INSTITUTO POLITÉCNICO DE BRAGANÇA
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Development of amphiphilic adsorbents for the stimulated uptake and release of polyphenols

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Abstract

This work was devoted to the development of amphiphilic molecularly imprinted polymers (MIPs) to target the stimulated uptake and release of polyphenols. MIP particles were synthetized through a free radical precipitation polymerization process with a hydrophobic crosslinker and different hydrophilic functional monomers. Polydatin was selected as the template polyphenol and anionic, cationic and neutral adsorbent particles were thus produced. The formation of microparticles (e.g. with size in the order of 1 μm) was evidenced by SEM. The incorporation of crosslinker and functional monomer in the final polymer networks was observed using FTIR. The adsorption capabilities of the materials synthetized were evaluated using solid phase extraction (SPE) with solvents of different amphiphilic character. Besides the individual retention, the competitive adsorption of a mixture of polydatin + resveratrol + gallic acid was also studied through HPLC (an Ascentis® C18 column was used). Some selectivity of the materials towards the different molecules was observed but the most relevant result obtained was the ability of the produced cationic networks to retain huge amounts of gallic acid. MIPs were also packed in HPLC columns and a continuous process with recycling was conceived through the use of an HPLC pump. Dynamics for the saturation and the release processes were thus measured and the role of the hydrophobic interactions in the different polyphenols adsorption/desorption was highlighted. These issues were further enhanced through the analysis of the retention and release of phenolic compounds in a sequence of two MIPs. A solvent gradient scheme was used and these ideas were applied to a created mixture containing gallic acid, tannic acid, polydatin and resveratrol. Portuguese Douro Region red wine was considered as a source of phenolic compounds and an extract from chestnut shell was also evaluated. The extract from chestnut shell was obtained through supercritical extraction with CO_2, using Bragança region fruits. Supercritical extraction with CO_2 was performed at T=50 °C and P=150 bar. Results obtained evidenced that optimized MIPs can be undoubtedly used to separate and concentrate polyphenols present in red wine, such as resveratrol or polydatin. These findings were further enhanced through the adsorption and release of the gallic acid + tannic acid + polydatin + resveratrol in the series of MIPs. Measurements for the shell chestnut extract obtained with supercritical conditions showed that phenolic compounds should not be present in an appreciable amount (in contrast with the red wine extract). Different extraction techniques (e.g. extraction at alkaline supercritical conditions) and chestnut plant components (leaves, burs, stalks, etc) should be considered in future researches to light this issue. At last, the synthesis of improved amphiphilic adsorbents through the RAFT grafting of functional brushes in the MIP particles surface was addressed. Preliminary results obtained seem to show that the grafted hydrophilic polymer brushes modify the pH/temperature triggered retention/release of polyphenols. However, future studies are needed to clarify these complex issues.

Keywords: Polyphenols, molecular imprinting, amphiphilic adsorbents, supercritical extraction

Համառոտագիր

Այս աշխատանքը նվիրված է ամֆֆիլիական մոլեկուլյար տպագրված պոլիմերների (ՄՏՊ) զարգացմանը, որի համար թիրախ է հանդիսանում պոլիֆենոլների խթանված յուրացումը և անրջատումը: ՄՏՊ-ի մասնիկները սինթեզվում են ազատ ռադիկալների պոլիմերացման պրոցեսի արագացման միջոցով՝ հիդրոֆոբիկ խաչանշորդով և տարբեր հիդրոֆիլային ֆունկցիոնալ մոնոմերներով: Պոլիդատինը ընտրվել է որպես պոլիֆենալ կաղապար և անիոնիկ, կատիոտիկ և բնական ադսորբենտ մասնիկները այդպես են արտադրվել: Միկրոմասնիկների (օրինակ՝ շուրջ 1 մկմ չափով) ձևավորումը հաստատվում է SEM-ի միջոցով: Խաչանշորդների և ֆունկցիոնալ մոնոմերների ներմուծումը պոլիմերային ցանցերի վերջում՝ հսկվում է FTIR-ի կիրառմամբ: Սինթեզված նյութերի կլանման հնարավորությունները գնահատվում են պինդ ֆազային անջատման (ՊՖԱ) կիրառմամբ՝ տարբեր ամֆֆիլական հատկություններով լուծիչների հետ միասին:Բացի դա, անհատական պահումը, պոլիդատին+ռեսվերատրոլ+գալիկ թթու խառնուրդի մրցակցային կլանումը ՝ նույնպես դիտարկվում է HPLC-ի միջոցով (կիրառվել է Ascentis® C18 սյունակը): Նյութերի որոշակի ընտրությունը տարբեր մոլեկուլների նկատմամբ՝ հսկվում է, սակայն ստացված ամենահամապատասխան արդյունքը դա կատիոտիկ ցանցի արտադրման ունակությունն է ՝ պահպանել մեծ քանակությամբ գալիկ թթու: ՄՏՊ-ն նույնպես տեղակայված է HLPC-ի սյունակներում և վերամշակումով շարունակական պրոցեսը կատարվում է HLPC-ի պոմպի կիրառման միջոցով: Այս կերպ չափվում են հագեցվածության դինամիկան և անջատման պրոցեսները, և հիդրոֆոբիկ փոխազդեցությունների դերը, տարբեր պոլիֆենոլների կլանման/անջատման գործում, բականին մեծ է: Այս արդյունքները հետագայում կզարգացվեն պահպանման անալիզների միջոցով և կանջատվի ֆենոլիկ միացություններ երկու ՄՏՊ-ների հաջորդականությամբ: Լուծույթների գրադիենտի սխեմայի գործածումը,ինչպես նաև այս գաղափարները՝ կիրառվում են ստեղծելու գալիկ թթու, թեննիկ թթու,պոլիդատին և ռեսվերատրոլ պարունակող խառնուրդ: Պորտուգալիայի Դուրո շրջանում կարմիր գինին համարվում է ֆենոլային միացությունների աղբյուր և շագանակի կեղևից ստացված էքստրակտը նույնպես գնահատվում է: Շագանակի կեղևի էքստրակտը ստացվում է CO_2-ով ընթացող սուպերկրիտիկական արդյունահանման միջոցով՝ կիրառելով Բրագանսա շրջանի մրգերը: CO_2-ով ընթացող սուպերկրիտիկական արդյունահանումը կատարվում է T=50 °C –ում և P=150 Բար: Ստացված արդյունքները վկայում են ,որ օպտիմալացված ՄՏՊ-ն անկասկած կարող է կիրառվել կարմիր գինում առկա պոլիֆենոլների առանձնացման և կենտրոնացման համար, այնպես ինչպես ռեզվերատրոլն ու պոլիդատինը: Այս հայտնագործությունները հետագայում կզարգացվեն ՄՏՊ-ի սերիաներում՝ գալիկ թթու+թեննիկ թթու+պոլիդատին +ռեսվերատրոլ –ի կլանման և անջատման միջոցով: Սուպերկրիտիկական պայմաններում ստացված շագանակի կեղևի էքստրակտի չափումները ցույց են տալիս, որ ֆենոլային միացությունները չեն կարող լինել մեծ քանակությամբ (ի տարբերություն կարմիր գինու էքստրակտի):

Արդյունահանման տարբեր մեթոդները (օրինակ սուպերկրիտիկական պայմաններում ալկալիների արդյունահանումը) և շագանակի բույսի բաղադրիչները (տերևները, փշերը, ցողունները և այլն) պետք է հաշվի առնել հետագա հետազոտություններում՝ դրական արդյունքների համար: Վերջապես, բարելավված ամֆֆիլիկ ադսորբենտների սինթեզը, ՄՏՊ-ի մասնիկների մակերևույթում ֆունկցիոնալ խոզանակների RAFT պատվաստման միջոցով, դիտարկված է: Ստացված նախնական արդյունքները ցույց են տալիս, որ պատվաստված հիդրոֆիլիկ պոլիմերային խոզանակները ձևավորում են պոլիֆենոլների թողարկումը: Այնուամենայնիվ, հետագա հետազոտությունները անհրաժեշտ են այդ բարդ հարցերի հստակեցման համար:

Հանգուցային բառեր՝ Պոլիֆենոլներ, մոլեկուլների տպագրում, ամֆֆիլիկ ադսորբենտներ, սուպերկրիտիկական արդյունահանում:

Resumo

Este trabalho foi dedicado ao desenvolvimento de polímeros molecularmente impressos (MIPs) anfifílicos visando a retenção e libertação estimulada de polifenóis. Partículas MIP foram sintetizadas através de polimerização radicalar por precipitação com um reticulante hidrofóbico e diferentes monómeros funcionais hidrófilos. A polidatina foi selecionada como polifenol molde e partículas adsorventes aniónicas, catiónicas e neutras foram assim produzidas. A formação de micropartículas (ex. com tamanho próximo de 1 μm) foi evidenciada por SEM. A incorporação de reticulante e monómero funcional nas redes de polímero foi observada por FTIR. A capacidade de adsorção dos materiais foi avaliada por extração em fase sólida (SPE) com solventes de diferente caracter anfifílico. Para além da retenção individual, foi também estudada a adsorção competitiva de uma mistura de polidatina + resveratrol + ácido gálico através de HPLC considerando uma coluna Ascentis® C18. Foi observada alguma seletividade dos materiais relativamente às diferentes moléculas, mas o resultado mais relevante é a capacidade das redes catiónicas para reter enormes quantidades de ácido gálico. Os MIPs foram também empacotados em colunas de HPLC e foi concebido um processo contínuo com reciclo usando uma bomba de HPLC. Foi medida a dinâmica de saturação e libertação e destacado o papel das interações hidrofóbicas na adsorção dos diferentes polifenóis. Esses aspetos foram adicionalmente desenvolvidos através do uso de uma sequência de MIPs. Foi usado um gradiente de solventes e essas ideias foram aplicadas a uma mistura de ácido gálico, ácido tânico, polidatina e resveratrol. Vinho tinto da região do Douro foi também considerado como uma fonte de compostos fenólicos, tal como um extrato de casca de castanha. O extrato de casca de castanha foi obtido com CO_2 supercrítico, usando frutos da região de Bragança. A extração supercrítica com CO_2 foi realizada a T=50 °C e P=150 bar. Os resultados obtidos mostram que os MIPs otimizados podem inequivocamente ser usados para separar e concentrar polifenóis presentes no vinho tinto, como a polidatina e o resveratrol. Estes resultados foram confirmados através da adsorção e libertação de ácido gálico + ácido tânico + polidatina + resveratrol na sequência de MIPs. As medições com extrato de casca de castanha indicaram que os compostos fenólicos não devem estar presentes em quantidade apreciável, nomeadamente em comparação com o vinho tinto. Diferentes condições de extração (ex. extração supercrítica em condições alcalinas) e partes do castanheiro (folhas, ouriços, galhos, etc) poderão ser considerados em trabalhos futuros para obter conclusões definitivas. Por fim, a síntese de adsorventes anfifílicos melhorados foi tentada através da enxertia RAFT de extensões funcionais na superfície das partículas. Resultados preliminares parecem mostrar que as extensões hidrófilas enxertadas modificam a retenção/libertação de polifenóis estimulada por alterações no pH/temperatura. No entanto são necessários estudos adicionais para clarificar estes processos complexos.

Palavras Chave: Polifenóis, impressão molecular, adsorventes anfifílicos, extração supercrítica

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List of abbreviation and symbols

AA	Acrylic acid
ACN	Acetonitrile
AIBN	2, 2′-Azobis (2-methylpropionitrile)
CL	Cross-Linker
CL/T	Cross-Linker/Template
DMAEMA	2-(dimethylamino) ethyl methacrylate
FM	Functional monomer
FTIR	Fourier Transform Infrared Spectroscopy
FRP	Free radical polymerization
I	Initiator
MIP	Molecularly imprinted polymer
M/T	Functional monomer/Template
NIP	Non-imprinted polymer
NVP	*N*-Vinylpyrrolidone
RAFT	Reversible addition-fragmentation chain transfer
S	Solvent
SEM	Scanning Electron Microscopy
TMTPA	Trimethylolpropane triacrylate
CPA	4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid
CDTPA	4-Cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoic acid
MAA	Methacrylic acid
PNIPA	Poly (N-isopropylacrylamide)
PMMA	Poly (methyl methacrylate)
EGDMA	Ethylene glycol dimethacrylate
SFE	Supercritical fluid extraction
SPE	Solid phase extraction
UV	Ultra violet

Chapter 1

1.1. Introduction

Polyphenols are polyhidroxylated phytochemicals which have common structures and can be categorized primarily into flavonoids and non-flavonoids. Additionally, polyphenols can also be subdivided in three main subclasses, such as, flavonoids, phenolic acids and stilbenoids. Polyphenols are the subject of increasing scientific interest because of their possible beneficial effects on human health. Different type of polyphenols such us trans-resveratrol, polydatin, catechin or ellagic acid find important applications in pharmaceutics, biotechnology or cosmetics. They provide a significant protection against development of several chronic diseases such as cardiovascular diseases (CVDs), cancer, diabetes, infections, aging, asthma etc, due to their anti-oxidant and anti-inflammatory activities. The antioxidant properties of polyphenols are mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. The antioxidant activity is influenced by high phenol content and by the presence of stronger phenolic antioxidants.

These active compounds are found in edible and non-edible plants. Indeed, they can be found in different vegetables and a growing activity concerning their efficient extraction, purification and concentration is nowadays observed. On other hand, many polyphenols present low bioavailability in humans when oral administration is used due to the low solubility in water and their fast metabolism and excretion. Thus, the development of vehicles for controlled uptake and release of polyphenols is also a key issue in this context.

In this work is proposed the development of molecularly imprinted particles to target different kinds of polyphenols, considering the precipitation polymerization technique along with the classical free radical and the reversible addition-fragmentation chain transfer (RAFT) mechanisms. The optimization of the molecular imprinting process should be studied through the change of the kind and relative amounts of the functional monomer (e.g. methacrylic acid, acrylamide, and 4-vinylpiridine) and cross-linker (e. g. ethylene glycol dimethacrylate, trimethylolpropane triacrylate, divinylbenzene, methylenebisacrylamide). Taking advantage of the RAFT polymerization process, the surface of the molecularly imprinted particles will be grafted with functional brushes (e.g. acidic/alkaline or hydroxyethyl methacrylate based brushes), leading to core-shell structures. Stimulation of the particles for uptake and release of polyphenols should thus be possible as a consequence of the change of the surrounding conditions (e.g. pH and/or temperature). Moreover, through the RAFT grafting process, the creation of amphiphilic particles is possible (e.g. hydrophobic imprinted core bearing a hydrophilic shell) which should be explored to increase the efficiency of the materials for the molecular recognition of polyphenols in aqueous systems.

The characterization of the molecular architecture of the networks and/or the linear counterpart's synthetized for molecular imprinting will be performed using size exclusion chromatography with multiple detection (e.g. refractive index, light scattering, ultraviolet, and viscosity) and FTIR-ATR. Morphology of the produced particles will be assessed by SEM. Performance of the molecularly imprinted particles concerning the stimulated uptake and release of polyphenols will be evaluated using different techniques, namely batch adsorption, solid phase extraction and chromatographic/frontal analysis. Within the later purpose, the produced particles will be packed in chromatographic columns (considering different bed lengths, ranging from 10 mm to 300 mm) and used as selective adsorbents for polyphenols. The amphiphilic behavior of the particles will be here explored for optimization of polyphenols uptake/release, considering eluents with different degrees of hydrophilicity. The development of materials with improved performance for the molecular recognition of polyphenols is an expected outcome of this research.

1.2. Organization of the Dissertation

This work is organized into eight chapters:

Chapter1. Intends to introduce the topic of this thesis in the current framework and present the primary aim of the project.

Chapter2. In this chapter is described the general concept of polyphenols. Why they are important and about molecular imprinting and applications of MIPs.

Chapter3. In this chapter is described experimental part of this project, about the reactants, the equipment and the techniques that I used in my work.

Chapter4. In this chapter are presented synthesis of molecularly imprinted polymers, using free radical polymerization, including the SEM images of my particles and also FTIR spectra of molecular imprinted particles.

Chapter5. In this chapter is presented characterization of molecular imprinted and non imprinted polymer using solid phase extraction (SPE) as well as theoretical and experimental of frontal analysis.

Chapter6. Are presented retention and release of phenolic compounds in a sequence of MIP adsorbents with application to natural extracts namely chestnut and red wine.

Chapter7. In this chapter are presented amphiphilic MIP adsorbents through the grafting of functional brushes in the particles surface by RAFT polymerization.

Chapter8. In this chapter are presented the main conclusions concerning the work performed.

Chapter 2

2.1 Structure and Classes of Polyphenols

Phenolic compounds have at least one aromatic ring with one or more hydroxyl groups and they occur in conjugated forms. They can be categorized as flavonoids and non-flavonoids [Lima.et. al, 2014]. Polyphenols are secondary compounds and have widespread in the plant kingdom [Gharras et. al, 2009].

Polyphenols represent a group of chemical substances common in plants, structurally characterized by the presence of one or more phenol units (see figure 2.1).

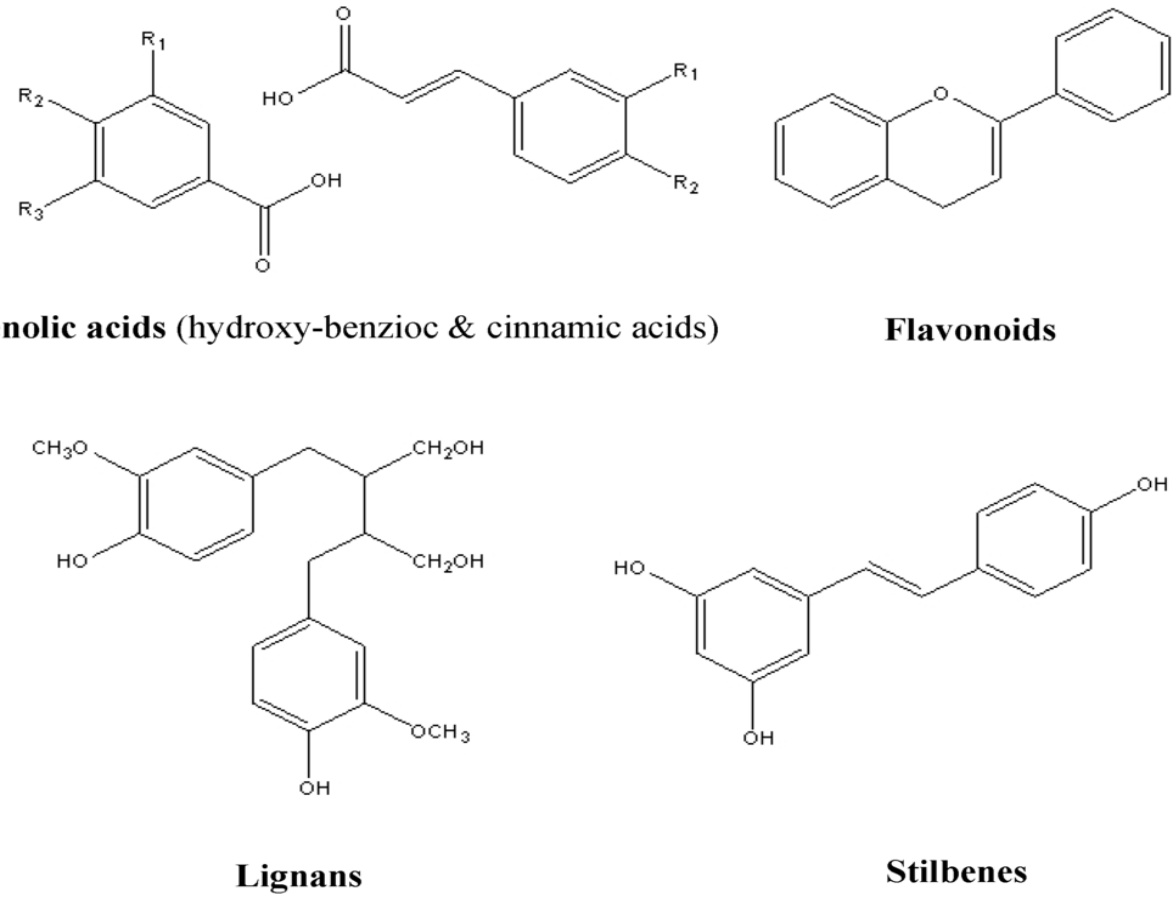


Figure 2.1: Structures of Polyphenols.
https://www.thepaleomom.com/polyphenols-magic-bullet-or-health-hype/

The polyphenols may divided into at least 10 different classes. It is due on their basic chemical structure [Lima et. al, 2014] as a function of the number of phenol rings that they contain and on the basis of structural elements that bind these rings to one another. The main classes include flavonoids, phenolic acid, stilbenoids and lignans [Knežević et. al, 2012].

The most common phenolic in human diet are phenolic acids, flavonoids and tannins. The flavonoids are most important group, which in turn is further divided in several categories (flavonols, flavnones, flavanols, flavones, anthocyanin, and isoflavones) [Lima et. al, 2014]. Polyphenols is the subject of increasing scientific interest due to their possible effect on human health [Pandey et. al, 2009]. They have a beneficial effect on human health and may provide health benefits by several mechanisms, including the elimination of free radicals, the protection and regeneration of other dietary antioxidants and the chelation of pro-oxidant metals [Lima et. al, 2014].

2.1.1 Polyphenols and Human Health

All health benefits of polyphenols depend on their metabolism and absorption including their conjugation with other phenolic, degree of glycosylation/acylation, molecular size and solubility. The metabolism and absorption in turn are determined by their structure [Ozcan et. al, 2014].

The process take place in different points during passage through the wall of the small intestine into the circulatory system and subsequent transport to the liver in the portal vein. The metabolites of polyphenols are quickly removed from plasma. Thus, in order to supply high metabolite concentrations in the blood, necessary daily consumption of plant products is needed [Ozcan et. al, 2014]. The available human studies of polyphenols can be divided into intervention studies, which are under defined circumstances applying nutritional intervention and measuring the biological outcome, and observational epidemiological studies. Strong epidemiological evidences indicate that moderate consumption of wine is associated with a significant reduction of cardiovascular events [Knežević et. al, 2012]. When circulating throughout our bodies, antioxidants like polyphenols may help or delay cell damage from free radicals that are unstable molecules that damage our cells and DNA.

Polyphenol metabolites circulate in the blood bound to proteins; in particular albumin represents the primary protein responsible for the binding. The important functions of polyphenols like, inhibition of pathogens and decay microorganisms, anti-deposition of triglycerides, reduce the incidence of non-communicable diseases such as cardiovascular diseases, diabetes, cancer and stroke, through processes involving reactive oxygen species [Pandey et. al, 2009]. The antioxidant action of polyphenols can potentially result in vasodilator, antithrombotic, anti-inflammatory, ant apoptotic, antiatherogenic effects associated with decreased cardio-vascular risk [Quinones et. al, 2013]. Thus the health beneficial effects of the polyphenols (See figure 2.2) depend upon both the intake and bioavailability [Pandey et. al, 2009].

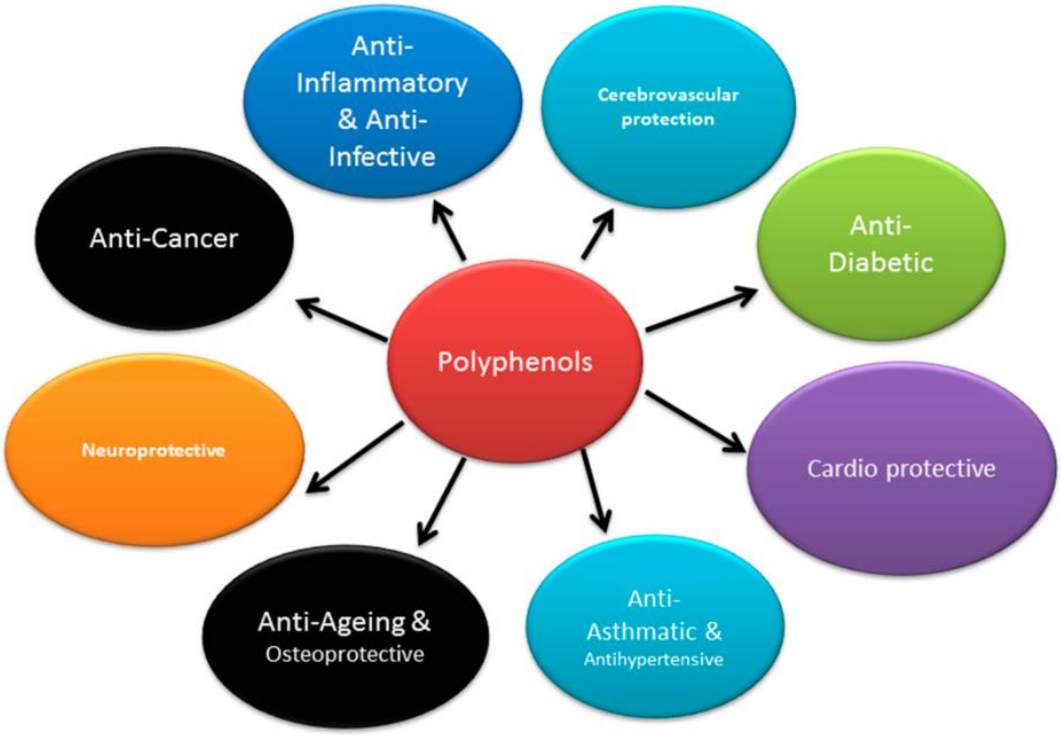


Figure 2.2: Role of polyphenols in humans.

http://www.mdpi.com/2072-6643/9/5/455/htm

2.1.2: Bioavailability of polyphenols

Bioavailability can be defined as the fraction of a nutrient or non-nutrient that is available for the human body for physiological functions and/or storage [http://publicationslist.org]. The health effects of polyphenols in human and in animal models depend on their absorption, distribution, metabolism and elimination. Absorption of polyphenols is followed by extensive conjugation and metabolism [Knežević et. al, 2012]. During the course of absorption, polyphenols are conjugated in the small intestine and later in the liver. This process mainly includes methylation, sulfation, and glucuronidation. This is a metabolic detoxication process common to many xenobiotic that restricts their potential toxic effects and facilitates their biliary and urinary elimination by increasing their hydrophilicity [Manach et. al, 2004].

The chemical structure of polyphenols determines their extent of absorption and rate as well as the nature of the metabolites present in the plasma and tissues [Knežević et. al, 2012]. These polyphenols reach the colon, where micro flora hydrolyze glycosides into aglycones and extensively metabolize these aglycones into various aromatic acids [http://publicationslist.org].

Accumulation of polyphenols in the tissues is the most important phase of polyphenol metabolism because this is the concentration which is biologically active for exerting the effects of polyphenols. Excretion

of polyphenols with their derivatives occurs through urine and bile. It has been observed that the extensively conjugated metabolites are more likely to be eliminating in bile, whereas small conjugates, such as mono sulfates, are preferentially excreted in urine. Amount of metabolites excreted in urine is roughly correlated with maximum plasma concentrations [Pandey et. al, 2009].

Polyphenols may also have an indirect effect on health because they are metabolized by the same pathways as various xenobiotic or endogenous hormones. More indirect effects of the diet on various parameters of gut physiology (pH, intestinal fermentations, biliary excretion, transit time, etc.) may have consequences on the absorption of polyphenols [Manach et. al, 2004].

2.1.3 Polyphenols in Fruits and Vegetables

Polyphenols can be found in fruits, herbs, vegetables, cereals, and other plant materials rich in phenolics. They are increasingly of interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food [Gharras et. al, 2009].

Fresh fruits and vegetables usually contain higher levels of polyphenols than food that has sat around for a few weeks. Some polyphenols are specific to particular food, whereas others, such as quercetinn are found in all plant products such as fruit, vegetables, cereals, leguminous plants, tea, and wine. However, generally foods contain complex mixtures of polyphenols. Beverages are concentrated, easily absorbed sources of polyphenol antioxidants. For example fruit juices that do not have added sugar. Pure pomegranate, blueberry, red grape, and unfiltered apple juice.

Tea and coffee are very rich sources of certain types of polyphenols. Caffeinated tea and coffee should be preferred, because decaffeination process may remove polyphenols along with the caffeine. Instead of other alcoholic beverages, we can drink red wine and beer.

Red wine is a very rich source of the famous polyphenol Resveratrol, which is present in high concentrations in the skins of wine grapes. Beer is also a great source of polyphenols, and contains a great variety of polyphenols [http://www.livestrong.com/]. Chocolate and cocoa are also of the richest sources of polyphenols, especially bitter, chocolate and unsweetened cocoa [http://wikihow.com]. Current dietary advice is that for optimum health people should consume at least five portions of fruit and vegetables every day, each portion of at least 80 grams [Archivio et. al, 2010].

In figure 2.3 are presented fruits such us berries, grapes, pomegranate and this type of fruits are very rich of polyphenols.

Figure 2. 3: Diets highest in Polyphenols

http://www.borecole.co.uk/polyphenols-in-the-diet-linked-to-longevity/

2.2 Flavonoids, Phenolic acids and Tannins

2.2.1 Flavonoids

They are describe as containing two or more aromatic rings, each bearing one or more phenolic hydroxyl groups, and connected by a carbon bridge. The basic flavonoid structure is the flavan nucleus, which consists of 15 carbon atoms arranged in three rings (C6-C3-C6), which are labeled A, B, and C, among the many classes of flavonoids [http://.biolinks.co.jp/].

Flavonoids are divided into three main classes. Flavonoids, isoflavonoids, and neoflavonoids, and those devided into subclasses, flavones, isoflavones and isoflavanes, flavanones, flavanols, anthocya nidins, chalcones and dihydrochalcones [Vihakas et. al, 2014].

2.2.2 Flavonoids and Human health

Flavonoids have biological and pharmacological activities. Flavonoids can to exert antimicrobial, cytotoxicity, anti-inflammatory as well as antitumor activities because act as powerful antioxidants which can

protect the human body from free radicals and reactive oxygen species. Flavonoids have received considerable attention because of their beneficial effects as antioxidants in the prevention of human diseases such as cancer and cardiovascular diseases, and some pathological disorders of gastric and duodenal ulcers, allergies, vascular fragility, and viral and bacterial infections [Yaoy et. al, 2013]. The capacity of flavonoids to act as antioxidants depends upon their molecular structure. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities.

Quercetin, the most abundant dietary flavonol is a potent antioxidant because it has all the right structural features for free radical scavenging activity [Saxena et. al, 2012].

2.2.3 Where can be found

Flavonoids generally occur in plants as glycosylated derivatives, and they contribute to the brilliant shades of blue, scarlet, and orange in leaves, flowers, and fruits. Flavonoids are found also in seeds, nuts, grains, spices, and different medicinal plants as well in beverages, tea, and beer [Pietta et. al, 2000]. Many of the berries are high in flavonoids, particularly red, blue and purple berries. Darker and riper berries tend to have higher flavonoid value and processing may reduce levels. Blackberries and black grapes are high in the flavonoids epicatechin [http://.livestrong.com/].

2.2.4 Phenolic acids

Phenolic acids are important components of the human diet, because they have antioxidant activity. Phenolic acids have been reported to have important biological and pharmacological properties and their can to diminish oxidative stress induced tissue damage resulting from chronic diseases, and their potentially important anticancer activities. The majority of phenolic acids are linked through ester, ether, or acetyl bonds either to structural components of the plant or to larger polyphenols, or smaller organic molecules or other natural products. Many phenolic acids like cinnamic and benzoic acid derivatives exist in all plant and plant-derived foods [Goleniowski et. al, 2013].

Phenolic acids are in fruits, vegetables, and whole grains. Sources include mangoes, berries, apples, citrus fruits, plums, kiwis, cherries, onions, tea, red wine, coffee, and wheat, corn, rice, and oat flours. They are extended in plant-based foods; humans consume phenolic acids on a daily basis. They are easily absorbed due to their simplicity [Goleniowski et. al, 2013].

2. 2. 5 Tannins

Tannins are light yellow or white amorphous powder or shiny, nearly colorless, loose mass. It has a characteristic strange smell and astringent taste. The ‘tannin’ (Figure 2.4) is means tanning substance and derived from the French word ‘tannin’ and is used for a range of natural polyphenols. Molecular weights of tannins, is from 500 to over 3000 [Ashok et. al, 2012].

Tannins can be divided into two groups, hydrolysable and non-hydrolysable or condensed tannins. Therefore, the term ‘hydrolysable tannins’ includes both the Gallo tannins and the ellagitannins. It should also be mentioned here that there are ellagitannins that are not hydrolysable, because of a further C–C coupling of their polyphenolic residue with the polyol unit, but are nevertheless for historical reasons classified as hydrolysable tannins [Khanbabaee et. al, 2001]. The term is widely applied to any large polyphenolic compound containing sufficient hydroxyls and other suitable groups to form strong complexes with proteins and other macromolecules [Ashok et. al, 2012]. The scientific word for these compounds is polyphenols. They are water-soluble polyphenols and we can find in many plant foods [http://vinepair.com/]. Tannins are found in tea and coffee and if we use too many these beverages without milk may lead to calcium and iron deficiency in the body and often lead to osteoporosis and anemia.

Tannins are supplemented to various processed foods, including ice-cream and caramel. They are used as refining materials to precipitate proteins in wines and beer; as tannins often lower the absorption of materials into the body.

Tannins are also often known as anti-nutrients. Tanning, tannins are also used in photography, dyeing, refining beer and wine as well as an astringent in medicines [Ashok et. al, 2012].

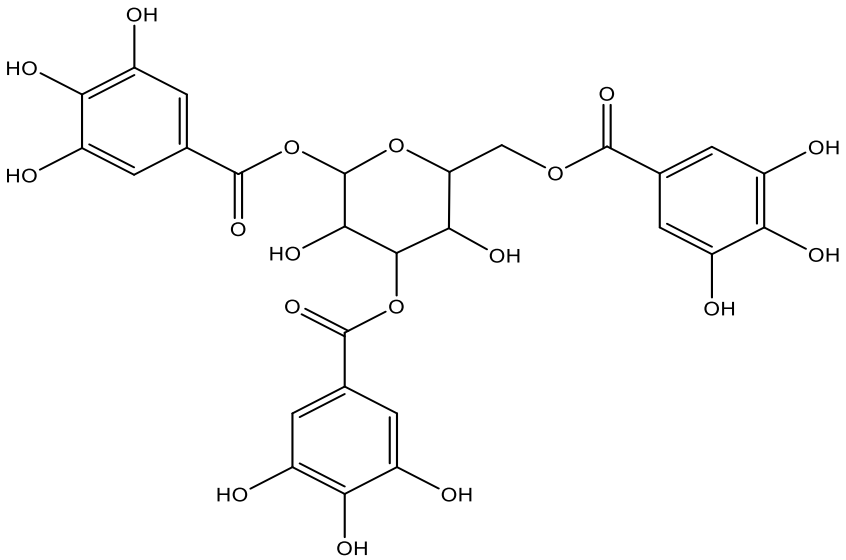


Figure 2.4: Basic structure of Tannin

2. 3 Resveratrol and Polydatin

2. 3. 1 Resveratrol

Resveratrol (3, 4′, 5,-trihydroxy-trans-stilbene) (figure 2.5) is a phenolic compound in the stilbenes group and naturally occurring polyphenol. It is particularly found in grape skin, pomegranate, and nuts, resveratrol is not water soluble [Ravagnan et. al, 2012].

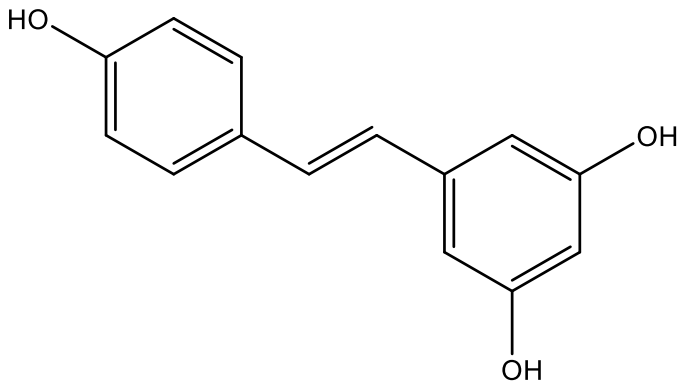


Figure 2.5: structure of resveratrol

It has been often linked to the health benefits often associated with red wines, and is not found in as much abundance in white wines [http:/.academicwino.com]. Resveratrol are contributes to the ability of polyphenols rich Mediterranean diet to reduce the incidence of age-related diseases such as coronary heart disease, cancer and dementia.

