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UNESP - Universidade Estadual Paulista
Instituto de Química - Araraquara
Departamento de Química Analítica

TESE DE DOUTORADO

Magnetic Molecularly Imprinted Polymers (MMIPs) - synthesis and characterization for the spectrophotometric determination of caffeine

Shahab Ali

Araraquara
2019

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Magnetic Molecularly Imprinted Polymers (MMIPs) - synthesis and characterization for the spectrophotometric determination of caffeine

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DEDICATION

To

My mother Zarbaha Begam and

My father Muhammad Anwar.

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ABSTRACT

Daily food items contain considerable amount of caffeine that is constantly released to the environment, causing major problems to living organisms that can shift the entire ecosystem resulting adverse public health and the environment. Secondly, many market samples of caffeinated products have found with extremely high amounts of caffeine to toxic level (300 mg – 400 mg) that must be regulated by rapid techniques to assure human health and safety.

An alternative method for the detection and quantification is the development of biomimetic sensors, using magnetic molecularly imprinted polymers (MMIPs) like sensing phase. The advantages of molecular imprinted materials are high selectivity, sensitivity and stability, and low cost. This work aimed to develop an efficient method of preparation of core-shell magnetic nanoparticles (MNPs) modified with molecularly imprinted polymers (MIPs) used as selective material, in order to determine caffeine in the food samples in a very smart magnetic fashion. Metacrylic acid (MAA) was used as functional monomers; ethylene glycol dimethacrylate (EGDMA) was used as cross-linking reagents, *2,2'-azo-bis* isobutironitrila (AIBN) as a radical initiator and ethanol as pororgenic solvent. Magnetic molecularly non-imprinted polymers (MNIPs) which served as a control for comparative studies, synthesized along with MMIPs.

Characterizations of MMIPs and MNIPs were performed by Vibrating sample magnetometry (VSM), Fourier Transform Infra-Red (FTIR) and Transmission Electron Microscopy (TEM). The adsorption capacity and selectivity of the materials were studied in detail, from the respective adsorption isotherms and its kinetics. The adsorption data was well described by Langmuir-Freundlich isotherm model with adsorption equilibrium constant (K) of 9.6×10^2 mol L^{-1} which is higher in comparison with the reported. The selectivity experiments revealed that prepared MMIP had higher selectivity toward caffeine compared to other molecules with comparable chemical structures of theophylline and xanthine

Keywords: Magnetic nanoparticles (MNPs), molecularly imprinted polymers (MIPs), caffeine.

RESUMO

Muitos alimentos de uso diário contêm uma quantidade considerável de cafeína que é constantemente lançada ao meio ambiente, podendo causar grandes problemas aos organismos vivos que podem prejudicar todo o ecossistema, resultando em um problema de saúde pública e do meio ambiente. Adicionalmente, em muitas amostras de produtos cafeinados, comercializadas no mercado, tem apresentado quantidades extremamente altas de cafeína, chegando a níveis tóxicos (300 mg - 400 mg) e que devem ser monitoradas e reguladas por técnicas confiáveis e rápidas para garantir a saúde e a segurança dos consumidores.

Um método alternativo para a detecção e quantificação é o desenvolvimento de sensores biomiméticos, usando como fase seletiva de sensoriamento polímeros magnéticos molecularmente impressos (MMIPs). As vantagens dos materiais impressos moleculares são alta seletividade, estabilidade e baixo custo. Assim, este trabalho teve como objetivo desenvolver um método eficiente de preparação de nanopartículas magnéticas core-shell (MNPs) modificadas com polímeros molecularmente impressos (MIPs) utilizados como material seletivo, a fim de determinar a cafeína nas amostras de alimentos. O ácido metacrílico (MAA) foi usado como monômero funcional; metacrilato de etilenoglicol (EGDMA) como reagente de reticulao, 2,2'-azo-bis-isobutironitrila (AIBN) como iniciador radicalar e etanol como solvente porogênico. Polímeros magnéticos não impressos (MNIPs) que serviram como controle para estudos comparativos, foram também sintetizados junto com MMIPs.

As caracterizações dos MMIPs e MNIPs foram realizadas por magnetometria da amostra vibrante (VSM), infravermelho por transformada de Fourier (FTIR) e microscopia eletrônica de transmissão (TEM). A capacidade de adsorção e seletividade dos materiais foram estudadas em detalhe, a partir das respectivas isotermas de adsorção e de sua cinética. Os dados de adsorção foram bem descritos pelo modelo isotérmico de Langmuir-Freundlich com constante de equilíbrio de adsorção (K) de 9,6 x 10^2 mol L^{-1}, que é maior em comparação com o relatado. Os experimentos de seletividade revelaram que o MMIP preparado apresentou maior seletividade em relação à cafeína em comparação com outras moléculas com estruturas químicas análogas de teofilina e xantina.

Palavras-chave: Nanopartículas magnéticas (MNPs), molecularmente imprimidos polímeros (MIPs), cafeína.

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LIST OF ABBREVIATIONS AND ACRONYMS

1VI: 1-vinylimidazole

ABDV: 2,2'-azo- *bis*- (2,4-dimethylvaleronitrile)

Abs: Absorbance

ACN: Acrylonitrile

AIBN: 2,2'-azo- *bis*- (2-methylpropionitrile)

AMPSA: 2-Acrylamido-2-methylpropane sulfonic acid

APM: 4,4'-diaminodiphenylmethane

BET: Brunauer, Emmett, Teller (Multimolecular Adsorption Theory)

BR: Britton-Robinson

HPLC: High Performance Liquid Chromatography

DNA: deoxyribonucleic acid

EGDMA: Ethylene Glycol Dimethacrylate

FEG-SEM: High-resolution scanning electron microscopy with field emission electron source

FTIR: Infrared with Fourier transform

IUPAC - International Union of Pure and Applied Chemistry

MAA: Methacrylic acid

MMIP: Magnetic Molecularly Imprinted Polymer

MNIP: Magnetic Nonprinted Polymer

SM: Structural monomer

FM: Functional monomer

MeOH: Methanol

MF: Functional monomer

MIP: Molecularly imprinted polymer

MPS: 3-methacryloxy-propyl-trimethoxysilane

NIP: Nonimprinted Polymer

SPE: Solid phase extraction

UV-Vis: Ultraviolet-Visible

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1 Introduction

Caffeine in the world's most widely consumed psychoactive drug classified as central nervous system(CNS) methyl xanthine class of stimulant.[1] It reversibly blocks the action of adenosine on its receptor and consequently prevents the onset of drowsiness induced by adenosine. Caffeine also stimulates certain portions of the autonomic nervous system[2].

