil

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IV.1.Abstract

The aim of this work was to prepare and evaluate molecularly imprinted polymers obtained by a sol-gel approach for the selective solid-phase extraction (SPE) of organophosphorus (OPs) pesticides from almond oil. The performances of molecularly imprinted silicas (MISs), prepared using different conditions of synthesis, were studied by applying different extraction procedures in order to determine the ability of the MISs to selectively extract ten target OPs. For this, the retention of OPs on MIS in pure media was compared with the retention on a non-imprinted silica (NIS), used as control sorbent, to prove the presence of specific cavities. The repeatability of the recovery yield of extraction on the most selective MIS was demonstrated both in pure and real

media. This MIS was able to selectively extract fenthion sulfoxide and dimethoate contained in almond oil after applying the optimized extraction procedure with recovery yields between 100 and 114%. The estimated limit of quantification (LOQs, S/N=10) between 1.2 and 4.6 µg/kg for those OPs in the almond fruits was more than 10 times lower than the Maximum Residue Levels (MRLs) established by the European Commission. This MIS therefore shows a high potential to selectively extract two OPs at trace levels from almond oils.

Keywords: solid-phase extraction; molecularly imprinted silica; organophosphorus pesticides; vegetable oils; liquid chromatography; mass spectrometry.

IV.2. Introduction

Almond oil (*Oleum amygdalae*) provides important health benefits such as reducing the incidence of obesity, cardiovascular diseases, diabetes or cancer. It is used in many fields in the food or pharmaceutical industries [1]. For instance, it is used to treat dry skin in psoriasis and eczema [2]. It is also largely employed in the cosmetic industry for its penetrating, moisturizing and restructuring properties. However, some pesticides, especially highly lipophilic ones, can be easily bio-accumulated in almond fruits and hence be transferred into the oil during the trituration process [3]. For this reason, the Maximum Residue Level (MRL), which is the highest level of a pesticide residue that is legally tolerated, was established by the European Commission to control the presence of these contaminants in raw materials such as oil seeds and fruits. Because MRLs on processed products are not yet established, a processing factor was proposed by FEDIOL (vegetable oil and protein meal industry association) that can be used to evaluate the corresponding contamination level in oils.

This study focused on organophosphorus (OPs) pesticides that are mainly used to protect plants [4]. However, these compounds are known as inhibitors of acetylcholinesterase [5]. OPs have a tendency to bind to this enzyme thus disturbing nerve function, which further results in paralysis and death [6]. Extraction of OPs from oil matrices containing a high content of triglycerides and the possible presence of lipophilic analytes [7] requires complicated sample treatment procedures before chromatographic analysis. In general, OPs are extracted from vegetable oil samples by using liquid-liquid extraction (LLE) [8–10] or low temperature extraction [11] with a clean-up step like gel permeation chromatography (GPC) [12], matrix solid-phase

dispersion (MSDP) including QuEChERs [7,8,13,14], or headspace solid-phase microextraction (HS-SPME) [14]. These techniques normally combined with performing chromatographic methods such as GC-MS/MS or LC-MS/MS allow, the target analytes that might be present in low quantities in this kind of sample to being extracted, identified and quantified. Solid-phase extraction (SPE) [15–17] is also still largely used as an extraction technique of OPs from oils. Nevertheless, conventional sorbents (such as Florisil, alumina or silica) that favor polar interactions in apolar solvents can also lead to the co-extraction of numerous interfering compounds. In order to decrease the incidence of this phenomenon and to increase selectivity of the sample treatment, molecularly imprinted polymers (MIPs) can be used as selective sorbents since they possess specific recognition sites based on the molecular recognition of the target OPs. Indeed, in common approach, the synthesis of MIPs is based first on the formation of a template-monomer complex by non-covalent interactions in a porogenic solvent. Then the radical polymerization around the template-monomer complex is induced by using a cross-linker in presence of a radical initiator. Finally, the template is removed from the resulting polymer by several washings in order to disrupt the interactions between the template and the monomers. The resulting imprinted polymer contains specific recognition sites that are sterically and chemically complementary to the template molecule, thus allowing the latter to being selectively recognized in real samples [18-21].

MIPs were applied as SPE sorbents [19–23] but also in other extraction techniques such as MSPD [22,23], dispersive solid phase extraction [24,25], SPME [26] or stir bar sorption extraction [27] to selectively extract OPs from several samples such as fruits [19,23], soils [24,25], vegetables [24,25]. However, to date, few works have used MIP sorbents in SPE to selectively extract OPs from oil samples. The first works were reported by *Bakas et al*. and the synthesized MIPs allowed only one target OP to being extracted at a time. Indeed, these studies focused on the extraction of methidathion [28], dimethoate [29] and fenthion [30], respectively from olive oil. Only one previous work reported by our group shows the possibility to extract several OPs from oil [31]. Indeed, after screening different conditions of synthesis, a MIP was selected for its capacity to extract selectively three OPs. After optimizing the extraction procedure, only three moderately polar OPs among the studied OPs were successfully selectively extracted (methidathion, malathion and diazinon). As OPs is belonging to a

wide range of structures and of polarities, it was complicated to trap the whole family of OPs.

As an alternative to radical polymerization, imprinted sorbent can be produced by a sol gel approach yielding molecularly imprinted silica (MISs) sorbents. They are generally synthesized by using 3-aminopropyl triethoxysilane (APTES) or phenyltriethoxysilane (PTMOS) (having respectively an amino or a phenyl group) as monomers that led to the formation of polar (hydrogen bonds, electrostatic interactions), hydrophobic and π-π interactions depending of the monomer and of the solvent used. The cross-linking agent is an alkoxysilane, *i.e*. tetraethoxysilane (TEOS) or tetramethoxysilane (TMOS) [32]. The synthesis of MISs is similar to those of MIPs. First the monomer and the cross-linker reacts mainly in aqueous solution to form silanol (Si-OH) groups through hydrolysis, then siloxane bonds (Si-O-Si) are formed by condensation reaction with the silanol groups around the template molecule with the help of an acidic or a basic catalyst [32]. The pH of the mixture will determine whether the dominant process is hydrolysis or condensation. The use of an acidic catalyst results in a slow hydrolysis step and a rapid condensation, the growth of the "polymer" being favored in comparison with the cross-linking. The polymer formed is then rather homogeneous, with small pores and a large specific surface area. Conversely, when a basic catalyst is used, the hydrolysis becomes the fast step and the condensation is the slow one. In this case, the polymer chain will rapidly cross-link and form particles that lead to a heterogeneous structure, obtained more rapidly than in acid catalysis. In these conditions, the specific surface area is smaller and the pores are larger, so the density of the "polymer" in basic catalysis will be lower [33, 34]. Sol gel reactions not only depend on the pH of the solution and the type of catalyst, then also depend on the temperature of the reaction, heating time or the solvent [35]. This sol gel technique was applied to the synthesis of MISs for SPME fibers using parathion ethyl [36] or diazinon [37] as template. These SPME fibers were used to extract templates and their structural analogs from aqueous matrices like fruits [36] or vegetable extracts [37]. This approach was also used for the synthesis of electrochemical sensors that exhibited a good selectivity in liquid phase for the parathion in pure media [38] or in real samples (rice) [39]. MISs have been used as SPE selective sorbents for different compounds such as a neurotoxic non-proteinogenic amino acid (β-N-methylamino-L-alanine) from cyanobacterial samples [40], nitroaromatic explosives from post-blast samples [32] or ibuprofen from

urine [33] but never for the OPs.

This work describes for the first time the synthesis of MISs for the extraction of OPs from almond oil. The targeted OPs were selected by taking into account the risk of their occurrence in such samples. Different conditions of synthesis were screened by varying the nature of the template, the monomer and the porogenic solvent. After this screening, the most promising MIS in terms of retention and selectivity was studied in more detail by investigating its behavior towards ten OPs in pure media. The repeatability of the molecularly imprinted solid-phase extraction (MISPE) procedure was demonstrated for a selection of compounds. Finally, in order to selectively extract these OPs at trace levels in almond oil, the extraction procedure was optimized. After this optimization, the limits of detection and quantification were determined and compared with the MRLs established by the European Commission.

IV.3.Materials and methods

IV.3.1.Chemicals

Organophosphorus (OPs) standards : dimethoate (DMT) 98%, fenthion sulfoxide (FSX) 99%, fenthion sulfone (FSN) 99%, methidathion (MTH) 98%, malathion (MAL) 99%, fenitrothion (FNT) 98%, diazinon (DIZ) 98%, pirimiphos-methyl (PIM) 99.5%, fenthion (FEN) 99% and chlorpyrifos-ethyl (CLE) 99.5% were supplied by Cluzeau Info Labo (Sainte-Foy-La-Grande, France). Individual stock solutions of each OP were made at a concentration of 100 mg/L in acetonitrile (ACN). A stock solution mixture containing 5 mg/L of each OP was prepared in ACN and stored at 4 °C until further use.

Monocrotophos (MCP), ammonium acetate for HPLC 99% (AAC), anhydrous n-hexane 95%, ethanol, 3-aminopropyl triethoxysilane (APTES) 99%, phenyltriethoxysilane, (PTMOS) 97% and tetraethyl orthosilicate (TEOS) 99.99% were supplied by Sigma-Aldrich (Saint Quentin Fallavier, France). Ammonium hydroxide (NH_4OH) solution at 32%, acetic acid (AA) and formic acid (FA) were purchased from VWR (Fontenay-sous-Bois, France).

HPLC-grade ACN, methanol (MeOH) and dichloromethane (DCM) were supplied from Carlo Erba (Val de Reuil, France). High purity water was dispensed by a Milli-Q purification system (Millipore, Saint Quentin en Yvelines, France).

IV.3.2.Apparatus and analytical conditions

The LC-MS/MS analyses were performed using a liquid chromatograph (UltiMate 3000®, Thermo Scientific, Illkirch, France) coupled with a Triple Stage Quadrupole Mass Spectrometer (TSQ Quantum Access MAX, Thermo Scientific, Illkirch, France) equipped with a heated electrospray ionization source (HESI2). The chromatographic separation was performed on an Accucore PFP column (150 x 2.1 mm, 2.6 µm, ThermoFisher Scientific, Villebon Courtaboeuf, France) thermostated at 32 °C with a column oven (Croco-cil, Interchim). Samples were analyzed using a linear gradient elution with water (A) and MeOH (B) both containing 0.1% (v/v) of FA and 4 mM of AC. The gradient started at 20% of B during 2.5 min, ramped up to 80% of B in 23.5 min, held for 2 min, and returned to the initial composition within 2 min where it was kept constant during 2 min to let the system equilibrate. The flow rate was set at 0.4 mL/min and the injection volume was 2 µL.

MS was operated in positive ion mode with MRM detection using a spray voltage of 3500 V and a skimmer offset of 5 V. Capillary and vaporizer temperatures were set at 280 °C and 295 °C, respectively. Sheath gas pressure and auxiliary gas pressure were set at 55 and 15 units, respectively. Nitrogen was used as nebulizer and desolvatation gas and argon as the collision gas at a pressure of 1.5 mTorr. For the optimization of MS detection, each OP was infused at a concentration of 5 mg/L in the mixture A/B (50/50, v/v). The quantification of the 10 OPs was performed in MRM mode using the specific transitions: 230 → 125 for DMT, 295 → 280 for FSX, 328 → 311 for FSN, 320 → 145 for MTH, 348 → 127 for MAL, 305 → 169 for DIZ, 306 → 164 for PIM, 352 → 200 for CLE. FEN and FNT both gave a very low signal intensity during infusion. A second transition was used for confirmation purposes and to avoid false positive responses. The tube lens and collision energies values corresponding to quantitation and confirming ions are summarized in the Annexe VI (Table 1).

The LC-DAD analyses were performed using a liquid chromatograph (LC) Agilent 1200 series (Agilent Technology, Massy, France) equipped with a binary pump, an auto sampler and a diode array detector (DAD) controlled by a Chemstation software. OPs were separated using the same column, flow rate and injection volume as for LC-MS/MS analysis. Samples were analyzed using linear gradient elution with water (A) and ACN

(B). The gradient started with 8% of B during 2.5 min and increased to 60% in 23.5 min, held for 2 min, returned to initial composition within 2 min and was maintained 2 min to let the system equilibrate. DMT, MTH, MAL were quantified at 210 nm, FSX at 240 nm, FSN at 230 nm, FNT at 270 nm, DIZ, PIM and FEN at 250 nm and CLE at 290 nm.

The calibration curves used for the quantification of the target OPs by LC-DAD and LC-MS/MS are summarized in the Annexe VII (Table 2) and Annexe VIII (Table 3).

IV.3.3.Synthesis of molecularly imprinted silica sorbents

Four MISs were synthesized using a template/monomer/cross-linker molar ratio of 1/4/20(Table IV.4-1) MCP and DIZ (0.25 mmol) were used as template, APTES and PMTOS (1mmol) as monomer, TEOS (5 mmol) as cross-linker, 2 mL of H_20 or of a H_20/ethanol mixture (70/30, v/v) as porogens and 400 µL of 32% NH_4OH solution as the catalyst. The template, weighed in a 4 mL glass vial, was dissolved in the porogen. Then, the monomer, the cross-linker and the catalyst were added to the glass vial containing the template molecule. The resulting solution was stirred thoroughly after adding each reagent and immersed in a silicone oil bath heated to 40 °C, stirred and kept there for 24 h. The obtained product was kept at room temperature for 3 h and heated at 120 °C in an oven for 18 h to evaporate the excess solvent and to dry the sorbent. Thereafter, the polymer was manually crushed and sieved. Only the particles between 25 µm and 36 µm were collected. A sedimentation step was performed 3 times using a 10 mL mixture of MeOH/H_20 (80/20, v/v) to remove the thinnest MIS particles before drying step during 24 h at room temperature. Afterwards, 22 mg of MIS particles were packed in a 1 mL disposable propylene cartridge (Interchim) between two polyethylene frits (20 µm, Sigma-Aldrich). To remove the template, the polymer was washed (with approximately 10 mL of MeOH) until the template could no longer be detected in the washing fractions by LC-DAD at 210 nm for MCP or 250 nm for DIZ. The washing fractions were analyzed directly to detect DIZ or evaporated and suspended before injection in LC-DAD in a mixture of MeOH/ACN/H_2O (40/10/50, v/v/v) to detect MCP. NIS sorbents were obtained by performing exactly the same procedure but in the absence of the template molecule.