Resveratrol are endowed with cardio protective, anti-microbial and chemo preventive properties [Ravagnan et. al, 2012]. It has received considerable attention for its anti-inflammatory, anti-tumorigenic, and anti-oxidant properties, and can to ability to increase lifespan in lower organisms and improve general health in mammals [Smoliga et. al, 2011].

2.3.2 Resveratrol in red wine

Red wine made from grapes it is strongly associated with resveratrol. The concentration of resveratrol in the red wine is depending of from the method of preparation of red wine. The concentration of resveratrol is higher in red wine, than in white wine. Red wine has resveratrol content (per 5 oz. glass) of 0.03 – 1.07 mg in contrast to the 0.01 – 0.27 mg. for white wines [http://www.news-medical].

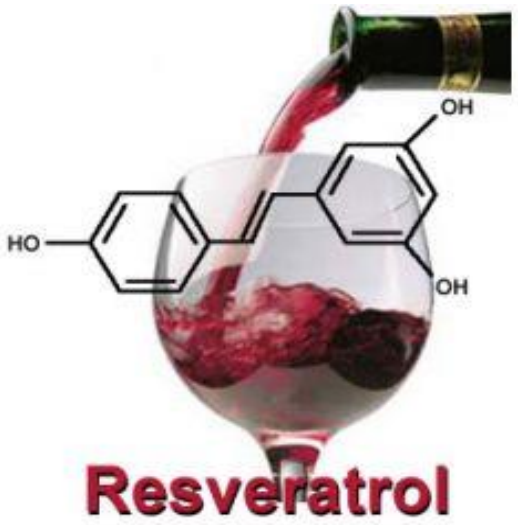


Figure 2.6: Resveratrol in red wine
http://www.natures-health-foods.com/Resveratrol.html

2. 3. 3 Polydatin

Polydatin is an active ingredient extracted from Polygonum cuspidatum, is a stilbenoid glucoside of resveratrol. A sugar molecule is jointed to the resveratrol structure, making polydatin more bioactive and powerful in antioxidant and anti-aging potency [http://herbnutritionals.com/].

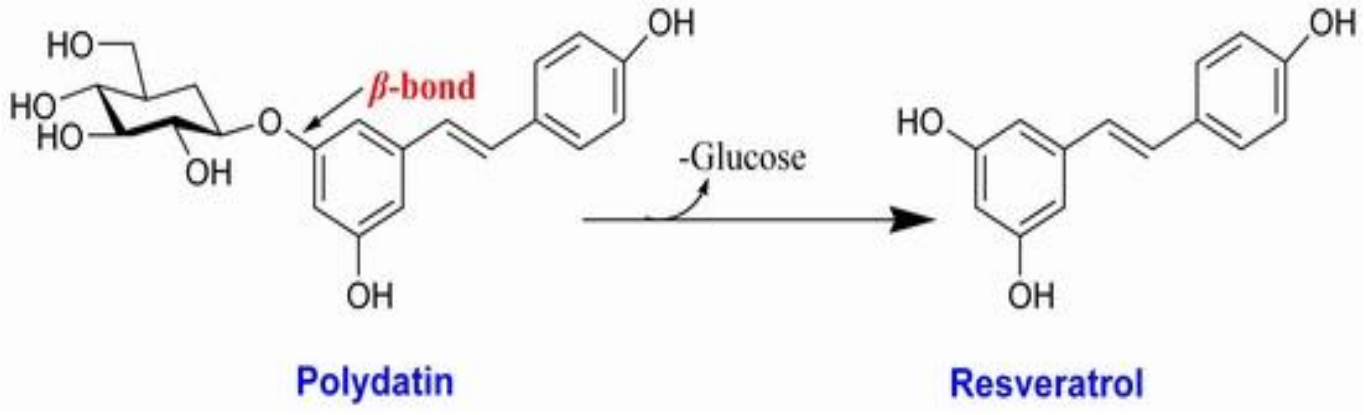


Figure 2.7: molecular structure comparison of polydatin and resveratrol
https://biofoundations.org/polydatin-precursor-to-resveratrol-with-higher-bioavailability-and-longer-half-life-than-resveratrol/

Polydatin is a type of polyphenolic phytoalexin which has many physiological and pharmacological effects, (anti-inflammatory and anti-oxidative activities). Polydatin is an effective candidate drug for the protection of photo-inflammation. Polydatin displays therapeutic potential for vascular dementia, which is due to anti-oxidant activity and the direct protection of neurons [http://sigmaaldrich.com/].

Polydatin, also known as pieced or trans-resveratrol-3-o-glucoside (see figure 2.8) is a stilbenoid glycoside of resveratrol [Ravagnan et. al, 2012]. Polydatin has all beneficial effects on human health that resveratrol:

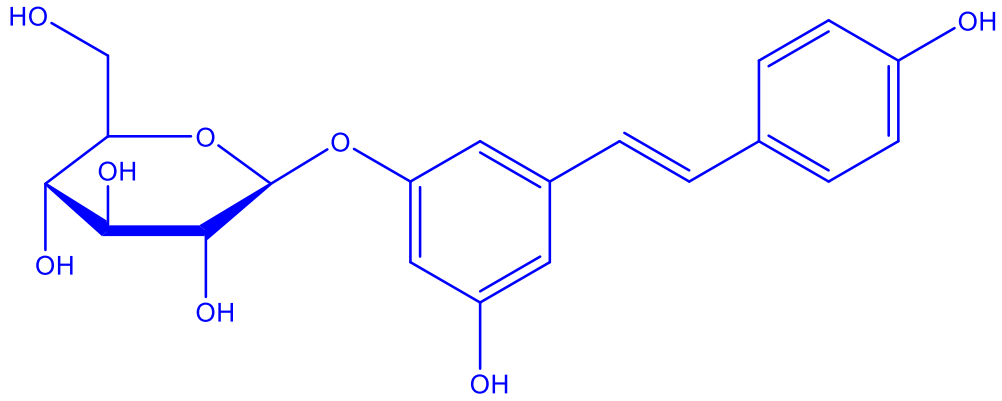


Figure 2.8: Structure of polydatin

Sometimes polydatin has a more advantages above trans-resveratrol.

Effects of polydatin:

- polydatin has effects on cardiovascular system;
- It has a protecting and nourishing effects on liver;
- Polydatin has antibacterial and antifungal effects;
- Polydatin own the effective of anti-oxidant and quench free-radicals. [Http://www. hnkeyuan. com/].

Polydatin is converted into resveratrol during the first pass metabolism. The normal resveratrol is destroyed during first pass metabolism; the same metabolism action is converting polydatin to trans-resveratrol and allowing it to stay much longer in plasma [http://biofoundations.org/].

2.4 Molecular Imprinting Technique (MIT)

2. 4.1 Molecular Imprinting Polymers

Molecular imprinting technique (MIT) was first applied to organic polymers. The start of molecular imprinting technology is marked in 1972. As reflected in the 80 original papers published in the field during 1997 molecular imprinting is currently attracting wide interest from the scientific community. Although interest in the technique is new, the concept has a long history [B. Sellergren]. Technique of molecular imprinting is used for preparing polymers with synthetic recognition sites having a predetermined selectivity for analyte of interest [Cao et. al, 2006].

Molecularly imprinted polymers (MIPs) are tailor-made synthetic receptors with high affinity and specificity toward the targeted analytes. They are typically prepared by the first copolymerization of a functional monomer and a crosslinking monomer in the presence of a template molecule and a suitable porogenic solvent and the subsequent removal of the template from the resulting cross-linked polymers. In the imprinting process, the functional monomers are arranged around the template, then "frozen" into position by polymerization with a high degree of cross-linking in the presence of a porogenic solvent. The functional monomers can interact either covalently or non-covalently with the template [Ma et. al, 2007]. The obtained MIPs have the imprinted binding sites (cavities) complementary to the shape, size, and functionality of the template and can thus specifically recognize the template molecule. This, together with their high stability, ease of preparation, and low cost makes them very promising synthetic substitutes for biological receptors [Zhao et. al, 2014]. This method of syntheses is the most preferred because MIPs have many outstanding advantages. MIPs are wielding high sensitivity and selectivity, ease and convenience of preparation, and robustness in solvents [Ma et. al, 2007].

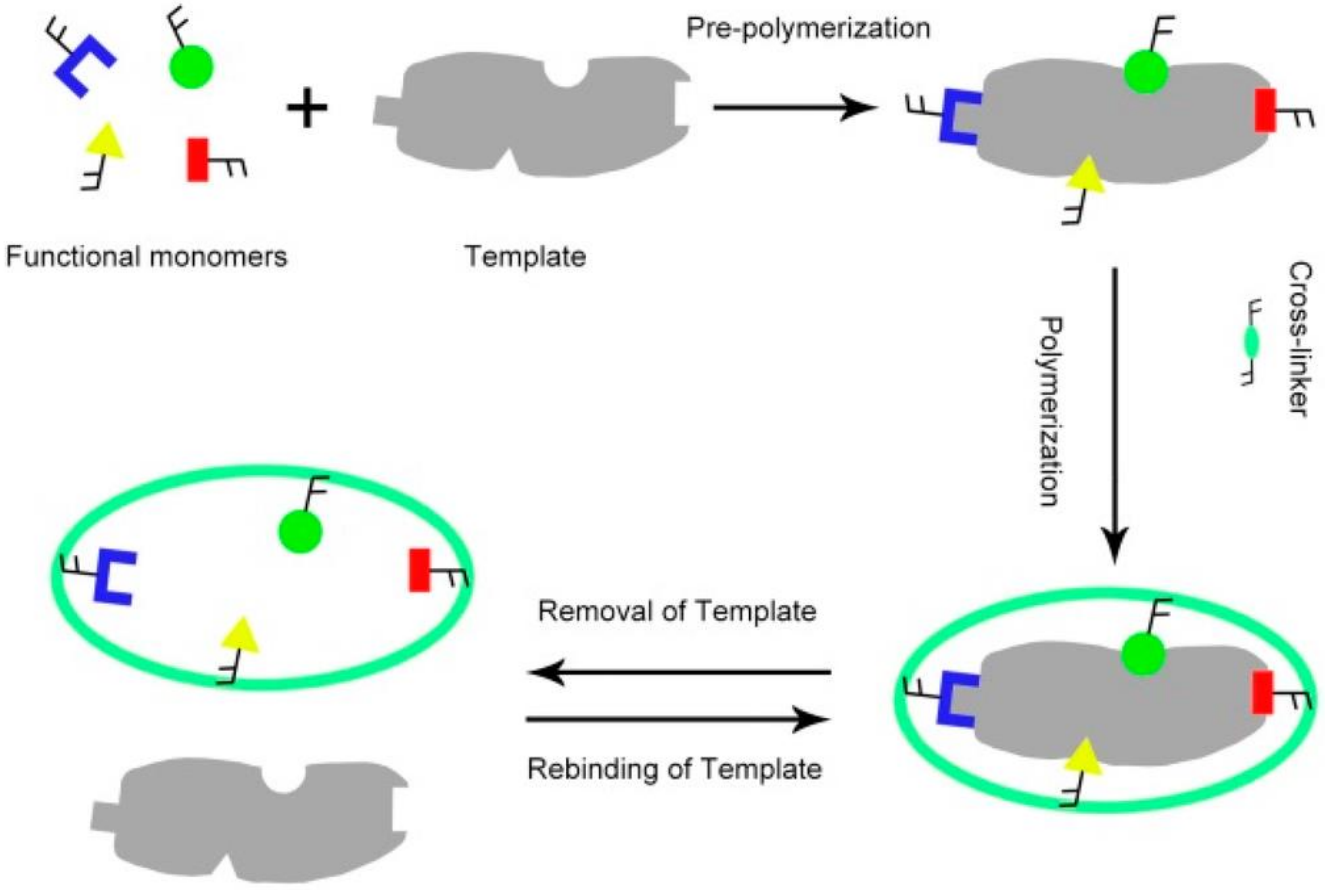


Figure 2.9: Molecular Imprinting scheme
http://www.mdpi.com/1422-0067/16/8/18328/htm

Molecular recognition ability is dependent on several factors, such as shape complementarity, functional complementarity, contributions from the surrounding environment. As for the functional complementarity, even though all non-covalent interactions are applicable to the molecular recognition between a target molecule and a molecular recognition site formed by a molecular imprinting, the nature of the template, monomers and the polymerization reaction itself determine the quality and performance of the polymer product. Moreover, the quantity and quality of the molecularly imprinted polymer recognition sites is a direct function of the mechanisms and extent the monomer–template interactions present in the pre-polymerization mixture. The recognition of the polymer constitutes an induced molecular memory, which makes the recognition sites capable of selectively recognizing the imprint species [Yan et. al, 2006]. Synthesis of MIPs is a more straight forward and inexpensive procedure [Cao et. al, 2006].

Imprinted polymers compared to biological systems such as proteins and nucleic acids, have higher physical robustness, strength, resistance to elevated temperature and pressure and inertness towards acids, bases, metal ions and organic solvents. In addition, keeping their recognition capacity is also possible for several years at room temperature [Vasapollo et. al, 2011]. These features make MIPs suitable in particular for a number of biochemical applications and recently the stability properties have attracted interest from the environmental sector since biomolecules are degraded in non-sterile environments while MIPs may be stable over extended periods of time. Therefore the molecular imprinting technique has been applied in various areas [Ma et. al, 2007].

2.4.2 Application of MIP

Molecular imprinting has now become an established method and has also been applied in the areas of biomedical and analytical chemistry. The peculiar properties of MIPs have made them a highly interesting tool for different application areas.

Molecular imprinting technology is considered a versatile and also promising technique. It is able to recognize both biological and chemical molecules as well as amino acids and proteins, nucleotide derivatives, pollutants, and food. Application areas of molecular imprinting technology include also separation sciences and purification, chemical sensors, catalysis, drug delivery, biological antibodies and receptors system. MIP has been used as chromatographic stationary phases for enantiomer separations, solid-phase extraction [Puoci et. al, 2010].

MIPs can serve also as artificial binding mimics of natural antibodies and can be used as recognition elements in immunoassay-type analyses [Vasapollo et. al, 2011].

2.4.3 Category of MIP

Molecular imprinting is classified according to the nature of the interactions between monomer and template during the polymerization process [Garcia et. al, 2011]. Essentially, two kinds of molecular imprinting strategies have been established based on covalent bonds or non-covalent interactions between the template and functional monomers. In both cases, the functional monomers, chosen so as to allow interactions with the functional groups of the imprinted molecule, are polymerized in the presence of the imprinted molecule.

The special binding sites are formed by covalent or, more commonly, non-covalent interaction between the functional group of imprint template and the monomer, followed by a crosslinker co-polymerization [Yan et. al, 2006]:

- Non-covalent protocol is easily conducted, avoiding the tedious synthesis of pre-polymerization complex.
- Removal of the template is generally much easier, usually accomplished by continuous extraction.
- A greater variety of functionality can be introduced into the MIP binding site using non-covalent methods [Garcia et. al, 2011].

2.4.4 Covalent Approaches

In covalent imprinting, (reorganized approach), covalent reversible and easily cleavable bonds, between the template molecule and the functional monomers, will be established leading to the formation of moderately homogeneous population of binding sites [Garcia et. al, 2011].

The binding of this type of polymer-relies on reversible covalent bonds [Yan et. al, 2006]. In this approach, the template-monomer complex is obtained without use of excess of functional monomer, minimizing non-specific interactions [Garcia et. al, 2011]. After polymerization they hydrolyzed the sugar moiety and used the polymer for selective binding and result shown that for covalent molecular imprinting, selectivity of MIP increases with maximization of cross linker. The requirements are different of covalent imprinting than in non-covalent imprinting, particularly with respect to ratios of functional monomer, cross linker, and template [Yan et. al, 2006].

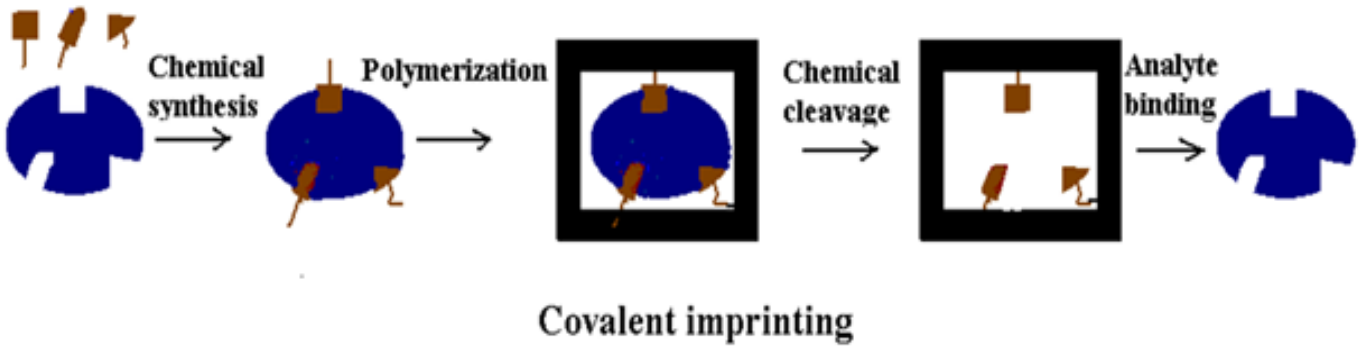


Figure 2.10: Schematic representation of covalent molecular imprinting procedures

http://www.mdpi.com/1422-0067/7/5/155/htm

2.4. 5 Non-covalent Approach

In non-covalent the methodology is far easier than covalent methods, and it produces higher affinity binding sites, versus covalent methods [Yan et. al, 2006]. Most widely used is the non-covalent procedure because it is relatively simple experimentally and the complication step during the synthesis is achieved by mixing the template with an appropriate functional monomer, or monomers, in a suitable porogen [Puoci et. al, 2010].

In this methodology, the functional monomers are used in excess compared to the template to displace the equilibrium to form the template-monomer complex since this interactions are governed by an equilibrium process. The non-covalent imprinting is characterized by weak interactions between the template molecule and the functional monomers, such as hydrogen bonds, Van der Waals forces, and ion or hydrophobic interaction [Garcia et. al, 2011].

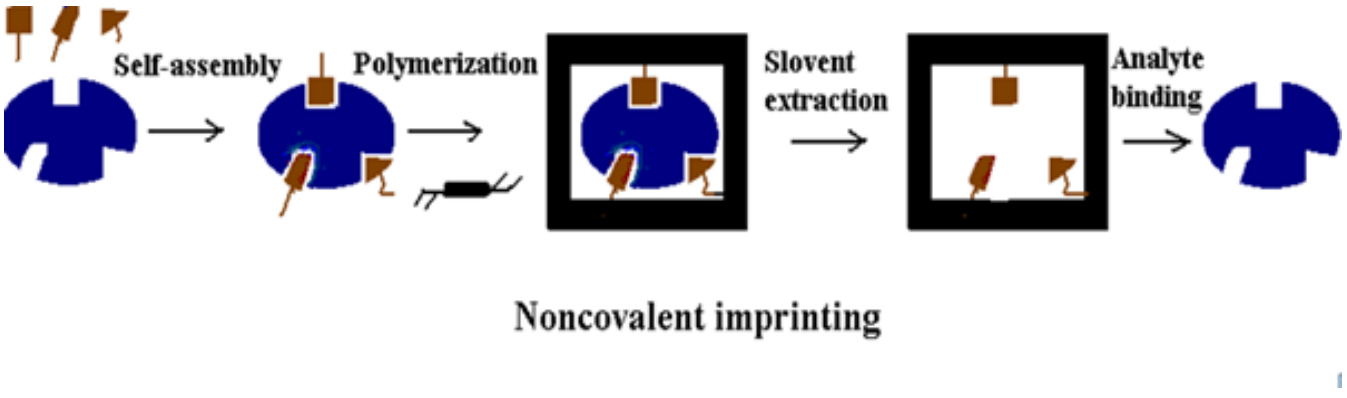


Figure 2.11: Schematic representation of non-covalent molecular imprinting procedures

http://www.mdpi.com/1422-0067/7/5/155/htm

2.4.6 Effecting of special molecular recognition

The synthesis of molecularly imprinted polymers is a chemically complex pursuit and demands a good understanding of chemical equilibrium, molecular recognition theory, thermodynamic and polymer chemistry in order to ensure a high level of molecular recognition.

The challenge of designing and synthesizing a molecular imprinted polymer can be a daunting prospect to the uninitiated practitioner, not least because of the sheer number of experimental variables involved, e.g. the nature and levels of template, functional monomer(s), cross-linker(s), solvent(s) and initiator, the method of initiation and the duration of polymerization [Rane et. al, 2015].

2.4.7 Template

Template is of central importance in all molecular imprinting processes. It directs the organization of the functional groups pendent to the functional monomers [Cormack et. al, 2004]. Optically active templates have been used in most cases during optimization. In these cases the accuracy of the structure of the imprint could be measured by its ability for racemic resolution, which was tested either in a batch procedure or by using the polymeric materials as chromatographic supports. Imprinting method is can be applied to a diverse range of analytes [Yan et. al, 2006], but not all templates are directly amenable to tinplating [Cormack et. al, 2004]. Three general conditions of ideal template molecule [Chen et. al, 2016]:

1. it should contain functional groups that do not prevent polymerization;
2. It should exhibit excellent chemical stability during the polymerization reaction,
3. It should contain functional groups that can form complexes with functional monomers.

2.4.8 Functional Monomer

Functional monomer is a form to form a pre-polymerization complex with the template by providing functional groups. Functional monomer can strongly interact with the template and form specific donor–receptor or antibody antigen complexes prior to polymerization. It is important to select a suitable functional monomer [Cormack et. al, 2004], because functional monomers are responsible for the binding interactions in the imprinted binding sites and, for non-covalent molecular imprinting protocols, are normally used in excess relative to the number of moles of template to favour the formation of template, functional monomer assemblies [Cormack et. al, 2004].

Functional monomer comprises two types of units [Chen et. al, 2016]:

1. Recognition unit,
2. Polymerizable unit.

2.4.9 Cross – linkers

Role of cross-linker in the polymerization process is to fix functional monomers around template molecules, thereby forming a highly cross-linked rigid polymer even after the removal of templates [Chen et. al, 2016]. It has three main functions:

1. The cross-linker is important in controlling the morphology of the polymer matrix;
2. It serves to stabilize the imprinted binding site;
3. It imparts mechanical stability to the polymer matrix [Yan et. al, 2006].

The type and the amount of cross-linker have profound influences on the selectivity and binding capacity of MIPs. Usually, a too low amount of cross-linker will result in unstable mechanical properties due to the low cross-linking degree, and an extremely high amount of cross-linker will reduce the number of recognition sites per unit mass of MIPs [Chen et. al, 2016].

2.4.10 Solvent (porogen)

Porogenic solvents have an important role in formation of the porous structure of MIP, which known as macro-porous polymers. The nature and level of porogenic solvents determines the strength of non-covalent interactions and influences polymer morphology which, obviously, directly affects the performance of MIP [Yan et. al, 2006]. The influence of the polymerization solvent has multiple roles [Rane et. al, 2015]:

1. It solubilizes all the monomers in the pre-polymerization mixture before polymerization;

2. It stabilizes template monomer pre-polymerization complexes;

3. It acts as a ‘porogen’ helping to control the porosity of the resulting polymer.

The polarity of porogens can affect the interaction between the template molecule and the functional monomer and therefore the adsorption properties of MIPs, especially in non-covalent interaction systems. Non-polar and less polar organic solvents, such as toluene, acetonitrile and chloroform, are often used for non-covalent imprinting to obtain good imprinting efficiency, since the adsorption properties and morphology of polymers are dependent on the types of solvents used. To evaluate the selection processes of monomers and

solvents for molecular imprinting and to have an insight into MIP selectivity, the use of theoretical calculations is very important [Chen et. al, 2016].

2.4.11 Initiators

A lot of chemical initiators with different chemical properties can be used as the radical source in free radical polymerization. They are used at low levels compared to the monomer, e. g. 1 wt. %, or 1 mol. % with respect to the total number of moles of polymerisable double bonds. The rate and mode of de-composition of an initiator to radicals can be triggered and controlled in a number of ways, including heat, light and by chemical/electrochemical means, depending upon its chemical nature [Yan et. al, 2006]. To ensure the polymerization reaction, removal of the dissolved oxygen from polymerization solutions immediately prior to polymerization is very important. Oxygen can be cleared by bubbling, an inert gas like nitrogen or argon [Chen et. al, 2016].

2.5 Why we are doing molecular imprinting of polyphenols

Consumption of fruits and vegetables rich in phytonutrients is known to promote human health and well-being. This region of Portugal, Trás-os-Montes e Alto Douro, it is rich in products that have these phytonutrients, like (chestnut, olive, grape, wine, almond etc.). These phytonutrients include polyphenols and the structurally more complex flavonoids. Therefore, there is much interest in exploring these products in this region.

The extraction, purification, concentration of polyphenols, actually present many challenges due to their low concentration, which their separation and extraction both costly and labor intensive. It is necessary the development of simple and robust methodologies than enable the characterization of these compounds in a quick and selective way. Molecular imprinting polymers using polyphenols with template is a technology that present these characteristics. It was reported the preparation of molecularly imprinted polymers, that which presented high selectivity towards the polyphenolic compounds [Schwarz et. al, 2016].

Chapter3 Materials and Equipments

3.1. Materials

The chemical structure, properties and amounts of different reagents (monomers, cross-linker, template, solvent, initiator, RAFT agent) used in synthesis are detailed presented in Table 3.1 to 3.2.

Table 3.1: Chemical structures, properties and amounts of the reagents used in the synthesis of Free radical polymerization.

Monomer	**Chemical Formula**	**MW (g/mol)**	**Density (g/ml)**	**Volume (ml)**	**Mass (mg)**	**n (mmol)**
AA	$C_3H_4O_2$	72.06	1.051	0.16	168.16	2.334
DMAEMA	$C_8H_{15}NO_2$	157.22	0.931	0.4	372.4	2.369
NVP	C_6H_9NO	111.14	1.04	0.256	266.24	2.396
Cross-Linker						
TMTPA	$C_{15}H_{20}O_7$	296.2	1.1	1.2	1320	4.455
Template						
POL	$C_{20}H_{22}O_8$	390.38	-	-	-	-
Solvents						
MeOH	CH_3OH	32.04	0.791	15.0	11865.0	0.370.0
ACN	C_2H_3N	41.04	0.781	48	37488	913.48
Initiator						
AIBN	C_2H_3N	164.21			70.2	0.428

Table 3.2: Chemical structures, properties and amounts of the reagents used in the synthesis of RAFT polymerization

Monomer	**Chemical Formula**	**MW (g/mol)**	**Density (g/ml)**	**Volume (ml)**	**Mass (mg)**	**n (mmol)**
MAA	$C_4H_6O_2$	86.09	1.015	15	1167.25	13.558
DMAEMA	$C_8H_{15}NO_2$	157.22	0.932	1.5	1398.0	8.892
NIPA	$C_6H_{11}NO$	113.16		1.015	1500.5	13.260
4VP	C_7H_7N	105.14	0.975	0.05	50.00	0.476
Cross-Linker						
EGDMA	$C_{10}H_{14}O_4$	198.22	1.051	0.35	367.850	1.856
Template						
POL	$C_{20}H_{22}O_8$	390.38				
Solvents						
MeOH	CH_3OH	32.04	0.791	15.0	11865.0	0.370.0
RAFT agents						
CPA	$C_{13}H_{13}NO_2\ S_2$	279.38	-	-	45.700	0.164
CDTPA	$C_{19}H_{33}NO_2\ S_3$	403.67	-	-	65.200	0.162

3.2 Equipment

In the experimental procedure were used different type of equipment and software that are presented in Table 3.3.

Table 3.3: Equipment used in the experimental procedure

Equipment	Model	Company	Software
Analitical balance	AS/ 220/C/2	RADWAG	-
GPC	GPC Max VE2001 TDA 203 UV detector 2520	VISCOTEK Knauer	OmniSEC 4.7.0
Soxhlet	HM01 Series	Labbox	-
FTIR	MB154s	BOMEM	Grams/32
UV Spectrophotometer	V-530	JASCO	VWS-580 Spectra Manager
Pump	Azura p 4. 1S	Knauer – Azura	-
Vacuum Pump	RE30022C	Stuart	-
Pressure reactor	Parr 4848	Parr instrument CO	Paralab

Chapter 4

4.1 Synthesis of Molecularly Imprinted Polymers

In this chapter is presented experimental work, for all synthesis of Molecular imprinted polymers particles (MIPs), and non-imprinted particles polymers (NIPs). These type of the particles were obtained using two different mechanisms, Controlled Radical Polymerization (CRP) by RAFT and free radical polymerization (FRP). Are also described the procedures used to isolate, clean and dry the MIP and NIP particles. To perform the syntheses presented in this section was used chemical compounds described in Table 3.1 to 3.3.

4.1.1 Free Radical Polymerization

Free radical polymerization (FRP) is performed under mild reaction conditions such us ambient temperatures and atmospheric pressures, in bulk or in solution, and is very tolerant to functional groups in the monomers and impurities in the system [Cormack et. al, 2004].

Free radical polymerization is consisting of from three main steps such as:

1. Initiation of the active monomer (steps of initiation such as dissociation and association steps)

$$I \xrightarrow{k_d} 2R$$

$$R + M \xrightarrow{k_i} P_1$$

$$R_i = 2\, f\, k_d\, [I]$$

2. Propagation or growth of the active chain by sequential addition of monomers (a process of monomer unit addition to the initiated monomer species.)

$$P_n + M \xrightarrow{k_P} P_{n+1}$$

$$R_p = k_p [P][M]$$

3. Termination of the active chain to give the final polymer product (three mechanisms of termination such as combination, disproportionation and chain transfer. This process perform when two propagating radical chains of arbitrary degrees of polymerization of x and y meet their free radical ends). [Juwono et. al, 2008].

$$P_n + P_m \xrightarrow{k_t} polymer$$

$$R_t = k_t [P]^2$$

4.1.2 Synthesis of MIPs using Free Radical Polymerization

Free radical polymerization (FRP) is using for synthesis of MIPs and NIPs and it is the most important synthetic method for the conversion of monomer into polymer. To synthesize the MIPs was added a template (Polydatin) in a monomer solution. The mixture it was placed in a ultrasounds bath during 30 minutes for promote the interaction with monomer template (hydrogen bonds). After this we was putting the others components (cross-linker, initiator) and the mixture it was purged with argon during 30 minutes. The final solution it was placed in paraffin bath, previously heating at 60ºC, and remained there during 24 hours. This synthesis was applied by batch reactor that is showed in Figure 4.1.

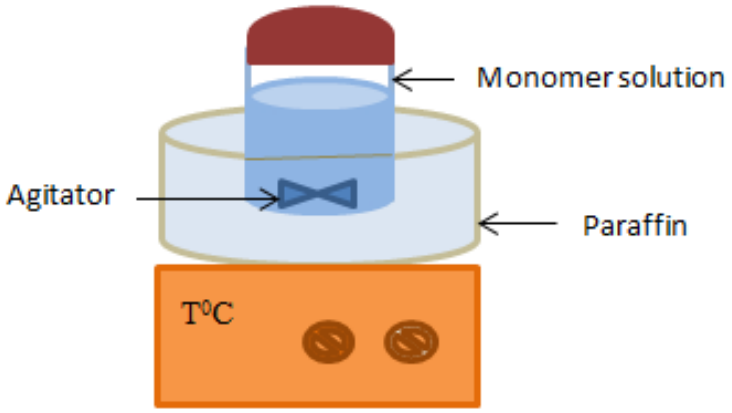


Figure 4.1: Scheme of the polymerization in batch reactor

The equations are presented to describe some of the parameters in Tables 4.1.

- Weight fraction of monomer:

$$Y_{CL}(\%) = \frac{n_{CL}}{n_M + n_{CL}} \times 100 \qquad (1)$$

- Initiator mole fraction:

$$Y_I(\%) = \frac{n_I}{n_M + n_{CL}} \times 100 \qquad (2)$$

- Cross-Linker mole fraction:

$$Y_{CL}(\%) = \frac{n_{CL}}{n_M + n_{CL}} \times 100 \qquad (3)$$

- Cross-Linker/Template mole ratio

$$Y_{CL/T} = \frac{n_{CL}}{n_T} \qquad (4)$$

- Mole ratio of functional monomer and template species:

$$Y_{M/R} = \frac{n_M + n_{CL}}{n_R} \qquad (5)$$

- RAFT agent mole ratio

$$Y_{M/RAFT} = \frac{n_M}{n_{RAFT}} \qquad (6)$$

- Functional monomer/Template mole ratio

$$Y_{M/T} = \frac{n_M}{n_T} \qquad (7)$$

In Figure 4.2, we can see the final product obtained in this type of polymerization (FRP)

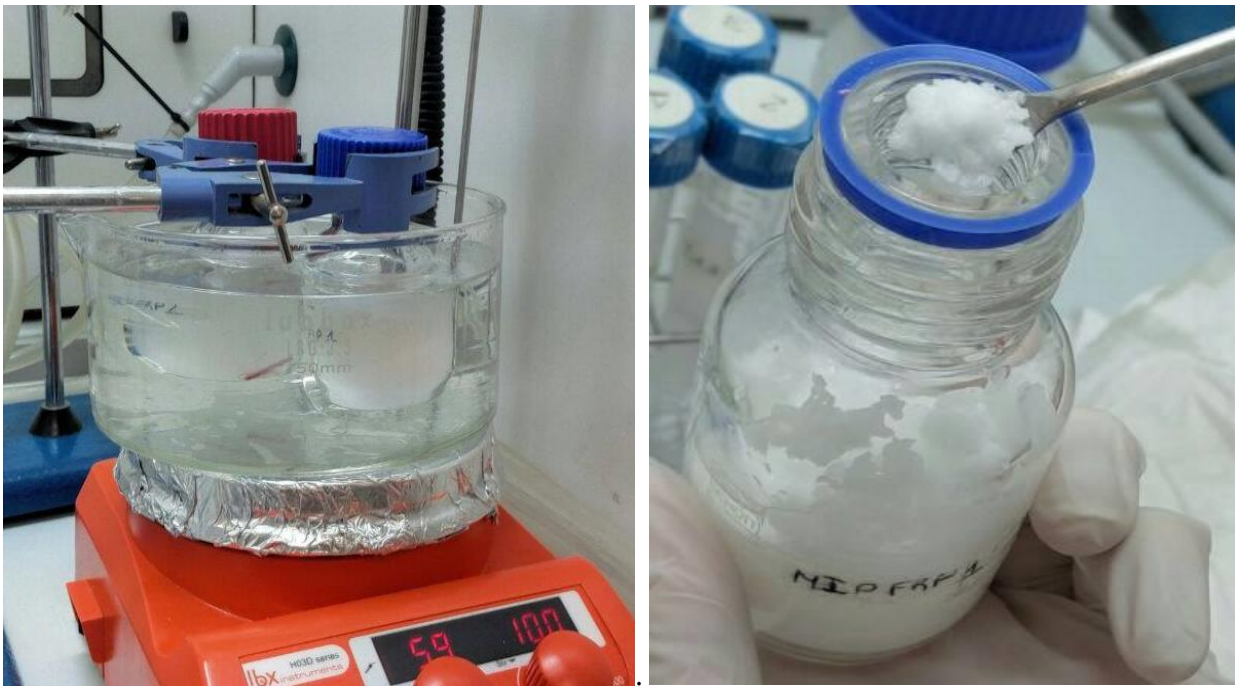


Figure 4.2: MIP and NIP Free Radical Polymerization (FRP) performed

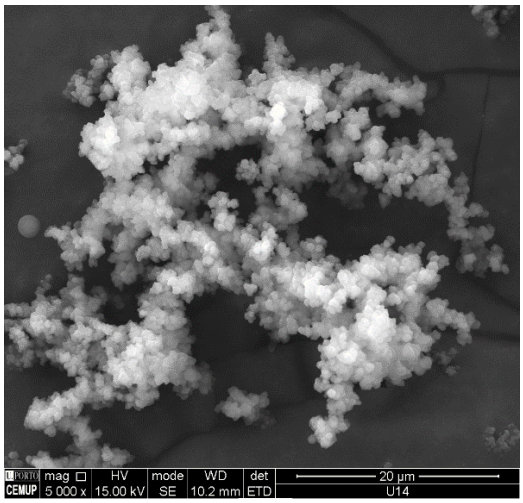
4.1.3 Template removal

After synthesis, for a good characterization of particles it is necessary to remove the template. With these goal, the particles were purified using a large excess of a MeOH/Acetic Acid (9/1 v/v), followed by centrifugation. This process was repeated until levels of template became too low to detect. This step it was monitored by UV spectrophotometer. Finally, the particles were washed in large excess of MeOH during 24 hours. After removal the MeOH, the particles were dried in a vacuum oven at 40 ºC.