Found commonly in the seeds, nuts, or leaves of a number of plants native to South America and East Asia. A well-known source of caffeine is the coffee bean, a misnomer for the seed of *Coffea* plants. Caffeine is extracted by steeping the plant product in water, a process called infusion. Caffeine-containing drinks, such as coffee, tea, and cola, are very popular (Figure 1.1). As source of 2014, 85% of American adults consumed some form of caffeine daily, consuming 164 mg on average.[3]

Figure 1-1 The caffeine content of some of our favorite drinks

Source: http://elaineallertondietitian.com/blog/caffeine. Accessed in March, 9th 2019.

History of coffee dates back to the 10th century, its origin thought to have been Ethiopia. The earliest substantiated evidence of either coffee drinking or knowledge of the coffee tree is from the 15th century, in the Sufi monasteries of Yemen.[4] By the 16th century, it had reached the rest of the Middle East, South India, Persia, Turkey, Africa. Coffee then spread to the Balkans, Italy and to the rest of Europe, to South East Asia and then to America.[5]

Caffeine is classified by the US Food and Drug Administration as "generally recognized as safe" (GRAS). Toxic doses, over 10 grams per day for an adult, are much higher than typical doses of under 500 milligrams per day. A cup of coffee contains 80–175 mg of caffeine, depending on what "bean" (seed) is used and how it is prepared (e.g. drip, percolation, or expresso). Thus, it requires roughly 50–100 ordinary cups of coffee to reach a lethal dose. However, pure powdered caffeine, which is available as a dietary supplement, can be lethal in tablespoon-sized amounts.

After water, coffee, tea, and soft drinks containing high amounts of caffeine are the most consumed beverages in our daily life. According to the study by US-FDA, the daily average American consumption of 300 mg caffeine[6], and metabolize about 95-98% into paraxanthine, theobromine, and theophylline. In highly populated areas, the excreted caffeine then goes through standardized wastewater treatment where, on average, 60-70% of the remaining caffeine, and is removed, and the rest enters the local streams and rivers intact.

In its natural plant caffeine functions as a pesticide, inhibits enzymes in insects' nervous systems, enduring reproductive harm, triggering paralysis and death in the more susceptible bugs[7]. Before dying, the adult and larval insects show, unnatural behaviors; for example, the larvae of mosquitoes may lose the ability to swim up to the water's surface and drown. Spiders fed caffeine-laced flies showed disorientation, incapable of creating symmetrical webs[8] (Figure 1.2).

Figure 1-2 Caffeine effects on spider

Source: https://owlcation.com/stem/Caffeine-Its-effects-on-animals-plants-and-the-environment. Accessed in March, 9th 2019.

Caffeine is more lethal when roughage and snails exposed to 0.5% caffeine solutions die within days, by increasing heart rate faster at concentrations of 0.1% and above, caffeine triggered deadly erratic and slowed pulse [9].

Larger forms of life succumb to the power of caffeine as well. By spraying caffeinated water on coqui frogs, the Hawaiian Department of Agriculture planned to perform mass amphibicide on the nuisance species with drug-induced heart attacks, forever silencing the amphibians' loud, shriek-like calls [10]. Luckily for the frogs, a lack of public support prevented the plan's actual implementation.

A wild parrot 20 gram caffeine in dark chocolate caused, irreparable damage of its liver, kidneys, and brain neurons[11]. A caffeine pill in German Shepherd displayed symptoms of overheating, an elevated heart rate, and agitated behavior before dying (for dogs the lethal dose is 140 mg per kilogram body weight)[12].

A major concern to environmentalists, for mollusks, tadpoles, snails, and other aquatic life. Caffeine molecule is significantly more toxic than it is to humans, harmful even at 0.097 mg L^{-1}.[2.] A recent study on inter-tidal salt-water mollusks, discovered that they produce significant

amounts of stress proteins when exposed to concentrations as low as 50 ng L^{-1}, and while caffeine does not bio-accumulate, reduced populations of these organisms could cause major shifts in the entire ecosystem.

Caffeine 1,3,7-trimethylxanthine (Figure 1.3), have the formula $C_8H_{10}N_4O_2$, molecular weight of 194.19 g mol^{-1}, a bitter taste, white color and melting point between 235–238 °C. It is moderately soluble in water; 2 g for each 100 mL, while very soluble in boiling water; 66 g for each 100 mL.[13.] Moderately soluble in ethanol 1.5 g/100 mL, weakly basic; pK_a of conjugate base of ~0.6, requiring strong acid to protonate it.[14] Caffeine not containing stereogenic centers[15] and hence is achiral. Its xanthine core contains two fused rings, a pyrimidinedione and imidazole. The pyrimidinedione contains two amide functional groups; all six of the atoms within the pyrimidinedione ring system are sp^2 hybridized and planar. Hence the fused 5,6 ring core of caffeine contains a total of ten pi electrons and according to Hückel's rule is aromatic.[16]

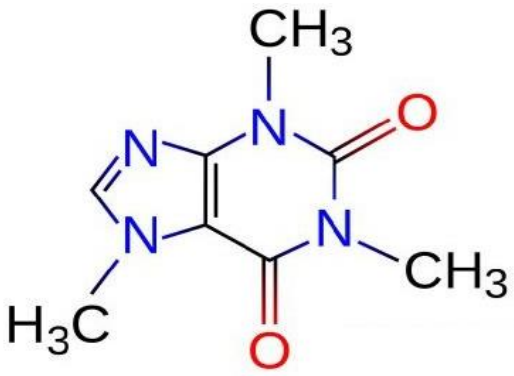


Figure 1-3 Caffeine Molecule structure.
Source: Own authorship

Caffeine mechanism of action principally is antagonist of adenosine[17]. In normal conditions when a person is alert in the absence of caffeine, little adenosine is present in the neurons of the CNS (Central Nervous System). Continued alertness over time accumulates adenosine in the neuronal synapse, binding and activating adenosine receptors on CNS neurons; causing the production of a cellular response that ultimately increases drowsiness. Caffeine antagonizes adenosine receptors; prevents adenosine from activating the receptor by blocking the

adenosine binding. This results, in a temporarily prevent or relieves drowsiness, and thus maintains or restores alertness[18].