IV.3.4.Characterization of four MISs in pure medium

The four synthesized MISs/NISs were evaluated in terms of selectivity and retention after applying the same SPE procedure to each MIS. The four MIS/NIS cartridges were first conditioned with 4 mL of hexane. Then, 1 mL of hexane spiked with six OPs (FSX, MAL, DIZ, FNT, FEN and CLE) at 1 mg/L was percolated through the MIS/NIS cartridges. Next, three washing steps were carried out: (W1) 1 mL of a mixture of hexane/DCM (95/5, v/v), (W2) 1 mL of a mixture of hexane/DCM (90/10, v/v) and (W3) 1 mL of a mixture of hexane/DCM (80/20, v/v). Finally, the OPs were eluted with 1 mL of ACN. Between the washing and the elution steps, the cartridges were dried by 5 mL of air. Each fraction resulting from each step was evaporated to dryness under a nitrogen stream and was suspended in 0.5 mL of ACN before injection in the LC-DAD system using the conditions described in Part IV.3.2.

The optimization of the extraction procedure for each MIS allowed us to reduce its length: a unique washing step was carried out and was applied to MIS to improve the recovery yields and the selectivity. For this, 1 mL of hexane spiked at 0.1 mg/L with eight OPs (DMT, FSX, FSN, MTH, MAL, DIZ, PIM and CLE) was percolated on each cartridge and a single washing step was performed with 1 mL of mixture of hexane/DCM (97/3, v/v) for MIS/NIS (1, 2 and 3) or 1 mL of mixture of hexane/DCM (70/30, v/v) for MIS 4. Finally, the OPs were eluted with 1 mL of ACN. The resulting elution fractions were directly analyzed by LC-MS/MS using the described conditions in Part IV.3.2.

After selection of the most promising MIS and in order to evaluate it for a larger number of OPs, the percolating solution (hexane) used on the MIS/NIS 1 was spiked with 1 mg/L of the ten target OPs and the washing step was carried out by using 1 mL of mixture of hexane/DCM (97/3, v/v). The OPs were eluted with 1 mL of ACN. The elution fractions were analyzed by LC-DAD to quantify FEN and FNT (both had a higher LOQ in LC-MS/MS) after evaporation and suspension of the fraction in 0.5 mL of ACN.

IV.3.5.MIS applied to almond oil extract

IV.3.5.1.Optimization of the extraction procedure

A LLE was first carried out using 3 x 1 mL of a mixture of ACN/DCM (90/10, v/v) for 200 mg of almond oil (Melvita) from organic agriculture. The oil extract was evaporated to dryness under a nitrogen stream, then diluted either with 1 mL or 10 mL of hexane spiked at 2 µg/L with the two OPs (DMT, FSX) (corresponding to a spiking

level of 10 and 100 µg of OPs by kilograms of oil, respectively). After conditioning the MIS/NIS with 4 mL of hexane, 1 mL of oil extract was percolated through both the MIS and the NIS cartridges. Then, 1 mL of hexane/DCM (97/3, v/v) was used for the washing step. Finally, the OPs were eluted with 1 mL of ACN. In both procedures, the elution fraction was evaporated to dryness under a nitrogen stream and suspended in 100 µL of ACN before injection in the LC-MS/MS system using the described conditions in Part IV.3.2.

For optimizing the volume of the washing step of the MISPE procedure, the oil extract was diluted with 10 mL of hexane and spiked at a concentration of 2 µg/L (corresponding to a spiking level of 100 µg/kg of oil) of the two selective OPs (DMT, FSX). After conditioning the MIS/NIS with 4 mL of hexane, 1 mL of oil extract was percolated through MIS/NIS cartridges. Then different volumes (0.4, 0.65 or 1 mL) of washing solution (hexane/DCM, 97/3, v/v) were tested.

The final extraction procedure applied to almond oil consisted in a first LLE step as previously described. The oil extract was evaporated to dryness under a nitrogen stream, diluted with 10 mL of hexane and spiked at 2 µg/L (corresponding to a spiking level of 100 µg/kg of oil) with two OPs (DMT, FSX). After conditioning the MIS/NIS with 4 mL of hexane, 1 mL of oil extract was percolated through both the MIS and NIS cartridges. Then, 0.65 mL of hexane/DCM (97/3, v/v) was used as a solution for the washing step. The rest of the procedure was as previously described.

Once the extraction procedure was optimized, it was applied to a non-spiked almond oil sample that was analyzed by LC-MS/MS after applying the full extraction procedure. FSX was detected at a concentration of 5 µg/kg, then the extraction recovery yields were corrected for this compound in all the extraction procedures applied to this sample.

IV.4.Results and discussions

IV.4.1.Choice of conditions of synthesis of MISs

The aim of this study was the preparation of MISs as an alternative to MIPs for the selective extraction of OPs from almond oil. As shown on Figure IV.4-1 the selected OPs present a wide structural variability and a wide range of physico-chemical properties. Therefore, different conditions of synthesis were screened as is described

Table IV.4-1. First of all, the effect of the template was tested. For this, two templates were used: a linear and polar one, MCP (log P= -0.22), used for the synthesis of MIS 1 and MIS 2 that should favor the extraction of the most polar OPs and a more hydrophobic one containing an aromatic ring, DIZ (log P= 3.69) used as template for MIS 3 and MIS 4 that should favor extraction of the most hydrophobic OP. In addition, two different monomers were selected: (i) APTES, that possesses an amino group that could generate polar interactions with the target OPs (MIS 1 to 3), and (ii) PMTOS, that possesses a phenyl group and that could develop hydrophobic or π-π interactions (MIS 4) with DIZ. The effect of the porogen was only studied with the most polar template, MCP, using H_20 (MIS 1) or a less polar mixture of H_20/ethanol (70/30, v/v) (MIS 2). As MISs prepared with a molar ratio 1/4/20 for template/monomer/cross-linker and base-catalyzed conditions using NH_4OH solution at 32% gave promising results in previous studies for targeting different types of compounds such as: a neurotoxic non-proteinogenic amino acid (β-N-methylamino-L-alanine) from cyanobacterial samples [40] or nitroaromatic explosives from post-blast samples [32]. Those conditions were fixed for the four syntheses of MIS. Once the four MISs were synthesized, an optimization of the SPE procedure was necessary to evaluate the performance of these supports in term of retention and selectivity.

Table IV.4-1. Synthesis conditions of four MISs, using NH4OH (32%) as catalyst and a molar ratio template/monomer/cross-linker of 1/4/20. NISs were synthetized in the same conditions without introducing the template.

Sorbent	Template	Monomer	Cross-linker	Porogen
MIS 1	MCP	APTES	TEOS	H_2O
MIS 2	MCP	APTES	TEOS	H_2O/Ethanol (70/30, v/v)
MIS 3	DIZ	APTES	TEOS	H_2O/Ethanol (70/30, v/v)
MIS 4	DIZ	PMTOS	TEOS	H_2O/Ethanol (70/30, v/v)

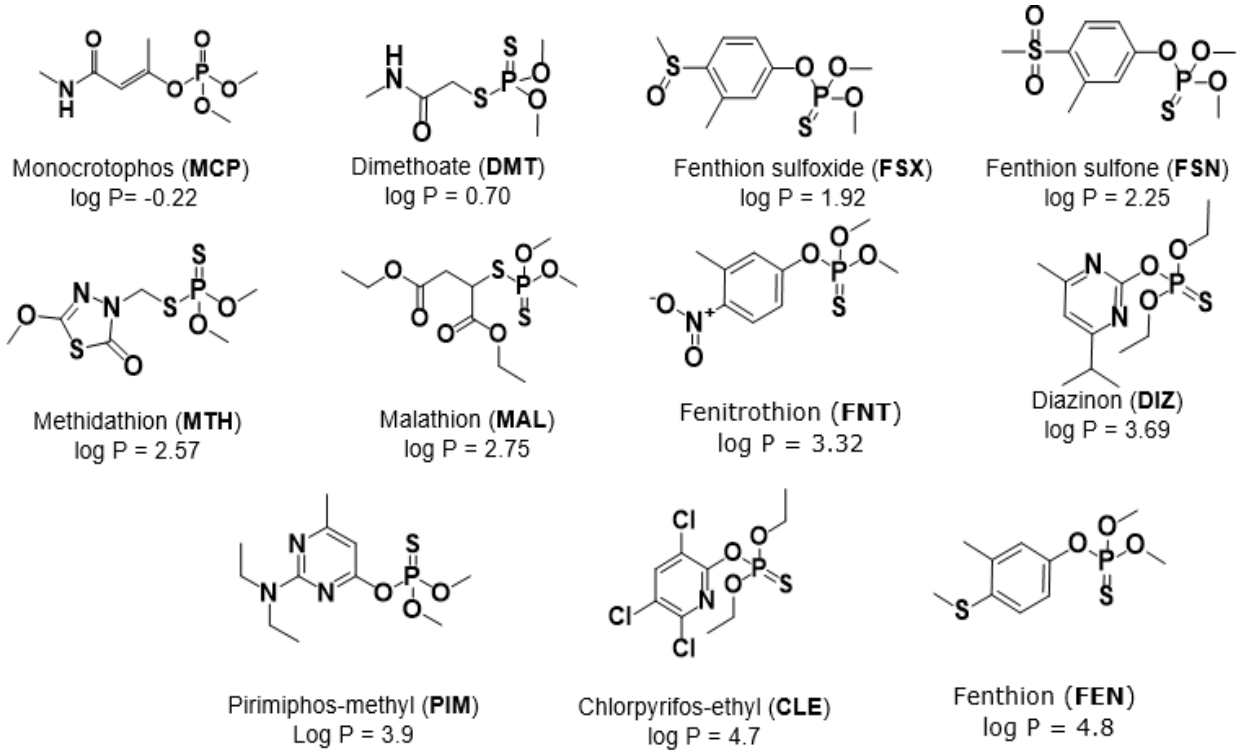

Figure IV.4-1. Chemical structure and partition coefficient of eleven OPs. Log P values are issued from Pesticide Properties DataBase from University of Hertfordshire.

IV.4.2.Comparison of the synthesized MISs

To evaluate the retention potential and the selectivity of the MISs, the extraction profile obtained on the NISs (synthesized in the same conditions as the MISs but without the introduction of the template molecule) were compared with the profile obtained on the MISs.

To limit data treatment, the four MISs/NISs were preliminary evaluated in term of retention and selectivity by analyzing the extraction profiles for only six OPs out of the ten. These six OPs (FSX, MAL, DIZ, FNT, FEN and CLE) were selected in order to cover the whole range of polarity, from the most polar (log P= 0.7) to the less polar (log P= 4.8) one. The recovery yields obtained for each OP for percolation, washing and elution fractions are presented on Figure IV.4-2 for each synthesized MIS/NIS.

The objective of this study being the selective extraction of OPs from oils, since hexane is commonly used as a solvent for oil extraction, it was selected as the percolation medium [28, 41]. In this solvent, polar interactions between monomers and analytes should be favored, so that in order to disrupt them, the polarity of the solvent used for the washing steps had to be increased. Therefore, three washing steps were

performed with an increasing elution strength: (W1) 1 mL of mixture of hexane/DCM (95/5, v/v), (W2) 1 mL of mixture of hexane/DCM (90/10, v/v) and (W3) 1 mL of mixture of hexane/DCM (80/20, v/v). An increase of the elution strength during the washing steps should allow the retention behaviors of OPs on MISs and on NISs to being differentiated. Indeed, if cavities are formed during the synthesis of the MISs, they must induce a stronger retention of OPs on the MISs than on the NISs that can only generate non-specific interaction of lower energy at its surface. The elution step was carried out with a more polar solvent *i.e* acetonitrile in order to disrupt the interactions formed between the monomers and the target analytes.

MIS 1 and MIS 2 were synthesized with the same template (MCP) and monomer (APTES), changing only the porogen (H_2O was used for MIS 1 and a mixture H_20/ethanol,70/30, v/v, for MIS 2). As shown on Figure IV.4-2, both MISs gave similar extraction profiles. The difference in polarity between the two porogens therefore seems to have no impact on the retention on MIS. More precisely, the most hydrophobic OPs (FNT, FEN and CLE) were neither retained on the MIS nor on the NIS and were lost during the percolation and washing steps. Conversely, the most polar OPs like FSX, MAL and DIZ showed a higher retention on the MIS than on the NIS, thus proving the presence of cavities that induce some selectivity in the retention process of these three compounds. However, the retention of these compounds was very low since they were lost mostly during the first two washing steps with the exception of FSX. This compound is among the most polar of the three compounds and was retained until the elution step. Its high retention was due to the polar interactions between its oxygen group and the amino group of the monomer (APTES) and as the retention was lower on the NISs, both MISs were selective for this OP. In conclusion, MIS 1 and 2 have shown potential for the selective extraction of the three most polar OPs tested (FSX, MAL and DIZ), but the extraction procedure had to be optimized in order to increase the retention of these OPs while maintaining MIS/NIS selectivity.

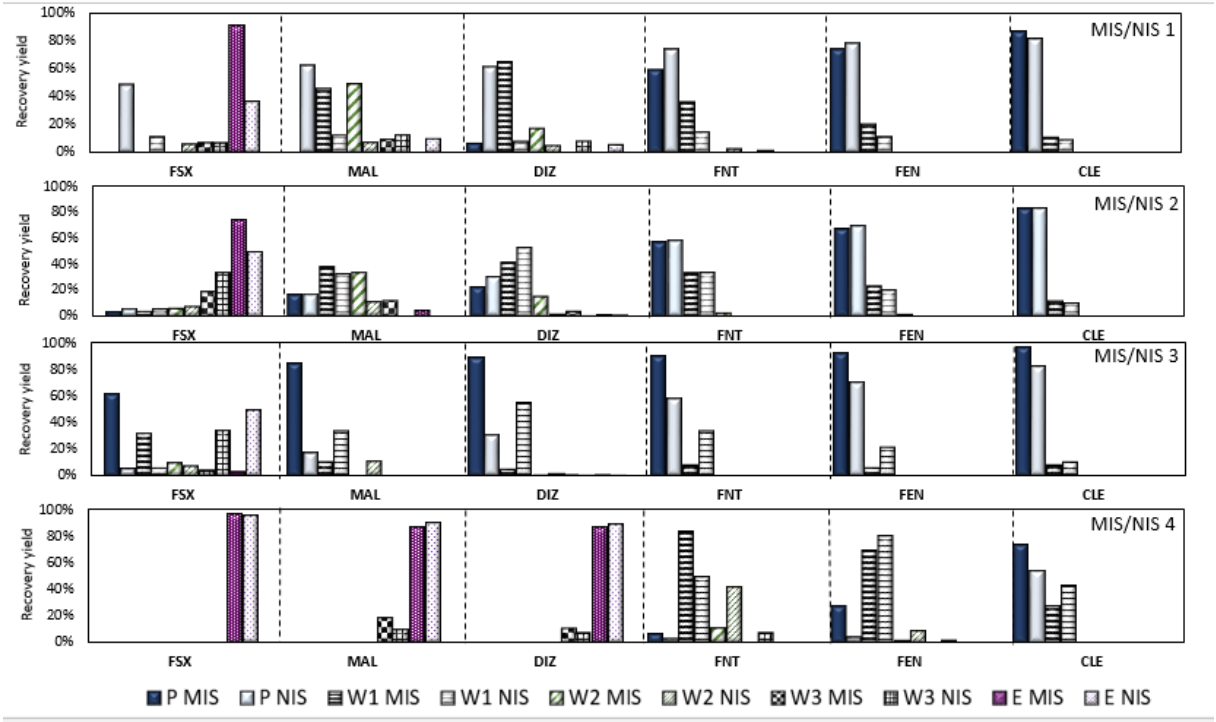

Figure IV.4-2. Extraction profiles of six OPs obtained on the four MISs/NISs by applying the screening extraction procedure including the percolation of 1 mL of hexane spiked with 1 mg/L of six OP, three washes with (W 1) 1 mL of hexane/DCM (95/5, v/v), (W 2) 1 mL of hexane/DCM (90/10, v/v) and (W 3) 1 mL of hexane/DCM (80/20, v/v) and an elution with 1 mL of ACN.