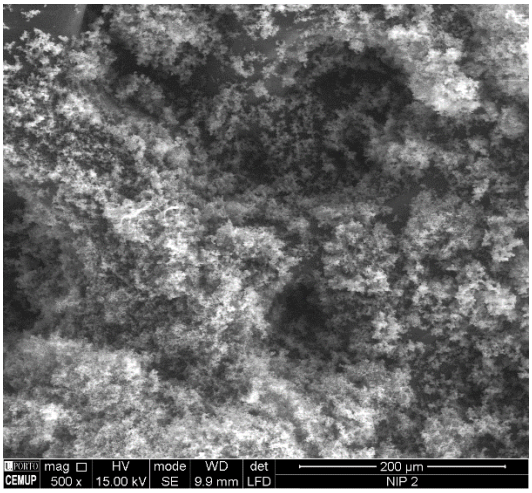
4.2 Analysis by Scanning Electron Microscopy (SEM)

The surface morphology and the particle size of both MIP and NIP were analyzed using scanning electron microscopy (SEM). The analysis by scanning electron microscopy was in microscopy center of University of Porto (CEMUP). Small particles were obtained, when synthetized by batch. The sizes of micro particles are about 1 μm.

The effect of the different polymerizations performed (e.g. different kinds monomers involved) can change the morphology of the particles**.** SEM analysis of the micro-particles (imprinted and non-imprinted) obtained in Free Radical polymerization are presented in Figure 4.3. Other SEM results are presented in Annex 1-3.



a



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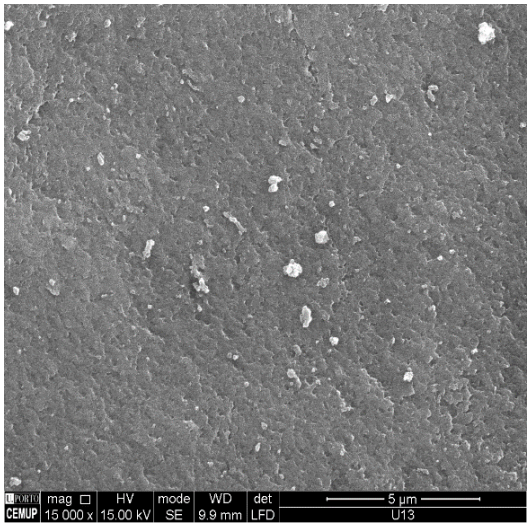
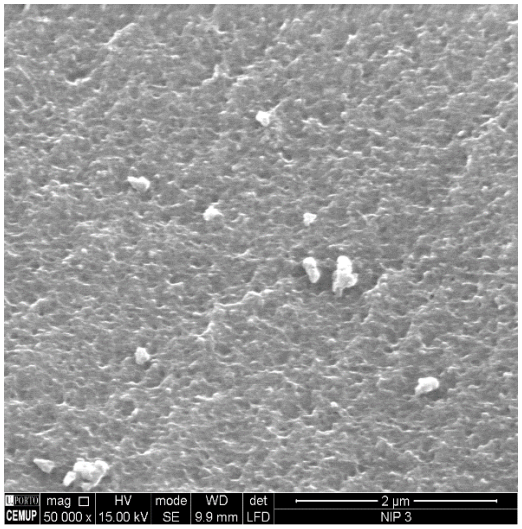


c



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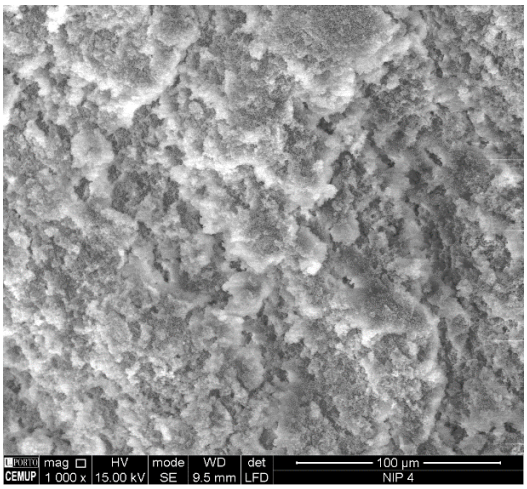

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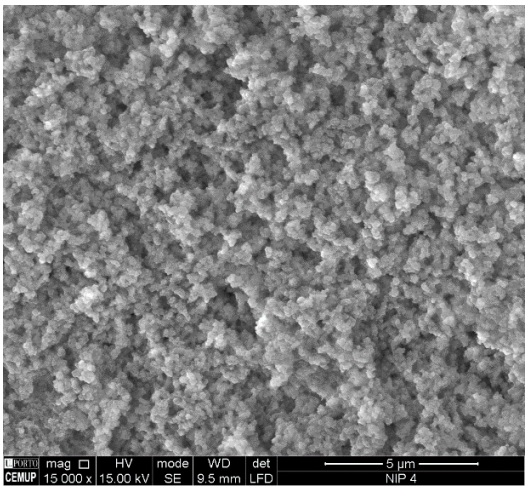


f

Figure 4.3: SEM micrographs of MIPs and NIPs produced a) MIP1, b) NIP1, c) MIP2 d) NIP2 and e), f) NIP3 Synthesized by Free Radical Polymerization

Table 4.1: Experimental condition used in batch reactor synthesis of NIP and MIP in ACN-MeOH 10/1

Material	Monomer (M)	Cross-linker (CL)	Template (T)	Initiator (I)	Solvent (S)	Y_M (%)	Y_I (%)	Y_{CL} (%)	$Y_{CL/T}$	$Y_{M/R}$	$Y_{M/I}$	$Y_{M/T}$
MIP1	AA	TMTPA	POL	AIBN	ACN/MeOH	4.55	3.8	65.62	15.71	-	0	8.23
NIP1	AA	TMTPA		AIBN	ACN/MeOH	4.55	3.81	65.62	-	-	0	-
MIP2	DMAEMA	TMTPA	POL	AIBN	ACN/MeOH	4.91	3.8	65.29	15.08	-	0	8.02
NIP2	DMAEMA	TMTPA		AIBN	ACN/MeOH	4.91	3.79	65.29	-	-	0	-
MIP3	NVP	TMTPA	POL	AIBN	ACN/MeOH	4.06	3.81	65.03	15.06	-	0	8.1
NIP3	NVP	TMTPA		AIBN	ACN/MeOH	4.06	3.78	65.03	-	-	0	-

4.3 Fourier Transform Infrared Spectrometer (FTIR)

The characterization of the molecular architecture of the networks and/or the linear counterpart's synthetized for molecular imprinting was performed FTIR spectroscopy.

Fourier Transform Infrared Spectroscopy (FTIR) is used namely in polymer science, organic synthesis, pharmaceutical industry, food analysis etc.

FTIR spectrometers have several prominent advantages such us:

1. The signal to-noise of spectrum is higher;
2. The accuracy of wavenumbers is high;
3. The scan time of all frequencies is short etc.

To analyze the polymer by FTIR, KBr granule polymer was incorporated. For this, was used about 2 mg of polymer and 200 mg of KBr. The polymer was mixed with a KBr and the mixture was crushed and ground to achieve a particle size of less than 0.01mm in an agate mortar and pestle. With these mixtures were prepared with 13 mm pellets, using the evacuable mold, hydraulic pump and a hydraulic press applying a pressure between 8 and 10 tons. Final appearance of the pellets produced is observable in Figure 4.3

Figure 4.3: Final Aspect of the pellets for analysis Infrared Spectroscopy with Fourier Transform (FTIR).

These pellets were submitted to the FTIR spectrometer, using a 13 mm disc holder. For the analysis of liquids (crosslinker and monomer) was the OMNI-CELL cell of Specac indicated with windows of crystals of NaCl, between which the liquid was placed. The analysis was performed in a range of frequencies between 1000 and 2000 cm^{-1}. Figures 4.4 and 4.5 are showing the spectrum of the functional monomer, the crosslinker, used in the synthesis of the respective polymer, and the spectrum of final product. In these spectra it is possible to see through the characteristics of the band that, there was the formation of the polymer for MIP1 and MIP2. FTIR spectra for the others polymers synthetized are presented in annex 6.

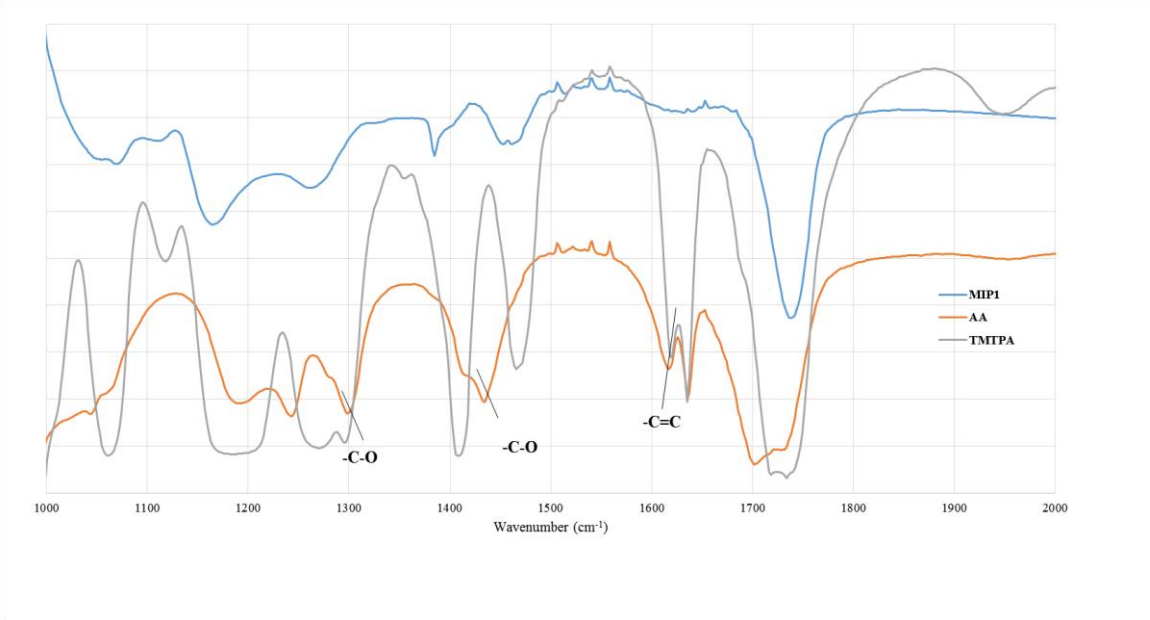


Figure 4.4: FTIR spectra of the MIP1, the functional monomer (AA) and the crosslinker (TMTPA) used in the synthesis of the MIP1.

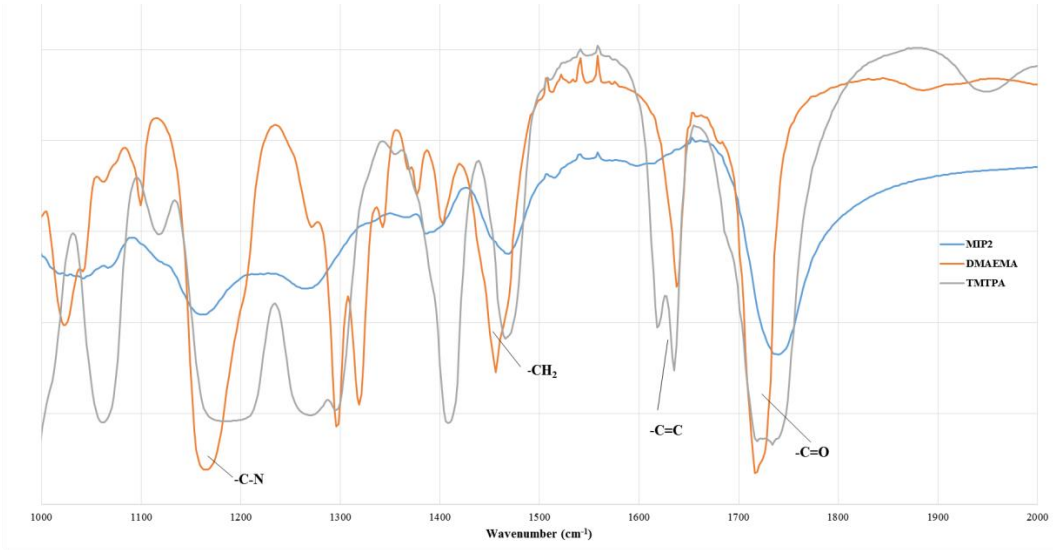


Figure 4.5: FTIR spectra of the MIP2, the functional monomer (DMAEMA) and the crosslinker (TMTPA) used in the synthesis of the MIP2

Chapter 5 Characterization of the Adsorption Capabilities for the Synthetized Molecular Imprinted Polymers

In this chapter is presented characterization of imprinted and non-imprinted polymers using solid phase extraction (SPE). It is described the experimental procedure used to perform the SPE evaluation of the different particles concerning the uptake and release of phenolic compounds, showed the experimental results obtained with the different materials in the three steps (loading, washing and elution).

Used equipments for characterization performed are presented in Table3.2.

5.1 Solid Phase Extraction

Solid phase extraction is a very popular method and is an extraction method for rapid and selective sample preparation. This method use the same type of stationary phases us are used in liquid chromatography columns. SPE method is use in many purposes, such as purification, trace enrichment, desalting, derivatization and class fractionation. It is depends from the versatility of the SPE.

SPE technique is based on the selective portioning of components between a liquid (sample matrix and or solvent with analytes) and a solid (sorbent) phase [Ferenc et. al, 2006].

Fundamental steps for SPE Adsorption

A typical solid phase extraction involves four steps (see Figure 5.1):

1. **Conditioning**

In this step we must prepare the sorbent for effective interaction with the compounds of interest.
The conditioning step it is important to create equilibrium between the aqueous sorbent and the solution.

2. **Loading**

In loading step analytes are retained on the sorbent. Solvent passes through the column using a vacuum pump and flow rate was very slowly (typical 1ml/minute). The analyte and some impurities are retained on the sorbent.

3. **Washing**

The washing step is performed after loading. In this process we used the solvent B to remove impurities from the loading step. Remove bound was less strongly than the compounds of interest.

4. Eluting

Elution step is a finally where we elute the analytes using a suitable extracting solvent C. This solvent needs to be appropriated to break the analyte-sorbent interaction and should be compatible with final analysis. Steps of Solid phase extraction are presented in figure 5.1

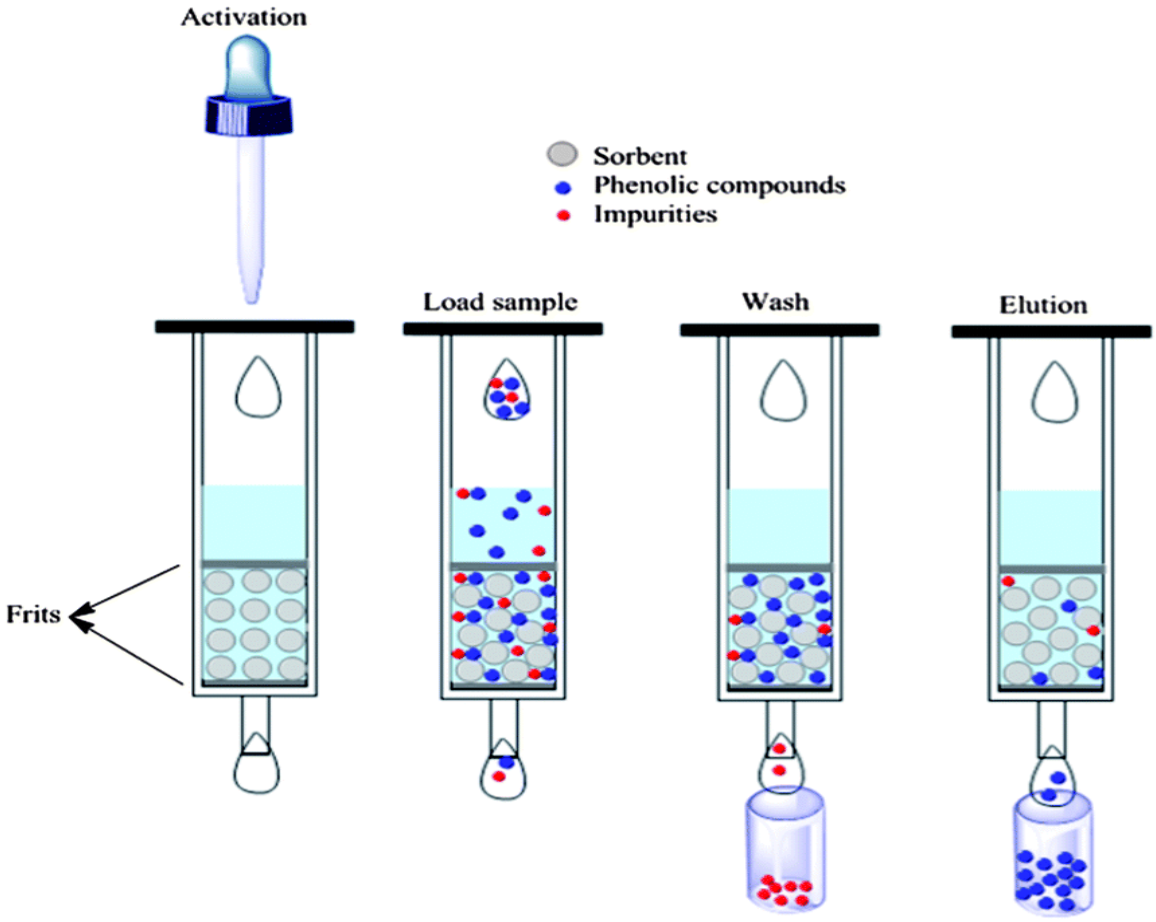


Figure 5.1: Steps of Solid Phase Extraction: Conditioning, Loading, Washing, Eluting http://pubs.rsc.org/en/content/articlelanding/2014/ay/c3ay41771a#!divAbstract.

The solid phase extraction has much more advantages than liquid-liquid phase extraction such us:

- high selectivity;
- reduced solvent usage;
- cleaner extract
- better reproducibility
- higher throughput due to automation etc. [http://theses.gla.ac.uk/].

5.1.2 Experimental procedure

In the solid phase extraction tests where used four steps that presented in Figure 5.1. Solvents and materials that we used are presented in Table 5.1 The experimental procedure was used to perform the SPE evaluation of the different particles concerning the uptake, release and separation of phenolic compounds. Within this purpose, was prepared 6 cartridges that contained the particles, MIPs and NIPs, about 50 mg. These particles were conditioned with a same solvent that we was used in adsorption of the phenolic compounds, during 24 hours, and then the solvent was removed with the vacuum pump connected in SPE system (see Figure 5.2).

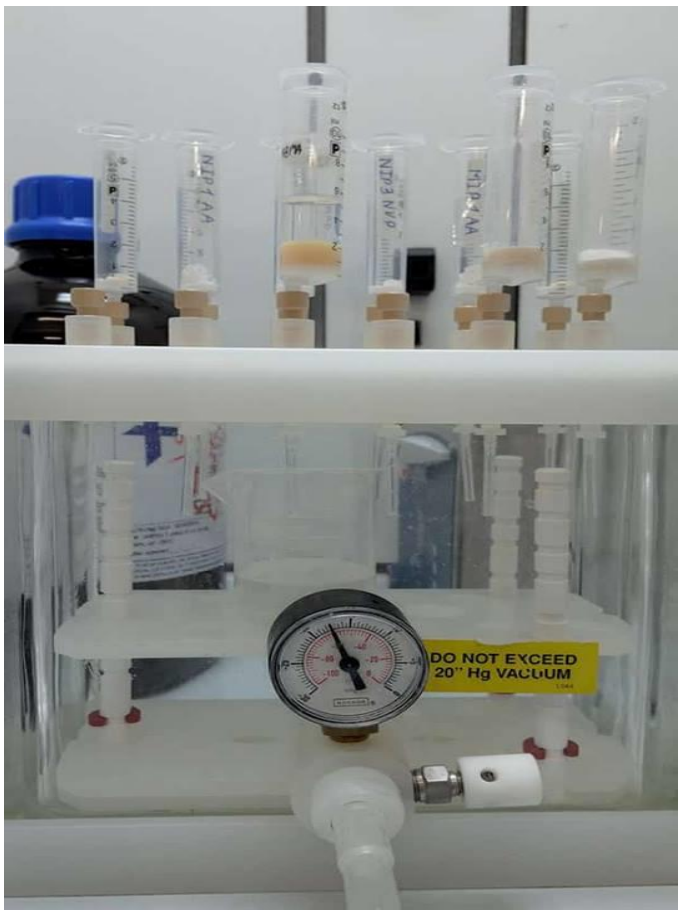


Figure 5.2: Solid phase extraction (SPE) system.

Thereafter, was prepared a solution containing phenolic compounds, following the loading step. In this step, 5ml of the solution at concentration of 0.06 mM were passed through the particles. The solution was collected in flask drop-by-drop with vacuum pump, for measurement in the UV spectrophotometer. Through this equipment we can measure the absorbance of each solution collected for calculate the fraction of phenolic

compounds which was retained in the particles. The fraction of phenolic compounds retained was obtained through the following equation:

$$\%\, retention = \frac{A_0 - A_1}{A_0} \quad (8)$$

Where:

A_0 – The UV-absorbance of mother solution

A_1 –The UV-absorbance after the loading process

The third step was washing, where we added 5 ml of solvent in the cartridge, the same solvent that we used in the loading step, then the solution it was collected and analyzed in the UV spectrophotometer for verified if there was phenolic compounds release. The last step was elution where we added 5 ml of a strong eluente (MeOH) with a objective to release the phenolic compounds that yet present in the particles. The solution colected it was analised in UV spectrophotometer (See in Figure 5.3). Afterward the particles were cleaning with MeOH/Acetic acid for recovery.

Table 5.1: Amount of materials and Solvents

Materials	**Weight (mg)**	**Solvent (ml)**	**V (ml)**	**Solvent (ml)**	**V (ml)**	**Solvent (ml)**	**V (ml)**
MIP1	50.5		5		5		5
NIP1	50.3		5		5		5
MIP2	50.5	ACN/MeOH	5	ACN/H2O	5	ACN/MeOH	5
NIP2	50.2	10/1	5	50/50	5	10/1	5
MIP3	50.6		5		5		5
NIP3	50.2		5		5		5

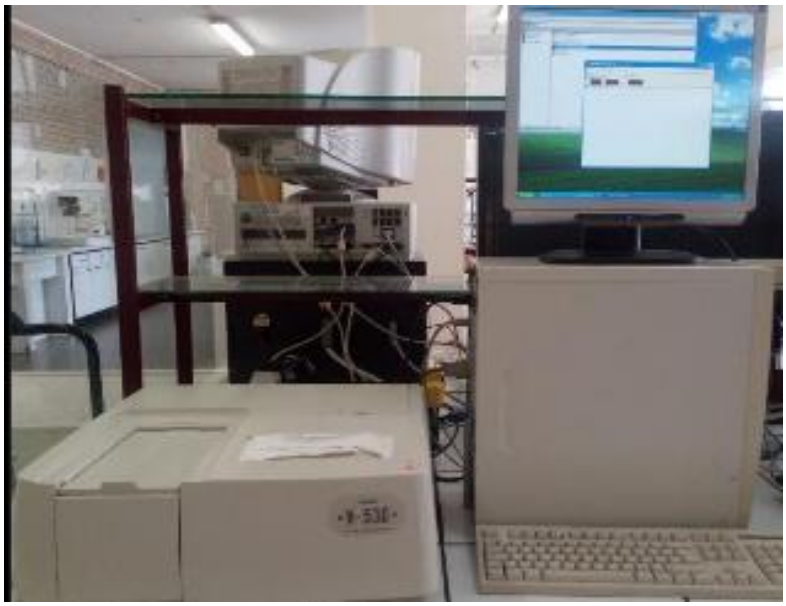


Figure 5.3: UV spectophotometer

5.2 Result and discussion

5.2.1 SPE adsorption of polydatin in the synthetized MIP and NIP materials

In order to evaluate the performance of the different particles synthetized (imprinted and non-imprinted) on molecular recognition, the SPE adsorption of polydatin (molecule used in molecular imprinting) was studied considering three different steps: loading, washing and elution. In the loading step, the solvent used was ACN/MeOH 10/1. This solvent was selected in order to minimize the hydrophobic interactions and then highlight the eventual molecular imprinting effect achieved. Note that the small amount of MeOH should be included in the mixture in order to allow the dissolution of the phenolic compounds. The washing step was also performed with ACN/MeOH 10/1 and pure MeOH was considered in the elution step: This strong solvent should in principle destroy the eventual hydrogen bonding between the template molecule and the functional monomer present in the imprinting cavities.

In Figure 5.4 are presented the results obtained in these measurements (three replicas were performed). These observations seems to indicate a very small (or even none) molecular imprinting effect on the MIPs produced. In fact, adsorption in NIPs is often higher that in MIPs which is compatible with a non-specific interaction between the template and the particles. A slight specific retention seems to occur only in MIP3. Results for the washing and elution steps are also consistent with this hypothesis because it is estimated the total release of the target molecule after these steps. A weak interaction between the functional monomers selected and polydatin during the polymer network formation should be at the source of this molecular imprinting

efficiency. Note that polydatin is a relatively high size molecule, which hinders its assembly in the molecular imprinting cavities.

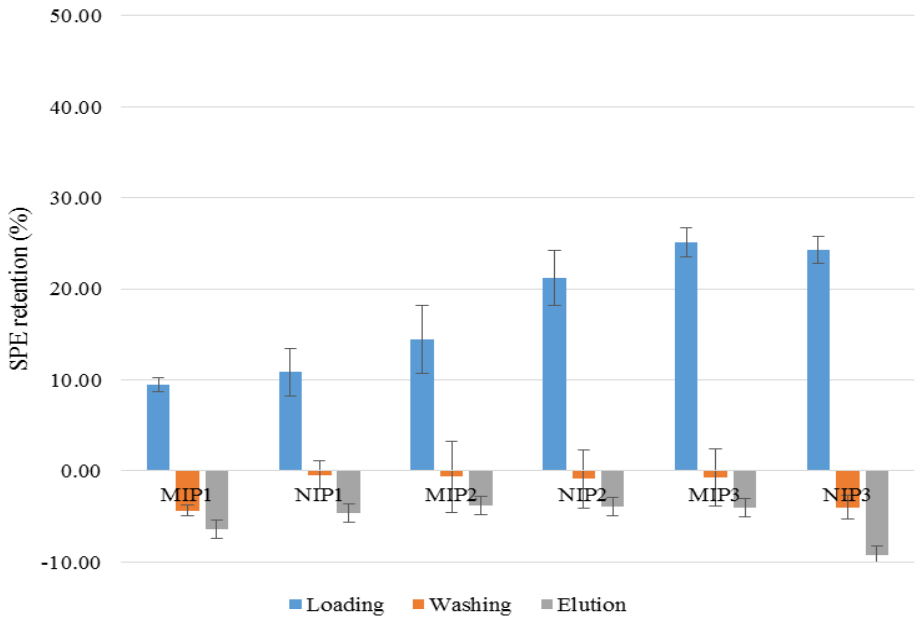


Figure 5.4: SPE adsorption of polydatin in the synthetized MIP and NIP materials. ACN/MeOH 10/1 was used as first solvent.

In order to confirm the existence of non-specific interactions in the different materials, SPE testing was performed in different conditions, namely considering a mixture of gallic acid, polydatin and resveratrol in ACN/H2O 50/50.

Washing was also performed with ACN/H2O 50/50 and elution with MeOH. Results just obtained are presented in Figure 5.5. These results are also consistent with the existence of non-specific interactions between the different molecules and the materials (it is not observed a clear difference between MIP and NIP particles inside each class). Note that the increase of global adsorption should be a consequence of presence of water in the solvent, causing hydrophobic interactions.

The unexpected negative values for the retained amounts after elution should be a consequence of the change of solvent relatively to the first step, which is used as reference, therefore affecting the UV measurements. The release of impurities present in the materials when MeOH was used is another possible cause for these observations.

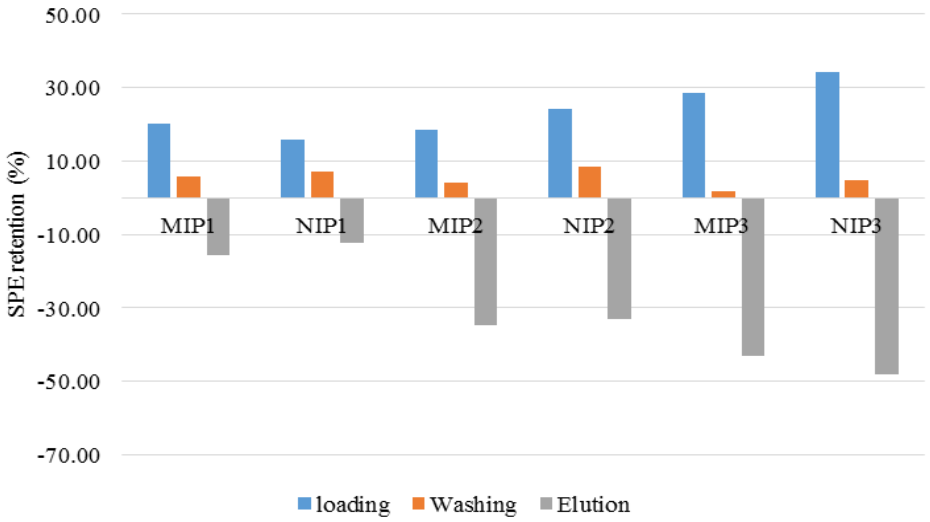


Figure 5.5: SPE adsorption of a mixture containing galic acid + polydatin + resveratrol on the synthetized MIP and NIP materials. ACN/H2O 50/50 was used as first solvent.

In Figure 5.6 are showed results for a similar experience with the mixture galic acid + polydatin + resveratrol but using ACN/MeOH 10/1 as solvent. Note that, in spite of the non-specific retention observed, interesting outcomes are here evidenced, namely in comparison with results of Figure 5.4. Indeed, washing and elution steps seem to show a stronger interaction of one or two of the other molecules (gallic acid and/or resveratrol) with the materials when the testing is performed under the present conditions. Due to these results, the competitive adsorption of the different molecules it was studied in deeper detail, namely by performing the HPLC analysis of the solutions after carrying out the SPE testing, as presented in the next sub-section.

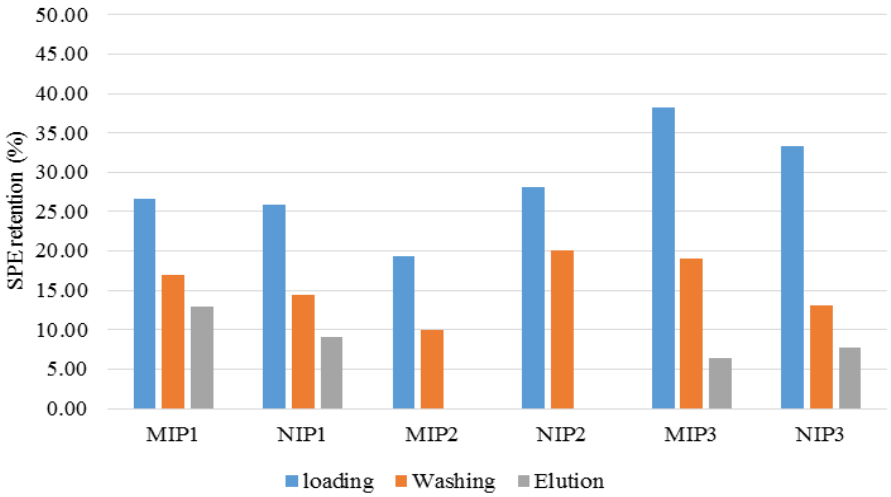


Figure 5.6: SPE adsorption of a mixture containing galic acid + polydatin + resveratrol on the synthetized MIP and NIP materials. ACN/MeOH 10/1 was used as first solvent.

5.3 Competitive SPE adsorption of polydatin+galic acid+resveratrol with HPLC analysis

Important limitations are found when detailed information on the adsorption/release processes are sought, especially when mixtures of molecules are considered, as briefly commented before. Thus, in order to get information concerning the competitive retention and release of polydatin, gallic acid and resveratrol in the different classes of MIP/NIP particles synthetized (anionic, cationic, non-ionic), the solutions resulting from each SPE step were analyzed using HPLC. All the SPE competitive experiments were performed with concentration C=0.02 mM for each phenolic compound. Higher precision measurements are also expected with this technique (e.g. decreasing the high errors observed in the elution step).

The required HPLC analysis were performed using an Ascentis® C18 column, 5 µ particle size and with dimensions: L × I.D. = 25 cm × 4.6 mm. This column was assembled in the Viscotek GPC max pumping system + the module Viscotek TDA 305. In this apparatus, the temperature of the column can be controlled and the UV signal is in-line available.

In Figure 5.7 is presented the HPLC analysis of the competivive SPE adsorption and release of the mixture polydatin+galic acid+resveratrol in MIP1. In this SPE testing, ACN/MeOH 10/1 was use as first solvent. The competitive adsorption of the three different molecules can be clearly observed in the loading step (evaluation of the peak areas allows the quantification of the relative amounts retained, as below explored).

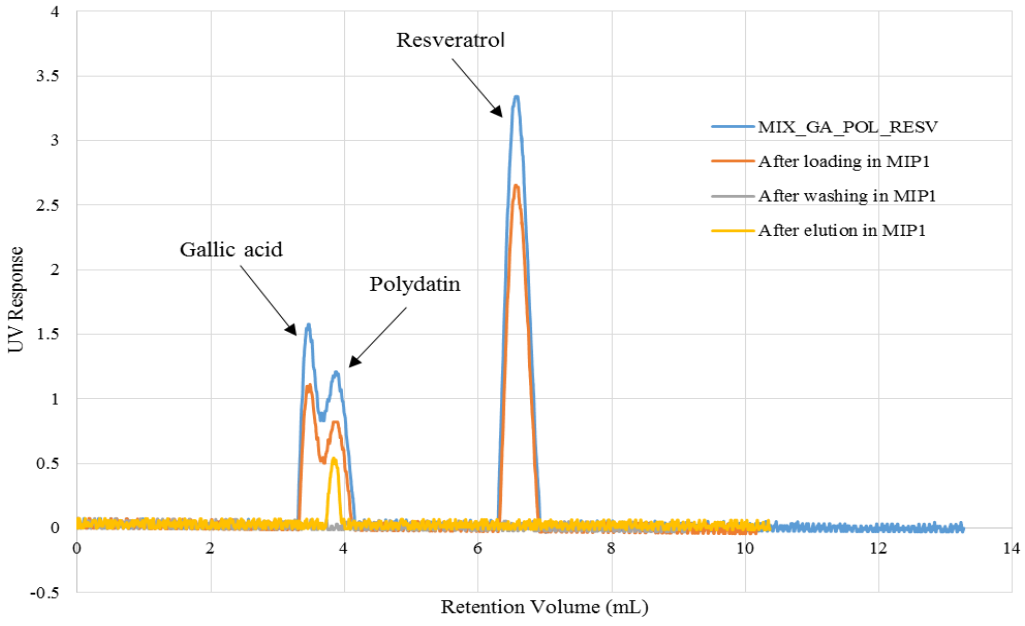


Figure 5.7: HPLC analysis of the competivive SPE adsorption and release of the mixture polydatin+galic acid+resveratrol in MIP1. ACN/MeOH 10/1 was use as first solvent.