Caffeine is both water- and lipid-soluble, and for this reason it readily crosses the blood–brain barrier. The caffeine molecule is structurally similar to adenosine (Figure 1-4), and is capable of binding to adenosine receptors on the surface of cells without activating them, thereby acting as a competitive antagonist[19].

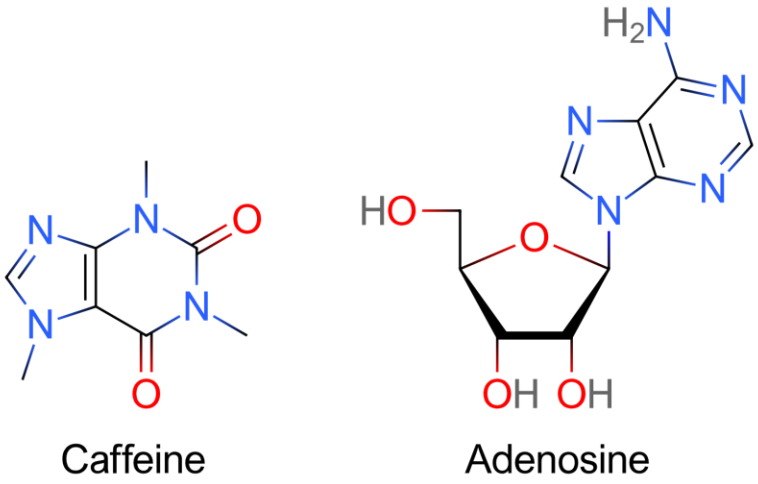


Figure 1-4: Caffeine and Adenosine Molecular Structures
Source: Own authorship

Caffeine antagonizes adenosine A2A receptors in the ventrolateral preoptic area (VLPO), thereby reducing inhibitory GABA neurotransmission to the tuberomammillary nucleus, a histaminergic projection nucleus that activation-dependently promotes arousal[20]. Disinhibition of the tuberomammillary nucleus is the chief mechanism by which caffeine produces wakefulness-promoting effects[21].

Caffeine from food is absorbed by the small intestine within 45 minutes of ingestion[22]. Peak blood concentration is reached within one hour and it is eliminated by first-order kinetics[23], [24]. Caffeine is metabolized in the liver by the *cytochrome P450 oxidase enzyme system* [25], in particular, by the CYP1A2 isozyme, into three primary metabolites:

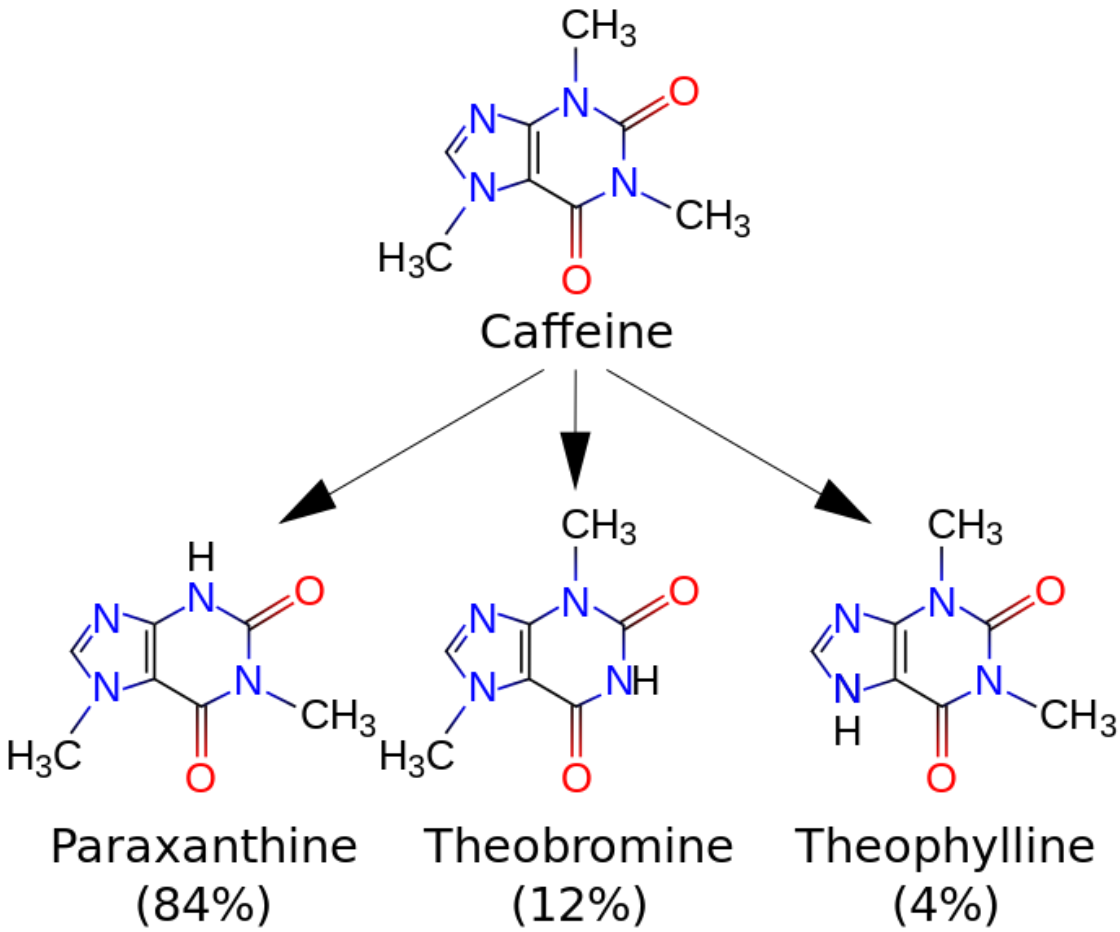


Figure 1-5: Caffeine and its immediate metabolites; CYP refers to the enzymes catalyzing the reactions.[26]

In which:

Paraxanthine: increases lipolysis, leading to elevated glycerol and free fatty acid levels in the blood plasma.