The synthesis of MIS 3 was performed in the same conditions as MIS 2 with the exception that MCP used as a template was replaced by a more hydrophobic compound, *i.e.* DIZ. A loss of retention and selectivity was observed as compared with MIS 1 and MIS 2. Indeed, the six OPs were lost during the percolation and washing steps. NIS 3 seemed even being more retentive than the corresponding MIS, especially for the more polar OPs. This phenomenon can be explained by a higher specific surface of the NIS (related to the absence of cavities) than of the MIS. At last, the replacement of APTES by PTMOS induced a higher retention on MIS 4 than on MIS 3 for all the compounds, especially for the more polar ones (FSX, MAL and DIZ) which can be explained by the expected π-π interactions between the phenyl ring of the monomers and of the compounds. Nevertheless, as for MIS/NIS 3, no selectivity was observed.

To confirm the highest selectivity obtained with MIS 1 and MIS 2 using the same extraction procedure for each MIS/NIS couple, the extraction was optimized for each MIS in order to promote retention and selectivity for a larger number of OPs. In order to confirm the behaviour of the MISs for the polar compounds, DMT and FSN were added to the rest of the studied analytes: these two compounds should have the same

behaviour on the MIS as FSX, which is the only compound that was selectively retained on the MISs. In contrast, the PIM, a hydrophobic compound, was added in order to verify the non-retention that was observed for CLE on the MISs. Finally, FNT and FEN were eliminated from the studied analytes since the LC-MS/MS analyses that follow the MISPE (a higher sensitivity was afforded for a large number of OPs) is not sensitive enough for these 2 compounds (Annexe VIII (Table 3)). The ten targeted OPs were then applied to the different MISs/NISs and the washing conditions were simplified by adapting the elution strength of a single washing fraction for each MIS/NIS using different proportions of DCM in hexane. This optimization was also performed in order to favor the retention and the selectivity for the larger number of OPs. For MIS 1 to 3, a percentage of 3% of DCM was shown to correspond to the best conditions to differentiate the MIS from the NIS. In contrast, for MIS/NIS 4 that demonstrate a higher retention, the elution strength was increased by adding 30% of DCM in hexane (1 mL). As showed on Figure IV.4-3, this modification of the washing step overall led to an increase in the extraction recovery yields in the elution fraction for each MIS/NIS couple for all the studied OPs, with the exception of CLE that was only slightly recovered in the elution fraction of MIS 2.

For MIS/NIS 1, the overall recovery yields were improved using these conditions for all the compounds and the selectivity was satisfactory for six out of the eight studied OPs despite a partial loss of selectivity for FSX. These washing conditions were chosen because they allow a selective extraction of the three most polar compounds with extraction recovery yields of 90-100% on MIS versus only 30-65% on NIS. As for MIS 1, MIS 2 showed an increase in the retention for six of the eight OPs. But the lowest elution strength of the washing solution led for this MIS/NIS 2 to a total loss of selectivity. Indeed, there were no differences in the recovery yields between MIS 2 and NIS 2 for DMT, FSX, FSN, and MTH. Finally, for MIS 3 and 4, a washing step with a higher elution strength did not allow any selectivity to being observed, the recovery yields on the MIS being similar or lower than those on the NIS. The final choice of synthesis and extraction conditions was made according to the selectivity obtained for the larger number of OPs simultaneously. Hence, MISs 2 to 4 were removed from this study and since showing the most promising in terms of retention and selectivity for six of the eight OPs, the couple MIS/NIS 1 was selected for the next experiments and renamed MIS/NIS for the last part of this paper.

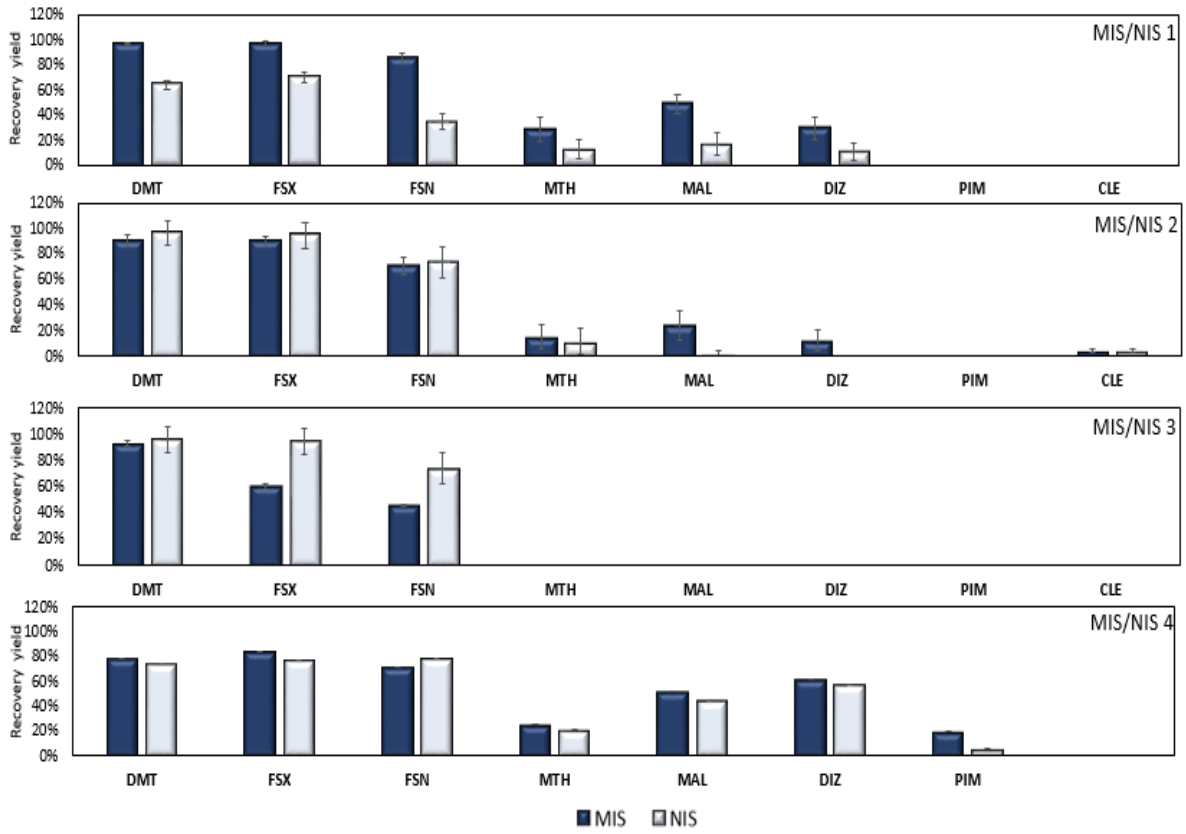

Figure IV.4-3. Recovery yield in the elution fraction obtained using four synthesis MISs/NISs by applying a short extraction procedure including the percolation of 1 mL of hexane spiked at 1 mg/L for MIS/NIS 1 or at 0.1 mg/L of eight OPs for MIS2 to 4, one washing step; 1 mL of mixture of hexane/DCM (97/3, v/v) on MIS/NIS 1 to 3 or 1 mL of mixture of hexane/DCM (70/30, v/v) on MIS/NIS 4. The average recovery yield % ± SD, (n= 3) was reported for MIS 1 to 3 and recovery yield (n= 1) for MIS 4.

IV.4.3.Repeatability of the extraction procedure

To evaluate more in detail the potential of the selected MIS (MIS 1), the developed extraction procedure was again applied to a spiked hexane sample but by introducing again the two OPs removed from the previous study for which a low retention but a slight selectivity were observed in the conditions of Figure IV.4-2. This implied again the use of LC-UV analysis for all the compounds and also to spike hexane at a higher concentration level (1 mg/L) to ensure their detection in the analyzed fraction. The extraction profiles showed that the behavior of these OPs can be divided into three different groups (Figure IV.4-4). A low retention was observed for the most hydrophobic OPs, *i.e.* FNT, FEN, PIM and CLE, those compounds being all recovered in the percolation and washing fractions. The second group is composed of MTH, MAL and DIZ (compounds having log P between 2.7 and 3.7) for which low recovery yields but selectivity were observed, as these recovery yields were higher in the elution fraction

using the MIS than the NIS. This recovery yields could be improved by decreasing the elution strength in the washing fraction (by lowering the proportion of DCM). Nevertheless, under these conditions the high selectivity obtained for the most polar compounds, *i.e*. the last group (DMT, FSX and FSN) would be affected. Then at last, for the most polar compounds recovery yields higher than 85% were obtained using the MIS versus between 35 and 70 % using the NIS thus confirming the selectivity of the MIS towards these compounds Also, the observed standard deviation values were between 1 and 10% (n= 3), which indicates the good repeatability of this MISPE procedure. The last part of the study was therefore focused on the evaluation of the potential of the MIS for extracting these compounds from real media.

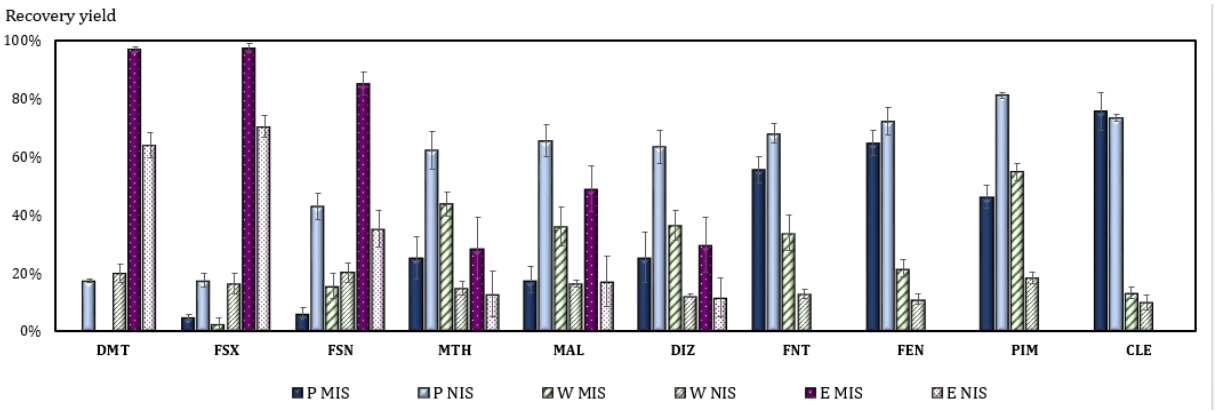

Figure IV.4-4. Extraction profiles obtained when percolating 1 mL of hexane spiked with 1 mg/L of ten OPs on MIS/NIS, washing with 1 mL of hexane/DCM 97/3 (v/v) and elution with 1 mL of ACN. The average recovery (%) ± SD (n= 3) was reported.

IV.4.4.Optimization of the extraction of OPs from almond oil

After the promising results obtained with the MIS synthesized in pure media, the performance of this sorbent was also evaluated in real media. Despite the selectivity shown for the three more polar compounds, according to the level of concentration targeted in real media, this part of the study only focused on the two OPs (DMT and FSX) that can be analyzed by LC-MS/MS. Indeed, FSN presented a high LOQ in LC-MS/MS (see the Annexe VIII (Table 3)). The applied SPE procedure was the same as for the spiked hexane sample but, due to the complexity of the sample, a LLE was considered as a previous step to the MISPE procedure. This LLE procedure was described by the ITERG (French Institute specialized in fats and oils) and used before the SPE step using a C18 sorbent [42]. In this work, the C18 sorbent was substituted by a MIS sorbent to get a selective clean up procedure. The LLE was carried out using 3 x 1 mL of ACN/DCM

(90/10, v/v) mixture for 200 mg of almond oil. The OPs were added directly into the oil extract obtained from the LLE and not before this step, since the aim of this work was the evaluation of the performance of MISPE. The MRLs values established by the most recent regulation (EC) No 1097/2009 for DMT and regulation (EU) No 310/2011 for FSX (Table IV.4-2) in almond fruits were taken as reference to set the spiking level of OPs in the almond oil to 10 µg/kg.

Table IV.4-2. Recovery yield obtained in the elution fraction using almond oil spiked with 100 µg/kg of DMT and FSX after LLE and SPE clean-up using MIS/NIS. LOQs correspond to S/N= 10.

Compounds (OPs)	(R ± SD)%, n= 3		MRLs[a] in almond seed (µg/kg)	LOQ in oil (µg/kg)	Processing factor [b]	Estimated LOQs [c] in seed
	MIS	NIS				
DMT	114 ± 10	93 ± 5	50	8 ± 1	1.7	4.6 ± 0.6
FSX	100 ± 16	70 ± 5	20	2 ± 1		1.2 ± 0.6

[a]: MRLs according to Reg. (EC) No 1097/2009 for DMT and Reg. (EU) No 310/2011 for FSX; [b]: processing factor from FEDIOL (vegetable oil and protein meal industry association); [c]: estimated LOQs according to FEDIOL processing factor.

After applying the procedure developed in pure media to an oil extract, lower recovery yields in the elution fraction were observed for DMT and FSX as compared with the pure media (Figure IV.4-5A). As showed on Figure IV.4-4 the recovery yield on MIS was 97 ± 2% for FSX in the spiked hexane sample, however it was only 52 % when applying the MIS to the oil extract diluted in hexane (Figure IV.4-5A). Moreover, for this compound there was no selectivity anymore. For DMT, the recovery yield was also drastically decreased (from 97% to 59%) although a slight selectivity was kept in real media. This large decrease in recovery yield could be explained by the matrix effects, *i.e.* the occurrence of a large amount of interfering compounds in the percolation fraction that modifies its elution strength. In order to reduce this matrix effect and to improve the recovery yields and the selectivity, the oil extract obtained from LLE was spiked at a concentration of 2 µg/L of OPs corresponding to 100 µg/kg in oil and was further diluted by a factor of 10 in hexane before the percolation of 1mL of the final diluted extract on the MIS/NIS. This dilution led to increased recovery yields and selectivity. As an example, DMT was recovered with 74 ± 5% on MIS and 58 ± 6% on NIS, the low RSD value attesting a good repeatability of the procedure (n= 3) (Figure IV.4-5B). However, these recoveries remained lower than in pure media. In order to obtain higher recovery

yields for the oil extracts, the volume used for the washing step during the MISPE procedure was further optimized.