A very interesting result is presented in figure 5.8. Indeed, with MIP2 the total retention of gallic acid was observed in the SPE competitive process. This should be a consequence of the ionic interaction between gallic acid (anionic specie) and the cationic polymer network containing DMAEMA.

The ability of this kind of materials to retain anionic species can be explored to simplify natural vegetable extracts where species like gallic acid or tannic acid are usually present in high amount, namely when compared with others valuable phenolic compounds. This issue will be enhanced in this work in upcoming sections.

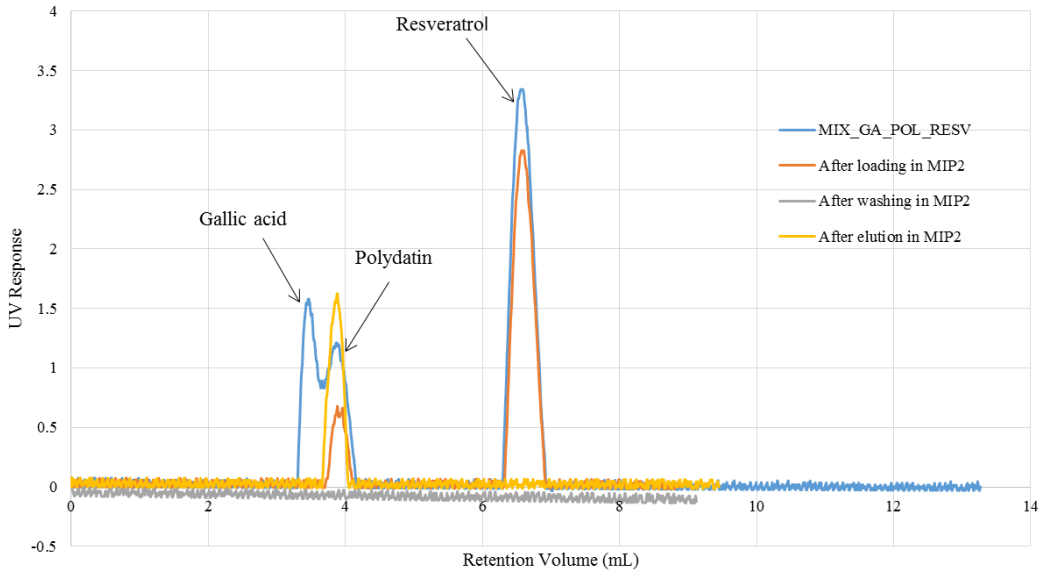


Figure 5.8: HPLC analysis of the competitive SPE adsorption and release of the mixture polydatin+galic acid+resveratrol in MIP2. ACN/MeOH 10/1 was use as first solvent.

Note that the high ability of MIP2 to retain gallic acid, as consequence of the ionic interactions above discussed, it is not a specific result of the molecular imprinting process. This issue is confirmed through the same testing with NIP2, as presented in Figure 5.9. Results for similar testing with other materials are presented in Annex 4.

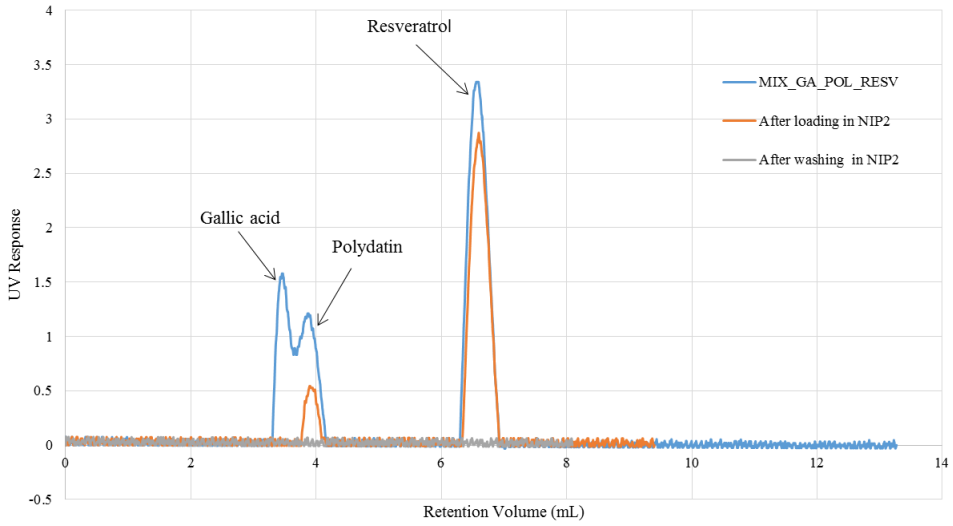


Figure 5.9: HPLC analysis of the competitive SPE adsorption and release of the mixture polydatin+galic acid+resveratrol in NIP2. ACN/MeOH 10/1 was use as first solvent.

The evaluation of the performance of the different materials for the competitive adsorption of phenolic compounds was also performed using ACN/H_2O 50/50 as first solvent. The results obtained are presented in Figures 5.10, 5.11, 5.12 and in annex 5. As main outcome, is again evidenced the ability of MIP2/NIP2 to adsorb a huge amount of gallic acid, namely in comparison with polydatin and resveratrol (see Figures 5.11 and 5.12).

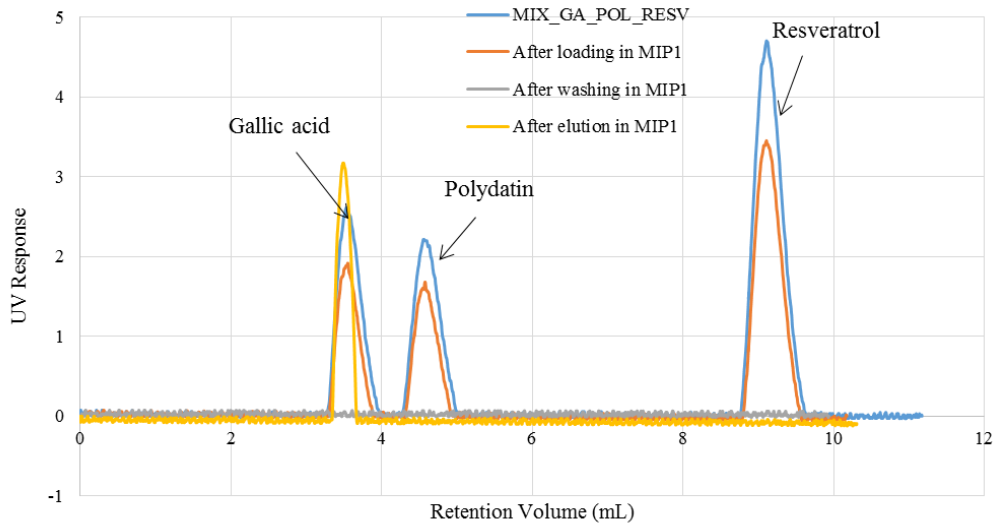


Figure 5.10: HPLC analysis of the competitive SPE adsorption and release of the mixture polydatin+galic acid+resveratrol in MIP1. ACN/H2O 50/50 was use as first solvent.

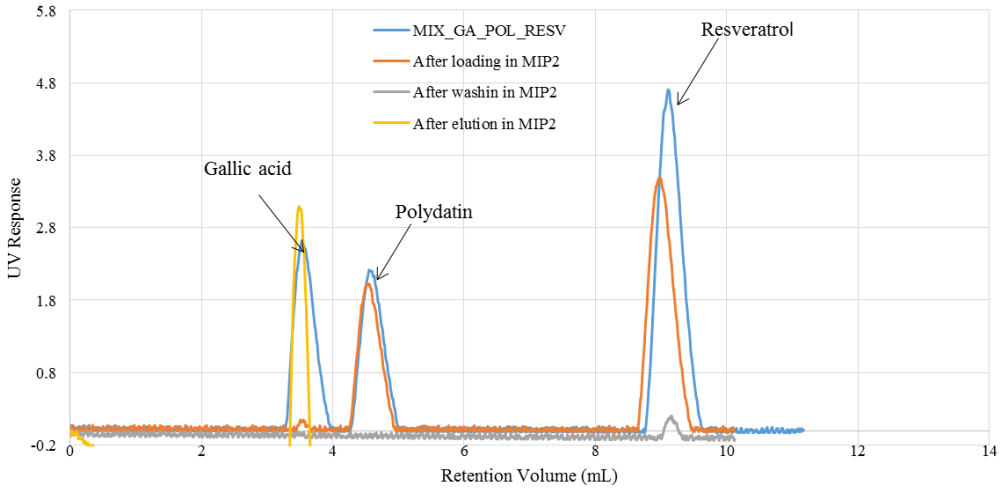


Figure 5.11: HPLC analysis of the competitive SPE adsorption and release of the mixture polydatin+galic acid+resveratrol in MIP2. ACN/H2O 50/50 was use as first solvent.

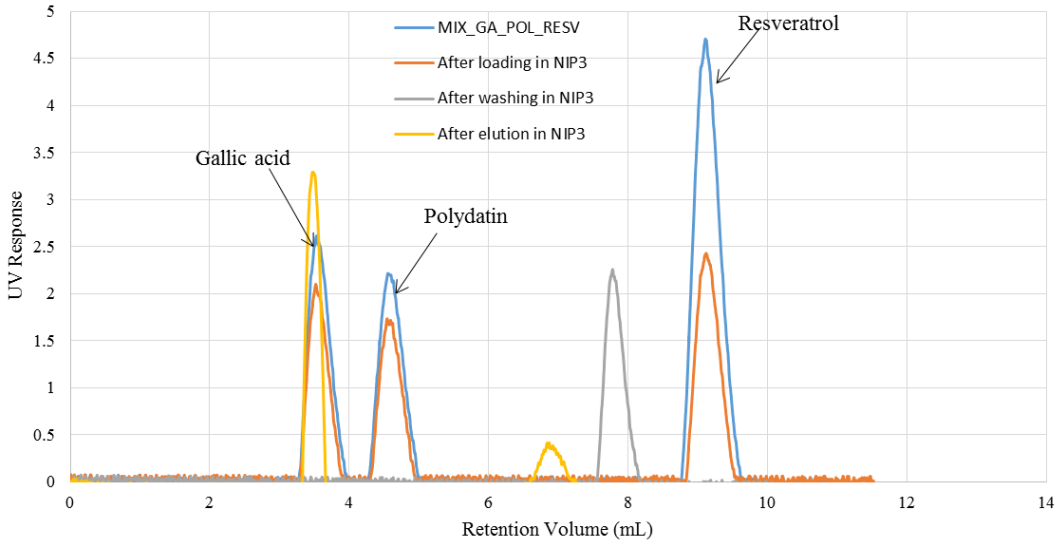


Figure 5.12: HPLC analysis of the competitive SPE adsorption and release of the mixture polydatin+galic acid+resveratrol in NIP2. ACN/H2O 50/50 was use as first solvent.

An important issue in the competitive adsorption testing performed is the optimization of the HPLC analysis conditions in order to allow the individual identification of the different phenolic compounds. As above mentioned, these analyses were performed Ascentis® C18 column and after an optimization procedure, the eluent ACN/H_2O/Acetic acid 30/70 at pH=3 was found to provide a reasonable separation of gallic acid, polydation and resveratrol. Note that a fast and simples analysis was sought (around 10 mL of elution volume are usually enough) and the lengthy HPLC gradient scheme was not here considered. It was also found that the temperature of T=45 °C for the column and the flow rate of Q=1 mL/min (pump pressure around 7.8 MPa)

allow to fulfil the conditions desired for the HPLC analysis. An additional important issue to optimize the HPLC analysis is the selection of the injected volume. In fact, in order to avoid the dilution of samples collected from the SPE in the different steps (loading, washing and elution), a low injection volume should be considered. In this way, the interference of the solvent coming from the SPE with the eluent of the HPLC analysis (ACN/H_2O/Acetic acid pH=3) is minimized. Indeed, in this condition, if a too high injection volume is used, the peak position for each phenolic compound is changed as a consequence of the SPE solvent introduced in the system. Optimized injection volume was found to be in the range 5 to 20 µL.

In Figure 5.13 is presented a representative example of this issue for the injection of polydatin in the HPLC system running with ACN/H_2O/Acetic acid 30/70 at pH=3 and the sample directly prepared in ACN/MeOH 10/1.

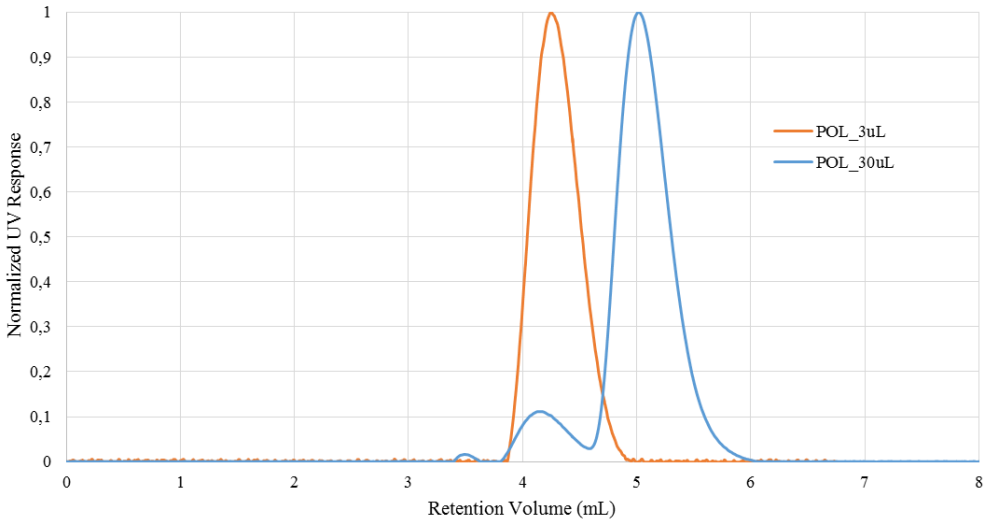


Figure 5.13: Effect of the injection volume in the peak position observed in the HPLC analysis. ACN/MeOH 10/1 was use as solvent in the preparation of the sample and ACN/H2O/Acetic acid 30/70 at pH=3 as HPLC eluent.

In Figures 5.14 and 5.15 are presented the global results for the competitive adsorption testing performed. These bars indicate the percentage of retention for each compound in the different materials and were calculated using the peak areas correspondent to the HPLC analysis. These results evidence again the high retention capability of MIP2/NIP2 for gallic acid retention with the two different solvents used in SPE. Additionally, increase in resveratrol retention is observed for all materials when the SPE solvent ACN/MeOH 10/1 is replaced by ACN/H2O 50/50 (see Figures 5.14 and 5.15). These results highlight the important effect of the hydrophobic interactions in resveratrol retention. Globally speaking, a low specificity for polydatin retention is observed when MIP and NIP materials are compared. The effect of the hydrophobic interactions are not so pronounced, namely in comparison with resveratrol. This outcome should be a consequence of the hybrid character of the polydatin molecule that contains hydrophilic and also hydrophobic domains.

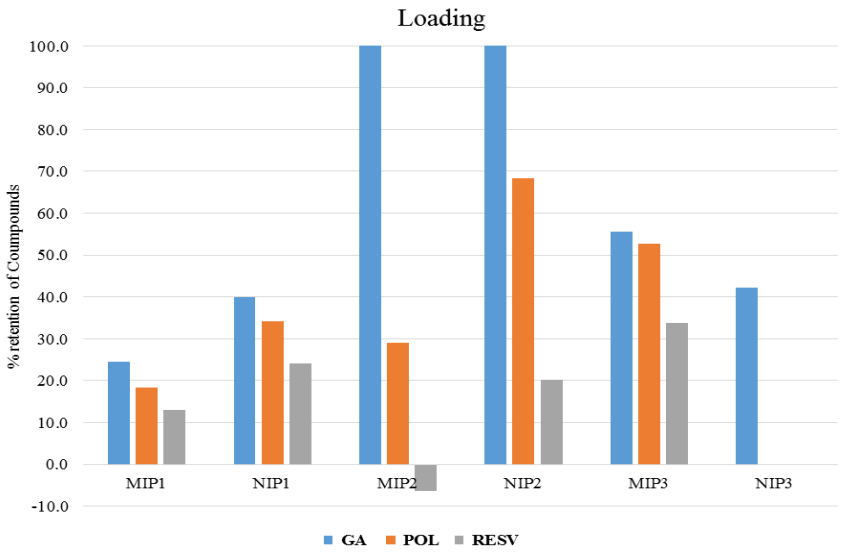


Figure 5.14: Global results for the SPE competitive adsorption of gallic acid, polydatin and resveratrol in different materials. Retention of each phenolic compounds was calculated using the peak areas correspondent to the HPLC analysis. The results with ACN/MeOH 10/1 in SPE are here provided.

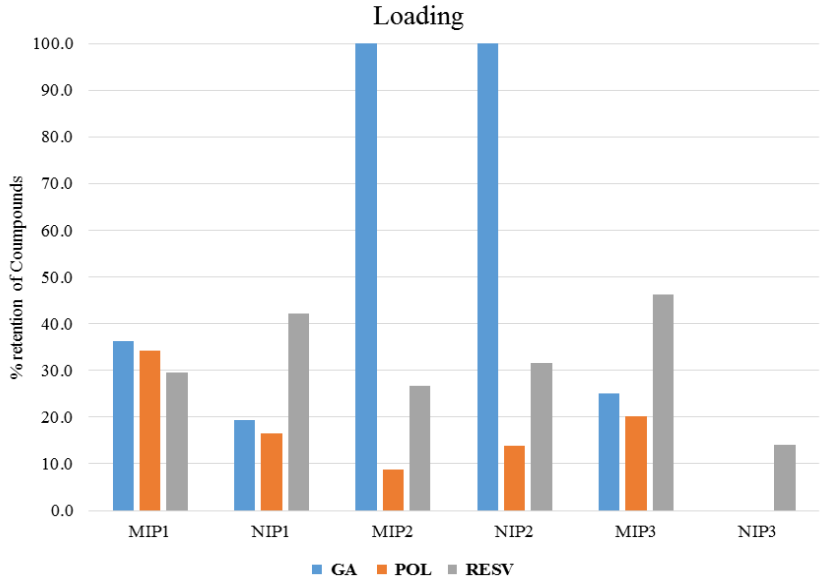


Figure5.15: Global results for the SPE competitive adsorption of gallic acid, polydatin and resveratrol in different materials. Retention of each phenolic compounds was calculated using the peak areas correspondent to the HPLC analysis. The results with ACN/H2O 50/50 in SPE are here provided.

5.4 Adsorption of Phenolic Compounds in a Continuous Process with Recycling

5.4.1 Theoretical Foundations of Continuous Processes and Frontal Analysis

Continuous adsorption processes are extremely important in for industrial and commercial purposes due to the increase of productivity. Moreover, using continuous techniques, such as frontal analysis, high precision sorption measurements can also be obtained. This method consists in replacing the current of the mobile phase to a solution containing the studied component with a known concentration. The "breakthrough" curve (elution curve) of the solute is registered on the outlet of the column (e.g. using a UV detector). A material balance of the solute (mass conservation) between the moment when the solution begins to flow through the column, and the instant it reaches saturation (concentration step) allows to calculate the amount adsorbed on the stationary phase (q*) that is in equilibrium with the mobile phase [Dias et. al, 2006].

In this section will be explored the use of MIP particles as sorbents for continuous processes that can be conceived for the retention and release of phenolic compounds. This analysis includes the packing of MIP particles in chromatographic columns and the assessment for the possibility of the stable running of a continuous adsorption/desorption process. In this context, a continuous process with recycling was designed. This operation mode was considered in order to simulate a possible treatment of a natural extract that demands for the total exhaustion of the phenolic compounds present in the solution.

5.4.2 Experimental Procedure

With an objective to evaluate the adsorption and release of different phenolic compounds in the synthesized materials, the experimental study of these processes in packed columns operating in continuous way was accomplished. For that, the column, shown in figure 5.16, it was packing with a predefined dry mass of MIP2, around 300 mg, using a vacuum pump in order to obtain a good compaction of the particle. The dimensions of the column are detailed in table 5.2

Figure 5.16: Packing columns used in experimental studies of the saturation and release polyphenols.

Column	
Internal length	Internal diameter
33.00 mm	4.60 mm
	

Table 5.2: Dimensions of columns used in experimental studies.

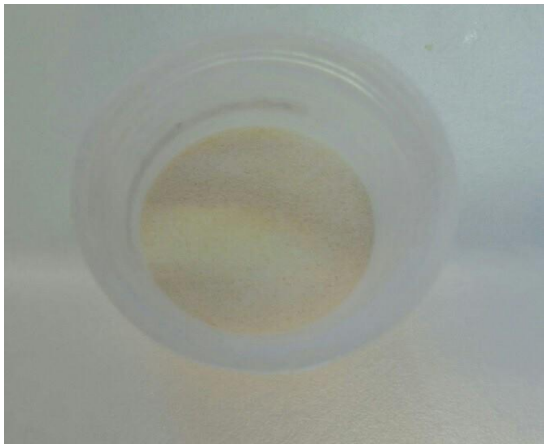


Figure 5.17: Amount of MIP2.

5.4.3 Saturation procedure

The continuous saturation process it was performed using a column that contained the material (MIP2), a HPLC pumps (KNAUER P4.1S) a reservoir with the solution containing the phenolic compound. In Figure 6.13 we can see this continuous process. The solution as prepared with H_2O/MeOH 50/50 at initial concentration=0.05 Mm. The column was plugged to the HPLC pump and the saturation procedure was started at flow-rate of 1 mL/min.

The UV Spectroscopy at 273 and 306 nm measures the retention of the drug in the MIP2 present in the column. Note that before starting the saturation process the column was conditioning with a H_2O/MeOH 50/50 (same solvent used in saturation process). Saturation process was performed until the concentration at the column exit becomes constant. After saturation process, we started release process.

5.4.4 Release of compounds

The release of phenolic compounds was performed in the reverse way: the pumping system was turned off, then we put column in oven were temperature was 50°C and flow rate was 0.1ml/min. After that eluent, (H_2O/MeOH (50/50) passed through the column, then started to release compounds from the column and every 10 min made testing. This process was also recorded in UV Spectroscopy and was finished when the concentration at the column exit became constant.

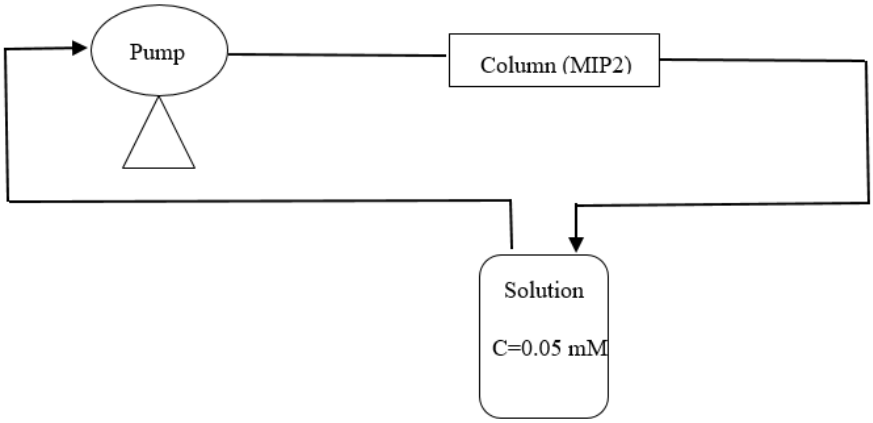


Figure 5.18: Scheme of Continuous process with recycling

5.5 Result and discussion

In Figures 5.19 to 5.21 are presented the results obtained in the framework of the continuous retention with recycling of gallic acid in MIP2. This material was selected due to the high retention of acidic phenolic compounds before discussed.

Dynamics for the change of concentration of gallic acid in the liquid solution and dynamics for the change of the adsorbed amount of this molecule in the particles are presented in Figures 5.19 and 5.20, respectively. A volume V=200 mL of solution at C0=0.05 mM was treated at Q=1 mL/min and at room temperature. Along the time, sampling of the liquid phase followed by UV absorption, allows the determination of the concentration in liquid phase and the adsorbed amount in the particles:

$$C(t) = \frac{UV(t)}{UV0} C0 \qquad (1)$$

$$q(t) = \frac{\big(C0 - C(t)\big)V}{m} \qquad (2)$$

In these conditions, in spite of some scattering of the experimental results, a good description of the retention process of the phenolic compound in the particles was achieved and saturation of the adsorbent seems

to be attained after around 24 hours of operation. Considering that the last measurement corresponds to the equilibrium of the adsorption process, the saturation of the particles is estimated to be at q*=16 µmol/g with concentration in the liquid phase C=0.025 mM (around 50% of solute was adsorbed).

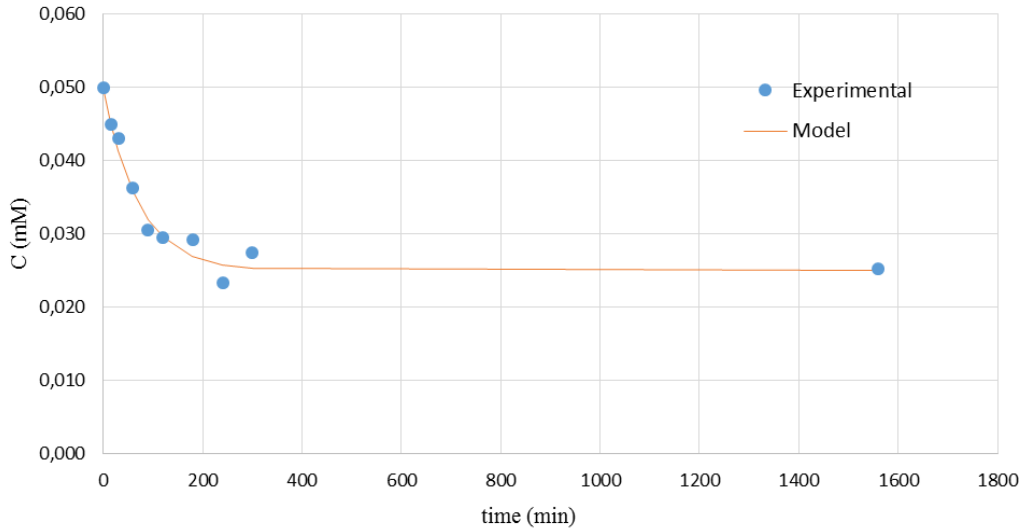


Figure 5.19: Experimental measurements and theoretical adjustement for the data obtained in the continuous retention with recycling of gallic acid. MIP2 was used as adsorbent. A volume V=200 mL of a solution of gallic acid in H2O/MeOH 50/50 at C0=0.05 mM was treated with Q=1 mL/min at room temperature. Change with time of concentration of gallic acid in the liquid solution is here presented.

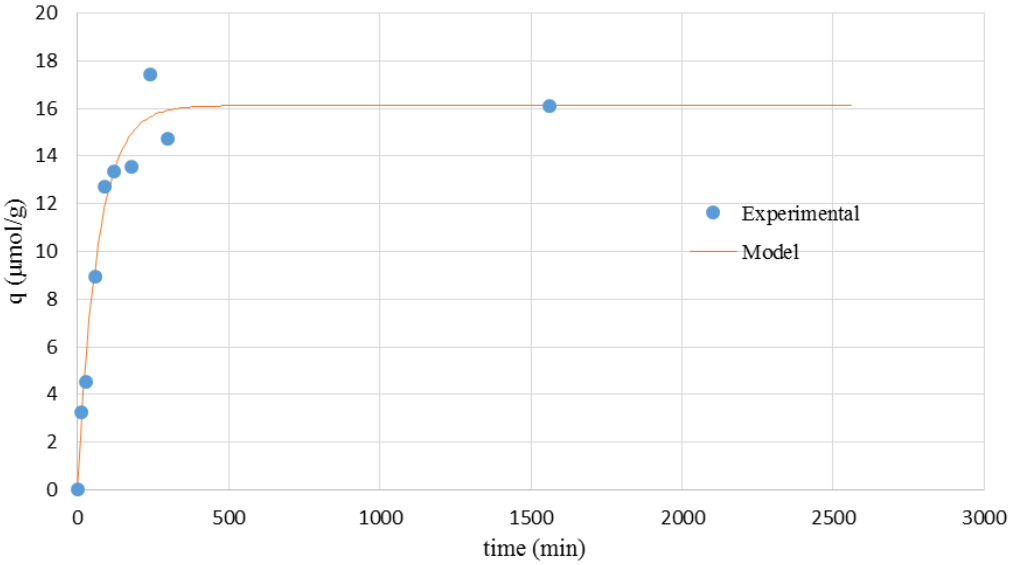


Figure 5.20: Experimental measurements and theoretical adjustement for the data obtained in the continuous retention with recycling of gallic acid. MIP2 was used as adsorbent. A volume V=200 mL of a solution of gallic acid in H2O/MeOH 50/50 at C0=0.05 mM was treated with Q=1 mL/min at room temperature. Change with time of the adsorbed amount of gallic acid in the particles is here presented.

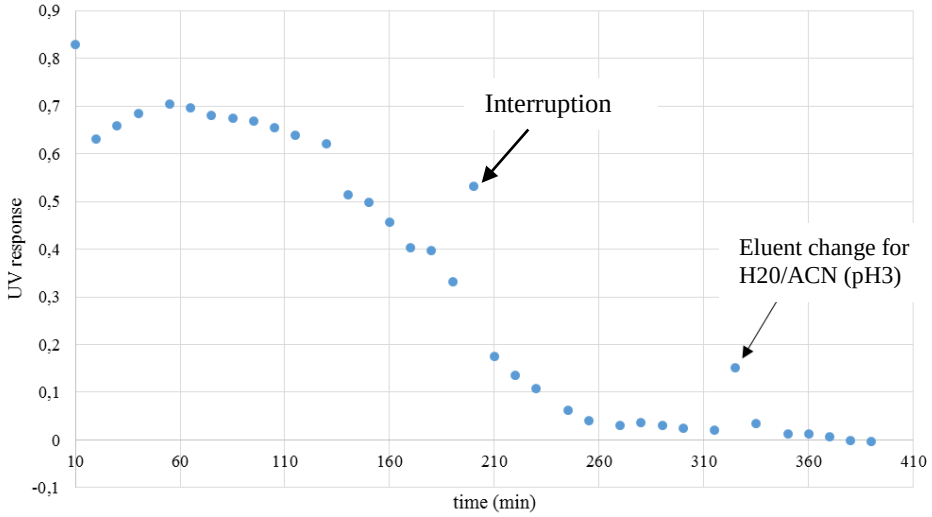


Figure 5.21: Release of gallic acid from the previous saturated column. Desorption temperature considered was T=50 °C in order to try the speed-up of this process at Q=0.1 mL/min. In the first stage, H2O/MeOH 50/50 was used as solvent, wich was then replaced by ACN/ H_2O 30/70 at pH=3

.

Analogue results are presented in Figures 5.22 and 5.23 for the retention of polydation in a continuous process with recycling. Note that, in similar operation conditions, a much lower retention efficiency is estimated in this case: after around 24 h, q*=8 µmol/g, C=0.037 (c.a. 25% retention of solute). This issue is a consequence of the non-specific interaction between gallic acid and the adsorbent (ionic interactions) that surpasses the molecular imprinting effect of polydatin.

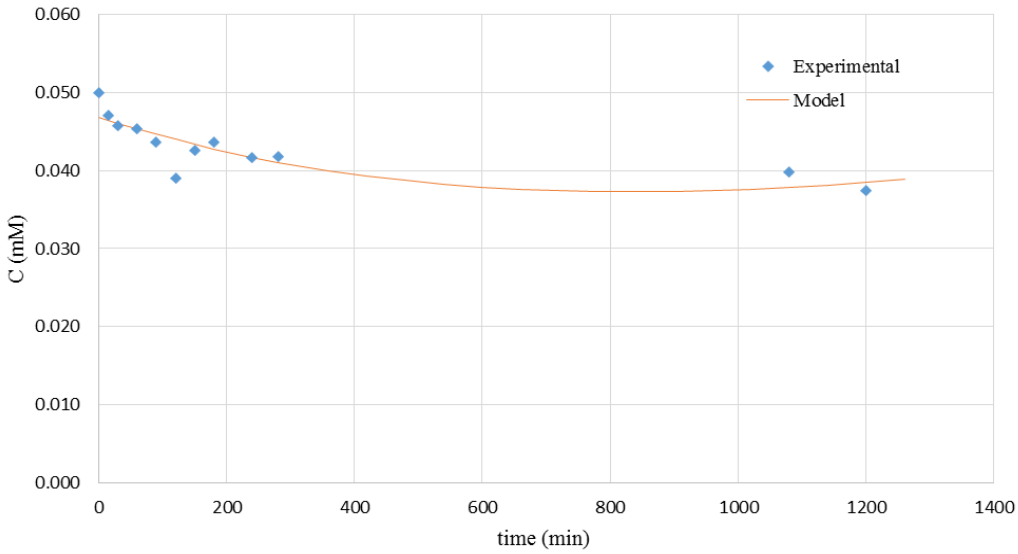


Figure 5.22: Experimental measurements and theoretical adjustement for the data obtained in the continuous retention with recycling of polydatin. MIP2 was used as adsorbent. A volume V=200 mL of a solution of polydatin in H2O/MeOH 50/50 C0=0.05 mM was treated with Q=1 mL/min at room temperature. Change with time of concentration of polydatin in the liquid solution is here presented.

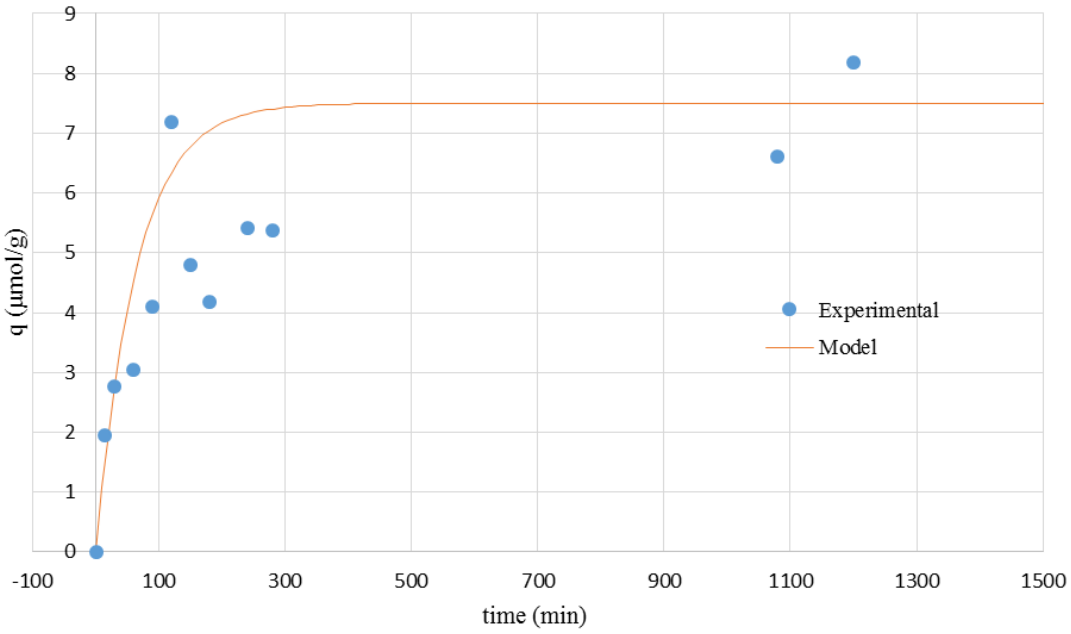


Figure 5.23: Experimental measurements and theoretical adjustment for the data obtained in the continuous retention with recycling of polydatin. MIP2 was used as adsorbent. A volume V=200 mL of a solution of polydatin in H2O/MeOH 50/50 at C0=0.05 mM was treated with Q=1 mL/min at room temperature. Change with time of the adsorbed amount of polydatin in the particles is here presented.

In Figures 5.21 and 5.24 are presented the results correspondent to the release of gallic acid and polydatin, after the saturation of the column in each case, as above described. The temperature of the process was considered as a parameter to enhance the release of the molecules. Runs were performed at T=50 °C with the column placed inside an oven.