Theobromine: dilates blood vessels and increases urine volume. Theobromine is also the principal alkaloid in the cocoa bean, and therefore chocolate.

Theophylline: Relaxes smooth muscles of the bronchi, and is used to treat asthma. These metabolites then further metabolized and then excreted in the urine[27].

Caffeine had some major health benefits and also some adverse effects on human body. High long-term caffeine consumption is associated with a lower risk of cardiovascular disease and diabetes and also reducing risk of Alzheimer's disease. Low doses of caffeine show

increased alertness and decreased fatigue. Caffeine has been shown to increase the metabolic rate. Caffeine is studied as a treatment for the Parkinson's disease motor symptoms. Caffeine may lower the risk of developing type 2 diabetes[28,29].

Caffeine can increase blood pressure in non-habitual consumers. High blood pressure is associated with an increase in strokes, and cerebral vascular disease, which in turn increase the risk of multi-infarct dementia. Caffeine may reduce control of fine motor movements (e.g. producing shaky hands). Caffeine can contribute to increased insomnia and sleep latency. Caffeine withdrawal can produce headache, fatigue and decreased alertness. High doses of caffeine (300 mg or higher) can cause anxiety[30].

Caffeine overdose can result in a state of central nervous system over-stimulation called caffeine intoxication (DSM-IV 305.90)[31]. This syndrome typically occurs only after ingestion of large amounts of caffeine, more than 400–500 mg at a time[32]. The symptoms of caffeine intoxication are comparable to the symptoms of overdoses of other stimulants: they may include restlessness, anxiety, excitement, insomnia, flushing of the face, increased urination, gastrointestinal disturbance, muscle twitching, a rambling flow of thought and speech, irritability, irregular or rapid heartbeat, and psychomotor agitation.

In cases of much larger overdoses, mania, depression, lapses in judgment, disorientation, disinhibition, delusions, hallucinations, or psychosis may occur, and rhabdomyolysis (breakdown of skeletal muscle tissue) can be provoked[33]. But, in extreme overdose can result in death.[34]

The median lethal dose (LD50) given orally in humans is dependent on individual sensitivity, but is estimated to be about 150 to 200 milligrams per kilogram of body mass (80 to 100 cups of coffee for an average adult)[35].

It is easier to reach high doses with caffeine pills, and the lethal dose can be lower in individuals whose ability to metabolize caffeine is impaired. Chronic liver disease is one factor that can slow the metabolism of caffeine[36].

To evaluate and monitor environmental impacts of caffeine together with qualitative and quantitative determination in food samples, it is important to develop materials and methods[37], that allow the selective detection of these molecules, as well as their quantification with high sensitivity with low cost speedy on spot analysis. For achieving these high characteristics, recently employed advance sensitive materials known as molecularly imprinted polymers (MIP) that mimicking the biological interaction of antigen antibody or enzyme substrate like

interactions[38], and can achieve the desired analytical results with a superiorly high selective and sensitive fashion[39].

1.1 Caffeine Determination

Caffeine determination in foods using various quantitative techniques, such as: UV-spectrophotometry[40], High-Performance Liquid Chromatography coupled to Mass Spectrometry (HPLC-MS)[41,42] and capillary electrophoresis.[37] Recently electrochemical methods have been proposed[43], for example Sharma et al. prepared an electrochemically polymerized sensor that gave results with low sensitivity, showed high selectivity due to the stability of electrochemically obtained molecular imprinting polymer (MIP).[44]

The innovative synthesis of molecularly imprinted polymers (MIPs) has proven to be a distinct method for selective determination and quantification of several molecules. These are polymers with high selective recognition sites in the form of cavities, that are complementary to the analytes structure functionality;[45] mimicking the affinity-based biological recognition system also being referred as biomimetic polymers, similar to the specific enzyme-substrate or antigen-antibody systems.

1.2 Molecularly printed polymers (MIPs)

The rationale uses of template (target molecule) to selectively bind to a specific recognition site was suggested by Mudd in 1932 and then Pauling in 1940[46]. In 1942, Linus Pauling described the preparation of artificial antibodies using antigen molecules as template. In 1949 through Frank Dickey carried out the first work using printed synthetic product (silica gel) in the selective adsorption of methyl orange.[47]The development of molecular imprinted synthetic polymers, better known as MIP (Molecularly Imprinted Polymers) has been used to obtain systems with biomimetic recognition capabilities similar to the specific enzyme-substrate and/or antigen-antibody systems,[48] as illustrated in Figure 1.6 below.

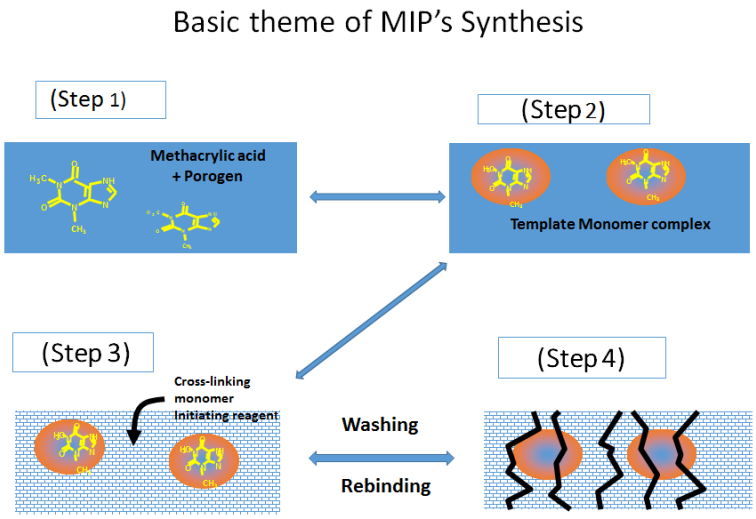


Figure 1-6 Basic theme of MIP's synthesis. Step 1: interaction of Template with Functional Monomer in pororgenic solvent, Step 2: formation of Template-Monomer Complex (TMC), Step 3: Polymerization of Cross-linker around TMC, Step 4: Template removal leaving imprinted cavities.