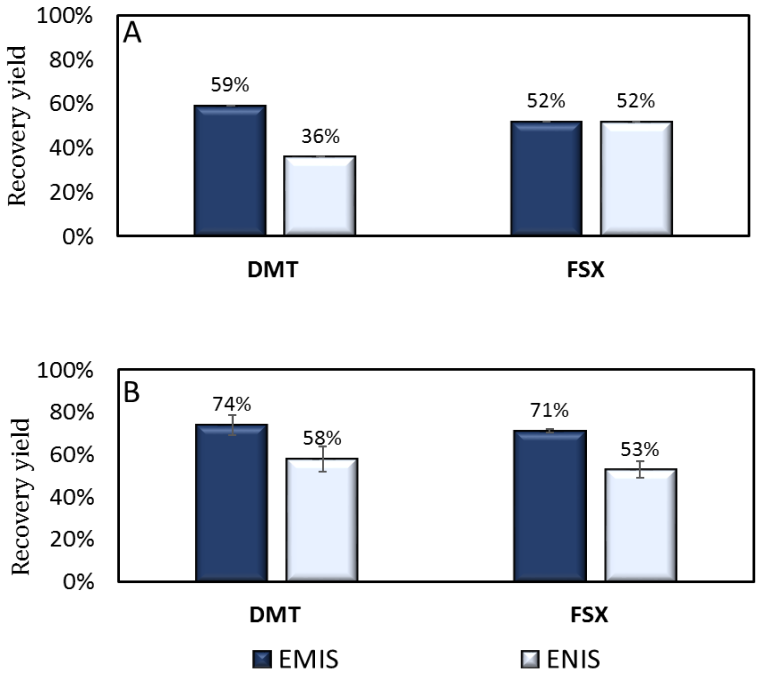

Figure IV.4-5 Recovery yield of DMT and FSX in the elution fraction using almond oil extract diluted in 1 mL and spiked with 10 µg/kg (A) or diluted in 10 mL and spiked with 100 µg/kg (B), 1 mL being percolated in both cases through the MIS/NIS. The average recovery (%) ± SD (n= 3) was reported for procedure (B) and n= 1 for the procedure (A).

For this, different volumes of washing solution (hexane/DCM, 97/3, v/v) were tested: 0.4, 0.65 or 1 mL (Figure IV.4-6). A volume of 0.4 mL in the washing step gave a good selectivity for DMT and FSX but was discarded because a probable high matrix effect led to recovery yields higher than 100 %, *i.e.* 122% for DMT and 131% for FSX These values could result from a too small volume used in the washing step, therefore the interferents were not removed and caused a matrix effect in LC-MS/MS analysis. A decrease in this matrix effect seems to have been obtained by increasing the washing volume to 1 mL, but the recovery and selectivity were lower than in pure media. By using a washing volume of 0.65 mL, recovery yields of 114 ± 10 % and 100 ± 16% were observed for DMT and FSX, respectively. This washing volume was selected since it appears as a good compromise in terms of recovery and selectivity, the recovery on the NIS being lower (93% and 70% for DMT and FSX, respectively).

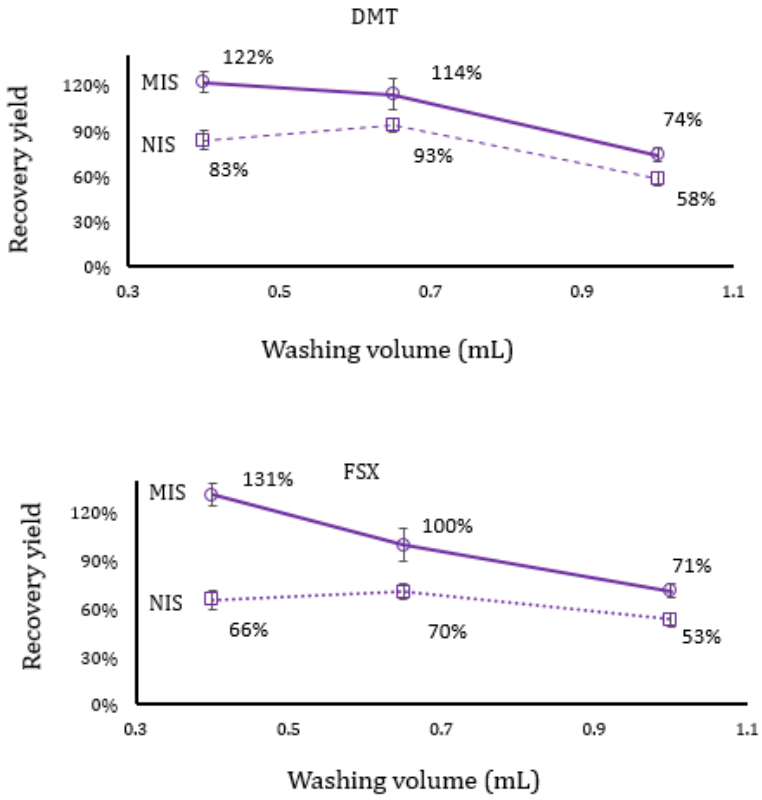

Figure IV.4-6. Recovery yield (%) ± SD (n= 3) in the elution fraction obtained after using different washing volumes (0.4, 0.65 or 1mL) of hexane/DCM (97/3, v/v).

IV.4.5.Influence of a LLE step prior MISPE

In order to simplify the whole extraction procedure, the necessity to use a LLE step before the MISPE clean-up was studied. This procedure (without LLE) was performed by directly diluting 200 mg of almond oil with 10 mL of hexane and by percolating 1mL of this solution through the MIS. The spiked concentration was the same (100 µg/kg of DMT and FSX in this oil) in order to work in the same conditions for both procedures. The recovery yields of the two OPs in the elution fraction of the MIS with or without a previous LLE step are presented on Figure IV.4-7. The extraction procedure was repeated in triplicate for both experiments. Lower recovery yields were obtained using only the MISPE clean-up alone by comparison with the use of LLE prior to MISPE clean-up (81 ± 14% and 82 ± 10% recovery for DMT and FSX, respectively). A loss of selectivity was also observed when the extraction procedure was applied without LLE, the recoveries on the MIS and on the NIS being similar for DMT and FSX. These results demonstrate that the introduction of LLE before MISPE was necessary since

recoveries and selectivity were less affected by the oil components, some of them being removed by the LLE step.

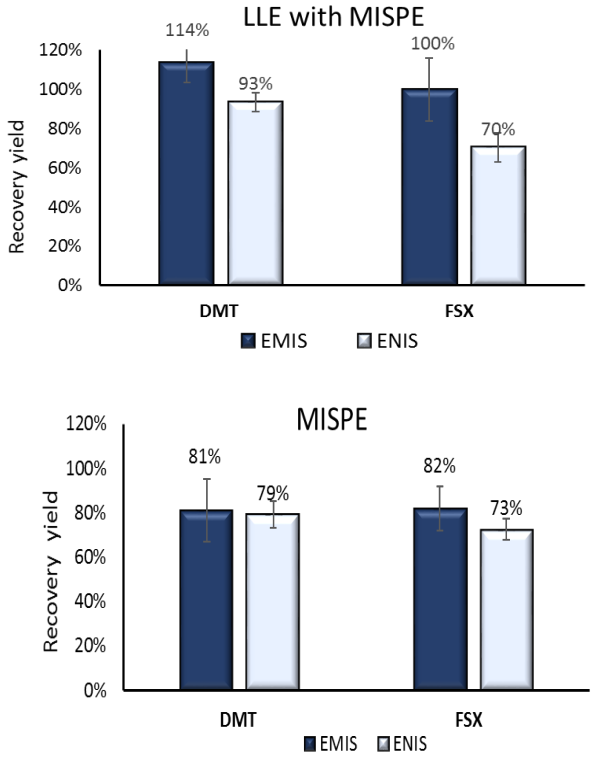

Figure IV.4-7. Recovery yield (%) ± SD (n= 3) of the DMT and FSX in the elution fraction of MISPE using 10 mL of almond oil extract spiked with 100 µg/kg, with and without a previous LLE step.

IV.4.6.Sensitivity of the applied analytical method

The LC-MS analysis in MRM mode of the elution fraction from the MIS (Figure IV.4-8) of the oil sample extract spiked at 2 µg/L of OPs corresponding to 100 µg/kg in oil, was used to estimate the LOQs. The calculated LOQs (S/N =10) for the two OPs were 2 and 8 µg/kg for DMT and FSX respectively (Table IV.4-2). As the MRLs for pesticides in oils are not set in the EU regulation, a processing factor proposed by FEDIOL was applied to compare the LOQs of pesticides in crude oils with the MRLs of pesticides in seeds or fruits. This processing factor is calculated by taking into account the oil content and the hydrophobicity of the OPs at the same time. For almond oil, the average oil content is 58% [1], thus the estimated processing factor is 1.7. This value was then used as a reference to estimate the LOQs of OPs in almond fruits (Table IV.4-2). The calculated LOQs in almond fruits were 1.2 ± 0.6 and 4.6 ± 0.6 µg/kg for DMT and FSX, respectively. These values are lower than the MRLs values of 20 and 50 µg/kg,

respectively. These results mean that this selective approach using the MIS allows two OPs in almond oil to being determined at a concentration level lower than their respective MRLs.

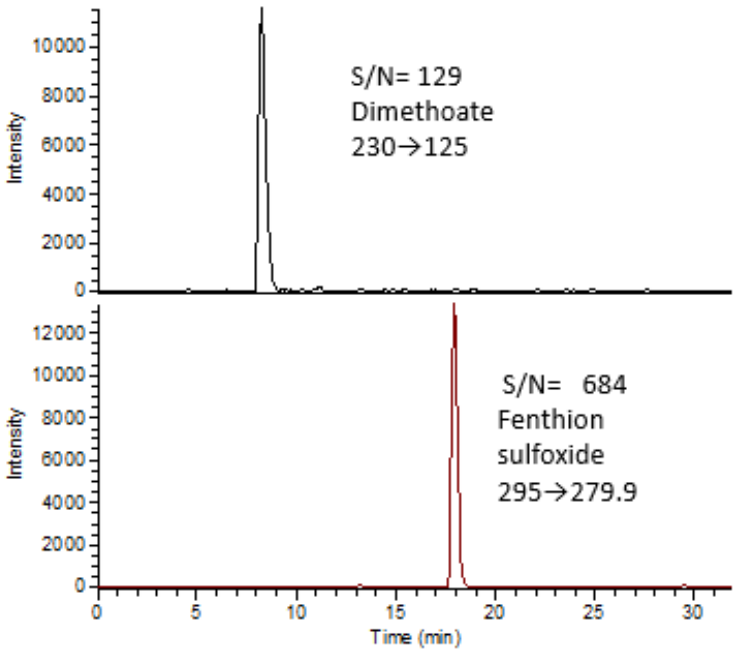

Figure IV.4-8. LC-MS chromatograms (MRM mode) of the elution fraction of almond oil extract spiked with 100 µg/kg of DMT and FSX issued of the MIS.

Moreover, it is important to mention the possible reusability of this sorbent. Indeed, it was used for more than 100 experiments without observing a decrease in its performances. Besides the selectivity and retention, reusability of the sorbent is an important factor that limits the use of reagents and the time of synthesis.

IV.5.Conclusions

Different conditions of synthesis were screened in order to determine those giving rise to a MIS able to selectively extract several OPs. Among the four synthesized MISs, the selected MIS obtained using monocrotophos as the template, 3-aminopropyl triethoxysilane as the monomer and tetraethyl orthosilicate as the cross-linker with a molar ratio of 1/4/20 respectively, was able to selectively trap six OPs (DMT, FSX, FSN, MTH, MAL, DIZ) in pure media, out of which three with high recovery yields (DMT, FSX, FSN).

After the study of the repeatability of the optimized MISPE procedure in pure media, the performance of this sorbent was evaluated also in real media, for two compounds that can be analyzed in LC-MS/MS with a high sensitivity. The optimization of the volume of oil extract before the MISPE and of the washing volume in MISPE procedure was performed to improve the recovery yields. Hence, this MIS was able to selectively extract DMT and FSX from almond oil with high recovery yields. The estimated LOQs, between 1.2 and 4.6 µg/kg of OPs from the almond fruit were lower than the MRLs (between 20-50 µg/kg) established for this matrix. MIS was able to selectively extract polar OPs such as DMT, FSX and FSN with high recoveries while the MIP allowed the selective extraction of moderately polar OPs such as MET, MAL and DIZ [31].

Acknowledgment

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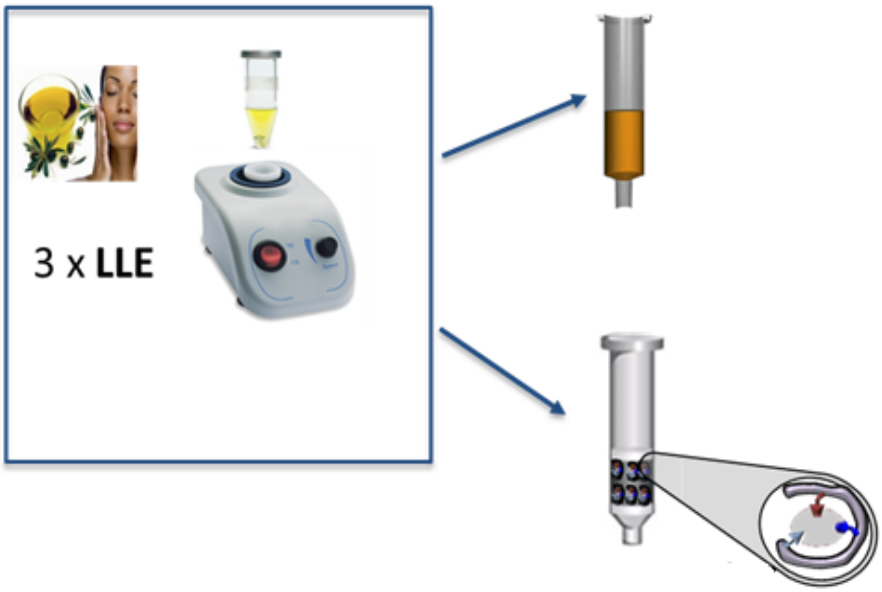
Chapter V: Reduction of matrix effects using molecularly imprinted silica applied to the extraction of organophosphorus pesticides from vegetable oils



Dans le chapitre précédent nous avons montré que le support MIS, synthétisé en utilisant le monocrotophos comme molécule empreinte, le 3-aminopropyl triéthoxysilane comme monomère et le tetraethyl orthosilicate comme agent réticulant avec un ratio molaire 1/4/20, pouvait extraire sélectivement deux OP polaires (dimethoate et fenthion sulfoxide) de l'huile d'amande. Ce chapitre III basé sur l'article « Reduction of matrix effects using molecular imprinted silica applied to the extraction of organophosphorus pesticides from vegetable oils » présente les avantages de l'utilisation de ce MIS pour extraire de trois huiles végétales différentes (amande, tournesol et olive) de façon sélective pour ces deux OP polaires.