A small flow rate Q=0.1 mL/min was considered in order to allow the thermal equilibrium of the eluent when collected by the pumping system at the reservoir at room temperature and flowing afterwards through the adsorbent. For gallic acid, when using H_2O/MeOH 50/50 in the desorption process, a lengthy release of the molecule is observed (see Figure 5.21) as a consequence of the strong interactions between the molecule and the adsorbent.

If the solvent is changed for ACN/H_2O 30/70 at pH=3 (acetic was used) the efficiency of release was increased, namely due to the effect of pH on the attenuation of the gallic acid – adsorbent ionic interactions. Note, however, that in spite of the measurement of a null UV signal at the outlet of the column, it is estimated that the complete release of the gallic acid was not achieved and stronger elution conditions should be needed. A fast release process was observed with polydatin, as presented in Figure 5.24 as a consequence of the weaker interactions with the adsorbent.

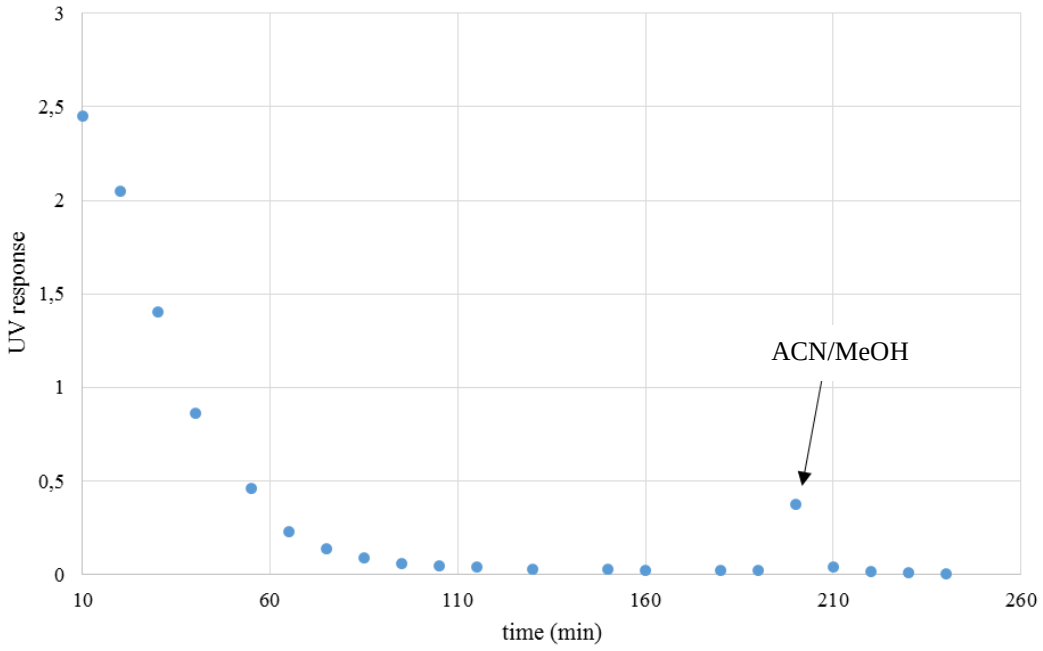


Figure 5.24: Experimental measurements and theoretical adjustment for the data obtained in the continuous retention with recycling of polydatin. MIP2 was used as adsorbent. A volume V=200 mL of a solution of polydatin in H_2O/MeOH 50/50 at C0=0.05 mM was treated with Q=0.1 mL/min. In the first stage, H_2O/MeOH 50/50 was used as solvent, which was then replaced by ACN/H_2O 30/70 at pH=3.

The exploratory results here presented concerning the adsorption and release of phenolic compounds in continuous processes with tailored adsorbents indicate that this approach can be eventually considered in the treatment of natural extracts (complex mixtures) if an optimized combination between adsorbent (one or more) and solvents, for retention and release, is found. Probably, a sequence of different adsorbents is needed with these complex mixtures and a gradient process including different solvents should be used. With these optimized conditions, should be possible to increase the efficiency on the separation and concentration of phenolic compounds present in natural extracts.

Results here presented also showed that, with the particles considered, the stable run of continuous chromatographic systems should be possible, which is important for commercial applications.

The issues just described will be additionally explored in the next chapter. Natural extracts prevenient from red wine (Portuguese Douro Region red wine) and from the chestnut shell (extract from Bragança region chestnut obtained with supercritical CO_2) will be considered for testing the retention and release of phenolic compounds with a sequence of different MIPs and a solvent gradient scheme.

Chapter 6 Retention and Release of Phenolic Compounds in a Sequence of MIP Adsorbents with Application to Natural Extracts

6.1 Introduction

In this chapter are presented results concerning retention and release of phenolic compounds with a sequence of different MIPs and a solvent gradient scheme. These ideas are applied to a created mixture containing gallic acid, tannic acid, polydatin and resveratrol, as well as to two different natural extracts. Red wine (Portuguese Douro Region red wine) is considered as a possible source of phenolic compounds and an extract from chestnut shell is evaluated with the same purpose. This extract from chestnut shell was obtained through supercritical extraction with CO_2, using fruits before collected at the Bragança region.

6.2 Supercritical fluid extraction from skin of chestnut

6.2.1 Supercritical fluid extraction (SFE)

Supercritical fluid extraction (SFE) is an interesting alternative for the extraction of natural thermo sensitive bioactive compounds that does not generate toxic residues due to its high selectivity, high extraction rate, and low critical point [Rodrigo et. al, 2012]. This method is very expensive due to the high investment cost and safety precautions of working at high pressure. Therefore SFE is profitable only when applied to high-added value to obtain ultra-pure products or of imposed by regulatory restrictions on residues.

The most important parameters of this method are pressure and temperature. Effect of these parameters is the supercritical fluid selectivity, yield and extraction rate, especially in the vicinity of critical point [Paula et. al, 2006].

6.2.2 Chestnut

These nuts grow in the forests of North Europe America, China and Japan. Chestnuts are sweet and they are very rich in nutrients, vitamins, minerals and protein. And they are containing far less fat than their nut relatives.

Comparison to other nuts, chestnut has fewer calories. 100 grams of chestnuts contain 213 calories. Chestnuts are an incredible source of protein with about 8 grams in 100 grams of this nut. Dietary fiber is excellent for the digestive system. It also helps the body lower the cholesterol [http://eathealthylivefit.com].

Figure 6.1: Chestnut fruit

http://szhelyi.blogin.hu/2016/10/

6.3 Experimental procedure

In this work, supercritical extraction was performed using a Parr unit assembled by Paralab (see Figure 6.2). This unit includes a 970 mL steel reaction/extraction vessel, a pumping system to supply carbon dioxide, control and purge valves, back pressure regulator (BPR) and a trap to collect the final extracts. This unit is automatically controlled by a computer application allowing defining the following set-points: temperature of the coil used to heat the inlet CO_2, temperature of the reactor/extractor, temperature of the BPR, temperature of the trap. The amount of CO_2 supplied to the vessel can be controlled using a Carioles flow-meter. Due to the abundance of chestnut trees in the Trás-os-Montes region, the skin chestnut fruit was considered in the extraction runs with supercritical CO_2.

In this way was intend to evaluate the possibility for valorization of residues from the chestnut processing namely as a source of phenolic compounds. An integrated research including the efficient extraction of polyphenols from natural vegetable resources, combined with the synthesis of MIPs to perform their molecular recognition, is thus sought. Hopefully, these materials can be useful to separate and concentrate valuable molecules present in complex extracts.

In a typical extraction experiment with supercritical CO_2, around 50 g of the skin of chestnut fruit (collect after the fruit processing) were placed in basket and afterwards inside the extraction vessel (see Figure 6.3). Ethanol (100 mL) was also feed to the extraction in the beginning of the batch process in order to improve the extraction efficiency due to the polar effects of this molecule (CO_2 is a non-polar solvent). This additional solvent is also helpful to collect the small amounts of extracts expected at the end. The set-points used for the extraction temperature and pressure were T=50 °C and P=150 bar, respectively. Extraction was performed during 1 hour and 30 minutes. At the end, CO_2 was released from the extraction vessel and the resulting extract was collected in the trap. The final extract is a yellow color ethanolic solution (see Figure 6.3)

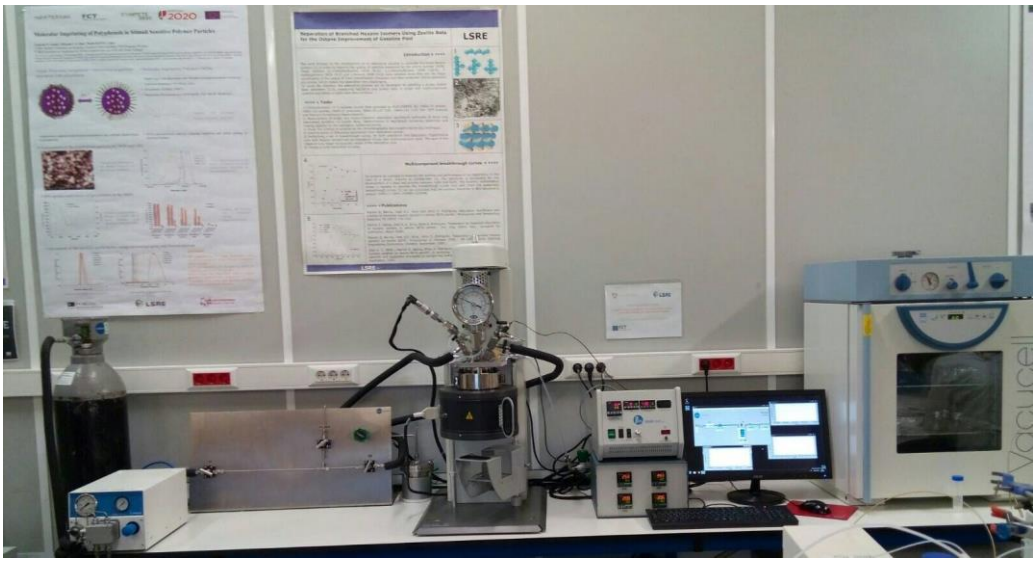


Figure 6.2: A view of the supercritical experimental unit used to obtain extracts from the skin of the chestnut fruit.

a b

Figure 6.3: a) Basket containing the skin of chestnut fruit that was placed inside the extraction vessel used with supercritical CO2. b) Final yellow colored ethanolic solution obtained through the supercritical CO_2 extraction of the skin of the chestnut fruit.

6.3.1 MIP adsorbent based on 4VP with Inverse-Suspension Synthesis

Considering the results previously presented, namely the low retention capability and the low selectively of the MIPs synthetized towards polydatin, it was decided at this point to use another MIP adsorbent developed in a parallel investigation in the framework of this research group. This MIP was produced considering 4-vinylpiridine (4VP) as functional monomer and considering an inverse-suspension synthesis process. Besides the possible stronger interaction of 4VP with polydatin (remember that AA, DMAEMA and NVP were used as

functional monomers in the above described molecular imprinting tasks with precipitation polymerization), a kind of surface molecular imprinting was enhanced with the inverse-suspension synthesis process due to the hybrid character of the template (polydatin contains hydrophobic and hydrophilic domains). The main parameters involved in the synthesis of this MIP (here named as MIP_4VP_IS) are presented in the following Table 6.1. This material was synthesized through Inverse suspension polymerization.

Table 6.1: Experimental conditions used in synthesis of MIP_4VP_IS

Material	Monomer (M)	Cross-linker (CL)	Template (T)	Initiator (I)	Oil	Sur	Y_M (%)	Y_I (%)	Y_{CL} (%)	$Y_{CL/T}$	$Y_{M/T}$	Y_O	Y_{SUR}
MIP_4VP_IS	4VP	EGDMA	POL	AIBN	n-heptane	Span 80	62.41	1.82	55.71	7.58	6.03	3.08	1.01

Y_O- mass ration between the oil phase and monomer phase

Y_{SUR}- mass ration between surfactant and oil (×100)

6.4 Rationale for the use of a Sequence of MIP Adsorbents

The approach here considered includes the use of MIP2 in series with MIP_4VP_IS. Due to the previously showed high ability of MIP2 to retain acidic phenolic compounds, the purpose of this material is to perform first the retention of molecules such as gallic acid and/or tannic acid that are often present in natural extracts (a first simplification of the complex mixture is then sought).

The solution obtained at the outlet of this material in then eluted through MIP_4VP_IS in order to retain other kinds of polyphenols, such as polydatin and resveratrol. In the release steps, a gradient of eluents (from hydrophilic to hydrophobic characters) is used in order to try the separation and the concentration of polyphenols. This strategy is depicted in the Figures below.

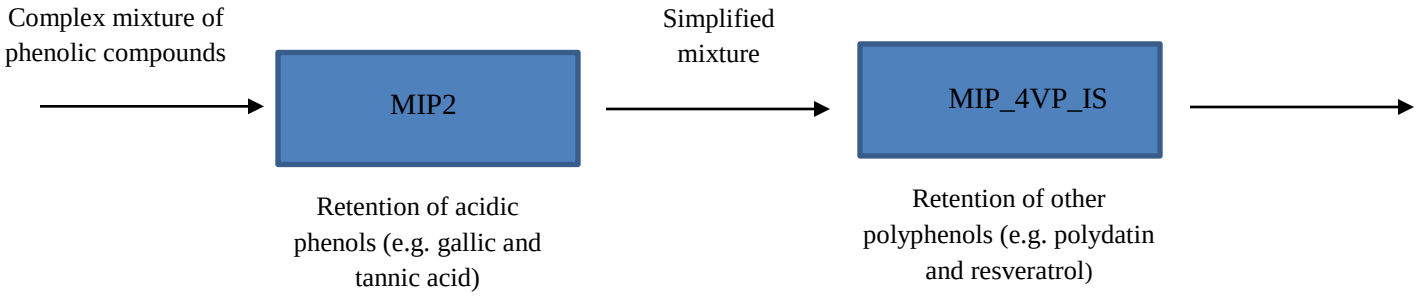


Figure 6.4: Strategy considered in the retention of a complex mixture of phenolic compounds (e.g. a natural extract) in a sequence of MIPs.

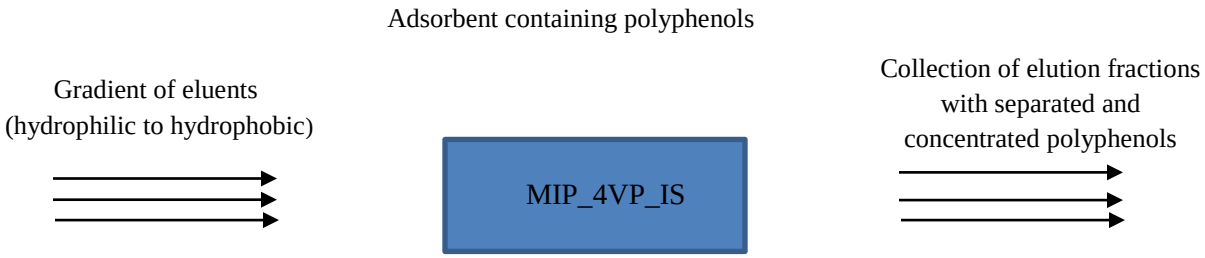


Figure 6.5: Strategy considered in the release of polyphenols from the saturated MIPs. A gradient of eluents is considered.

Note that some similar process can in principle be conceived for the release of the molecules retained in MIP2, exploring (e.g.) a gradient of solvents with different pH values.

6.5 HPLC analysis of the natural extracts based on red wine and chestnut shell

In Figures 6.6 and 6.7 are presented the results concerning the HPLC analysis of the natural extracts based on red wine and chestnut shell. The HPLC analysis conditions are the same above described for the SPE testing with polyphenols (Chapter 5). Both chromatograms (measured at 273 and 306 nm) show that the extracts are complex mixtures. The first elution region should be associated with the presence of compounds such as gallic and tannic acid. Other polyphenols, such as resveratrol, should present higher elution volume, as showed before (see Chapter 5). Note that phenolic compounds such as polydatin and resveratrol present higher UV absorption at 306 nm while gallic and tannic acid have stronger absorption at 273 nm (two wavelengths were considered in order to explore this effect).

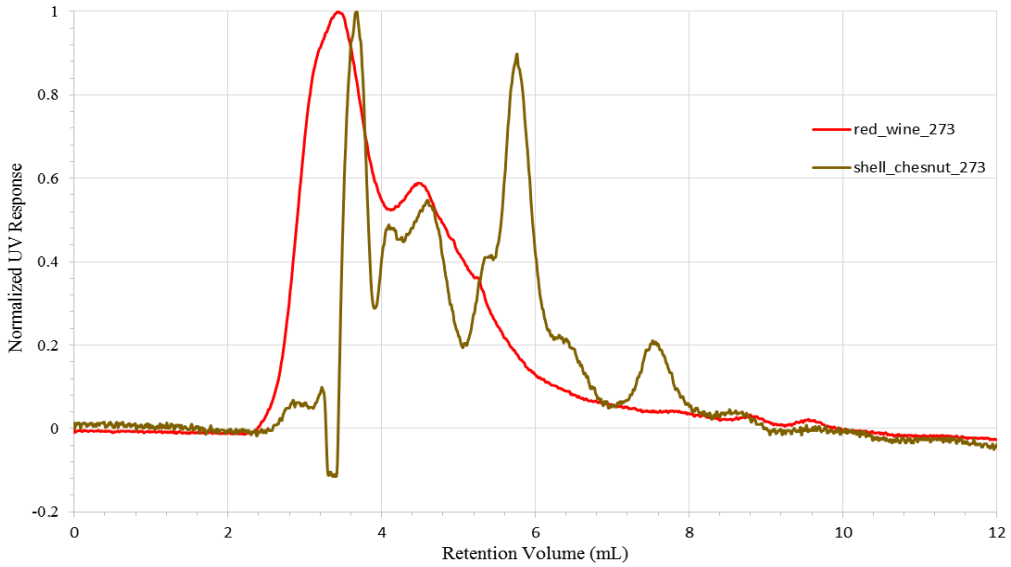


Figure 6.6: HPLC chromatograms correspondent to the original red wine extract solution and to the original shell chestnut extract solution. Measurements were performed at 273 nm.

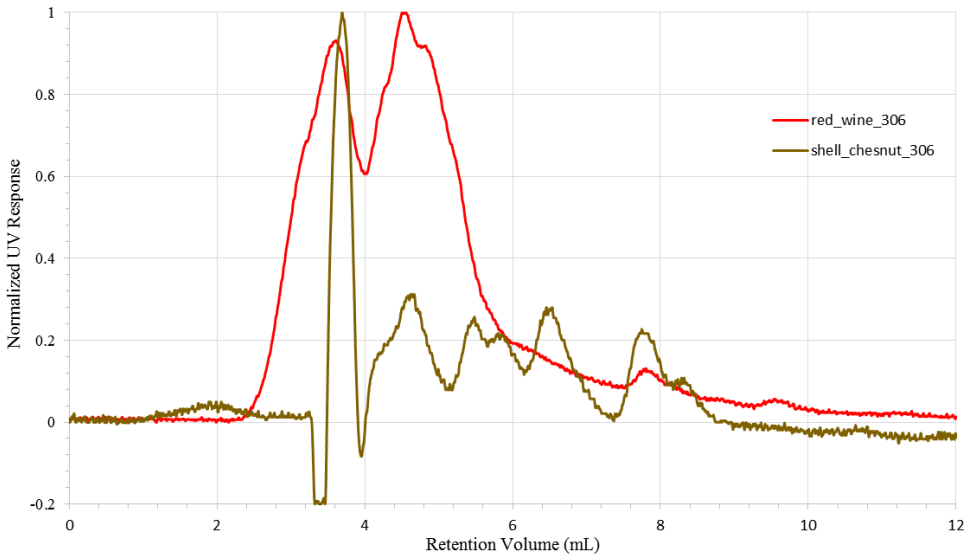


Figure 6.7: HPLC chromatogram correspondent to the original red wine extract solution and to the original shell chestnut extract solution. Measurements were performed at 306 nm.

6.6 Adsorption and release of a red wine based extract in the optimized MIP

In order to show the performance of MIP_4VP_IS in polyphenols retention, in Figures 6.8 and 6.12 are presented the HPLC chromatograms for the original red wine extract and the HPLC chromatograms for the adsorption and the release processes after the saturation of the material with the extract. This saturation was performed through the continuous recycling of the wine extract (direct dilution of red wine in H_2O/ACN 70/30 at the concentration 0.02% v/v of red wine in the solvent) in a column packed with MIP_4VP_IS (200 mg were used). This process is totally similar to those described in Chapter 5 and a volume V=400 mL of red wine solution was considered. Afterwards, the release of the adsorbed compounds was performed using a solvent gradient starting with H_2O, then H_2O/ACN 50/50 and finally ACN/MeOH 10/1.

Results presented in Figure 6.8 highlight the high capacity of MIP_4VP_IS to retain compounds present in the red wine extract. A very relevant decrease in UV absorption in observed for the samples collected at the reservoir at different instants, as can be observed in Figure 6.8. Moreover, a change in the shape of the chromatograms correspondent to different sampling moments is also observed, which is compatible with some selectivity in the adsorption process.

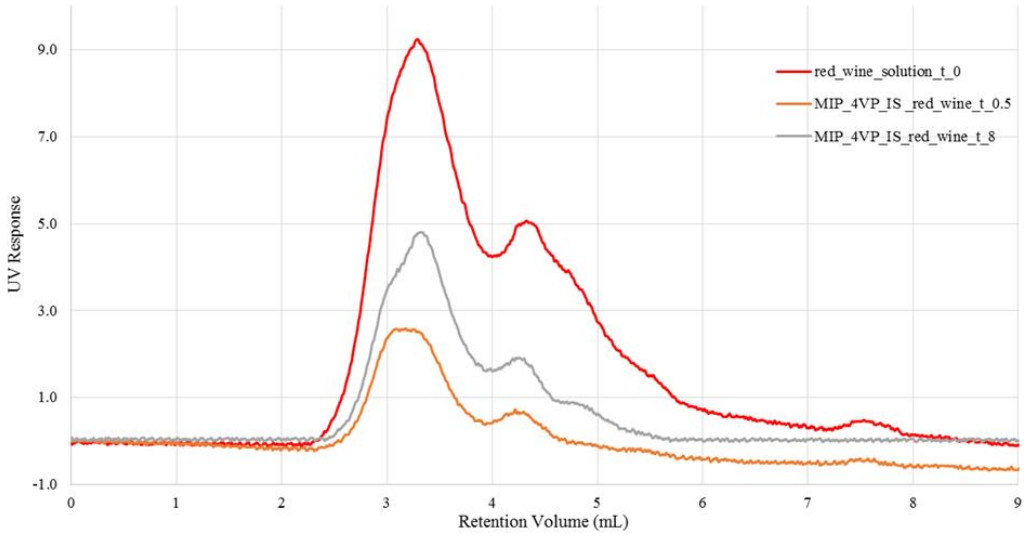


Figure 6.8: HPLC chromatograms correspondent to the original red wine extract solution and the chromatograms correspondent to the outlet of the column at different moments of the adsorption process. Measurements were performed at 273 nm.

In Figure 6.9 are presented results concerning the desorption process (release) when H_2O is used as eluent. The hydrophilic compounds should be mainly released at this stage. However after the second fraction of 2 mL, the UV response starts to be very low, indicating that it not possible to get more species at these conditions.

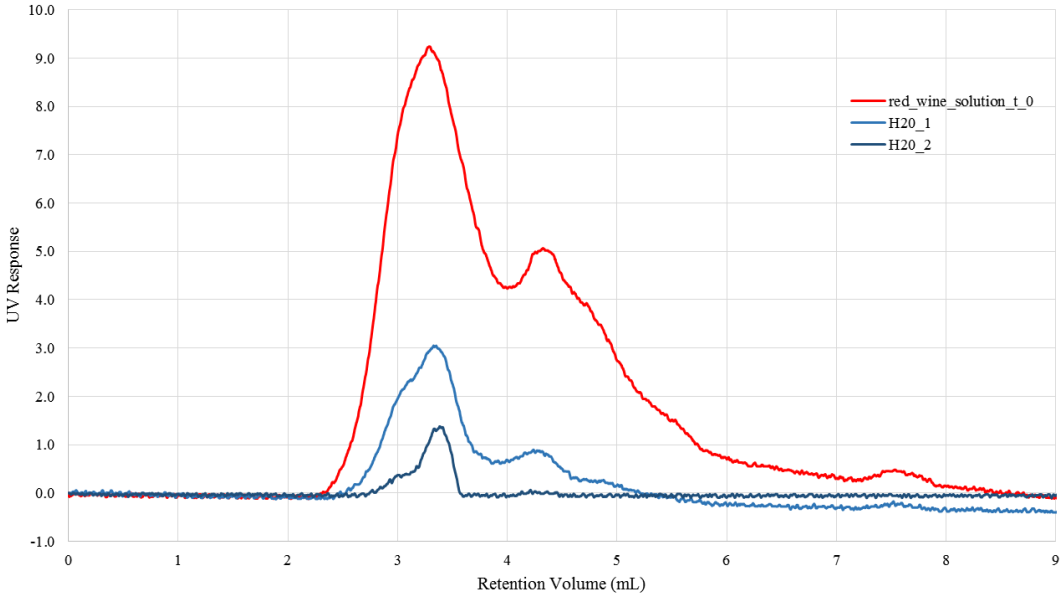


Figure 6.9: HPLC chromatograms correspondent to the original red wine extract solution and the chromatograms correspondent to the release process with H_2O as eluent (two release fractions of 2 mL are showed). Measurements were performed at 273 nm.

In Figure 6.10 are showed similar results with H_2O/ACN 50/50 and a high UV response in the eluent collected at the outlet of the column is measured. Moreover, new peaks that cannot be observed in the original extract appear in the eluted fractions. This should be compatible with the ability of the adsorbent to concentrate some specific species present in the red wine that can be afterwards eluted if a compatible eluent is used.

The release process with ACN/MeOH as eluent is illustrated in Figure 6.11 and the appearance of new peaks in the chromatogram can be observed, mainly at around 6.5 mL retention volumes.

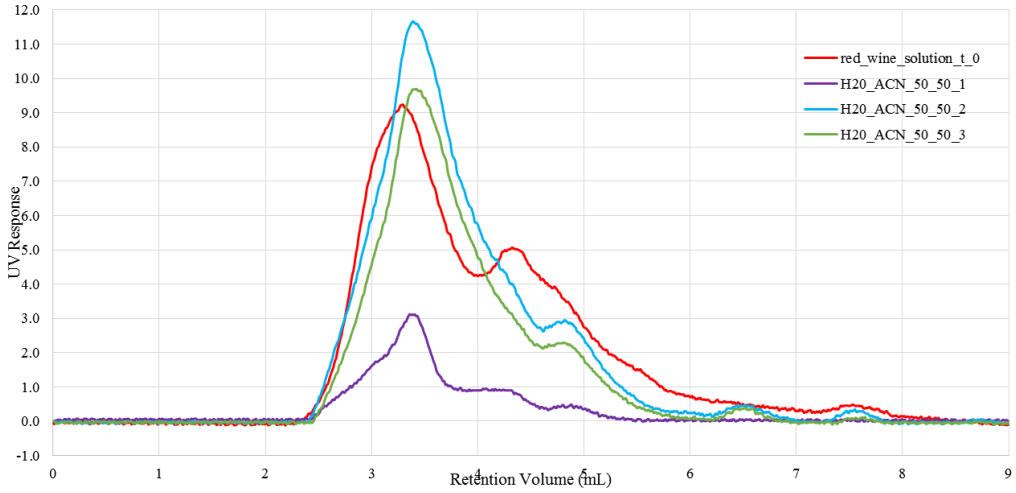


Figure 6.10: HPLC chromatograms correspondent to the original red wine extract solution and the chromatograms correspondent to the release process with H_2O/ACN 50/50 as eluent (three release fractions of 2 mL are showed). Measurements were performed at 273 nm.

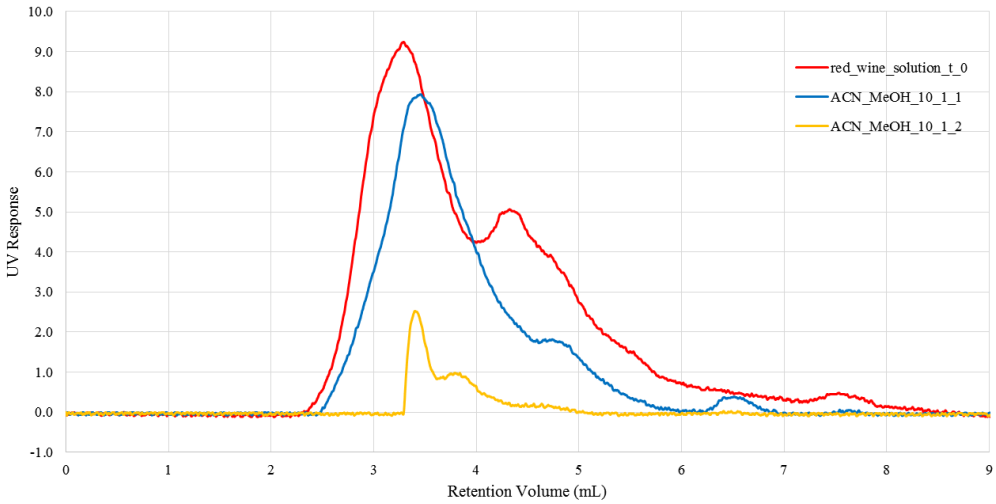


Figure 6.11: HPLC chromatograms correspondent to the original red wine extract solution and the chromatograms correspondent to the release process with ACN/MeOH 10/1 as eluent (two release fractions of 2 mL are showed). Measurements were performed at 273 nm.

In order to identify plausible polyphenol compounds present in the eluted fractions, the samples were spiked with resveratrol and polydatin. A very interesting result is presented in Figure 6.12 where the chromatogram of the original red wine extract is compared with that correspondent to a fraction eluted with H_2O/ACN 50/50 and the same fraction spiked with polydatin and resveratrol.

The new peak present in the eluted fraction at 6.5 mL retention volume matches with the peak correspondent to resveratrol. Thus, it is very plausible that MIP_4VP_IS was able to concentrate resveratrol present in red wine in a very low concentration. A similar effect can be speculated for polydatin, as can be observed in Figure 6.12. Indeed, a pronounced peak in the polydatin region is observed in the eluted fraction but not in the original extract. All of these results show that this adsorbent is a promising material for the concentration and separation of polyphenols present at very low concentration in natural extracts.

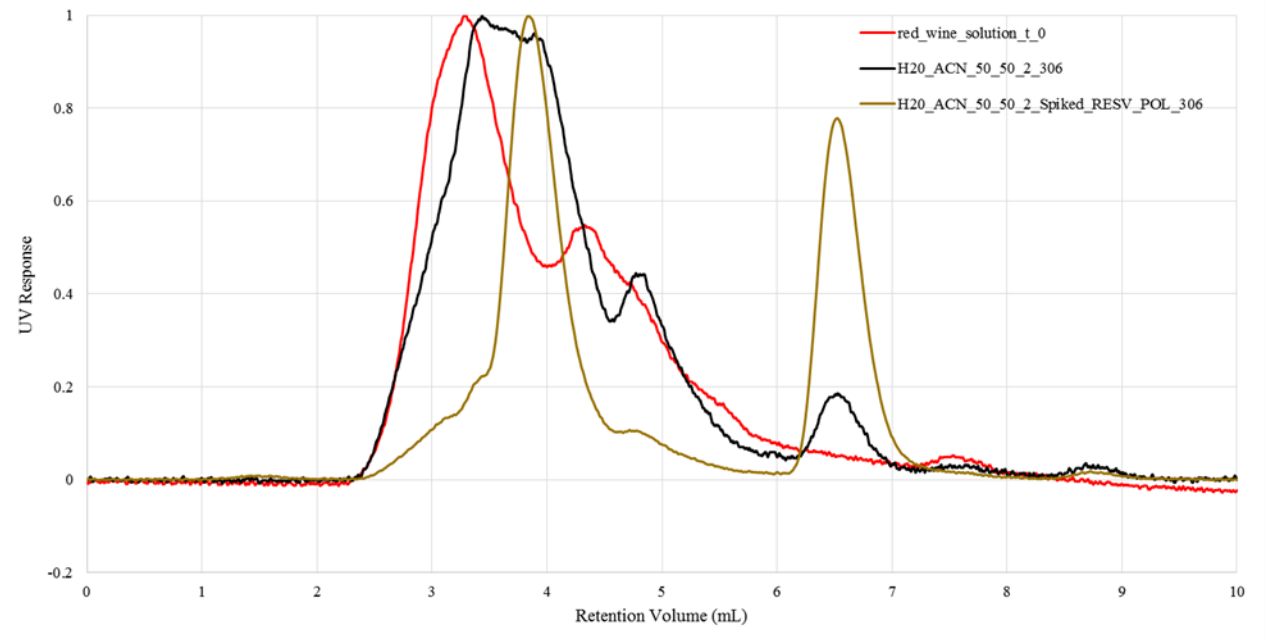


Figure 6.12: HPLC chromatograms correspondent to the original red wine extract solution, a released fraction with H_2O/ACN 50/50 and this release fraction spiked with polydatin and resveratrol. Measurements were performed at 306 nm. Results clearly show that the MIP adsorbent was able to concentrate the resveratrol present in red wine extract at very low concentration. The effect is also plausible for polydatin.

6.7 Adsorption and release of the mixture of phenolic compounds in a sequence of MIPs

The results presented in the last subsection show that an important issue in the treatment of natural extracts is their simplification, namely by separating the part correspondent to gallic and/or tannic acid (usually

present at high amount) from the other kinds of polyphenols (e.g. polydatin and resveratrol). So, it was decided to evaluate the performance of the sequence MIP2 + MIP_4VP_IS to perform this task (see also Figure 6.4 and 6.5 above). In order to have clear results on this issue, a mixture of gallic acid + tannic acid + polydatin + resveratrol was produced in ACN/MeOH 10/1 at concentration of 0.02 mM in each compound.

The formed solution was passed in a SPE sequence of the two materials and the composition of the different eluted solutions was analyzed by HPLC. Note that, besides the loading steps, different elution steps were performed for the two adsorbents, considering different types and volumes of solvents.

In Figure 6.13 it is presented the HPLC chromatogram correspondent to the original mixture of gallic acid + tannic acid + polydatin + resveratrol that was used as the inlet in MIP2. In the same figure is also included the HPLC chromatogram of the solution collected at the outlet of MIP2. These two chromatograms show that MIP2 is able to retain a higher amount of gallic acid and tannic acid, namely in comparison with polydatin and resveratrol. This strategy can be used to simplify natural extracts, namely concerning the removal of acidic phenolic compounds from these complex mixtures.

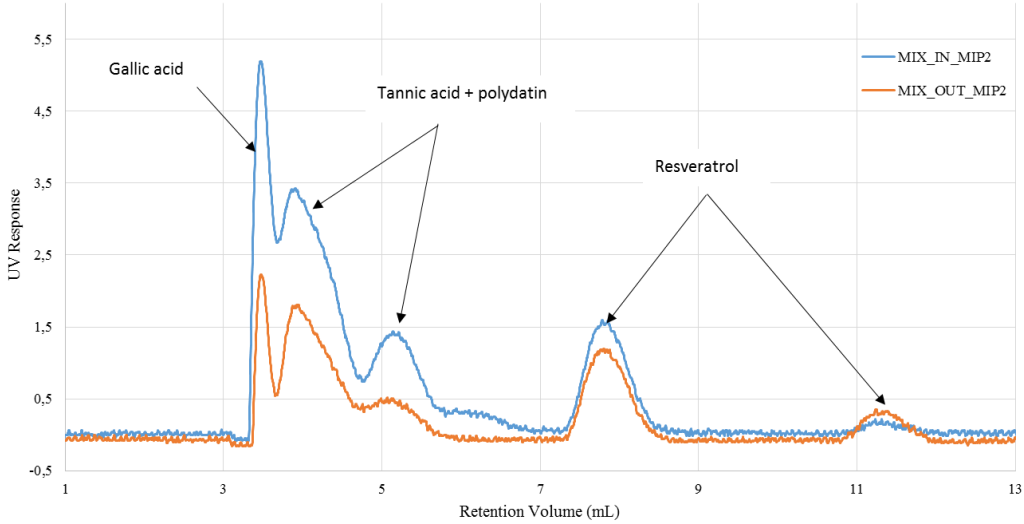


Figure 6.13: HPLC chromatogram correspondent to the original mixture of gallic acid + tannic acid + polydatin + resveratrol (inlet of MIP2) and the HPLC chromatogram of the solution after the adsorption process (outlet of MIP2). Measurements were performed at 273 nm. These results show the ability of MIP2 to retain a higher amount of gallic acid and tannic acid.