Source: Own authorship

Among the advantages offered by MIP can be mentioned: low cost; possibility of synthesis in situations where no biomolecule (receptor or enzyme) exists; resistance to adverse environments in which natural biomolecules would not withstand as in the presence of acids, bases, organic solvents and high temperatures.[49]

Molecular imprinted polymers (MIP) have been reported to exhibits significantly high affinity for the molecules used as the template than for similar molecules including closely related isomers. In recent years, MIPs have attracted much attention due to their outstanding advantages, such as predetermined recognition ability, stability, relative ease and low cost of preparation, and potential application to a wide range of target molecules. MIPs can, not only concentrate but also selectively separate the target analytes from complex samples.[50,51]

MIP synthesis involves, analyte molecule used as a template interacts by covalent or non-covalent bonds with the molecules of one or more type of functional monomers, forming first the Template-Monomer Complex (TMC). Subsequently polymerizing agent is added to the medium that promotes crosslinking and form a rigid polymer matrix around TMC. The polymerization reaction starts by employing heat, light, and the addition of a radical initiator. After completing polymerization, template molecules then removed from the polymer matrix, leaving behind

complementary cavities also known as recognition sites, by washing with solvent or by means of chemical cleavage if the molecule establishes covalent attachment with the monomer. The resulting polymer having uniform sized cavities capable of selectively holding the template molecule present in a complex sample. Non-imprinted Polymers (NIP) synthesized simultaneously sidewise utilizing exact materials and methods except not using the template (analytes free) as a standard for comparative analysis of MIP to serve as a control polymer.[52] MIPs emphasizing its worth employed as biomimetic compared to usual analysis techniques. Below is an illustrative schematic of MIP synthesis and the removal of the template molecule as shown in Figure 1.7 bellow.

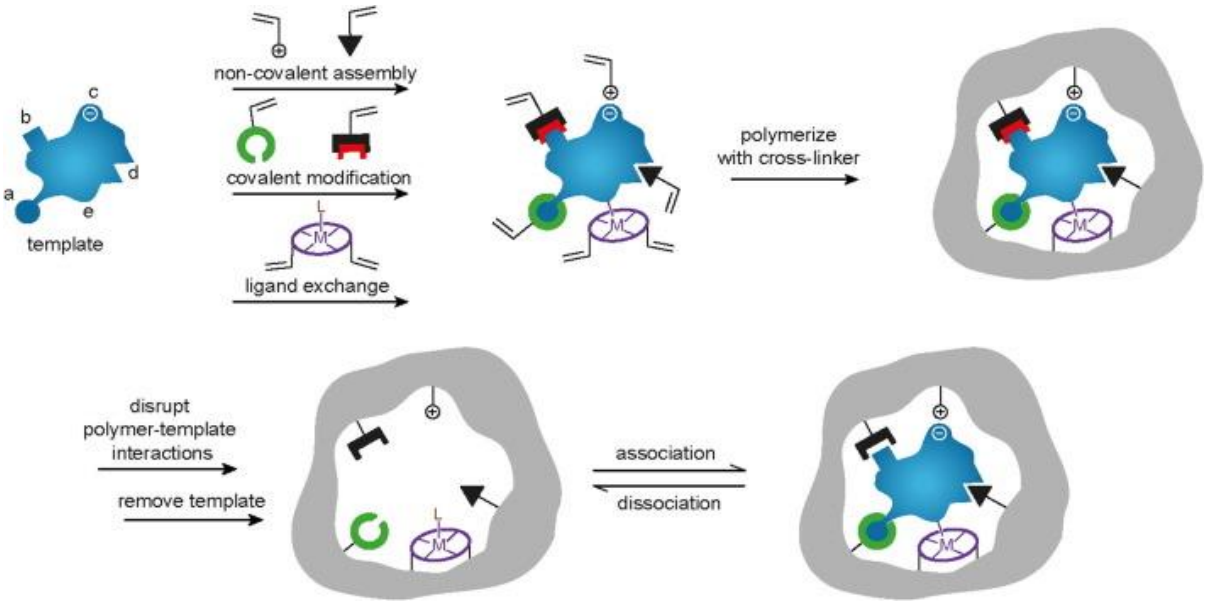


Figure 1-7 MIP synthesis illustration.
Source: H.F. EL-Sharif ET AL, 2015[53]

Schematic representation of the imprinting process, synthetic strategy involves one or more of the following interactions: (a) reversible covalent bonds, (b) covalently attached polymerizable binding groups that are activated for non-covalent interaction by template cleavage (sacrificial spacer or semi-covalent strategy), (c) electrostatic interactions, (d) hydrophobic or van der Waals interactions, (e) co-ordination with a metal center. Each, (a–e)

respectively are formed with complementary functional groups or structural elements of the template. Subsequent polymerization in the presence of a cross-linker produces a porous matrix in which template sites are located. For MIP synthesis, it is important to evaluate the choice of the reagents. The template molecule is responsible for the definition of the spatial organization of the functional groups with the functional monomers.[54]

The functional monomer is responsible for the interactions that will form between the polymer and analyte in the specific cavity formed.[55] The main purpose of the structural monomer is to stabilize the binding sites with molecular recognition and mechanical stabilization of the polymer matrix[56], and the type and amount of these reagents has great impact on the physical and chemical characteristics of the MIP.[57] Hence the cavities formed are contrary identical to the functional groups of molecular structure of that template, which is the main bases for templet selective recognition, and resulted MIP are stable structures which can be stored for long time and can be utilized in various different environments.