Tout d'abord la répétabilité de la procédure d'extraction en milieu pur a été évaluée et des coefficients de variation inférieure à 10% ont pu être observés. Ensuite la capacité de ce support a été déterminée (plus de 10 µg d'OP pour 20 mg de support) et à permit de confirmer que ce support MIS permet l'analyse des OP dans les huiles à des niveaux de concentrations très élevés. Après cette caractérisation en milieu pur, ce support a été évalué pour extraire les deux OP polaires de trois huiles végétales différentes (amande, olive et tournesol). Les résultats montrent que la rétention des deux OP cibles sur le MIS est très différente selon la nature de l'huile utilisée. En effet, si l'extraction dans les huiles d'olive et de tournesol conduit à des rendements d'extraction similaire à ceux obtenus en milieu pur (proche de 80% pour les deux OP), les rendements obtenus pour l'extraction de l'huile d'amande semblent être inférieur (respectivement 72 et 45% pour le DMT et le FSX). Néanmoins, l'évaluation de l'effet de matrice lors des analyses en LC-MS/MS a montré que l'utilisation de ce support permet de réduire significativement les effets de matrice par rapport à l'utilisation de supports classiques (C18), d'un facteur compris entre 2 et 10 dans l'huile d'amande. Et donc d'améliorer de façon importante les limites de quantification correspondantes. En effet, leur calcul pour les OP a conduit à des valeurs de 6 à plus de 100 fois inférieures aux limites maximum résiduelles (LMR) comprises entre 10 et 3000 µg/kg pour les amandes, les olives et les graines de tournesol. Par conséquent, ce MIS présente un réel potentiel pour extraire sélectivement ces deux OP polaires présent à l'état de traces de différentes huiles en réduisant les effets matrice.

Afin d'élargir la gamme d'OP extrait des huiles végétales par ces supports imprimés et au vu des similarités entre les procédures d'extraction optimisées sur le MIS et le MIP, un couplage des deux supports pourrait être envisagé pour permettre

l'extraction d'un plus grand nombre de composés OP en ajustant légèrement la procédure d'extraction.

V.Article 3

Reduction of matrix effects using molecularly imprinted silica applied to the extraction of organophosphorus pesticides from vegetable oils

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V.1.Abstract

Vegetable oils are largely used in food but also in the cosmetic industry for their ability to moisturize, protect and strengthen the skin. However, the use of pesticides on crops, such as organophosphorus compounds (OPs) may cause health risks to humans. Hence, their analysis at trace levels in such a complex matrix requires a selective extraction prior to the chromatographic analysis. According to a previous work, a molecularly imprinted silica (MIS) sorbent was synthesized and used for the selective solid-phase extraction (SPE) of polar OPs from several oils (almond, olive and sunflower oils). The repeatability of the extraction procedure on this newly synthesized MIS was first evaluated in pure media. Its capacity was studied and was found higher than 10 µg of fenthion sulfoxide for 20 mg of support. The extraction recoveries from these three oils ranged 72-86% for dimethoate and 45-68% for fenthion sulfoxide. Matrix effects were studied in detail showing that the MIS allowed us to reduce them by a factor of 2 to 10, as compared to the use of classical sorbents (C18). Finally the estimated limits of

quantification (LOQs, S/N=10), ranging 0.2-10 µg/kg for OPs, were lower than the Maximum Residue Levels (MRLs) established by the European Commission, that are comprised between 10 and 3000 µg/kg in almond, olive and sunflower seeds.

Keywords: solid-phase extraction; molecularly imprinted silica; organophosphorus pesticides; vegetable oils; matrix effects; liquid chromatography; mass spectrometry.

V.2.Introduction

Vegetable oils are widely used for cooking and processing in the food industry since they are rich in saturated and unsaturated fatty acids, triglycerides, antioxidants, and other fat-soluble vitamins [1]. These oils are also well known in the cosmetic domain for moisturizing, protecting and strengthening the skin. Vegetable oils are usually extracted from crops by mechanical pressure or organic solvents. Therefore, pesticides used on these crops may contaminate the vegetable oils, thus explaining the necessity to strictly monitor their concentration in these matrices. Hence, the European Commission (EC) regulation No 396/2005 have established the Maximum Residue Level (MRL) as the highest level of a pesticide residue that is legally tolerated in raw materials such as oil seeds and fruits. However, MRLs on processed products are not established yet. Hence, a 'processing factor' was proposed by FEDIOL (vegetable oil and protein meal industry association) to estimate the corresponding contamination level in oils.

Among all pesticides applied to crops, organophosphorus compounds (OPs) are found in vegetable oil, sometimes in high concentrations. The OPs are neurotoxic compounds, through inhibiting the activity of acetylcholinesterase (AChE) [2,3]. Therefore, the analysis of OPs pesticides in such matrices becomes necessary. However, high amount of lipids in vegetable oils can co-extract with the analytes of interest and can seriously affect the extraction efficiency and performance of analytical instruments. Indeed, a small amount of lipids could cause significant damage to the column, source and detector [4]. Therefore, methods of sample pretreatment are required to remove the lipids that can co-extract with the analytes prior to chromatography and/or mass spectrometry analysis. In general, liquid-liquid extraction (LLE) [5–9] step, low temperature precipitation [7], gel permeation chromatography [10], QuEChERs methods [8,9,11,12], matrix solid-phase dispersion (MSDP) [13] or solid-phase microextraction

(SPME) [14] are used to extract OPs from vegetable oils prior to their chromatographic analysis. Solid-phase extraction (SPE) [5,15,16] is also largely used as extraction technique of OPs from vegetable oils. However, the use of conventional sorbents lacks of selectivity and leads to the co-extraction of many interfering compounds. Hence, other sorbents, *i.e.* molecularly imprinted polymers (MIPs), based on molecular recognition mechanism, were proposed as selective sorbents since possessing specific recognition sites for the target OPs [17–22]. In common approach, their synthesis is based first on the formation of template-monomer complex by non-covalent interactions in a porogenic solvent. The polymerization is then produced around the complex by using a cross-linker in the presence of an initiator [23–25]. Finally, the template is removed, leaving a polymer with cavities that are complementary to the template in size, shape and position of the functional groups.

To date, few studies reported the use of MIPs for the selective extraction of OPs in vegetable oils. *Bakas et al.* have reported the selective extraction using MIP sorbents in SPE focusing on one OP from olive oil in each study, *i.e.* methidathion [26], dimethoate [27] and fenthion [28], respectively. Up to now, only one work made by our group reported the selective extraction for several OPs in different oil samples, using a MIP [29]. In this work, after screening different conditions of synthesis, a selected MIP was able to extract the moderately polar OPs (methidathion, malathion and diazinon) among the studied OPs from almond, sunflower and olive oils. However highly polar and non-polar OPs that are also frequently present in oils were not selectively extracted by this MIP. Hence, as an alternative to radical polymerization, imprinted sorbent based on sol gel approach, molecularly imprinted silica (MISs) sorbents can be prepared. Their synthesis is similar to those of MIPs. First, the monomer, an organosilane with a functional group (amino, phenyl) and a cross-linker, an alkoxysilane, react in aqueous solution to form silanol (Si-OH) groups by hydrolysis, leading siloxane (Si-O-Si) bonds to being formed by a condensation reaction with the silanol groups around the template molecule, with the help of an acidic or alkaline catalyst [30,31]. MISs were already proposed for the selective extraction of different neurotoxic compounds from cyanobacterial samples [32], nitroaromatic explosives from post-blast samples [33] or ibuprofen from urine [34] and also to extract OPs in samples such fruits, vegetable or rice, by applying MIS in SPME [35,36] or as sensors [37]. As for the extraction of OPs from oil samples, our group recently prepared a MIS with the objective to extract OPs of

different polarities [38]. Again, after the screening of different conditions of synthesis, a MIS was found able to extract six OPs from pure media out of which three with high yields of recovery–(dimethoate, fenthion sulfone and fenthion sulfoxide) from almond oil, showing a good complementarity in term of selectivity with the previously developed MIP [29].

Hence, this work aimed at again preparing a MIS in the same conditions of synthesis, using monocrotophos as template, 3-aminopropyl triethoxysilane (APTES) as monomer, tetraethoxysilane (TEOS) as cross-linker, in water and with ammonia as catalyst to selectively extract polar OPs from three different oils (almond, olive and sunflower oils) and to compare the performances of the MIS in this real conditions with those of the conventional sorbent, the C18 silica, in terms of limit of quantification and of matrix effects.

V.3.Materials and methods

V.3.1.Chemicals

Standard pesticides, dimethoate (DMT) 98%, fenthion sulfoxide (FSX) 99% and fenthion sulfone (FSN) 99%, were supplied by Cluzeau Info Labo (Sainte-Foy-La-Grande, France). Individual stock solutions from each OP were prepared at a concentration of 100 mg/L in acetonitrile (ACN). A stock solution mixture containing 5 mg/L of each OP was prepared in ACN and stored at 4 °C prior to use.

Monocrotophos (MCP) 98.5%, ammonium acetate for HPLC 99.0% (AC), anhydrous n-hexane 95%, 3-aminopropyl triethoxysilane (APTES) 99%, and tetraethyl orthosilicate (TEOS) 99.99% were supplied by Sigma-Aldrich (Saint Quentin Fallavier, France). HPLC-grade ACN, methanol (MeOH) and dichloromethane (DCM) were supplied by Carlo Erba (Val de Reuil, France). High purity water was dispensed by a Milli-Q purification system (Millipore, Saint Quentin en Yvelines, France). Ammonium hydroxide (NH_4OH) solution at 32%, acetic acid (AA) and formic acid (FA) were purchased from VWR (Fontenay-sous-Bois, France).

Vegetable oils issued of organic farming (almond, olive and sunflower oil) were supplied from organic supermarket in Paris, France.

V.3.2.Apparatus and analytical conditions

The LC-MS/MS analyses were performed using a liquid chromatograph (UltiMate 3000®, Thermo Scientific, Illkirch, France) coupled with Triple Stage Quadrupole Mass Spectrometer, TSQ Quantum Access MAX (Thermo Scientific, Illkirch, France) equipped with a heated electrospray ionization source (HESI2). The chromatographic separation was performed on an Accucore PFP column (150 x 2.1 mm, 2.6 µm, ThermoFisher Scientific, Villebon Courtaboeuf, France) and maintained at 32 °C with a column oven (Croco-cil, Cluzeau Info Labo, Sainte-Foy-La-Grande, France). Samples were analyzed in the same conditions as previously described [29] using water (A) and MeOH (B) both containing 0.1% (v/v) of FA and 4 mM of AC. Briefly, the gradient started at 20% of B for 2.5 min then increased to 80% of B in 23.5 min, held for 2 min, and returned to the initial composition within 2 min followed by a further 2 min to let the system equilibrate. The flow rate was set at 0.4 mL/min and the injection volume was 2 µL.

MS was operated in positive ion mode with MRM detection using an electrospray voltage of 3500 V and a skimmer offset of 5 V. The capillary and vaporizer temperatures were set at 280 °C and 295 °C, respectively. The sheath gas pressure and auxiliary gas pressure were set at 55 and 15 units, respectively. Nitrogen was used as nebulizer and desolvatation gas and argon as the collision gas at a pressure of 1.5 mTorr. For the optimization of the MS detection, each OP was infused at a concentration of 5 mg/L in the mobile phase A/B (50/50, v/v). The quantification of the 3 OPs was performed in MRM mode using the specific transitions. A second transition was used for confirmation purposes and to avoid false positive responses. The m/z values, tube lens and collision energies values corresponding to quantitation and confirming ions are summarized in the Annexe IX (Table 1).

The LC-DAD analyses were performed using a liquid chromatograph (LC) Agilent 1200 series (Agilent Technology, Massy, France) system equipped with a binary pump, an auto sampler and a diode array detector (DAD) controlled by a Chemstation software. OPs were separated using the same column, flow rate and injection volume as for LC-MS/MS analysis. Samples were analyzed using linear gradient elution with water (A) and ACN (B). The gradient started at 8% of B during

2.5 min then increased to 60% of B in 23.5 min, held for 2 min, returned to the initial composition within 2 min followed by a further 2 min to let the system equilibrate. DMT and FSX were quantified at 210 and 240 nm, respectively.

V.3.3.Synthesis of molecularly imprinted silica (MIS) sorbents

As previously described [38], the MIS was synthesized with MCP (0.25 mmol) used as template, APTES (1mmol) as monomer, TEOS (5 mmol) as cross-linker, H_20 (2 mL) as porogen and NH_4OH solution at 32% (400 µL) as the catalyst. Briefly, the template was dissolved in the porogen. Then, the monomer, cross-linker and catalyst were added to the glass vial containing the solution of the template molecule. The resulting solution was immersed into a silicone oil bath heated to 40°C, stirred and kept for 24 h. The obtained product was left at room temperature for 3 h and heated at 120 C in an oven for 18 h to evaporate the solvent in excess and to dry off the sorbent. Thereafter, the polymer was crushed and manually sieved. Only particles between 25 µm and 36 µm were collected. The sedimentation was performed 3 times, using 10 mL of MeOH/H_20 (80/20, v/v) mixture to remove the thin particles and dried off for 24 h at room temperature. Next, 20 mg of polymer were packed in a 1 mL disposable cartridge of propylene (Interchim) between two polyethylene frits (20 µm, Sigma-Aldrich). To remove the template, the polymer was washed until the template could no longer be detected in the washing fraction (with approximately 10 mL of MeOH). NIS sorbents were prepared by performing the overall procedure in the absence of template.