In Figure 6.14 it is presented the HPLC chromatogram of the solution collected at the outlet of MIP2 that was afterwards loaded in MIP_4VP_IS. The chromatogram of the solution collect at the outlet of MIP_4VP_IS is also presented in the same plot. The comparison of the two chromatograms allows concluding that MIP_4VP_IS is a good adsorbent for the retention of polydatin and resveratrol. Thus, the sequence of MIP2 + MIP_4VP_IS seems to have promising features concerning the treatment of natural extracts. Acid phenolic compounds are preferentially retained in the first MIP of the sequence (MIP2) while the other compounds are retained at higher amount in the second material

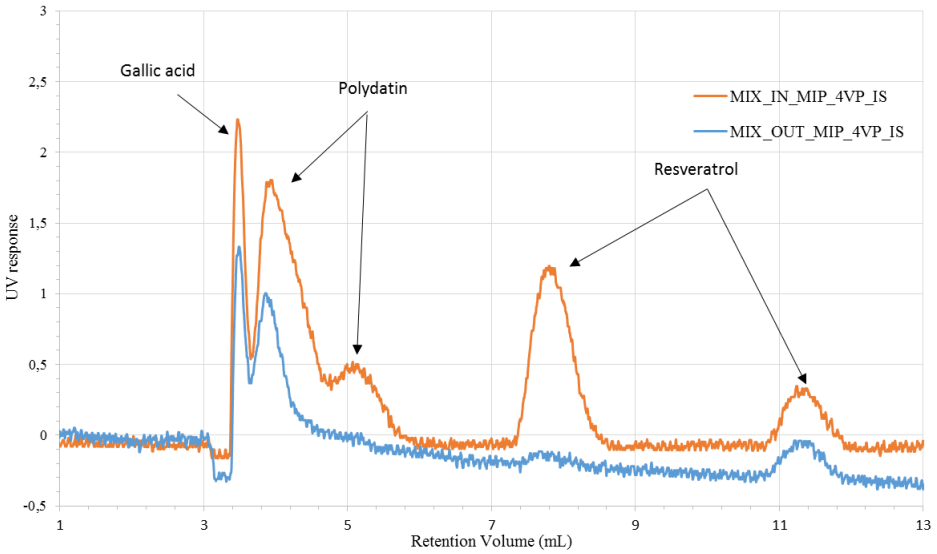


Figure 6.14: HPLC chromatogram correspondent to the solution after adsorption in MIP2 (outlet MIP2=inlet MIP_4VP_IS) and the HPLC chromatogram of the solution after the adsorption process in MIP_4VP_IS (outlet of MIP_4VP_IS). Measurements were performed at 273 nm. These results show the ability of MIP_4VP_IS to retain a higher amount of polydatin and resveratrol.

Results concerning the release of phenolic compounds from MIP_4VP_IS (previously loaded as above described) are showed in Figure 6.15. This first release step was performed using H2O/ACN 50/50 as eluent. Comparison of the chromatogram corresponding to loading step with that one relative to the release step, allows to conclude that polydatin is preferentially released when compared with resveratrol. This result is also a consequence of the amphiphilic solvent (H2O/ACN 50/50) is the elution step that enhances the compatibility with polydatin.

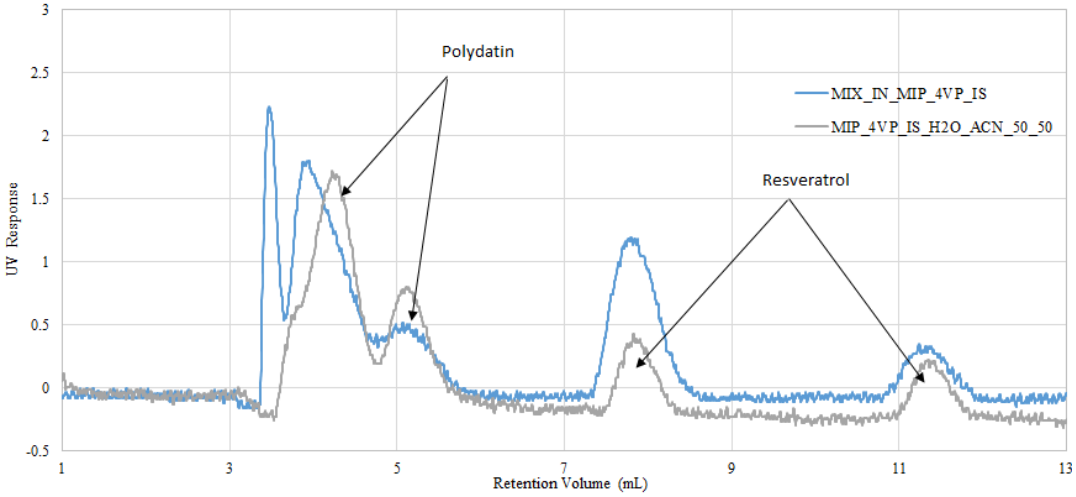


Figure 6.15: HPLC chromatogram correspondent to the solution adsorbed in MIP_4VP_IS (inlet MIP_4VP_IS) and the HPLC chromatogram of the solution released from the adsorbent with H_2O/ACN 50/50 as eluent. Measurements were performed at 273 nm. These results show the ability of MIP_4VP_IS to retain polydatin and resveratrol and after release preferentially the first phenolic compound with an amphiphilic solvent.

Results for the following release step performed, using again the same H_2O/ACN 50/50 eluent, are presented in Figure 6.16. The results for the second fraction collected evidence the decrease of the amount of polydatin release, showing that a gradient process can help in the separation and concentration of phenolic compounds using MIPs.

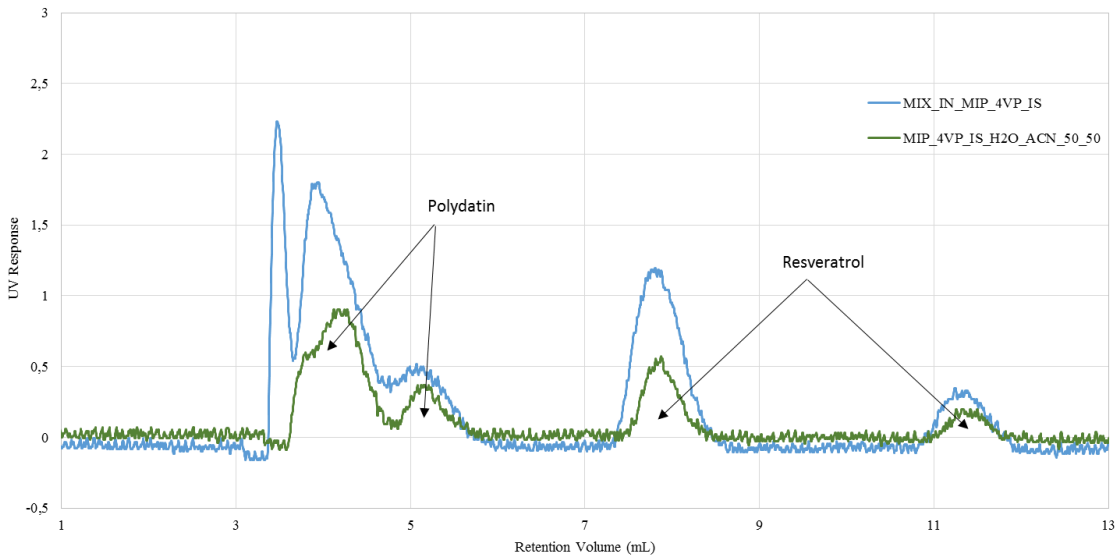


Figure 6.16: HPLC chromatogram correspondent to the solution adsorbed in MIP_4VP_IS (inlet MIP_4VP_IS) and the HPLC chromatogram of the solution released from the adsorbent with H_2O/ACN 50/50 as eluent (second fraction). Measurements were performed at 273 nm. In comparison with Figure 6.15 a lower amount of polydatin is now released, showing the effect of the gradient process.

The last release step was performed with ACN/MeOH 10/1 as eluent and the obtained results are presented in Figure 6.17. Note that, is this third fraction, almost only resveratrol is released, which confirms the usefulness of the proposed strategy to separate and concentrate phenolic compounds.

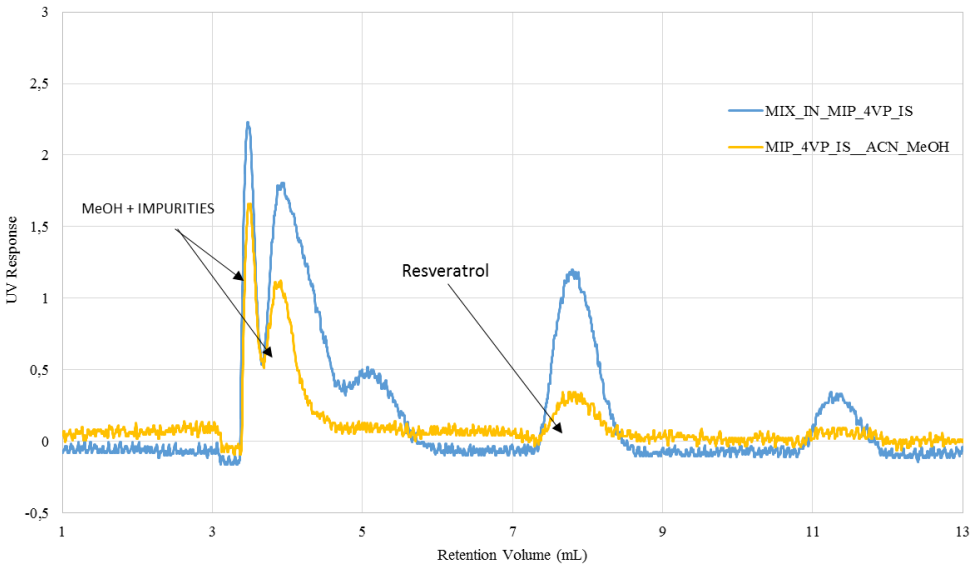


Figure 6.17: HPLC chromatogram correspondent to the solution adsorbed in MIP_4VP_IS (inlet MIP_4VP_IS) and the HPLC chromatogram of the solution released from the adsorbent with ACN/MeOH 10/1 as eluent (third fraction). Measurements were performed at 273 nm. In comparison with previous Figures, the possibility to obtain nearly pure resveratrol at the final stage is here highlighted.

Figure 6.18 compares the chromatogram of this third fraction but with measurements at 273 and 306 nm. These results confirm that the peak at around 8.5 mL retention volume should be resveratrol.

Additionally, it is also showed that the initial peaks starting at around 3 mL retention volume is relative to impurities and not to gallic acid, tannic acid, polydatin or resveratrol. In fact, these two peaks, they also appear in blank injections.

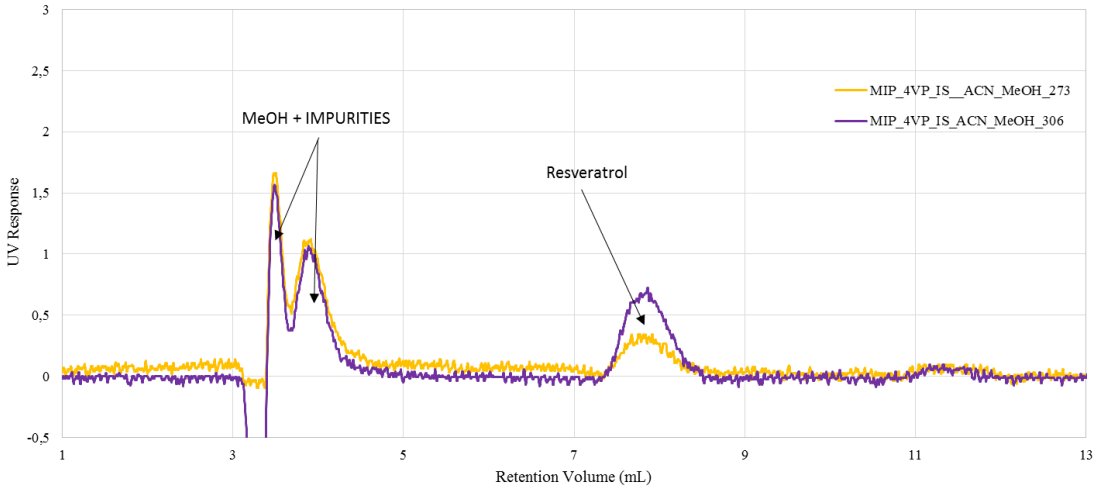


Figure 6.18: HPLC chromatogram of the solution released from the adsorbent with ACN/MeOH 10/1 as eluent (third fraction). Measurements were performed at 273 nm and 306 nm. This plot shows that the first peaks are correspondent to impurities (also appear in blank injection) and that the peak at around 8 mL retention volumes is referent to resveratrol.

6.8 Adsorption and Release of Shell Chestnut Extracts in a Sequence of MIPs

The extracts of the shell of the chestnut fruit obtained through the supercritical process with CO_2, as above described, were submitted to different testing in the sequence of MIPs before detailed (see previous section). The goal of such experiments was to assess the usefulness of these materials in the identification/separation/concentration of polyphenols that eventually are present of such extracts.

In Figures 6.19 to 6.22 are presented the results thus obtained, considering ACN/MeOH 10/1 in the retention process. Two different release steps (with water and water/ACN 50/50) were performed. These results highlight that, under the conditions used, the retention of species present in the chestnut extract is very small in both materials. Release steps also confirm this observation and only a small peak at around 5.8 mL retention volume can be identified as a consequence of some adsorbed molecules. Note that the adsorption process was performed with ACN/MeOH 10/1, which seems to be too much hydrophobic to allow the retention of the species present in the chestnut extract. Probably, these species are more hydrophobic than the polyphenols before tested and therefore cannot be retained in the conditions of the testing described in Figures 6.19 to 6.22.

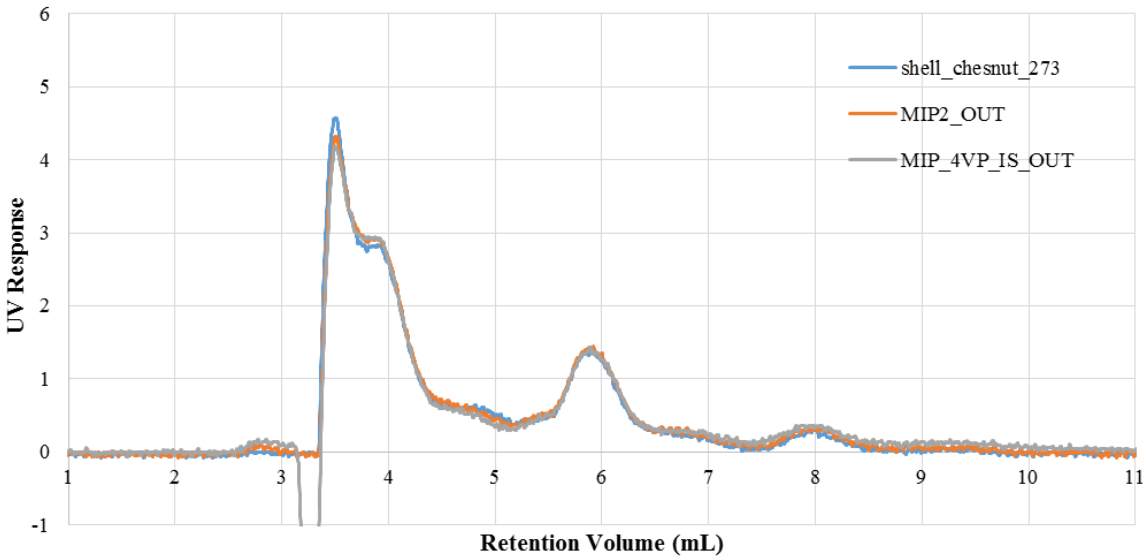


Figure 6.19: HPLC chromatograms correspondent to the original shell chestnut extract (obtained by extraction in supercritical CO2) and HPLC chromatograms correspondent to the outlet of the MIP_2 and MIP_4VP_IS columns (after the adsorption process). The mixture ACN//MeOH 10/1 was used as solvent in the retention steps. Measurements were performed at 273 nm.

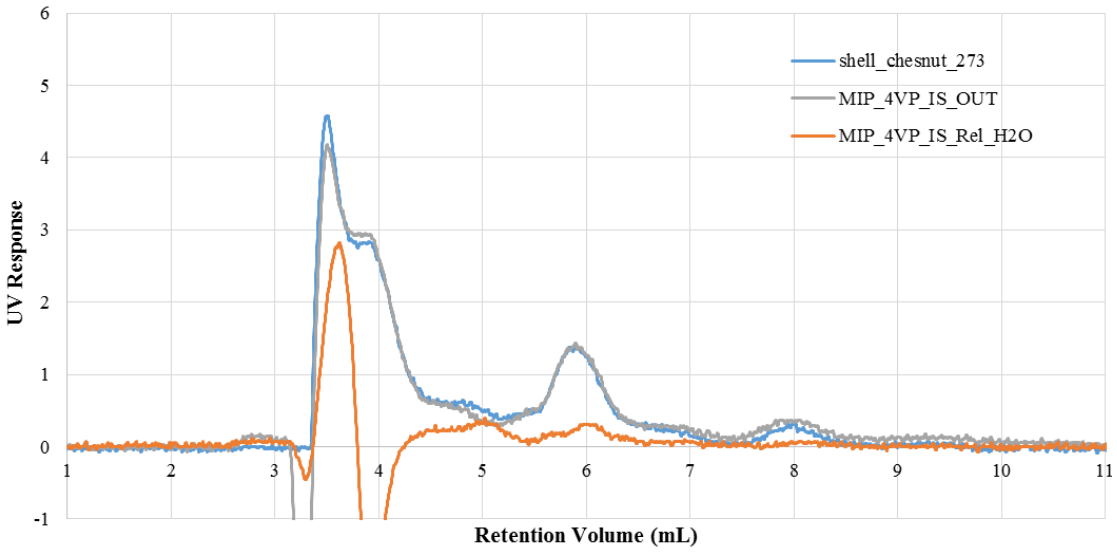


Figure 6.20: HPLC chromatograms correspondent to the original shell chestnut extract, outlet of MIP_4VP_IS after retention with ACN/MeOH 10/1 and outlet of MIP_4VP_IS after release with H2O. Measurements were performed at 273 nm.

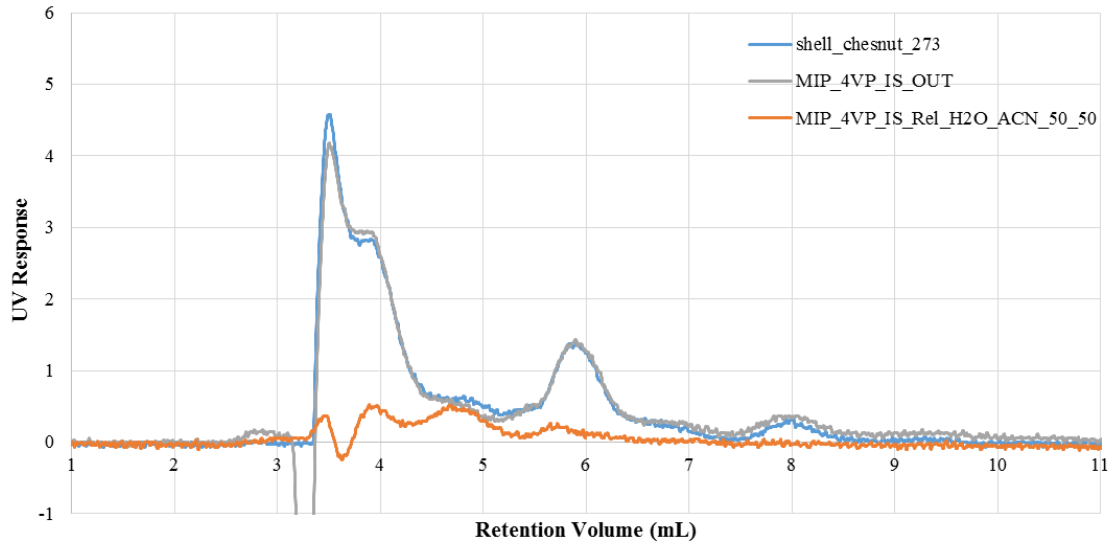


Figure 6.21: HPLC chromatograms correspondent to the original shell chestnut extract, outlet of MIP_4VP_IS after retention with ACN/MeOH 10/1 and outlet of MIP_4VP_IS after release with H2O/ACN 50/50. Measurements were performed at 273 nm.

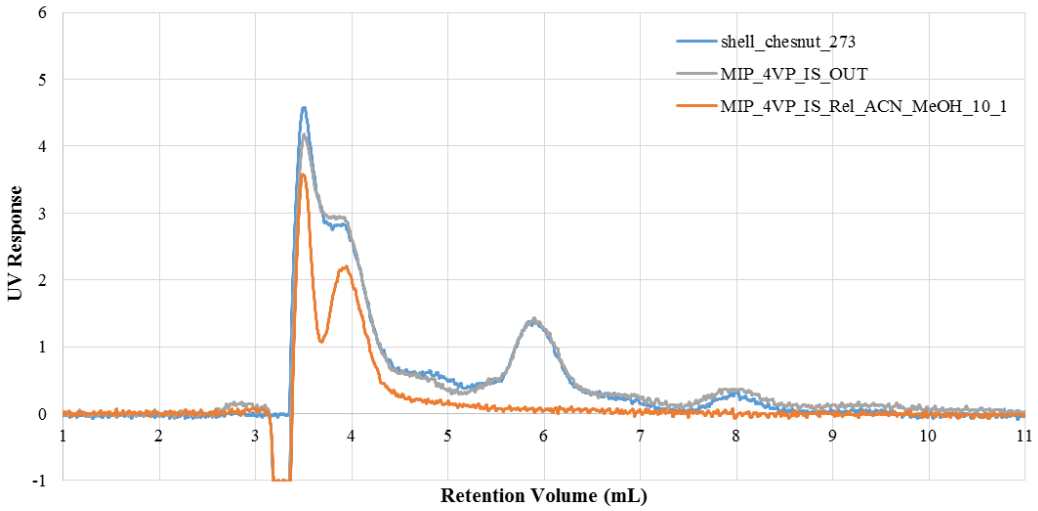


Figure 6.22: HPLC chromatograms correspondent to the original shell chestnut extract, outlet of MIP_4VP_IS after retention with ACN/MeOH 10/1 and outlet of MIP_4VP_IS after release with ACN/MeOH 10/1. Measurements were performed at 273 nm.

Confirmation that the structure of the molecules present in the chestnut extract should be very different from the kinds of polyphenols before used is provided in Figures 6.23 to 6.25. The original extract of the shell of the chestnut fruit was spiked with gallic acid and resveratrol and after submitted to the same testing. Results show that the sequence of MIPs is able to retain the gallic acid and resveratrol present in the modified extract. These molecules can be afterwards recovered, similarly to the studies presented in the previous sections.

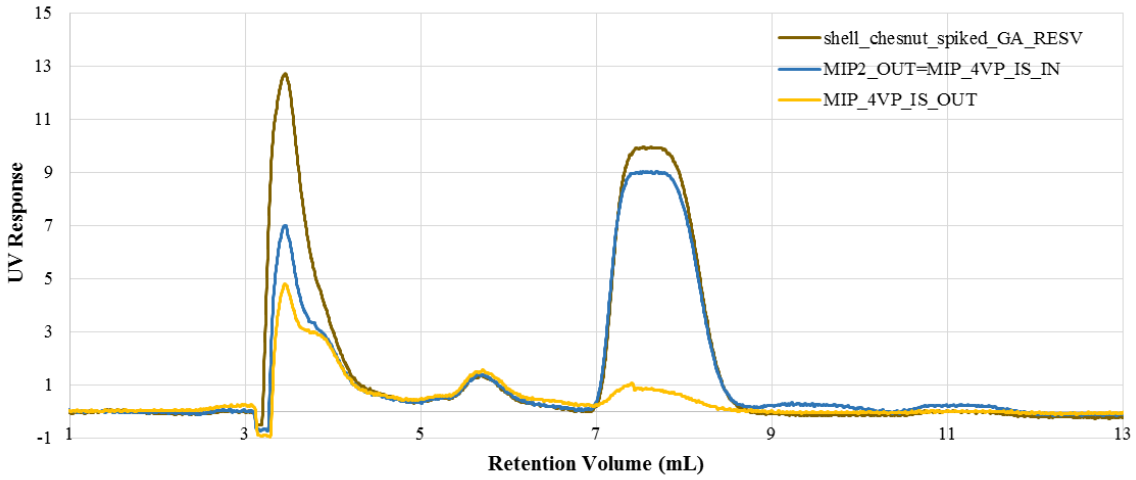


Figure 6.23: HPLC chromatograms correspondent to the original shell chestnut extract (obtained by extraction in supercritical CO2) with spiking of gallic acid and resveratrol and HPLC chromatograms correspondent to the outlet of the MIP_2 and MIP_4VP_IS columns (after the adsorption process). The mixture ACN/MeOH 10/1 was used as solvent in the retention steps. Measurements were performed at 273 nm.

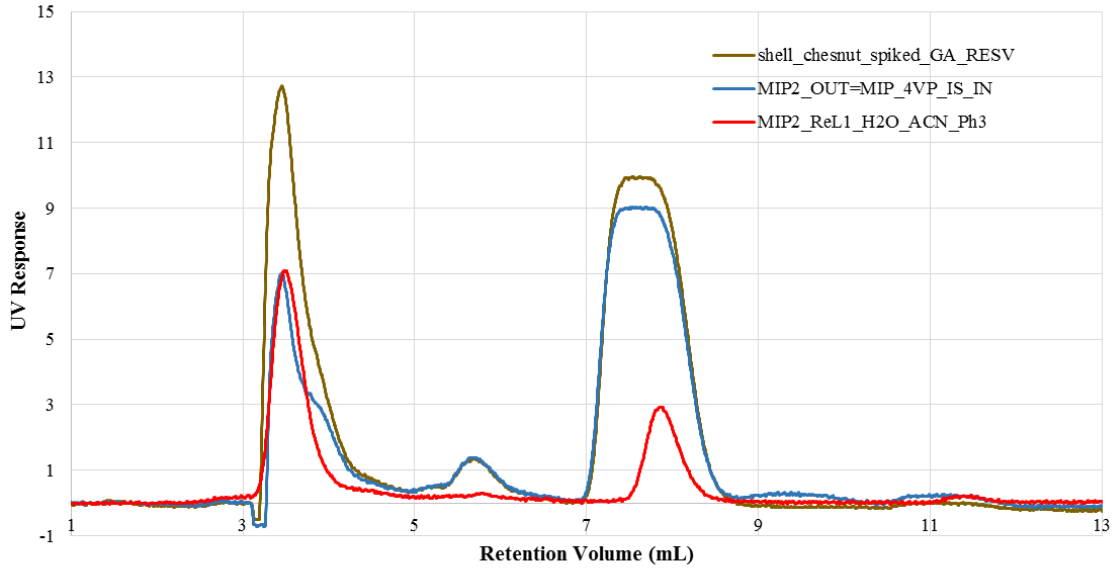


Figure 6.24: HPLC chromatograms correspondent to the original shell chestnut extract with spiking of gallic acid and resveratrol, inlet of MIP_4VP_IS for retention with ACN/MeOH 10/1 and outlet of MIP_4VP_IS after release with H2O/ACN 70/30 at pH=3. Measurements were performed at 273 nm.

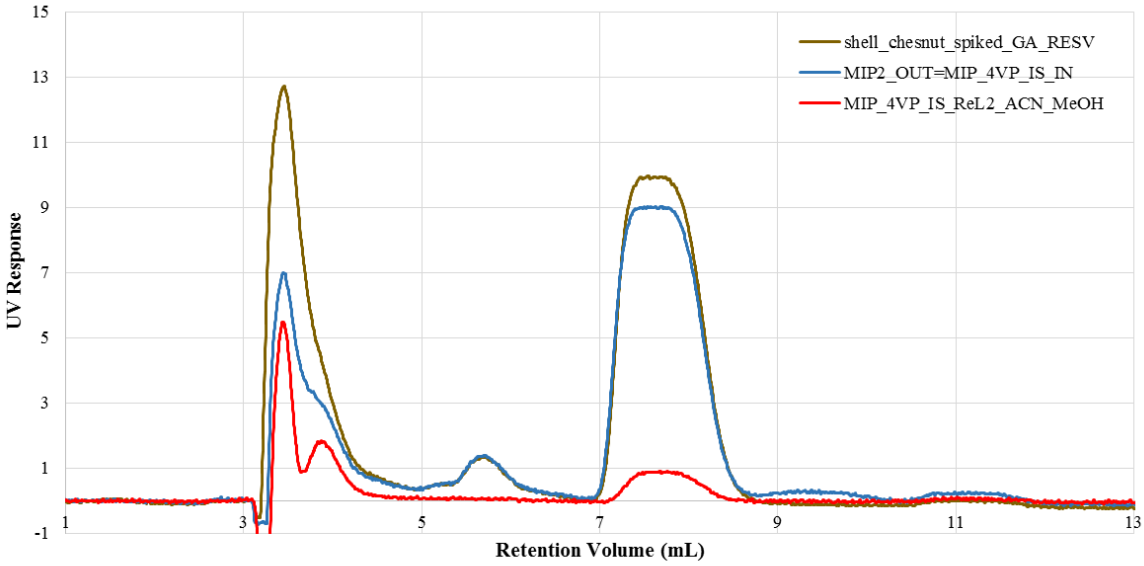


Figure 6.25: HPLC chromatograms correspondent to the original shell chestnut extract with spiking of gallic acid and resveratrol, inlet of MIP_4VP_IS for retention with ACN/MeOH 10/1 and outlet of MIP_4VP_IS after release with H2O/ACN 70/30 at pH=3. Measurements were performed at 273 nm.

In order to investigate the possible influence of the solvent used in the retention of the compounds present in the chestnut extract, similar experiments were performed with H2O/ACN 70/30. Some results obtained for the loading and release steps are presented in Figures 6.25 to 6.27 and practically nil retention was also observed in these new conditions. Moreover, the chestnut extract was spiked with catechin (molecule often considered to be present in the chestnut) and the modified extract was also analyzed. Results thus obtained are presented in Figure 6.28. It is observed that the sequence of materials is also able to retain this kind of polyphenol if present in the extract. This result is also indicative of the absence of phenolic compounds in the obtained extract or that these molecules are present at extremely low concentration. Note the important contrast with the results for red wine extract above presented, showing in this case a clear presence of phenolic compounds.

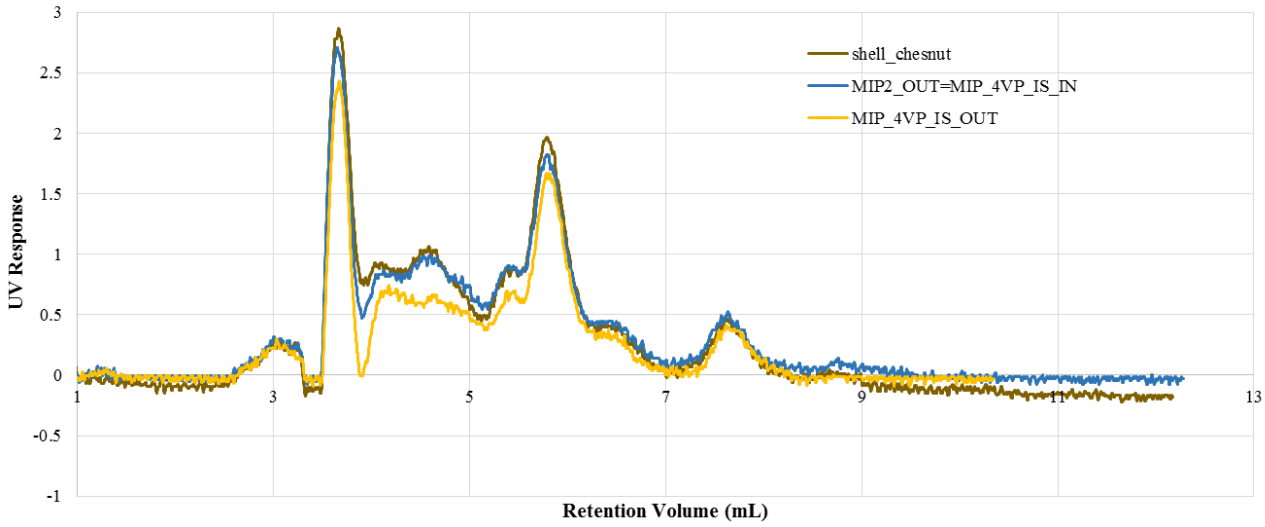


Figure 6.26: HPLC chromatograms correspondent to the original shell chestnut extract (obtained by extraction in supercritical CO2) and HPLC chromatograms correspondent to the outlet of the MIP_2 and MIP_4VP_IS columns (after the adsorption process). The mixture H2O//ACN 70/30 was used as solvent in the retention steps. Measurements were performed at 273 nm.

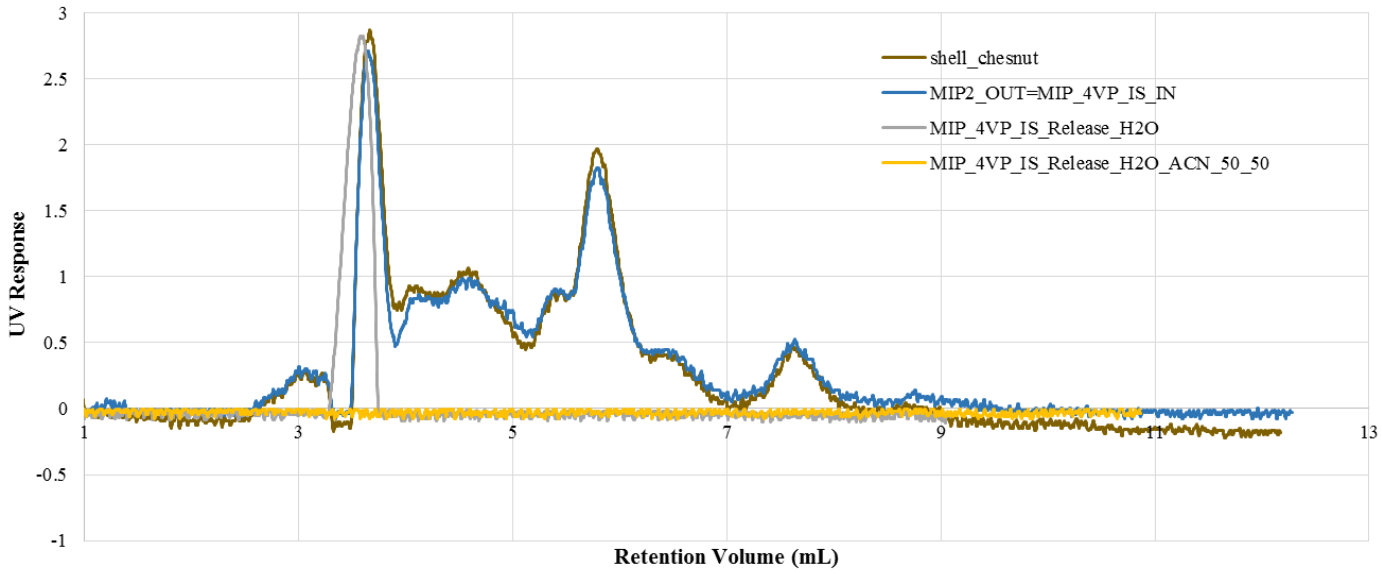


Figure 6.27: HPLC chromatograms correspondent to the original shell chestnut extract, inlet of MIP_4VP_IS for retention with H2O/ACN 70/30 and outlet of MIP_4VP_IS after release with H2O and with H2O/ACN 50/50. Measurements were performed at 273 nm.