The radical initiator will initiate the free radical polymerization reaction basically in two ways: thermal or UV radiation.[31] The porogenic solvent of the medium should not interfere with the analyte-monomer interaction and is also responsible for the formation of pores in the polymer.[54]

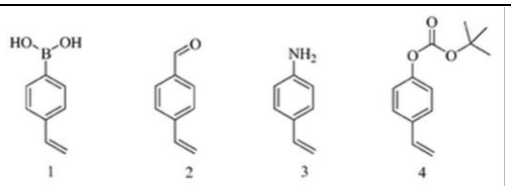
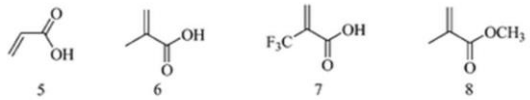
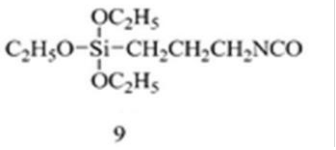
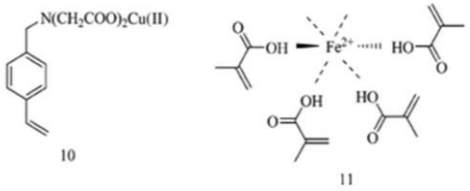
The idea of covalent bonding involving template and functional monomer was introduced by Wulff and Sarchan.[32] In this work the template is removed from the polymer by cleavage of the covalent bonds. The high stability of the bonds formed between template and monomers leads to the formation of a large number of homogeneous binding sites, minimizing the existence of nonspecific binding sites. It should be noted that when the template is covalently attached to the functional monomer, in its subsequent application, the interactions are expected to be of the non-covalent type.

The concept of non-covalent interaction was introduced by Arshady and Mosbach.[33] Non-covalent interactions occur between specific functional groups on the polymerizable monomers and the template in the pre-polymerization mixture. These interactions can be of the type hydrogen bonds, ionic, hydrophobic interactions, among others. The non-

covalent procedure is considered the most widely used molecular imprinting method and has been applied to a wide variety of molecules. This is due to the fact that this type of interaction presents high versatility and applicability.

Among the various monomers and crosslinking agents reported in the literature, few were used in most studies. Usually the most commonly used functional monomers given in Table 1 include methacrylic acid, 2- and 4-vinylpyridines, trifluoromethylacrylic acid, acrylamide and hydroxyethylmethacrylate.

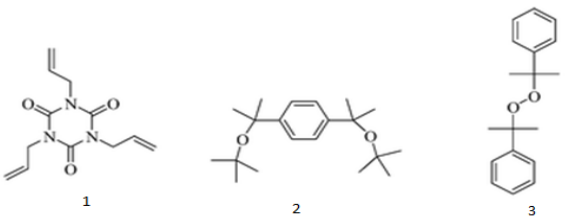
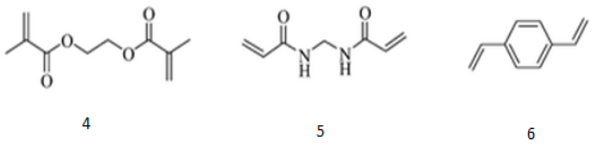
Table 1: Functional Monomers classified on types of bonding generally carried out[58]

Common Types	Functional monomers	Molecular Structure
(A) Covalent	**(1)** 4-Vinyl benzene boric acid; (**2**) 4-vinyl benzaldehyde; (**3**) 4-vinyl aniline; (**4**) *tert*-butyl *p*-vinylphenylcarbonate.	
(B) Non-covalent	(**5**) acrylic acid (AA); (**6**) methacrylic acid (MAA); (**7**) trifluoromethyl acrylic acid (TFMAA); (**8**) methyl methacrylate (MMA);	
(C) Semi-covalent	(**9**) 3-Isocyanatopropyltriethoxysilane (IPTS)	
(D) Ligand exchange	(**10**) Cu (II)-iminodiacetate-derivatized vinyl monomer; (**11**) Fe^{2+}/MAA complex	

Crosslinking agents (Table 2) include glycol dimethacrylate (EGDMA), divinylbenzene and trimethylolpropyl trimethylacrylate (TRIM).

On the other hand, it should also be visualized that the efficiency in the formation of the binding sites in the MIP, is highly influenced by the chemical nature of the template. For this, the template must be stable and inert during polymerization and have functional groups that can interact with the functional groups of the monomer(s). The functional monomer plays an important role in the synthesis of MIP, since it is closely related to the formation of the binding sites with the template molecule.

Table 2: Common Cross-linkers classified on types of bonding generally carried out [59]

Common Types	Cross-linkers	Molecular Structure
(A) Covalent.	(1) triallyl isocyanurate (TAIC); (2) bis(1-(tert-butylperoxy)-1-methylethyl)-benzene (BIPB); (3) dicumyl peroxide (DCP);	 1 2 3
(B) Non-covalent	(4) ethylene glycol dimethacrylate (EGDMA); (5) N,N′-methylenediacrylamide (MBAA); (6) divinylbenzene (DVB)	 4 5 6

It is reported in the literature that the best interaction between monomer and template occurs when there is complementarity, and the monomer with acid functional groups reacts with template with basic functional groups and vice-versa.[58] In all molecular imprinting processes the template molecule plays a central role since it directs the organization of functional groups of the monomers. Complementarity interactions between the template molecule, functional monomer and crosslinking agent are necessary to create a molecular organization.

In fact, for all this to occur, a porogenic solvent must be chosen to promote the solubilization of the reagents, allowing the interactions between template and monomer to occur efficiently, thus contributing to the formation of a MIP with high porosity. Thus, the evaluation of the functional groups of the monomers and the template is important for choosing the best monomer for the synthesis. It should also be understood that the maximum efficiency of the

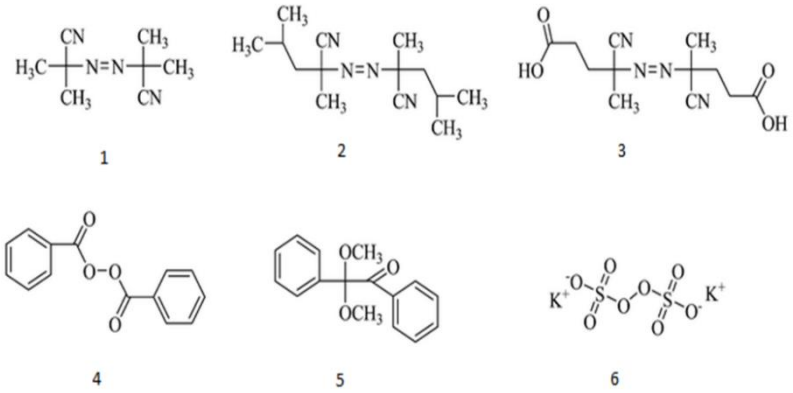
formation of the impression occurs when the polymerization reaction is performed using an appropriate solvent so that the solubility of the printed species is not compromised.