V.3.4.Solid phase extraction procedure in pure media

To confirm the repeatability of both the extraction procedure and the synthesis, the same extraction procedure was applied as described in our previous work [38]. The MIS/NIS sorbents were conditioned with 4 mL of hexane. Then, 1 mL of hexane solution spiked with 1 mg/L of each OP was percolated through the MIS/NIS and 1 mL of hexane/DCM (97/3, v/v) mixture was further used for the washing step. Finally, the target OPs were eluted with 1 mL of ACN. The elution fraction was evaporated to dryness under a nitrogen stream, reconstituted with 0.5 mL of ACN and directly analyzed by LC-DAD. The analytical conditions are described in Part V.3.2.

The same procedure was applied to samples of hexane spiked with 1, 5 and 10 mg/L of FSX to evaluate the capacity of the MIS.

V.3.5.Extraction procedure of OPs from vegetable oils

Two OPs were extracted from almond, olive and sunflower oils by applying first a LLE by using 3 times 1 mL of ACN/DCM (90/10, v/v) mixture for 200 mg of oil. This LLE procedure was described by the ITERG (French Institute specialized in fats and oils) and used before a clean-up step using a C18 sorbent, based on the percolation of spiked solution in ACN/DCM (90/10, v/v) and an elution with methanol [39]. In this work, a MIS sorbent was used as clean-up sorbent in replacement of C18 silica. For this, the oil extract was evaporated to dryness and then reconstituted in 10 mL of hexane spiked at 2 µg/L (equivalent to 100 µg/kg in oil) with DMT and FSX. Afterwards, a SPE with the MIS/NIS was carried out. First the cartridges were conditioned with 4 mL of hexane, then 1 mL of the oil extract was percolated through the silica sorbent. Then the MIS/NIS sorbents were washed with 0.65 mL of hexane/DCM (97/3, v/v) and the OPs were eluted with 1 mL of ACN. The elution fraction was evaporated to dryness and reconstituted in 100 µL of ACN before LC-MS/MS injection.

Non-spiked blank oils were analyzed by LC-MS/MS after applying the full extraction procedure. FSX was detected at concentrations of 5 and 6 µg/kg in the almond and sunflower oils, respectively. The extraction recoveries were therefore corrected for FSX by taking into account these initial contents.

V.4.Results and discussions

V.4.1.Repeatability of the MIS synthesis

In the previous work, a high retention, a good selectivity and a good repeatability of the extraction procedure were obtained for three polar OPs (DMT, FSX and FSN) among the ten evaluated compounds with a MIS prepared using MCP, APTES and TEOS with a 1/4/20 molar ratio [38]. A MIS was then synthesized in the same conditions and its capacity to extract two polar OPs (Figure V.4-1) with a high selectivity and high recoveries was first checked. FSN was removed from this study since bringing a low signal in LC-MS/MS analysis. The new synthesized MIS was evaluated first in pure media by applying the same extraction procedure developed in

the previous work. Results are reported on Figure V.4-2. High recoveries (79% and 89%) for FSX and DMT, respectively were obtained again on the MIS with a standard deviation between 2 and 10% (n=3) showing also a good repeatability of the extraction procedure. The selectivity was also demonstrated by the lower extraction recoveries (38 and 70 %) obtained on the NIS for FSX and DMT, respectively. The recoveries on both sorbents are slightly lower than those obtained on the MIS/NIS previously synthesized, likely explained by the use of 20 mg of MIS instead of 22 mg while applying the same percolated and washing volume during the extraction procedure.

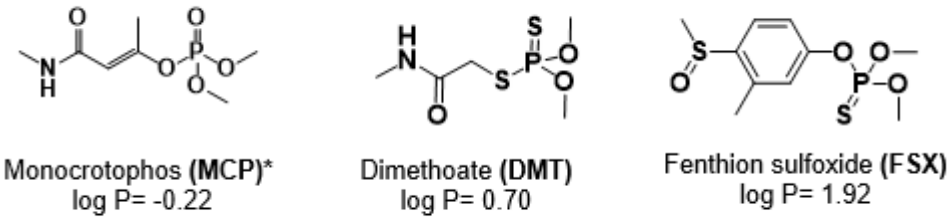

Figure V.4-1. Chemical structure and partition coefficient of two OPs and of the template. Log P values are issued from Pesticide Properties Data Base from University of Hertfordshire.*

After confirming a similar behavior of the studied compounds on both newly synthesized sorbents and a good repeatability of the extraction yields, the potential of this MIS was evaluated in terms of capacity and of selective extraction of OPs from several oil extracts.

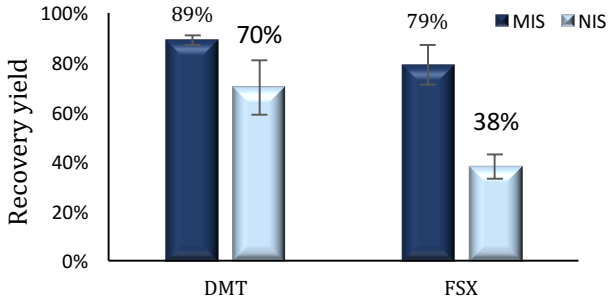

Figure V.4-2. Recovery yield (%) ± SD (n= 3) of DMT and FSX in the elution fraction obtained on MIS/NIS. Extraction procedure: percolation of 1 mL of hexane spiked with 1 mg/L of each OP, washing with 1 mL of hexane/DCM 97/3 (v/v) and an elution with 1 mL of ACN.

V.4.2.Study of the capacity of the MIS in pure medium

The capacity of the MIS was studied in order to complete the characterization of this MIS in a pure medium. The capacity of an imprinted sorbent can be defined as the largest amount of target molecule that can be selectively retained by the cavities of this sorbent in given conditions of use with a constant recovery. It is thus related to the number of specific cavities of the MIS. Hence, FSX was used to evaluate the capacity of this MIS because of the highest selectivity of the MIS towards this compound. Three amounts (1, 5 and 10 µg corresponding to the percolation of 1mL of hexane spiked at 1, 5 and 10 mg/L) of FSX were percolated through the MIS/NIS cartridges (Figure V.4-3) and the recovery yields were reported as a function of the percolated amount of FSX. The recovery yield remained constant for the three spiking levels. This was in agreement with the recoveries reported on the Figure V.4-2, using the same procedure with average recoveries of 79% and 40% on the MIS and the NIS, respectively, for these three spiking levels. These constant recoveries reflect the fact that the capacity was not reached. Higher spiking levels were not studied because of the limited solubility of the polar FSX in hexane. Moreover, a contamination of 10 mg/L in the oil extract already represents a very high level of contamination for this kind of samples. This value of capacity higher than 10 µg of FSX for 20 mg of MIS (higher than 0.5 mg/g, *i.e.* 1.7 µmol/g of MIS) is in good agreement with the range of capacity values reported by our group for other MISs synthesized for a polar neurotoxin (0.34 µmol/g [32]) or for explosives (17 µmol/g [33]). It is also in the range of capacity values reported for MIPs (produced by radical polymerization of organic monomers) for OPs (ranging from 0.5 µmol/g [26] to 3.31 µmol/g [29]). This value therefore offers the possibility to apply this sorbent to highly contaminated real oil samples.

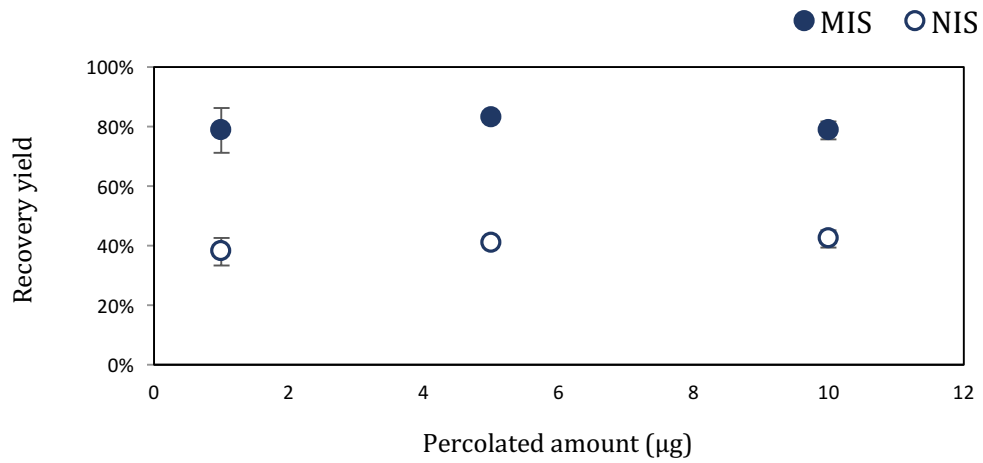

Figure V.4-3. Recovery yields obtained after the percolation of 1 mL hexane spiked with various amounts of fenthion sulfoxide on MIP and on NIP; n= 3 for the spiking level 1 and 10 mg/L and n= 1 for the spiking level 5 mg/L.

V.4.3.Extraction of DMT and FSX from various vegetable oils

In order to evaluate the potential of the MIS for the extraction of OPs from real media, the latter was applied to the extraction of two OPs (DMT and FSX) from three organic vegetable oils (almond, olive and sunflower). Oils were first treated by LLE and the resulting extracts were diluted in 10 mL of hexane spiked at 2 µg/L (equivalent to 100 µg/kg in oil) with the two OPs before percolation through the MIS/NIS according to our previous work showing the necessity to dilute the extract before passing it through the MIS [38]. 1 mL of this diluted fraction was then percolated through the MIS/NIS: the recovery yields of the elution fractions are reported in Table V.4-1 and compared to those previously obtained in a spiked pure medium. In addition, the selectivity was maintained for the three oil samples as recoveries on MIS were still higher than on NIS. Moreover, the recovery yields obtained for olive and sunflower oil samples on the MIS are quite similar to those obtained in pure spiked hexane. However, the recovery yield of almond oil extracts were lower than for the two other oil extracts, which could be explained by a matrix effect that affects the retention of the target OPs. This different behavior was confirmed by experiments carried out in triplicate for this oil sample that led to low RSD values of 4-10%. These results thus highlight the necessity to first evaluate the recoveries for each type of oil,–since the presence of matrix constituents could have an effect on the retention of OPs on the imprinted sorbent. Once the recoveries were determined, the extraction procedure was reliable, as shown by the low RSD values obtained for almond oil samples.

Table V.4-1. Recovery yields (%) obtained in the elution fraction of the MIS/NIS after the percolation of almond, olive and sunflower oil extracts spiked with DMT and FSX at 2 µg/L (equivalent to 100 µg/kg oil) and compared to recoveries obtained in a spiked pure medium.

OPs	Pure medium (n=3)		Olive oil (n = 1)		Sunflower oil (n = 1)		Almond oil (n = 3)	
	MIS	NIS	MIS	NIS	MIS	NIS	MIS	NIS
DMT	89 ± 2	70 ± 11	86	67	81	53	72 ± 6	50 ± 10
FSX	79 ± 8	38 ± 5	80	73	68	48	45 ± 10	18 ± 4

V.4.4.Evaluation of the matrix effects

After studying the effect of sample constituents on recovery yields, the matrix effects, that can affect the quantification of compounds at trace levels in LC-MS/MS, were studied more in detail and compared to those that could be encountered using C18 silica [39]. For this, an almond oil extract obtained by LLE was diluted in the appropriate solvent and percolated through the MIS or C18 silica, used as clean-up sorbent. The final extracts resulting from the use of each sorbent were spiked at an equivalent of 100 µg/kg of oil with the two OPs prior to the LC-MS/MS analysis. To evaluate the matrix effects, the MS signal of each compound was compared to the MS signal observed after direct injection of the standard solution of OPs [40]. In parallel, recoveries using C18 silica were estimated, as previously for MIS/NIS sorbents, by spiking the LLE extract with the two OPs.

As shown in Table V.4-2, the recoveries obtained with C18 silica were higher than with the MIS. Indeed, recovery yields of 100% were obtained using C18 silica, thus highlighting the fact that components from the oil sample do not seem to affect the retention of OPs on this sorbent contrarily to the MIS. However, concerning the quantification of OPs in LC-MS/MS, the matrix effects were 3 or 10 times higher with C18 silica than with the MIS. This indicates that the use of the MIS as selective sorbent allows reducing most of the matrix effects that may interfere with the quantification of OPs by LC-MS/MS whose specificity of the signal, when working in MRM mode, could let think that the extracts are clean. The higher content in matrix components of the C18 extract compared to the MIS extract is also illustrated by the LC-DAD analysis of the elution fraction obtained using MIS and C18. Whether this method cannot be used for the quantification of OPs at this low level of concentration, the chromatogram reported

on Figure V.4-4 corresponding to the use of C18 shows that the C18 extract contained more matrix constituents than the MIS extract.

Table V.4-2. Recovery yields and matrix effect in LC-MS/MS quantification obtained when analyzing an almond oil extract spiked with DMT and FSX (spiking level equivalent to 100 µg/kg) after LLE and SPE clean-up using MIS or C18.

Compounds (OPS)	Sorbent	Recovery yield (%) n= 3	Matrix effect (%) n= 3
DMT	MIS	72 ± 6	1 ± 4
	C18	103 ± 1	10 ± 9
FSX	MIS	45 ± 10	3 ± 4
	C18	112 ± 3	8 ± 11

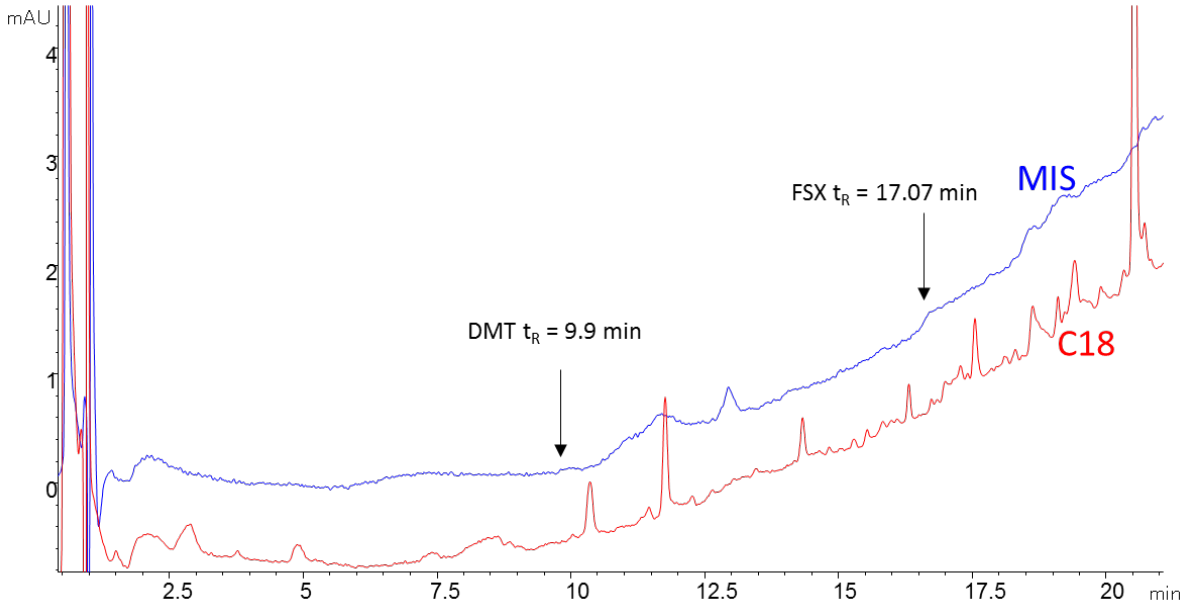

Figure V.4-4. LC-UV (210 nm) analysis of the elution fraction of almond oil extract spiked with two OPs using C18 or MIS after LLE (spiking level equivalent to 100 µg/kg).