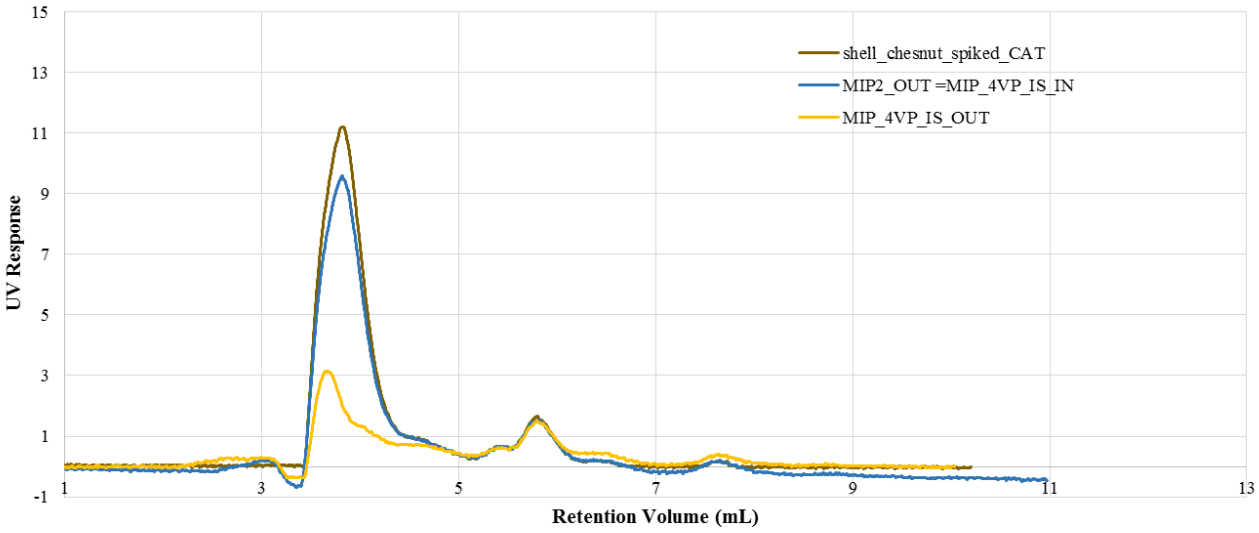


Figure 6.28: HPLC chromatograms correspondent to the original shell chestnut extract with spiking of catechin, outlet of MIP2=inlet of MIP_4VP_IS and outlet of MIP_4VP_IS. H2O/ACN 70/30 was used and solvent in the retention steps. Measurements were performed at 273 nm.

Results here presented show that the shell chestnut extract obtained with supercritical conditions should not contain phenolic compounds at an appreciable amount (or even be absent of these kinds of molecules). Indeed, nearly nil retention in the two MIP materials was observed for these extracts considering different

uptake conditions (e.g. adsorption with ACN/MeOH 10/1 or H2O/ACN 50/50 to assess the effect of the hydrophilicity of the solvent). Moreover, the shell chestnut extract was spiked with phenolic compounds such as gallic acid, resveratrol and catechin and the ability of the adsorbent materials to retain these molecules was proved. A clearly higher amount of phenolic compounds was retained by these materials when a red wine extract was considered, as above described, Different extraction techniques (e.g. extraction at alkaline conditions) should be considered in future researches in order to evaluate the effect of the conditions used on the composition of the extracts. Moreover, other parts of the chestnut plant (leaves, burs, stalks, etc) can be used with supercritical or conventional extraction. A deeper knowledge on the usefulness of the materials here developed to exploit these vegetable components as an important source of phenolic compounds can thus be achieved.

Chapter 7 Amphiphilic MIP Adsorbents through the Grafting of Functional Brushes in the Particles Surface by RAFT Polymerization

7.1 Introduction

In the last few years, an important research effort on materials chemistry and engineering was devoted to the development of amphiphilic structures, namely amphiphilic polymer networks. These networks include hydrophilic and hydrophobic units that are covalently bonded in a tridimensional arrangement. In this way, is theoretically possible to synthetize materials containing two micro-domains with different polarities (polar and apolar). Thus, should be possible that each micro-phase (micro-domains) function in independent way due to the favorable interaction with other micro-domains (or molecule or medium) of the same polarity. Thereafter, each kind of micro-phase present in the amphiphilic material will swell in compatible solvents and with the concomitant preferential adsorption of molecules with similar polarity. This behavior of amphiphilic materials encourage their use in several different kinds of applications, such as controlled drug release, special adsorbents, tissue engineering, biomedical materials, advanced bio-sensors, etc. [Zhou et. al, 2014, Puoci et. al, 2007, Zhang 2013, Pan et. al, 2009, Ma et. al, 2012, Zhao et. al, 2014, Zhang 2014].

MIP and NIP particles synthetized in this work, as before described, are in their nature amphiphilic materials because they include in the formed networks an apolar micro-phase, correspondent to the organic crosslinker (e.g. TMPTA or EGDMA), and also polar micro-domains due to the functional monomers selected (e.g. AA, MAA, NVP, DMAEMA or, at a much lower extent, NVP). However, due to the smaller amount of functional monomer usually considered (e.g. 20% or even less to improve the molecular imprinting effect), the resulting adsorbents are generally unbalanced in their polar/apolar magnitudes. A possible strategy to improve the amphiphilic character of MIP particles is through the grafting of hydrophilic functional brushes in the particles surface, considering an appropriated controlled radical polymerization mechanism, as explored recently in several research works [Liu et. al, 2014, Pan et. al, 2011, Pan et. al, 2010, Dias et. al, 2016, Zhao et. al, 2010, Achilleos et. al, 2008, Han et. al, 2009].

In the present work, the improvement of the amphiphilic character of MIP particles was tried thought the RAFT surface grafting of hydrophilic functional brushes in the surface of previously RAFT precipitation-synthetized molecularly imprinted particles. Polymer brushes improving compatibility with aqueous systems were considered within this purpose, namely poly (MAA), poly (DMAEMA) and poly (NIPA). Besides the improvement of the polar character of the particles (thus enhancing wettability of the materials), the stimulation of these brushes is possible through the change of parameters such as pH and/or temperature. Stimulated

uptake/release of specific templates processes should thus be possible with MIPs bearing surface grafted functional brushes.

Bellow, are presented the details concerning the two-step RAFT polymerization method considered in this work to obtain amphiphilic MIP adsorbent particles. Additionally, a preliminary evaluation of these materials in the framework of the pH/temperature stimulated retention and release of phenolic compounds is also discussed.

7.2 MIP particles with surface extensions to improve sensitivity

The main goal of this last part of the work it is generically presented in Figure 7.1. Note that besides the improvement of the amphiphilic character of the particles, their stimulation by pH/temperature it is also in principle possible.

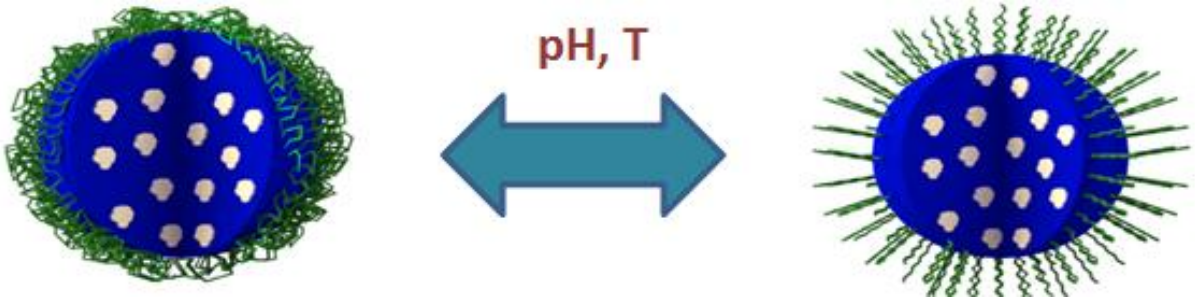


Figure 7.1: Generic representation of the final molecularly imprinted particles with surface grafted hydrophilic functional brushes. Besides the improvement of the amphiphilic character of the materials (hydrophobic core and hydrophilic shell), the stimulation by changes in parameters like pH and/or temperature it is in principle possible.

7.3 Experimental procedure

The experimental production of such kind of advanced particles is based on the use of the RAFT polymerization technique (main concepts presented in Figure 7.2) with a two-step synthesis procedure. In the first step, molecular imprinting is performed in the presence of a RAFT agent that will be immobilized in the surface of the particles. In the second step, these RAFT groups are reactivated and a second polymerization is performed to make the growing of functional brushes in the particles.

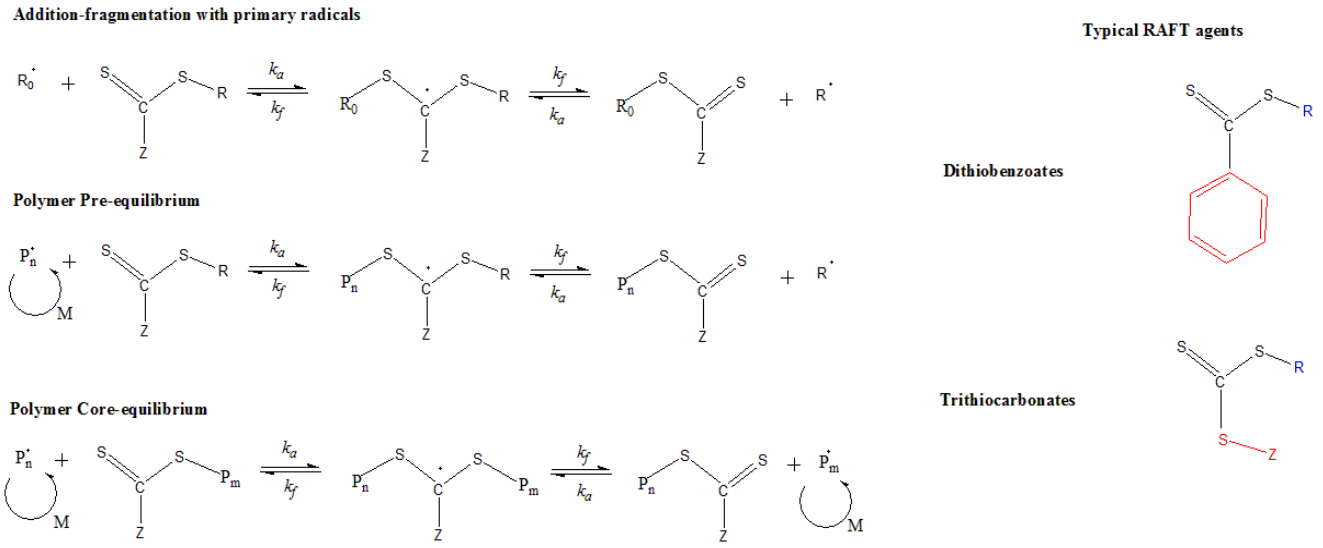


Figure 7.2: RAFT Scheme

7.4 Molecular Imprinting of polydatin using RAFT agents

The schemes below presented are a brief illustration of the two synthesis steps above mentioned. Several different combinations between functional monomer considered for molecular imprinting (4VP was here used), template, cross-linker, solvents, initiator and RAFT agent are possible. Details on the chemicals used and main features of the products are also presented in these schemes. Concerning the functional brushes grafted, different kinds of stimulation can be conceived. Here were explored anionic and cationic pH-sensitive polymers (pMAA and pDMAEMA, respectively), as well as the temperature-sensitive pNIPA polymer.

1) Molecular Imprinting of polydatin with the system 4VP/EGDMA (precipitation polymerization in MeOH/H2O) in the presence of the RAFT agent CPA

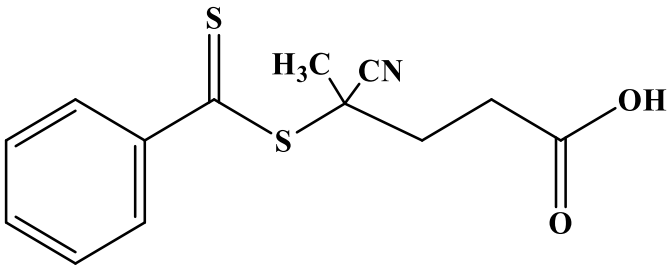
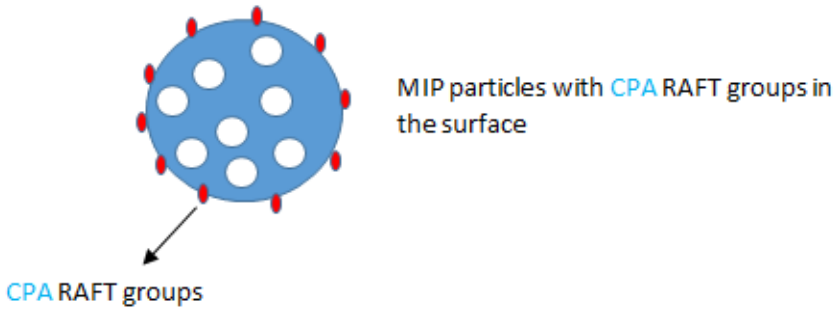
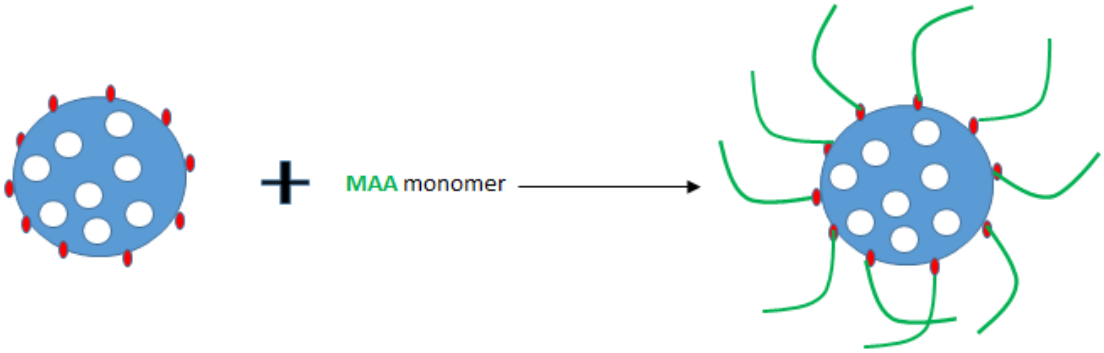


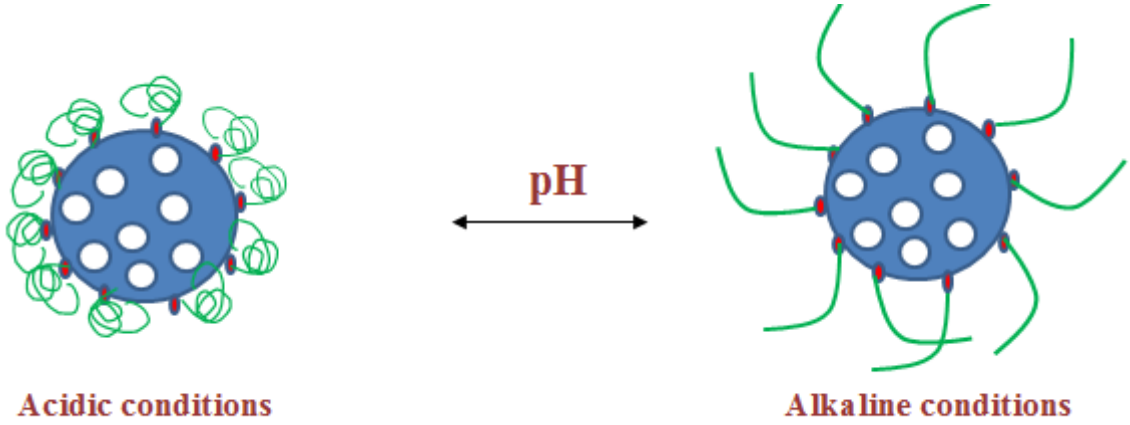
Figure 7.3: Basic structure of 4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid (CPA)



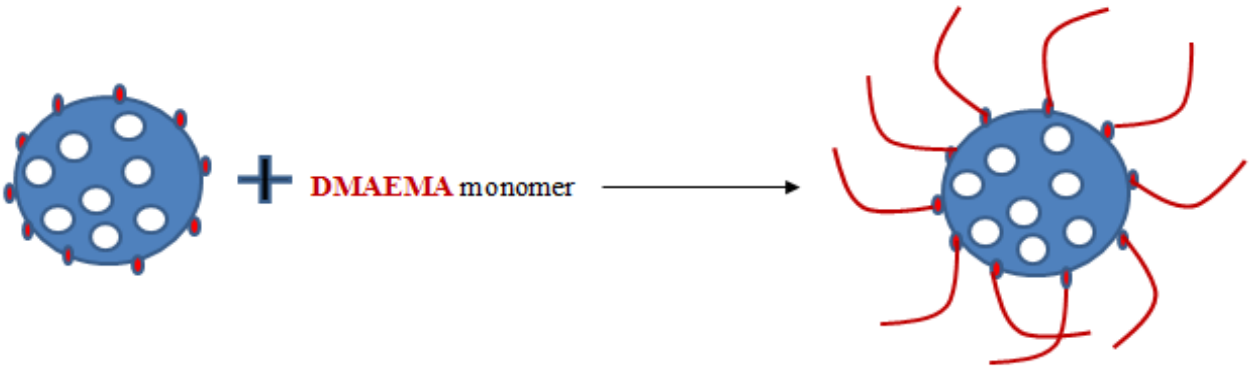
2. Grafting of **PMAA** brushes on the surface of the particles through the **CPA** RAFT groups



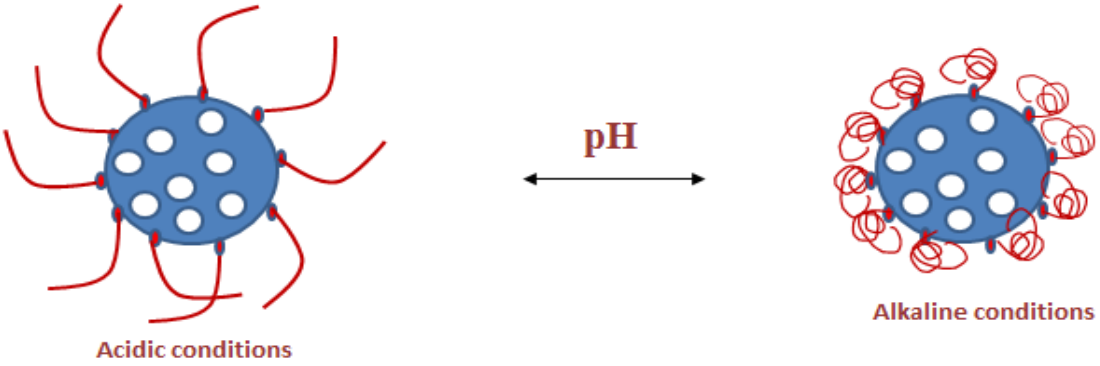
The resulting particles should be stimulated by pH change due to the swelling/collapsing of PMMA chains in alkaline/acidic conditions:



3. Grafting of **DMAEMA** brushes on the surface of the particles through the RAFT groups.



The resulting particles should be stimulated by pH change due to the swelling/collapsing of DMAEMA chains in acidic/alkaline conditions:



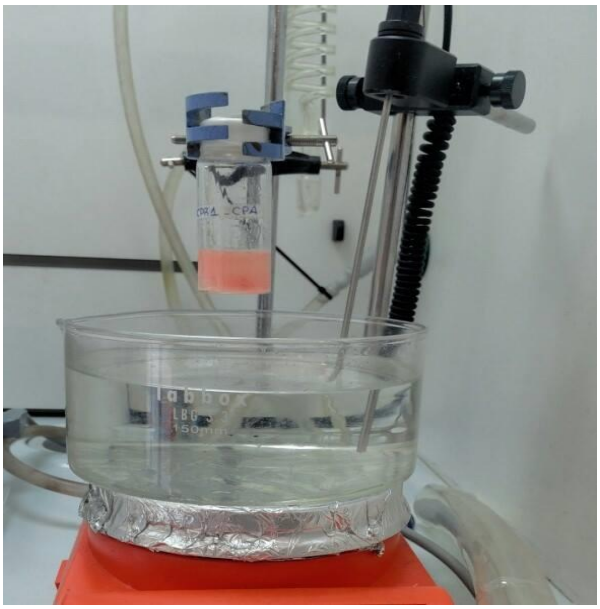


Figure 7. 4: Precipitation polymerization of the RAFT agent CPA.

4. Molecular Imprinting of polydatin with the system 4VP/EGDMA (precipitation polymerization in MeOH/H2O) in the presence of the RAFT agent CDTPA.

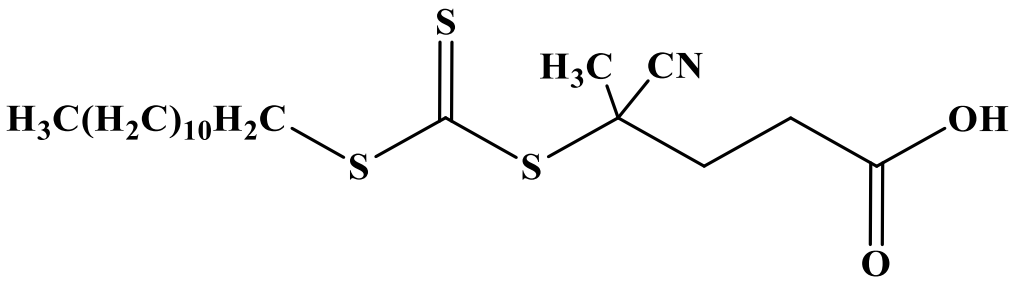
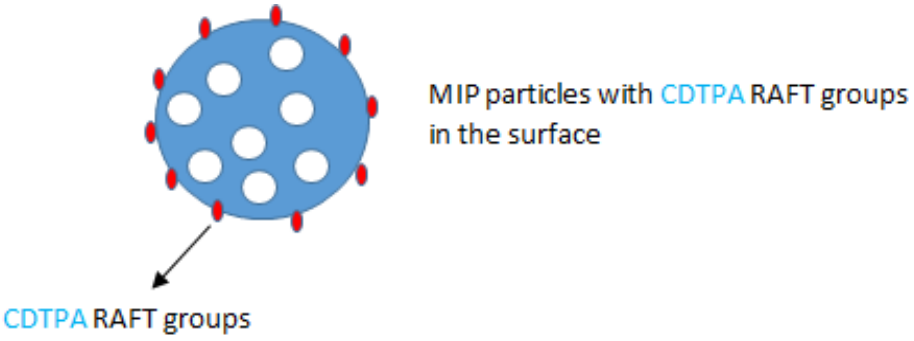
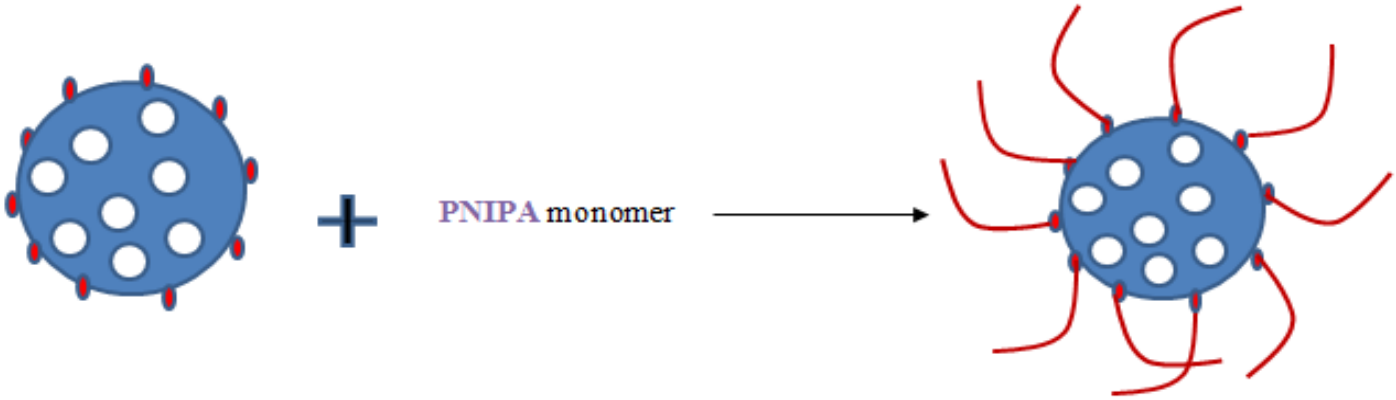


Figure 7. 5: Basic structure of 4-Cyano-4-[(dodecylsulfanylthiocarbonyl) sulfanyl] pentanoic acid (CDTPA)



5. Grafting of PNIPA brushes on the surface of the particles through the RAFT groups



The resulting particles should be stimulated by Temperature change due to the swelling/collapsing of PMMA chains in acidic/alkaline conditions:

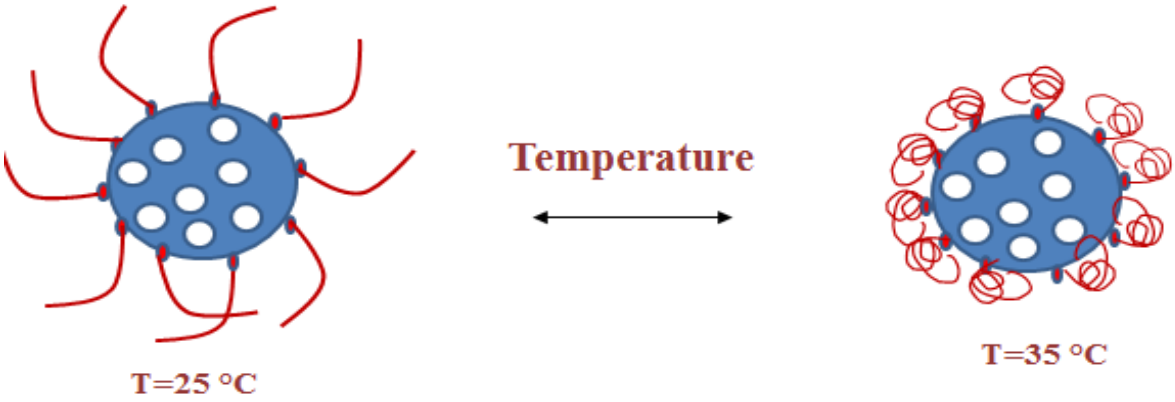


Figure 7.6: Precipitation polymerization performed presence of the RAFT agent CDTPA

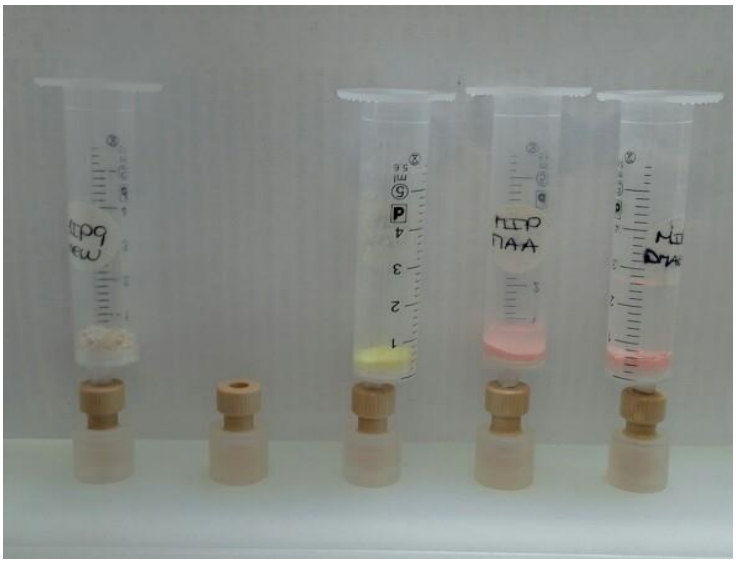


Figure 7.7: Solid phase extraction (SPE) of RAFT polymerization

Table 7.1: Experimental conditions used in the molecular imprinting of POL in Raft synthetized polymer particles considering 4VP as a functional monomer. Precipitation polymerization performed in H2O/MeOH (1/4) at 60°C using EGDMA as crosslinker and AIBN as a thermal initiator.

Material	Monomer (M)	Cross-linker (CL)	Template (T)	Initiator (I)	Solvent (S)	RAFT (R)	Y_M (%)	Y_I (%)	Y_{CL} (%)	$Y_{CL/T}$	$Y_{M/R}$	$Y_{M/I}$	$Y_{M/T}$
MIP	4VP	EGDMA	POL	AIBN	H_2O/MeOH	CPA	4.71	1.92	79.60	24.07	25.60	2.03	6.17
MIP	4VP	EGDMA	POL	AIBN	H_2O/MeOH	CDTPA	4.71	1.95	79.60	24.15	25.96	1.98	6.19

Table 7.2: Experimental conditions used in the particles surface RAFT grafting of functional brushes. Polymerization performed at 70°C using AIBN as a thermal initiato

Material	Monomer (M)	Initiator (I)	Solvent (S)	RAFT (R)	m particles (mg)	Y_M (%)	Y_I (%)	Y_P (%)	$Y_{R/I}$	$Y_{M/R}$
MIP	DMAEMA	AIBN	MeOH	CPA	150.3	9.0	0.07	1.25	3.25	428.32
MIP	MAA	AIBN	MeOH	CPA	150.2	9.0	0.07	1.25	3.32	430.45
MIP	NIPA	AIBN	DMF	CDTPA	180.2	9.6	0.07	1.26	3.43	399.45

7.5 Assessment of polydatin adsorption and release in MIP particles with surface grafted functional brushes

After cleaning and drying the MIP particles with surface grafted hydrophilic functional brushes, these materials were submitted to batch adsorption and release testing. Polydatin (the imprinted molecule) was used as a representative polyphenol is these experiments. Batch adsorption was considered at this preliminary stage due to the lower amount of adsorbent required and to avoid the possible leakage of very small particles (nanoparticles) through the filters when continuous processes are used (causing possible errors in the UV measurements). Water/acetonitrile 70/30 was used as solvent and the concentration of polydatin in the initial solution was settled C_0=0.02 mM. In order to study the eventual stimulation of the MIPs through the pH change, solutions at pH=3 and pH=10 were also considered in the experiments (HCl and NaOH were used to adjust the pH at the desired value). Stimulation of the particles by the temperature change was assessed by studding the retention and the release process at room temperature (~25 °C) and also at T=40 °C. In each experiment performed, 20 mg of adsorbent and 2 mL of solution were considered. The amount of polyphenol retained or release was estimated by measuring the UV absorption of the initial and final solutions.

In Figure 7.8 are presented the results obtained for the batch retention experiments performed with the MIP particles bearing different pH sensitive surface grafted functional brushes (MIP_MAA and MIP_DMAEMA). Similar amounts of polydatin are retained in both materials when a DI water solution is used but some differences are observed in the measurements performed at pH=3 and pH=10 (see Figure 7.8). At pH=3 a higher amount of polyphenol is adsorbed in the MIP_MAA material comparatively to the MIP_DMAEMA particles.

This order is inverted when the measurements are performed at pH=10. Thus, the effect of the pH on the retention of analyte seems to be clear. However, it is hard to work out an explanation for these results considering only the effect pH on the ionization of the functional brushes present at particles surface (p(MAA) brushes ionized in alkaline conditions and the reverse for p(DMAEMA)). Indeed, besides the ionization of the functional brushes, these observations should also include the effect of the ionization of the polydatin when pH is changed.

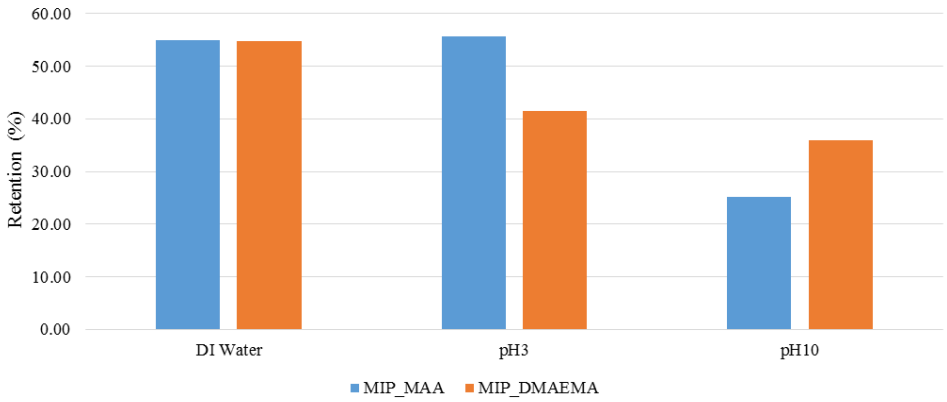


Figure 7.8: Results for the batch adsorption experiments performed with the MIP particles bearing different pH sensitive surface grafted functional brushes (MIP_MAA and MIP_DMAEMA). Water/ACN solutions containing polydatin at C0=0.02 mM were used. Besides the DI water solution, testing at pH=3 and pH=10 were also performed.

In Figure 7.9 are presented the results concerning the assessment of the effect of the temperature on the polydatin retention in the MIP particles bearing different kinds of surface grafted functional brushes, namely p(MAA), p(DMAEMA) and p(NIPA). For the MIPs with p(MAA) and p(DMAEMA) brushes a decrease on the adsorbed amount is measured when temperature is changed from ~25 to 40 °C. This observation is compatible with the low sensibility of these brushes to temperature changes and the well-known weakening of adsorption process when the temperature grows up (e.g. due the decay of hydrogen bonding). However, for the MIP bearing the temperature-sensitive polymer brushes (p(NIPA)), an increase in the adsorption amount was measured when temperature was raised from ~25 to 40 °C (see Figure 7.9). Thus, an additional impact on the effect of temperature on the retention process seems to be possible with the grafting of p(NIPA) brushes on the MIPs surface.

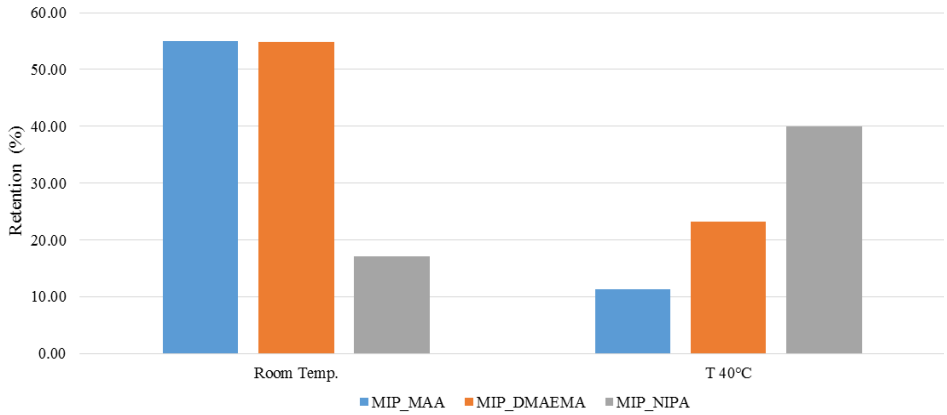


Figure 7.9: Results for the batch adsorption experiments performed with the MIP particles bearing different surface grafted functional brushes (MIP_MAA, MIP_DMAEMA and MIP_DMAEMA). Water/ACN solutions containing polydatin at C0=0.02 mM were used. Results concerni ning the effect of the temperature (T=25 and T=40 °C) on the adsorption of polydatin are here reported.

In Figure 7.10 are presented the results concerning release of polydatin from MIP particles preloaded with this polyphenol, according the description performed for the results presented in Figure 7.8. The effect of pH in the polyphenol release is also evidenced in the measurements presented in Figure 7.10. Nevertheless, a very small difference is observed when the two different kinds of materials (bearing p(MAA) or p(DMAEMA) brushes) are compared. However, a boost in the release process is reported at pH=10, which should be a consequence of the higher solubility of the polyphenol at alkaline conditions.