The porosity of the MIP will depend on the degree of solubility of the reactants, because the lower the solubility, the larger the non-selective pores and the low surface area will form. When high solubility occurs, small pores, the size of the target molecule, and large surface area will form.

The rigidity of the template in the MIPs is performed using a crosslinking reagent or crosslinking agent, the most commonly used being ethylene glycol dimethacrylate (EGDMA), which must be in excess during the polymerization, generally following a ratio with the template from 1:20 to 1:200 (mol/mol). The crosslinking agent used in obtaining an MIP performs many functions such as control of polymer matrix morphology, stabilization of binding sites with molecular recognition ability and stabilization mechanical properties of the polymer matrix. During synthesis has been used a high amount of crosslinking agent, so that not only obtain macroporous materials such as mechanically stable materials also possible to maintain the complementary three-dimensional structure. Accordingly, the functional groups will be in an optimal configuration for the re-assembly of the template molecule, allowing the recipient to recognize the original substrate and consequently the high specificity of the polymer. As a rule, the polymers have grading degrees greater than 80%.

The polymerization reaction begins with the use of a radical initiator under heating that can occur with the use of a water bath or UV radiation in the absence of oxygen. The best-known radical initiators given in Table 3 are: (AIBN)2,2'-azo-bisisobutyronitrile, (ABDV) azo-bis-dimethylvaleronitrile, benzoyl peroxide (BPO) and 4,4'-aza-*bis*-(4-cyano pentaenoic acid).

Table 3: Common polymerization initiators used in the synthesis of MIPs[59]

Initiators	Molecular Structure
(1) Azobisisobutyronitrile (AIBN); (2) azobisdimethylvaleronitrile (ADVN); (3) 4,4′-azo(4-cyanovaleric acid) (ACID); (4) benzoylperoxide (BPO); (5) dimethylacetal of benzyl (BDK); (6) potassium persulfate (KPS).	

Polymerization of MIP involves three important steps: initiation, propagation and termination.

Initiation: Once the formation of the polymer chain is initiated, it propagates in a short time (possibly one or two seconds), giving rise to a high molecular weight molecule before the termination process occurs, even when the amount of monomer consumed is low. Normally, the initiator promotes the formation of free radicals by remaining active throughout the polymerization process. Many initiators, with different chemical properties, can be used as source of radicals in polymerization processes. Usually, their concentration is low in comparison to that of the monomer, for example, 1% relative to the total number of moles of polymerizable double bonds[60].

The initiation is accomplished by homolytic cleavage yielding two active free radicals, followed by the double bonds in the monomer that react with the unpaired electrons of the radical. In this reaction, the active center of the radical strikes the double bond of the monomer or the crosslinking agent, leaving an unpaired electron in the monomer as a new active center.[61,62]. The activation of this reaction is usually carried out by thermal decomposition or photolysis. Below we have a simplified scheme of the reactions performed in this step, where is represent the initiator, R the activated radical of the initiator, M the monomer and M the activated monomer.

$$I \longrightarrow 2R^{\bullet} \qquad \text{(reaction 1)}$$

$$R^{\bullet} + M \longrightarrow M^{\bullet} \qquad \text{(reaction 2)}$$

Propagation: After initiation, the growth of the polymer chain occurs at a very rapid propagation rate. The polymer chains increase significantly reaching a high molecular weight at the end of this step.[63]

Termination: The termination occurs when the polymer terminates the growth through free electrons of two growing chains that join together and form a single one. In the scheme below C_a and C_b represent growing chains active, and C ab the final chain after the termination process.

$$\mathbf{C_a + C_b \longrightarrow C_{ab}} \qquad \text{(reaction 3)}$$

1.3 Core-shell Magnetic Molecularly Imprinted Polymer

Preparation of the polymer with the selective character (as previously described selectively adsorb analyte), MIPs demonstrate advantages in terms of adsorption capacity, chemical stability and reproducibility. In view of these advantages, the immobilization of MIPs on various surfaces of solid supports, such as core@shell[64], nanowire[65], optical fiber[66], electrodes[60] have been reported. The development of chemical sensors, becoming more interesting. In this way, the MIP will act as the sensing phase conferring high selectivity to the system. In addition, to being possible to perform extractions, pre-concentrations and clean-up samples in a sensitive way.[67]

Nanoparticles are solids up to 100 nm in size and have characteristics of high surface area in relation to its volume.[68] These nanoparticles are mostly used as nuclei for the MIP core@shell. Among these, iron oxide magnetic nanoparticles with superparamagnetism and high stability is the best choice materials.[69] [70] The most commonly reported synthesis of magnetite methods being coprecipitation,[71] hydrothermal,[72] sonication and polyol. Co-precipitation is widely used easy and fast method; salts of iron (II) chloride and iron (III) precipitated in alkaline medium to form magnetite.[73]

Among several advantages presented, the principal is its surface characteristics that allow its easy encapsulation and modification to protect them from degradation, while preserving their magnetic response.[74] The modification of the magnetic cores became necessary prior to polymerization, to avoid loss of the polymer layer around magnetic cores. Silanizing agents, such as tetraethylorthosilicate (TEOS) extensively applied to the magnetite coating by the sol-gel method.[75] Thus high density silane layer of terminal functional groups is achieved, allowing various types of covalent coupling on its surface, reducing the leaching problem of the polymer layer.

In analytical separation magnetic MIP is applied directly to the sample, without the need for previous treatment of the same. In addition, the polymer is readily separated from the test solution by the application of an external magnetic field, such as a magnet.[76]

Magnetic MIPs have been recently employed for several complex matrices such as river waters containing different contaminants – dyes, antibiotics, herbicides, using spectrophotometry, liquid chromatography, HPLC as well as electrochemical detection methods.