V.4.5.Sensitivity of the applied method on the three vegetable oils

The sensitivity of the method was assessed for the three oils to evaluate the performance of the developed method for the determination of these OPs at concentration level lower than the MRLs. However, the MRLs were established for these pesticides in raw material only (seeds) and not for the oils. Thus, in order to compare the LOQs (S/N= 10) obtained with this method with the MRLs established by the EU regulation (Table V.4-3), their estimation was necessary. It was proposed by FEDIOL to take into account a concentration factor between oil and seeds to calculate LOQs in seeds from the estimated LOQs in oils. To calculate this concentration factor, the oil content of the seeds has to be considered. The estimated oil content of the analyzed

samples were 58% [41], 20% [42] and 50% [43] for almond, olive and sunflower seeds, respectively. The estimated processing factor is usually applied to hydrophobic compounds (log P > 3), but it was also applied, in this case, to the target analytes despite their higher polarity (log P of 0.7 and 1.9 for DMT and FSX, respectively) to estimate the LOQs in almond. The LOQs were estimated by taking into account the LC/MS chromatograms in MRM mode of the elution fraction from the MIS (Figure V.4-5) for the three oil samples (spiking level equivalent to 100 µg/kg). The LOQ values after applying the processing factor ranged 2.3 to 10 µg/kg for DMT and 0.2 to 1.5 µg/kg for FSX in the three oils were lower than the MRLs. These results mean that this analytical method allows these OPs to being determined at concentrations lower than their MRLs (between 10 to 3000 µg/kg for DMT and between 10 to 20 µg/kg for FSX).

Of note, it is also important to mention that the MIS was used more than 50 times without observing a decrease in recoveries: reusability is an important parameter when assessing the global cost of a sorbent.

Table V.4-3. Estimated LOQs (S/N= 10) of DMT and FSX obtained thanks to the three oils spiked at 100 µg/kg.

Oils	Compounds (OPs)	MRLs[a] in seed (µg/kg)	LOQ in oil (µg/kg)	Oil content in seed	Processing factor [b]	Estimated LOQs [c] in seed (µg/kg)
Almond n= 3	DMT	10	3.9 ± 0.6	58%	1.7	2.3 ± 0.4
	FSX	20	0.3 ± 0.1		1.7	0.2 ± 0.1
Olive	DMT	3000	24	20%	4	6
	FSX	10	2		4	0.5
Sunflower	DMT	10	20	50%	2	10
	FSX	20	3		2	1.5

[a]: MRLs according to Regulation (EU) No 2017/1135 and No 310/2011; [b]: processing factor from FEDIOL (vegetable oil and protein meal industry association); [c]: estimated LOQs according to FEDIOL processing factor.

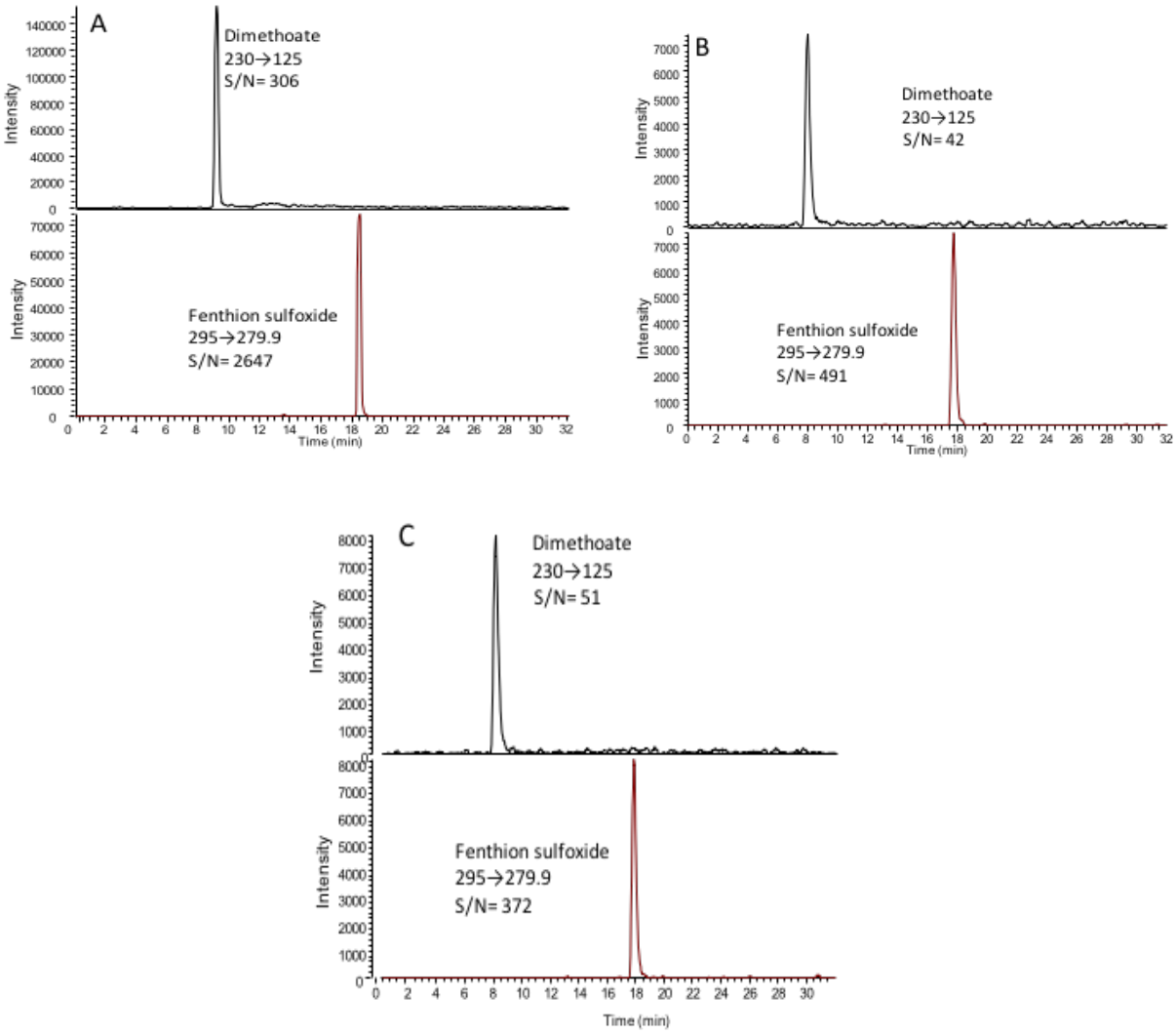

Figure V.4-5. LC-MS/MS analysis (MRM mode) of almond (A), olive (B) and sunflower (C) oil extracts spiked with two OPs and cleaned-up using the MIS (spiking level equivalent to 100 µg/kg).

V.5.Conclusions

This work confirms that a MIS synthesized using monocrotophos as template, 3-aminopropyl triethoxysilane as monomer and tetraethyl orthosilicate as cross-linker, was able to selectively trap two polar OPs, dimethoate and fenthion sulfoxyde from pure media and from oil extracts. The repeatability of the extraction procedure was satisfactory for both media with RSD values lower than 10% for oil extracts. The retention of the two target OPs on the MIS were quite different depending on the nature of the used oil (olive, sunflower or almond). Nevertheless, for their determination by LC-MS/MS at trace levels in oil extract, the use of this sorbent allows matrix effects to being reduced by comparison with the use of classical sorbents (C18), by a factor of 2 to 10

with almond oil. The estimated limits of quantification (LOQs, S/N=10) between 0.2 to 10 µg/kg for OPs were lower than the Maximum Residue Levels of 10 to 3000 µg/kg established by the European Commission in almond, olive and sunflower seeds. Therefore, this MIS shows a high potential to selectively extract these two polar OPs at trace levels from different oils by reducing matrix effects.

Acknowledgment

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Conclusions et perspectives

L'objectif de ce travail a été de développer des supports capables d'extraire sélectivement plusieurs pesticides organophosphorés (OP), présentant des disparités structurales importantes et ayant une gamme de polarité assez large (log P compris entre 0,7 et 4,7), dans des d'échantillons d'huiles végétales. Pour cela, deux approches de synthèse de supports générant un mécanisme de rétention basé sur la reconnaissance moléculaire ont été étudiées.

Dans la première approche, des polymères à empreintes moléculaires (MIP) ont été synthétisés par polymérisation de monomères organique, autour d'une molécule empreinte, initiée par voie radicalaire donnant lieu à des cavités complémentaires de la molécule empreinte en forme et en fonction chimique. Différentes conditions de synthèse ont été criblées pour identifier celles conduisant au MIP le plus sélectif et performant par rapport à sa capacité à piéger le plus grand nombre d'OP. La sélectivité de ces polymères a été évaluée en étudiant en parallèle la rétention des OP en milieu pur sur ces MIP et sur des polymères non imprimés (NIP) obtenus par la même voie de synthèse mais sans introduire la molécule empreinte. Le support MIP le plus prometteur a été obtenu en utilisant le monocrotophos comme molécule empreinte, l'acide méthacrylique en tant que monomère et le diméthacrylate d'éthylène glycol comme agent réticulant. Ce MIP a permis d'extraire sélectivement cinq OP modérément polaires : methidathion, malathion, diazinon, fenitrothion et fenthion (ayant des log de P compris entre 2,5 et 3,7) d'un milieu pur proche du solvant utilisé pour diluer les huiles. Après avoir étudié la répétabilité de la procédure SPE optimisée et de la synthèse MIP en milieu pur, les performances de ce polymère ont été évaluées en milieu réel. La rétention des OP sur le MIP était similaire en utilisant trois huiles différentes (olive, tournesol et amande). Une optimisation de la procédure d'extraction sur l'huile d'amande a donc été réalisée afin d'améliorer les rendements pour trois OP (methidathion, malathion et diazinon). Des rendements compris entre 73 et 99% en utilisant le MIP et de seulement 34 à 75% en utilisant le NIP ont été obtenus, témoignant de la sélectivité de la procédure en milieu réel. Ce MIP nous a également permis de réduire les effets de matrice d'un facteur de deux à trois. Ces effets de matrice sont de 7 et 11% en utilisant le MIP et de 21 et 35% en utilisant le support de silice C18 pour un échantillon d'huile d'amande

enrichie à 100 µg/ kg. De plus, les LOQ obtenues pour les graines d'amande, entre 0,3 et 2 µg/kg, sont inférieures aux teneurs maximales résiduelles (LMR, comprises entre 20 et 50 µg/kg) établies pour ces graines par le règlement 396/2005 de l'Union Européenne.

Cependant, vu la difficulté de piéger toute la famille des OP ciblés à cause de leurs disparités en structure et en polarité, une seconde approche de synthèse par voie Sol-Gel, a été étudiée. Cette approche consiste à utiliser des organosilanes qui par hydrolyse puis condensation autour d'une molécule empreinte conduisent également à la formation de cavités spécifiques. Tout comme pour les MIP, différentes conditions de synthèse ont été criblées afin d'identifier celles conduisant à un support imprimé à base de silice (MIS) capable d'extraire sélectivement plusieurs OP. Le MIS sélectionné a été obtenu en utilisant le monocrotophos comme molécule empreinte, le 3-aminopropyl triéthoxysilane comme monomère et le tetraethyl orthosilicate comme agent réticulant. Ce support a permis de piéger sélectivement six OP (dimethoate, fenthion sulfoxide, fenthion sulfone, methidathion, malathion et diazinon) en milieu pur, et notamment d'obtenir des taux de récupération élevés sur les trois composés les plus polaires, au log P entre 0,7 et 2,2 (dimethoate, fenthion sulfoxide, fenthion sulfone). Après l'étude de la répétabilité de la procédure d'extraction optimisée en milieu pur et de la synthèse, comme pour le MIP, les performances de ce support ont été évaluées en milieu réel, pour le dimethoate et le fenthion sulfoxide qui pouvaient être analysés à faible teneur en LC-MS/MS. Une optimisation de la procédure SPE a été effectuée pour améliorer les rendements en milieu réel. Il est apparu que la rétention des deux OP cibles était très différente selon la nature de l'huile utilisée pour cette étude, à savoir les huiles d'olive, de tournesol et d'amande. Néanmoins, l'utilisation de ce support nous a permis de réduire les effets de matrice par rapport à l'utilisation de supports classiques (C18), par un facteur compris entre 2 et 10 comme montré pour l'huile d'amande. Les limites de quantification (LOQ, S/N = 10) estimées entre 0,1 et 13,6 µg/kg pour les OP se sont avérées inférieures aux limites maximums résiduelles (LMR), fixées entre 10 et 2000 µg/kg pour les graines d'amandes, olives et de tournesol. Par conséquent, ce MIS a présenté un potentiel élevé pour extraire sélectivement ces deux OP polaires à l'état de traces dans différentes huiles en réduisant les effets matrice.

Ces deux support MIP/MIS appliqués en SPE après une étape nécessaire d'extraction liquide/liquide pour diminuer les effets de matrice, ont présenté une

complémentarité en termes d'extraction sélective des OP visés. En effet, les OP les plus polaires ont été extraits sélectivement par le MIS alors que les OP modérément polaires ont été extraits sélectivement par le MIP. Concernant les OP plus hydrophobes : pirimiphos-methyl, fenthion, chlorpyriphos-ethyl et chlorpyriphos-methyl, ils n'ont pas été retenus sélectivement avec les procédures d'extraction développée sur le MIP ou sur le MIS. Une autre synthèse de polymère imprimé utilisant une autre empreinte et/ou une autre procédure d'extraction devront donc être développés pour tenter de piéger sélectivement ces composés.

Finalement, pour élargir la gamme d'OP piégé et au vu des similarités entre les procédures d'extraction optimisées sur le MIS et le MIP, un couplage des deux supports doit pouvoir être envisagé pour simplifier l'étape d'extraction, en ajustant légèrement la procédure d'extraction.

Annexe I. Chapter II

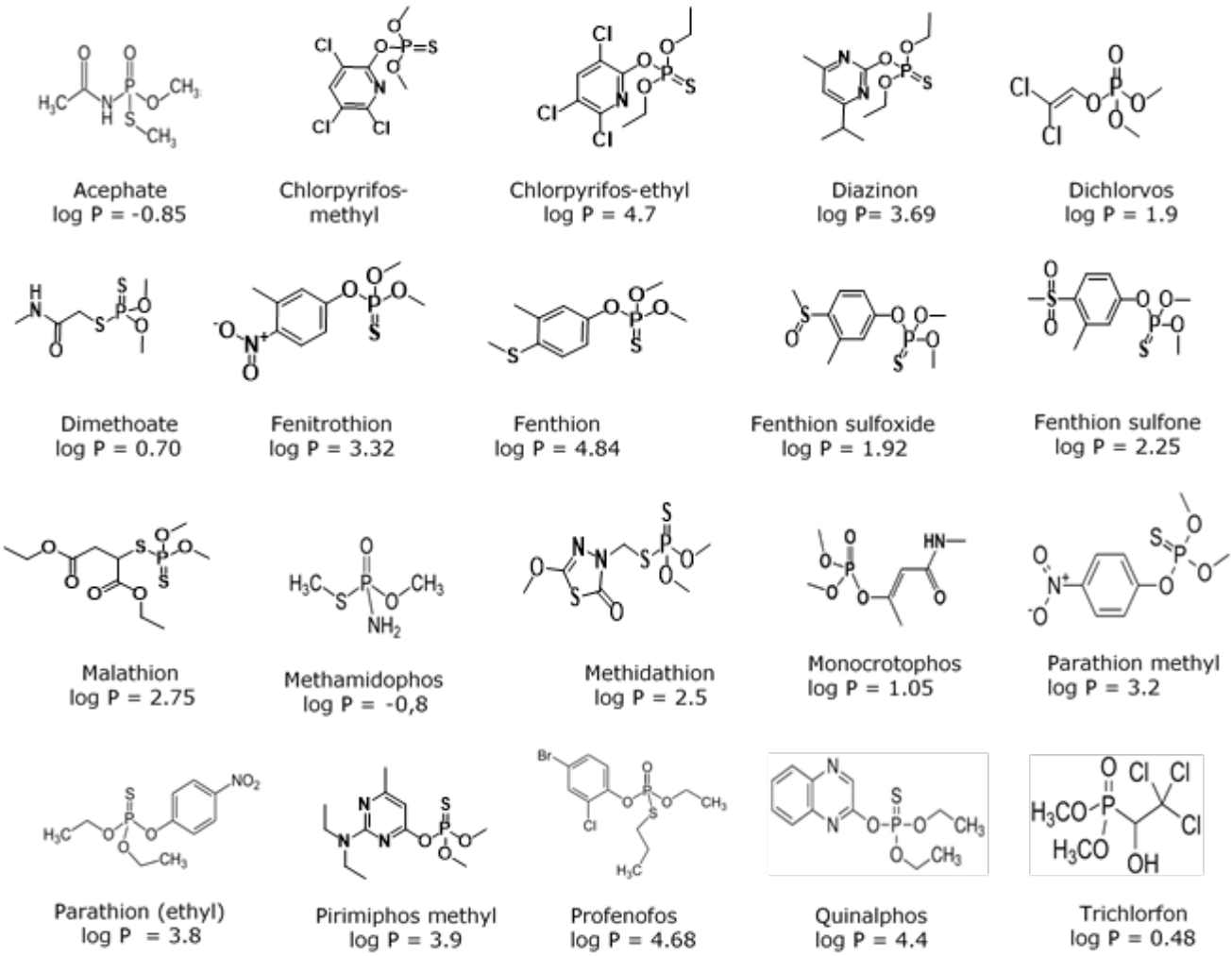

Figure 1. Structure and log P values of organophosphorus pesticides.

Annexe II. Chapter III

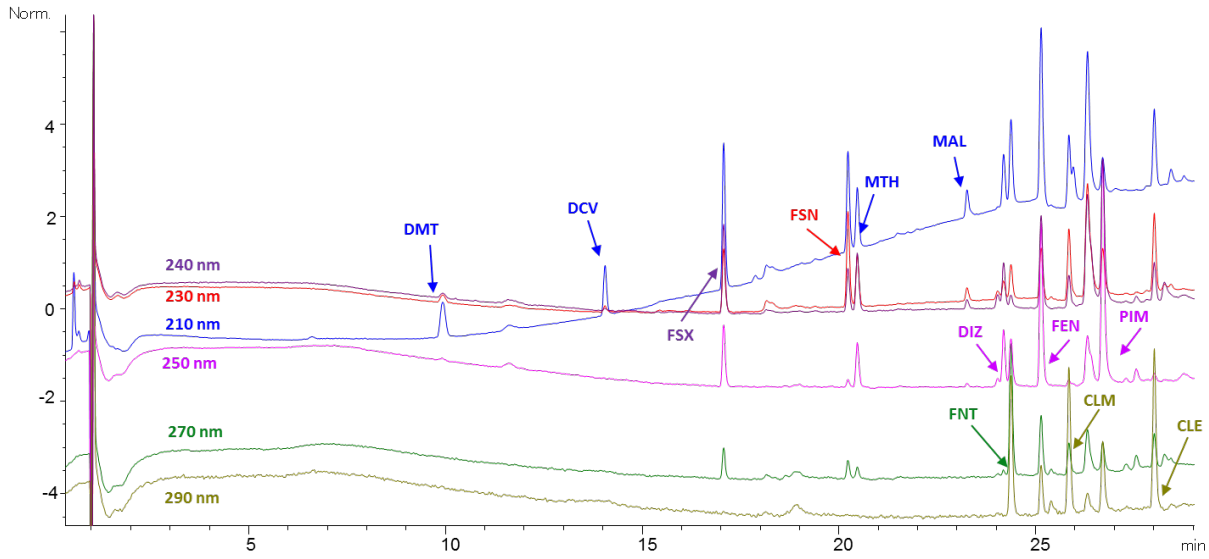

Figure 1. Séparation des OP à 1 mg/L en ACN a 210,230, 250,270 et 290 nm par LC-UV.

Annexe III. Chapter III

Table 1. Gamme de linéarité mesurée de 0,5 à 5 mg / L dans l'ACN et coefficients de corrélation correspondants (R^2) et le temps de rétention (t_R) en utilisant une longueur d'onde λ (nm) différente dans DAD.

Composés (OPs)	λ (nm)	Droite d'étalonnage	R^2	t_R (min)
DMT	210	y = 7,3482x + 0,1293	0,9999	9,93
FSX	240	y = 5,7938 + 0,0178	1	17,07
FSN	230	y = 9,3715 - 0,1244	1	20,23
MTH	210	y = 7,445 – 0,1089	1	20,47
MAL	210	y = 3,3801 – 0,1914	0,9995	23,26
DIZ	250	y = 3,956 – 0,3606	0,9955	24,16
FNT	270	y = 5,2063 – 0,0245	0,9998	24,37
FEN	250	y = 10,82 + 0,152	0,9999	25,14
PIM	250	y = 17,46 – 0,0486	0,9961	26,68
CLE	290	y = 4,1651 – 0,0007	0,9999	28,01

Annexe IV. Chapter III

Table 2.

Compounds (OPs)	Precursor Ion m/z (Da)	Tube lens (V)	Quantitation ion m/z (Da)	Collision energy of quantitation ion (V)	Confirming Ion m/z (Da)	t_R (min)
DMT	$[M+H]^+$= 230	90	125	22	170	7.7
FSX	$[M+H]^+$= 295	116	280	18	109	17.7
FSN	$[M+NH_4]^+$= 328	85	311	12	125	19.0
MTH	$[M+NH_4]^+$= 320	60	145	13	85	19.5
MAL	$[M+NH_4]^+$= 348	81	127	17	99	22.12
DIZ	$[M+H]^+$= 305	96	169	21	153	23.9
PIM	$[M+H]^+$= 306	96	164	22	108	24.8
CLE	$[M+H]^+$= 352	112	200	21	97	26.3

Tube lens values corresponding to quantitation and confirming ions and collision energies corresponding to quantitation ions.

Annexe V. Chapter III

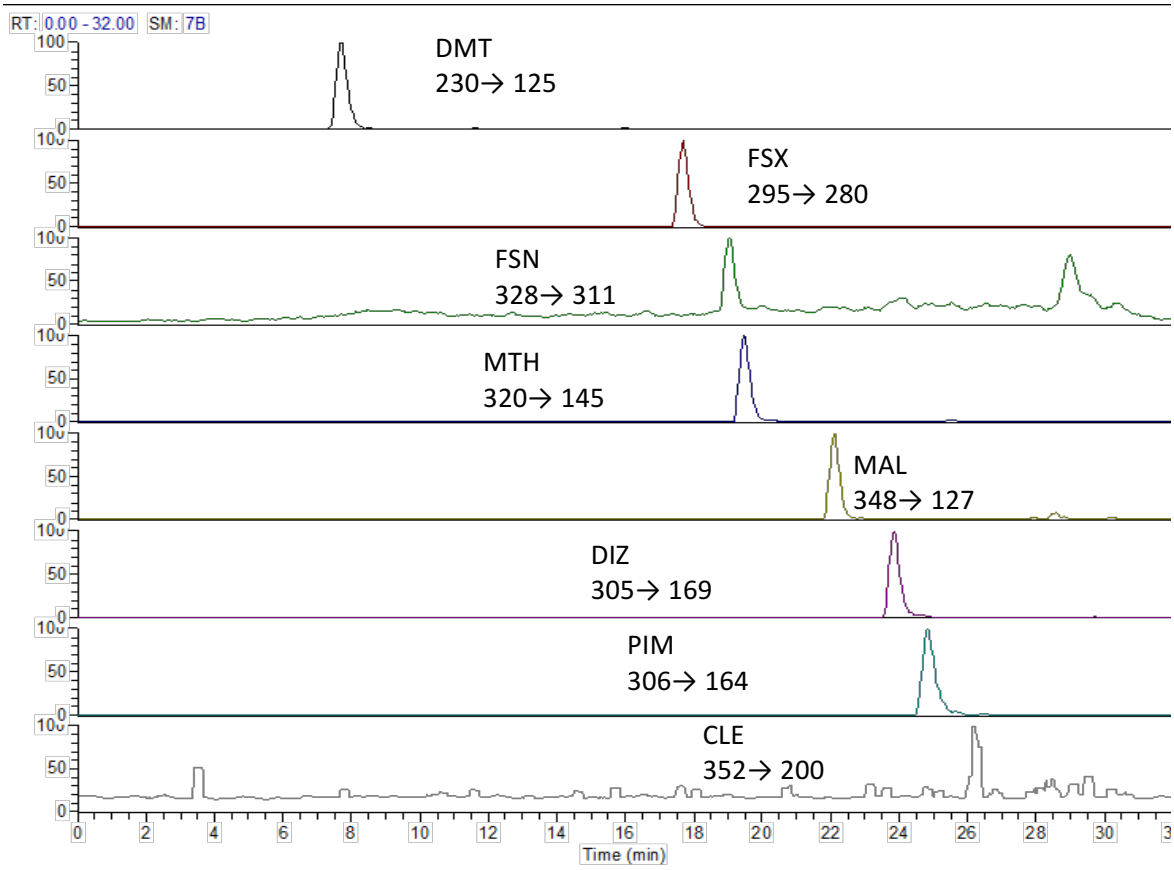

Figure 1. LC-MS chromatograms (MRM mode) of the elution fraction of an almond oil extract spiked at 100 µg/kg with eight OPs issued of the MIP. Extraction procedure was described in part III.3.5.2 (washing volume of 0.65 mL).

Annexe VI. Chapter IV

Table 1.

Compounds (OPs)	Precursor Ion m/z (Da)	Tube lens (V)	Quantitation ion m/z (Da)	Collision energy of quantitation ion (V)	Confirming Ion m/z (Da)
DMT	$[M+H]^+$= 230	90	125	22	170
FSX	$[M+H]^+$= 295	116	280	18	109
FSN	$[M+NH_4]^+$= 328	85	311	12	125
MTH	$[M+NH_4]^+$= 320	60	145	13	85
MAL	$[M+NH_4]^+$= 348	81	127	17	99
DIZ	$[M+H]^+$= 305	96	169	21	153
PIM	$[M+H]^+$= 306	96	164	22	108
CLE	$[M+H]^+$= 352	112	200	21	97

Tube lens values corresponding to quantitation and confirming ions and collision energies corresponding to quantitation ions.

Annexe VII. Chapter IV

Table 2. Linear calibration curves measured from 5 to 100 µg/L in ACN. Corresponding correlation coefficients (R^2) and retention time (t_R) obtained by using LC-MS/MS. The linearity of fenthion was measured from 250 - 1000 µg/L in ACN.

Compounds (OPs)	Calibration curves	R^2	t_R (min)
DMT	y = 37824x + 20949	0.999	8.2
FSX	y = 26707x + 20213	0.9992	17.9
FSN	y = 17804x + 25557	0.998	19.2
MTH	y = 32622x + 30081	0.9987	19.7
MAL	y = 1211.9x + 25668	0.9945	22.2
DIZ	y = 95196x + 64560	0.999	23.9
FEN	y = 203246x - 0904	0.9482	23.8
PIM	y = 18764x + 308 10	0.9974	24.8
CLE	y = -4659.1x + 2703.3	0.9962	26.3

Annexe VIII. Chapter IV

Table 3. Estimated LODs (S/N= 3) and LOQs (S/N= 10) by injecting spiked OPs at 200 µg/L in LC-DAD and with 5 µg/L in LC-MS (except for FEN, 1000 µg/L).

	LC-UV		LC-MS	
Compounds (OPs)	LOD (µg/L)	LOQ (µg/L)	LOD (µg/L)	LOQ (µg/L)
DMT	50	160	0.6	2.2
FSX	20	70	0.1	0.4
FSN	2	10	2.1	6.9
MTH	50	170	0.3	0.9
MAL	90	300	0.2	0.8
DIZ	50	160	0.08	0.3
FNT	20	50	No signal	No signal
FEN	10	30	300	1000
PIM	20	60	0.2	0.8
CLE	30	90	0.4	1.3

Annexe IX. Chapter V

Table 1.

Compounds (OPs)	Precursor Ion m/z (Da)	Tube lens (V)	Quantitation ion m/z (Da)	Collision energy of quantitation ion (V)	Confirming Ion m/z (Da)
DMT	$[M+H]^+$= 230	90	125	22	170
FSX	$[M+H]^+$= 295	116	280	18	109
FSN	$[M+NH_4]^+$= 328	85	311	12	125

Tube lens values corresponding to quantitation and confirming ions and collision energies corresponding to quantitation ions.

Liste des figures

Liste des tableaux