1.4 Kinetic studies in MIPs

One of the ways to evaluate MIP binding sites is through kinetic studies that rely on an adsorption study of the template in MIP at certain time intervals. In this method, a fixed amount of MIP is brought into contact with solutions containing different amounts of caffeine for a specific analysis time. After contact for any given time, the solution is separated from the polymer by filtration. Next, the solution is used to measure the concentration of free (remaining) template molecule and is determined by reference analytical methods (such as HPLC-UV) and by difference the amount bound (adsorbed) in the MIP. In this type of study, it is possible to find the best adsorption time of the template in the polymer.

1.4.1 Adsorption isotherms obtained for template affinity studies with MIP

Adsorption isotherms are used to determine the maximum amount of template adsorbed on the surface of adsorbent materials (MIP and NIP in this case) at a constant temperature, in order to determine parameters associated with adsorption.

There are several mathematical models for adjusting the experimental data of the adsorption of an analyte to the adsorbent support (isotherms), among them the Langmuir and Freundlich.

One of the characteristics of the Langmuir isotherm is to assume the formation of a monolayer on the adsorbent material and assume an approximation of the adsorption limit quantity (Equation 1).[77,78].

$$q_e = \frac{K_L \times C_e}{1 + a_L \times C_e} \qquad (Equation\ 1)$$

Where: q and is the amount of adsorbed template at equilibrium (mg g^{-1}), C_e corresponds to the template concentration at equilibrium (mg L^{-1}), K_L and a_L are constants in the Langmuir isotherm.

The Langmuir equation can also be represented by the linearized form (Equation 2), which is used to obtain the parameters of this adsorption model.

$$\frac{C_e}{q_e} = \frac{1}{K_L} + \frac{a_L}{K_L} C_e \qquad (Equation\ 2)$$

The Freundlich isotherm, on the other hand, assumes the existence of a multilayer adsorption and predicts an exponential distribution of several adsorption sites with different energies. It is represented by Equation 3: [77,78].

$$q_e = K_F \times C_e^{b_F} \qquad (Equation\ 3)$$

Where: q e is the amount of template adsorbed at equilibrium (mg g^{-1}), C_e is the concentration at equilibrium (mg L^{-1}), K_F and b_F are parameters corresponding to the Freundlich isotherm. The linear form of the Freundlich equation is given by Equation 4: [77,78].

$$\ln q_e = \ln K_F + b_F \ln C_e \qquad (Equation\ 4)$$

1.5 Application of Molecularly Imprinted Polymers (MIP)

The solid phase extraction method (SPE) is a widely used method for the extraction, clean up and concentration of analytes when they are present at low concentration levels. Through this methodology it is possible to carry out the removal of the analyte of interest, separating it from interfering compounds in complex matrices. Therefore, SPE is considered a very important method for the preparation of samples.

The use of MIP as adsorbent material in the solid phase extraction technique has been shown to be of high selectivity and chemical and thermal stability when compared to other materials such as modified silica (C18), ion exchange resins and immunosorbents, besides reproducibility in the preparation of the polymer. The technique is referred to as solid phase extraction with molecular imprinting (MISPE). MISPE functions as a conventional SPE procedure, except for the substitution of common stationary phases for the printed polymer. Thus, it comprises the steps of conditioning, loading, washing and eluting the cartridges.[78].

Initially it should be considered that the solvent in which the samples are to be percolated should not interfere in the interactions formed between the polymer and the analyte in order that the retention of the analytes is the largest possible. The characteristics of the analyte used as a template are important for the choice of the best washing solvent. They are usually used low polar solvents such as dichloromethane, toluene and chloroform or high polar solvents such as methanol. Thus, in this type of analysis the conditions of pH, polarity and volume of solvent must be optimized, so that the template separation of the interfering compounds is made. The efficiency of this method as well as the applicability of this material in different matrices will depend on several factors, such as amount of MIP used, MIP efficiency in adsorption, interaction of the template with the active site and the way the analysis is performed.

Thus, this thesis was aim to develop a MMIP selective to caffeine and its application in various methods and techniques, for caffeine analysis in quality control labs for different market samples and food items, containing caffeine and, also for online on spot analysis of environmental studies.

5 Conclusions - Perspectives

In this thesis we demonstrated, the great potential of molecularly imprinted polymers as selective receptors in “real world” applications. The main challenge was to design a MIP that could efficiently capture the target molecules in an extremely complex and unfavorable environment. Secondly MMIP could be easily separated from the complex medium after interaction by its super paramegnatic nature. Here demonstrated a systematic study in order to synthesize various magnetic selective materials to caffeine.

Our first attempts, where the MIP was prepared acrylonitrile (ACN) and EGDMA were not much successful, as very small or even no binding was. Also, polymers synthesized used the structural monomer TRIM similarly provided unsatisfactory results. The polymer synthesized with MAA in the proportion 1:4:10 (template: monomer: cross-linker) shown very low binding as compared to higher proportion (1:8:20) respectively.

The porogenic solvent, also was essential to obtain the best MMIP polymer. In this sense, acetonitrile provided a MMIP with superior properties than those synthesized used ethanol.

The non-covalent MMIP, synthetized with MMA in acetonitrile in a 1:8:20 ratio presenting specific selective adsorption capacities for caffeine with respect to theophylline and xanthine, the affinity constant presented was greater for the MMIP, showed than the MMIP synthesized was highly selective, since both molecules have very similar chemical structures.
In application of the MMIP synthesized more studies should be carried out to achieve satisfactory results. Since, as the group experience the unsatisfactory results are due to the inadequate treatment to samples, and not due to the MMIP synthesized.

This PhD thesis has allowed us to better understand the significant advantages of MIPs as tailor-made synthetic receptors, with emphasis on recognition in complex environments. The field of molecular imprinting has been developed remarkably since the pioneering studies of Wulff and Mosbach, owing to the intensive research performed in the recent years. Extensive efforts towards the optimization of synthetic protocols and the rational design of MIPs have allowed to circumvent some of the issues related to MIP synthesis such as low affinity and selectivity in complex environments as well as the successful targeting of biomacromolecules.

Hence, it is evident that MIPs can be more than an alternative to antibodies, being more versatile, robust and cost-effective. Nowadays, MIPs feature outstanding recognition

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