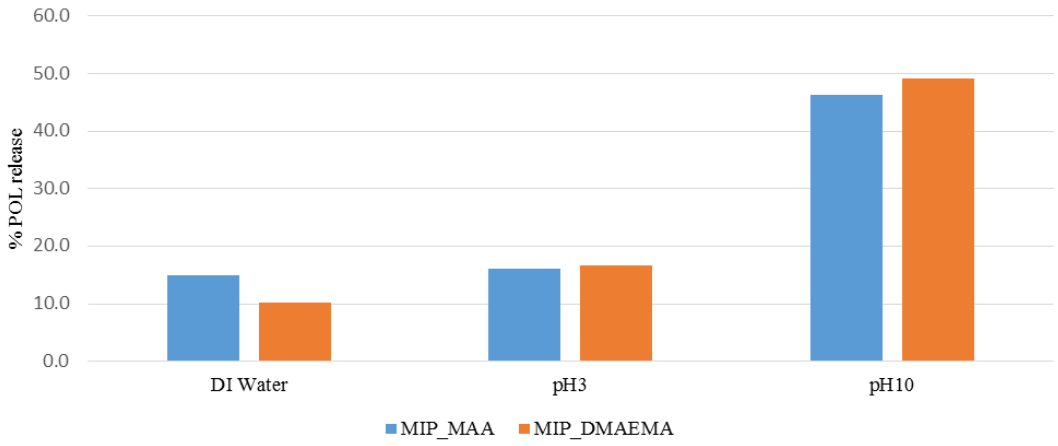


Figure 7.10: Results for the batch release of polydatin from preloaded MIP particles bearing different pH sensitive surface grafted functional brushes (MIP_MAA and MIP_DMAEMA). Release was performed in the same solvents used in the loading process, namely water/ ACN solutions with DI water (without adjusting the pH) and also with the adjustment of the pH at pH=3 and pH=10.

The study of the effect of the temperature on the release of the template molecule was also tried, as presented in Figure 7.11. A very high release amount was measured for the three materials at T=40 °C, which is consistent with the enhancement of the desorption process at high temperatures. At room temperature, low release amounts were observed with MIPs bearing p(MAA) or p(DMAEMA) brushes while a very high release of the polyphenol was estimated for the MIPs with p(NIPA) brushes. Again, the grafting of p(NIPA) brushes on the MIPs surface seems to have also an important impact on the release process. However, additional experimental work is needed in order to confirm these effects.

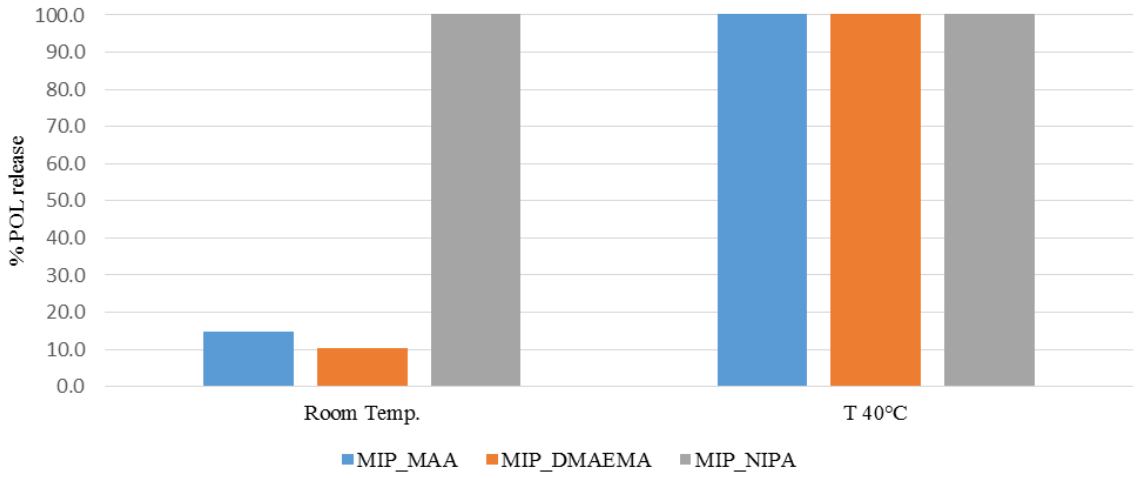


Figure 7.11: Results for the batch release of polydatin from preloaded MIP particles bearing different pH sensitive surface grafted functional brushes (MIP_MAA, DMAEMA and MIP_NIPA). Release was performed in the same solvents used in the loading process, namely wat er/CAN solution (without adjusting the pH) at T=25 and T=40 °C.

Globally, preliminary results here presented show that the grafting of hydrophilic polymer brushes on the MIPs surface, thus improving the amphiphilic character of the materials; modify the pH/temperature triggered retention/release of polyphenols. However, further studies are needed in order to clarify if the observed changes are only due to the grafting of the brushes. Indeed, the change of parameters such as pH and temperature modify the solution properties of polyphenols (e.g. solubility) which can also have an impact on the results obtained.

Chapter 8

8.1 Conclusion

This work was devoted to the development of amphiphilic molecularly imprinted polymers (MIPs) to target the stimulated uptake and release of polyphenols. In the first stage of the research, MIP particles were synthetized through a free radical (FRP) precipitation polymerization process in a mixture of acetonitrile (ACN) and methanol (MeOH). Trimethylolpropane triacrylate (TMPTA), a hydrophobic molecule, was used as crosslinker and different hydrophilic functional monomers were tried in order to obtain amphiphilic materials with particular features for the stimulated molecular recognition of polyphenols. Acrylic acid (AA), 2-(dimethylamino) ethyl methacrylate (DMAEMA) and N-vinylpyrrolidone (NVP) were specifically used as functional monomers in molecular imprinting experiments with polydatin as template. Anionic, cationic and neutral MIPs were sought using AA, DMAEMA and NVP as functional monomers, respectively. Polydatin was selected as template due to the hybrid characteristics of this molecule concerning the simultaneous presence of hydrophobic and hydrophilic domains.

Morphology of the products synthetized was analyzed by SEM and the formation of microparticles (e.g. with size in the order of 1 μm) was evidenced. However, it was showed that the morphology of the MIPs and NIPs (analogue non-imprinted materials) is strongly dependent on the monomers involved, possibly due to the phase separation phenomena during the network formation. Products were also analyzed through Fourier Transform Infrared (FTIR) spectroscopy and the total vinyl double bond (C=C) conversion as well as the incorporation of crosslinker and functional monomers in the networks can be confirmed.

The adsorption capabilities of the different MIPs and NIPs synthetized was evaluated using solid phase extraction (SPE) experiments with solvents of different amphiphilic character (e.g. ACN/MeOH or ACN/H2O). Different phenolic compounds were also considered in these studies, namely polydatin, resveratrol and gallic acid. Results obtained, namely the comparison of the MIP/NIP performances, showed that the non-specific interactions between the molecules and the materials prevail in the conditions tested.

The competitive adsorption of a mixture of polydatin + resveratrol + gallic acid in these MIP and NIP materials was also studied through the HPLC analysis of the different SPE eluted fractions. An Ascentis® C18 column was used within this purpose. Some selectivity of the materials towards the different molecules was observed but the most relevant result obtained was the ability of the materials based on DMAEMA to retain huge amounts of gallic acid. This capacity is due to the ionic interaction between the gallic acid (anionic specie) and the polymer network containing DMAEMA (cationic material). Hydrophobic interactions, namely in resveratrol retention, were also highlighted with these studies.

The use of the synthetized MIPs was also explored for the adsorption of phenolic compounds in continuous processes. MIP particles were packed in small HPLC columns and a continuous process with recycling was conceived through the use of an HPLC pump. The stable running of the created set-up during a long operation time was showed. Dynamics for the saturation and the release processes were thus measured. Note that this configuration can be especially useful for the retention, separation and concentration of phenolic compounds present in natural vegetable extracts. The results obtained show that this approach can be useful in practice if an optimized combination between adsorbents (one or more) and solvents (eventually in a gradient process) can be engineered in order to enhance the treatment of complex mixtures prevenient from natural extracts.

These issues were further enhanced through the analysis of the retention and release of phenolic compounds in a sequence of different MIPs. A series of two MIPs was considered in the framework of these studies, including the cationic material based on DMAEMA and an optimized material based on the 4-vinylpiridine functional monomer and synthetized by inverse-suspension. A solvent gradient scheme was used and these ideas were applied to a created mixture containing gallic acid, tannic acid, polydatin and resveratrol, as well as to two different natural extracts. Red wine (Portuguese Douro Region red wine) was considered as a possible source of phenolic compounds and an extract from chestnut shell is evaluated with the same purpose. This extract from chestnut shell was obtained through supercritical extraction with CO2, using fruits before collected at the Bragança region. This supercritical extraction with CO2 was performed at T=50 °C and P=150 bar. Results obtained evidenced that optimized MIPs can be undoubtedly used to separate and concentrate polyphenols present in red wine, such as resveratrol or polydatin. Spiking of natural extracts with these compounds and the analysis through HPLC in an Ascentis® C18 column were considered within this purpose. These findings were further enhanced through the adsorption and release of the gallic acid + tannic acid + polydatin + resveratrol in the series of MIPs. The usefulness of the synthetized materials to simplify (e.g. performing first the retention of the acidic phenolic compounds) and separate/concentrate some target polyphenols (e.g. polydatin and resveratrol) was showed.

Measurements for the shell chestnut extract obtained with supercritical conditions showed that phenolic compounds should not be present in an appreciable amount (in contrast with the red wine extract). Indeed, the ability of the MIPs to retain gallic acid, resveratrol and catechin when spiked in the chestnut extract was undoubtedly evidenced. However, almost nil retention was observed with the original extract. Different extraction techniques (e.g. extraction at alkaline supercritical conditions) should be considered in future researches in order to evaluate the effect of the conditions used on the composition of the extracts. Moreover, other parts of the chestnut plant (leaves, burs, stalks, etc) can be used with supercritical or conventional extraction.

In the last part of this research was addressed the synthesis of improved amphiphilic adsorbents through the grafting of functional brushes in the MIP particles surface. The RAFT polymerization mechanism was considered in the framework of these studies. In the first production step, the molecular imprinting was performed using a precipitation polymerization process in the presence of a RAFT agent (4-vinylpiridine was used as functional monomer and polydatin as template). The resulting MIP particles bear out RAFT groups in their surface that were reactivated in the second synthesis step. Thereafter, hydrophilic functional brushes were grafted out in the particles considering another RAFT polymerization reaction. Different kinds of polymer brushes improving compatibility with aqueous systems were considered within this purpose, namely poly (MAA), poly (DMAEMA) and poly (NIPA). Besides the improvement of the polar character of the particles (thus enhancing wettability of the materials), the stimulation of these brushes is possible through the change of parameters such as pH and/or temperature. The final materials were submitted to batch adsorption and release testing. For stimulation of the MIPs through the pH change, solutions at pH=3 and pH=10 were considered in these experiments. Stimulation of the particles by the temperature change was assessed by studding the retention and the release process at 25 and 40 °C. Results obtained show that the grafting of hydrophilic polymer brushes on the MIPs surface, thus improving the amphiphilic character of the materials; modify the pH/temperature triggered retention/release of polyphenols. However, further studies are needed in order to clarify if the observed changes are only due to the grafting of the brushes and/or other solution properties of polyphenols, such as the change of their solubility with pH and temperature.

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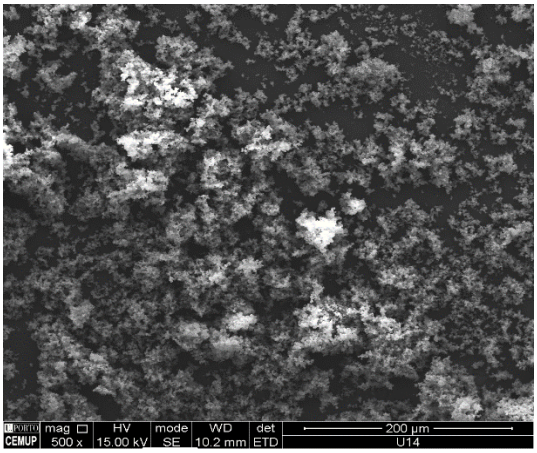
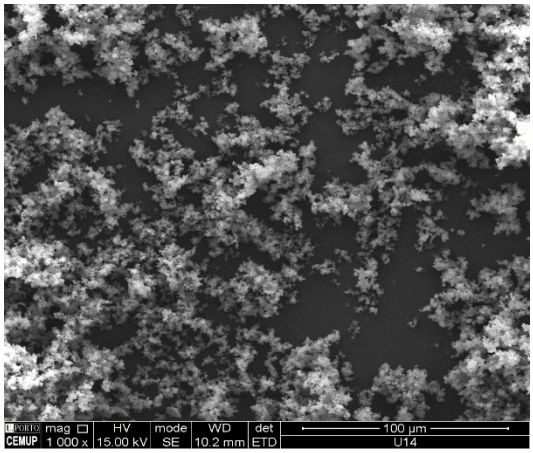
ANNEXES

ANNEX 1.

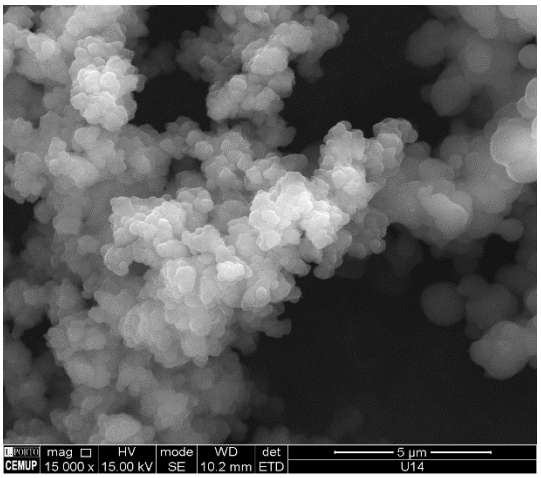
SEM micrographs of MIPs and NIPs produced a),b), c), d), e) MIP1, f), g), h), i), NIP1 obtained Free Radical Polymerization by batch where functional monomer was (AA).



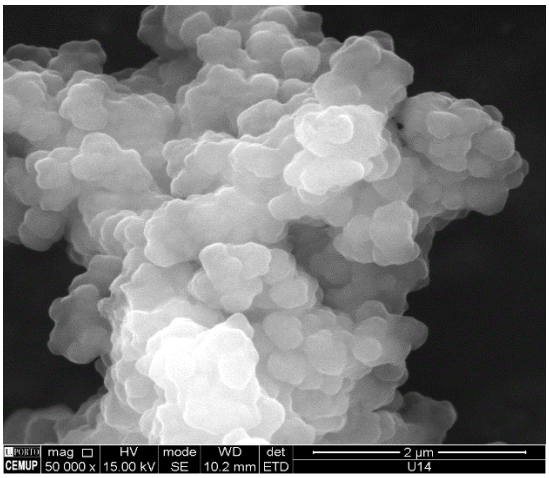
a



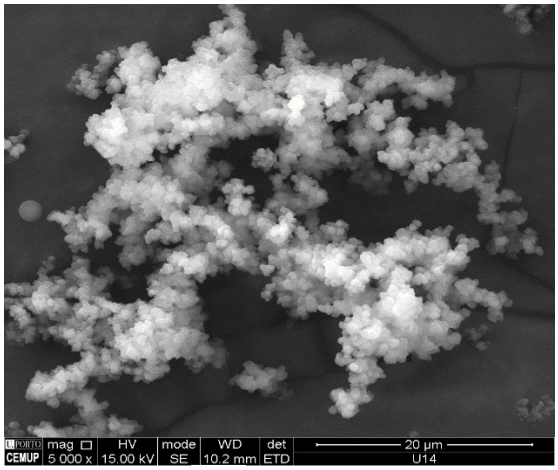
b



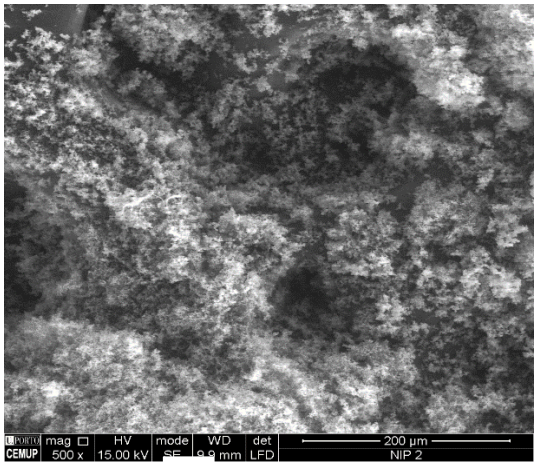
c



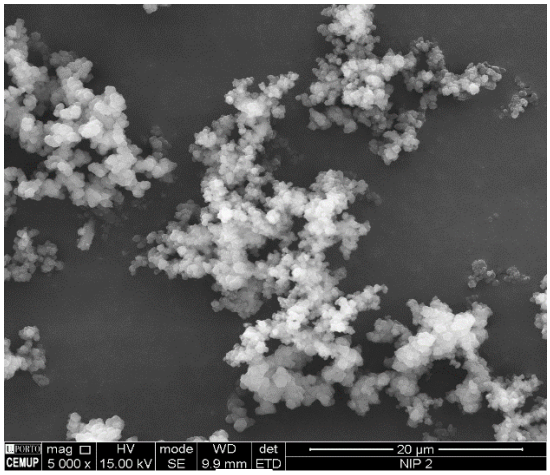
d



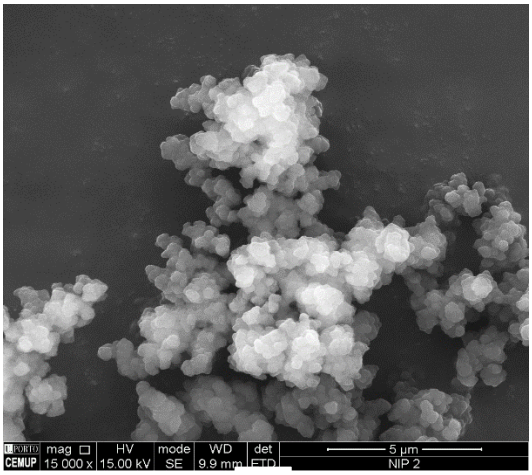
e



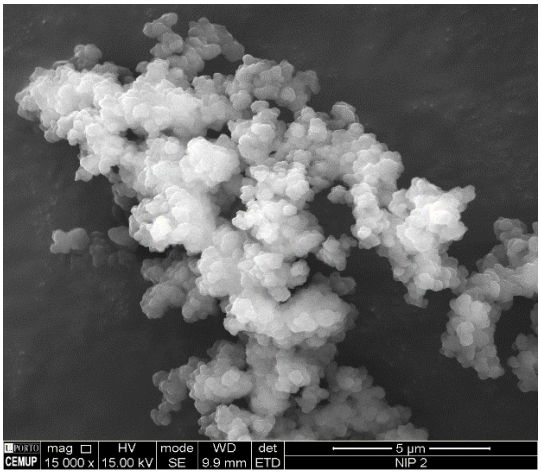
f



g



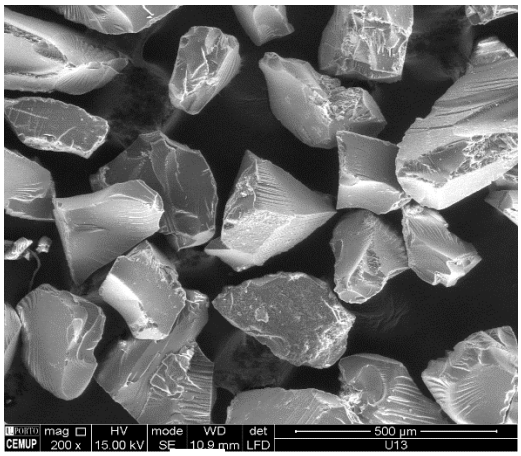
h



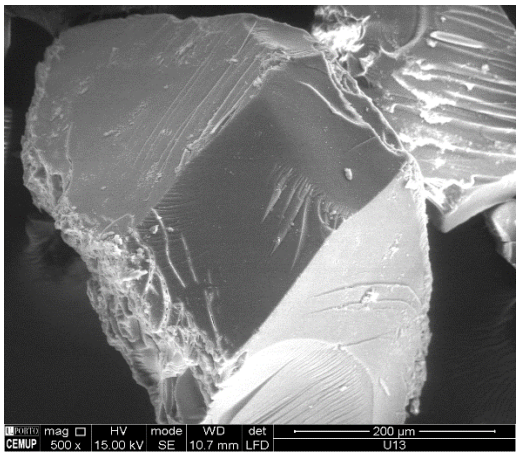
i

ANNEX 2.

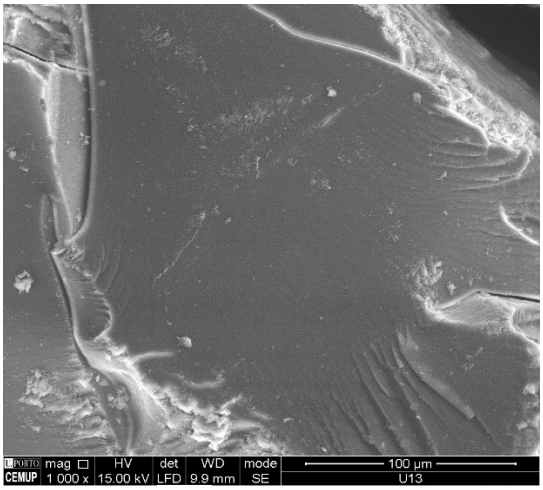
SEM micrographs of MIPs and NIPs produced a),b), c), d), e) MIP2, f), g), h), i), j) NIP2 obtained Free Radical Polymerization by batch where functional monomer was (DMAEMA).



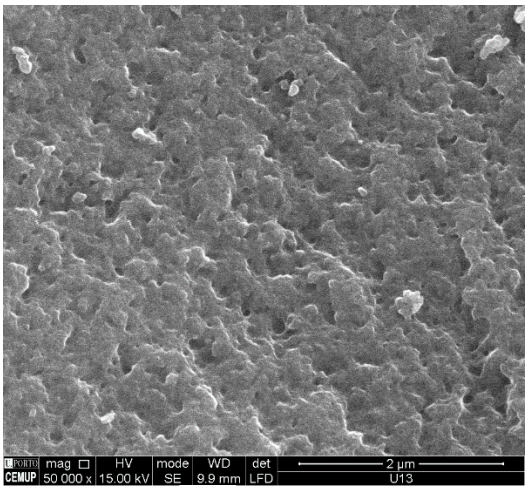
a



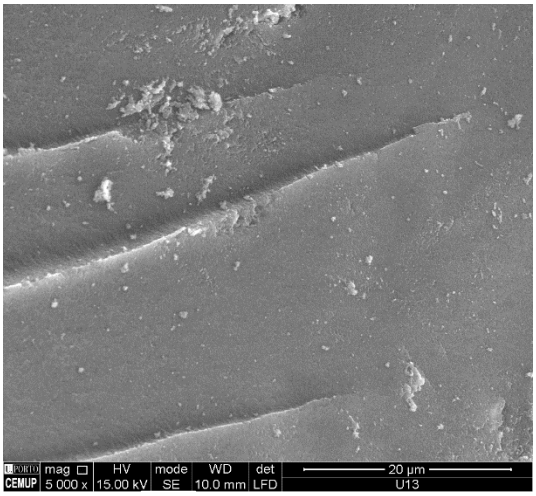
b


mag HV det WD mode
1 000 x 15.00 kV LFD 9.9 mm SE
100 µm
CEMUP
U13

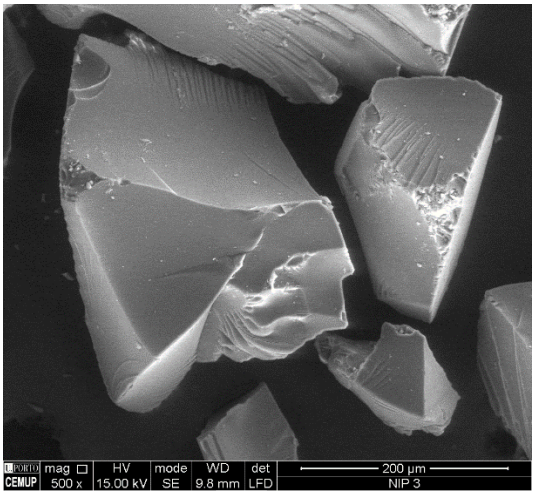
c


mag HV mode WD det
50 000 x 15.00 kV SE 9.9 mm LFD
2 µm
CEMUP
U13

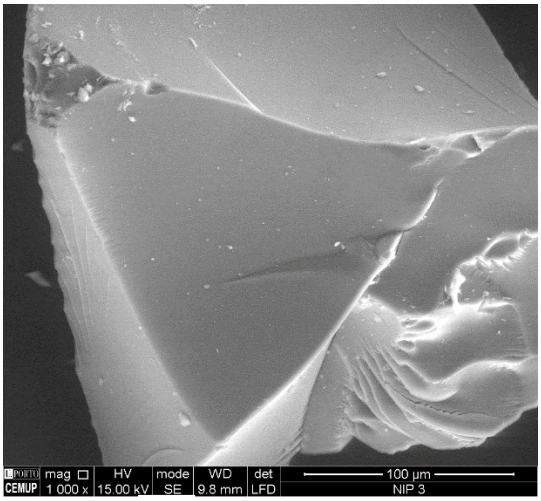
d


mag HV mode WD det
5 000 x 15.00 kV SE 10.0 mm LFD
20 µm
CEMUP
U13

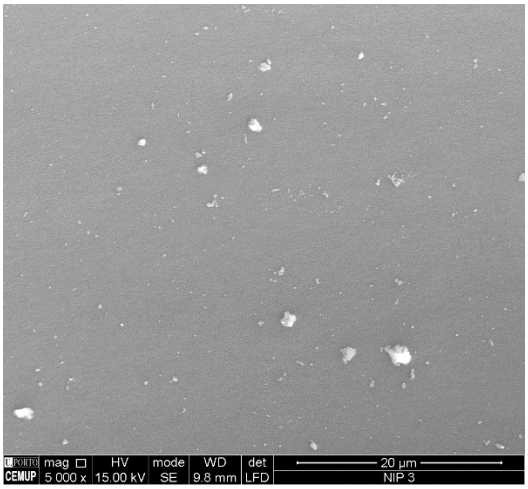
e


mag HV mode WD det
500 x 15.00 kV SE 9.8 mm LFD
200 µm
CEMUP
NIP 3

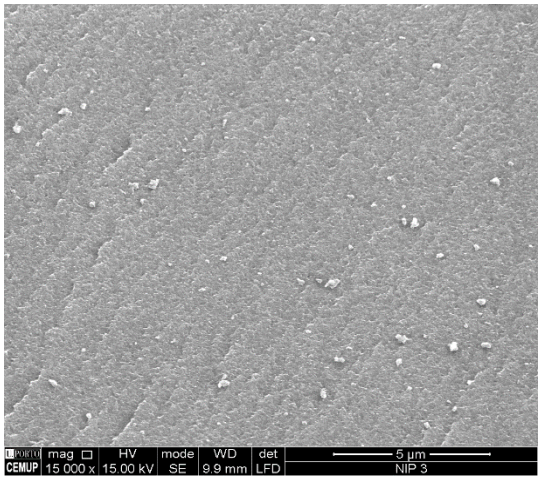
f


mag HV mode WD det
1 000 x 15.00 kV SE 9.8 mm LFD
100 µm
CEMUP
NIP 3

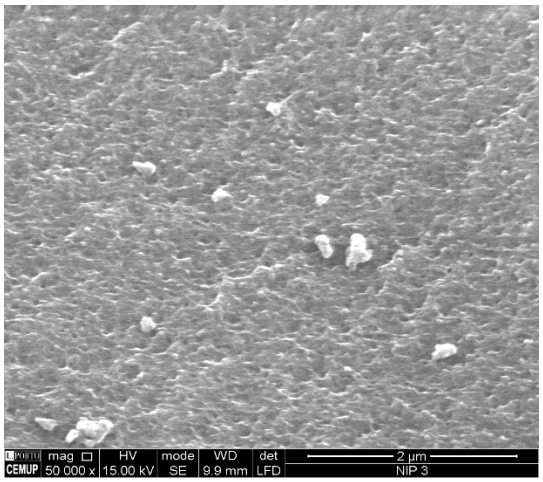
g


mag HV mode WD det
5 000 x 15.00 kV SE 9.8 mm LFD
20 µm
CEMUP
NIP 3

h



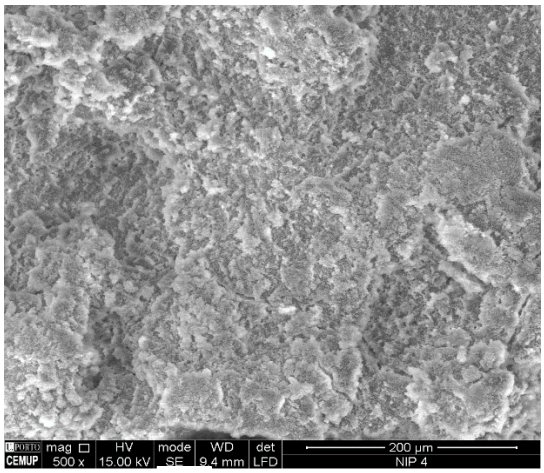
i



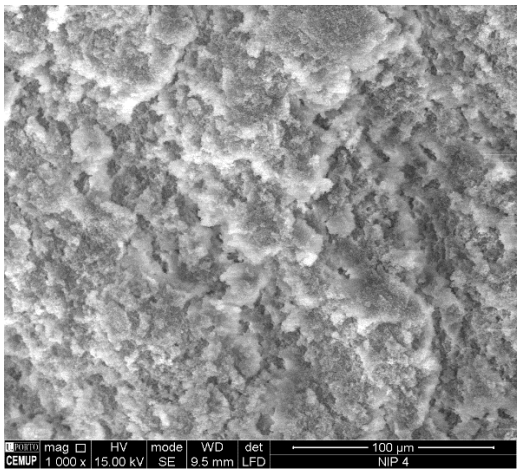
j

ANNEX 3

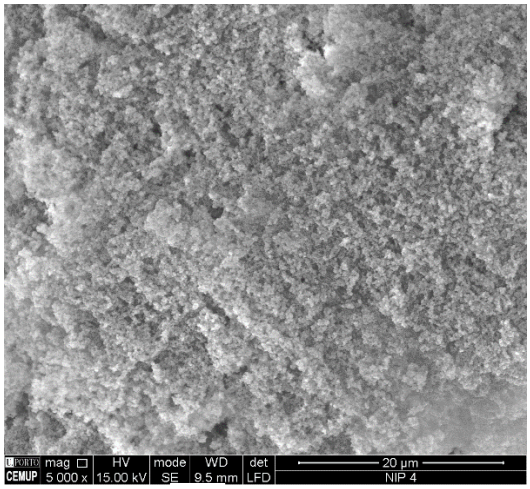
SEM micrographs of NIPs produced a), b), c), d), e) NIP3, obtained Free Radical Polymerization by batch where functional monomer was (NVP).



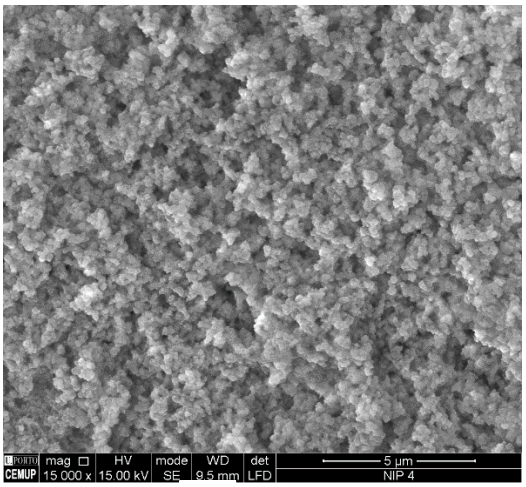
a



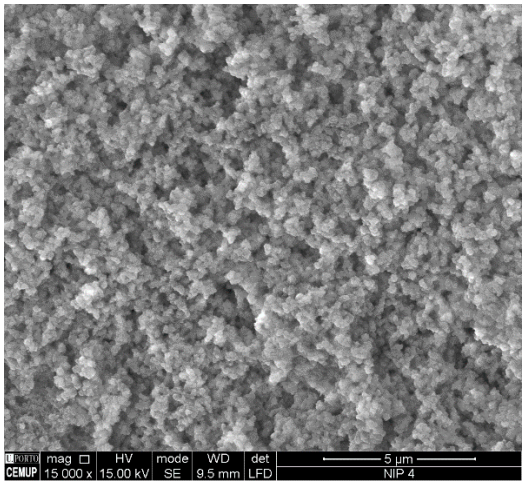
b



c



d



e

ANNEX 4

HPLC Analysis of the competitive SPE adsorption and release of the mixture polydatin+galic acid+resveratrol in ACN/MeOH 10/1.

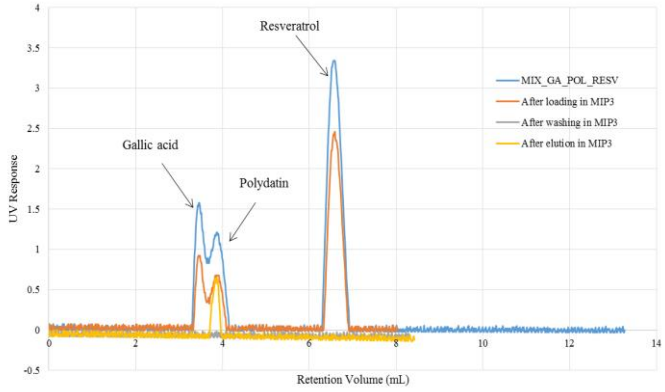


Figure 4.1: HPLC analysis of the competitive SPE adsorption and release of the mixture polydatin+galic acid+resveratrol in MIP3. ACN/MeOH 10/1 was use as first solvent.

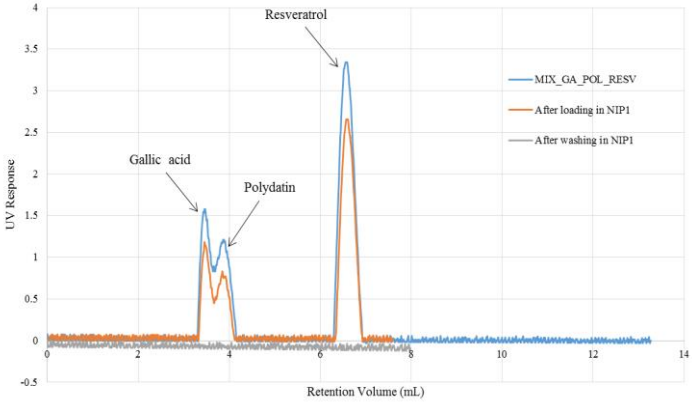


Figure 4.2: HPLC analysis of the competitive SPE adsorption and release of the mixture polydatin+galic acid+resveratrol in NIP1. ACN/MeOH 10/1 was use as first solvent.

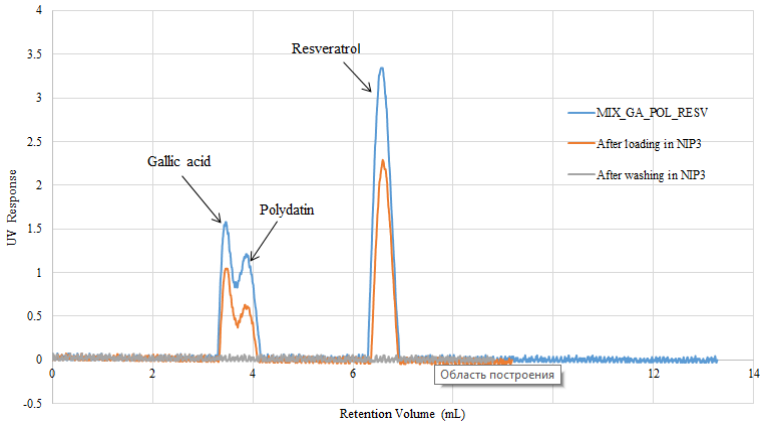


Figure 4.3: HPLC analysis of the competitive SPE adsorption and release of the mixture polydatin+galic acid+resveratrol in NIP3. ACN/MeOH 10/1 was use as first solvent

ANNEX 5

HPLC Analysis of the competitive SPE adsorption and release of the mixture polydatin+galic acid+resveratrol in ACN/H_2O 50/50.

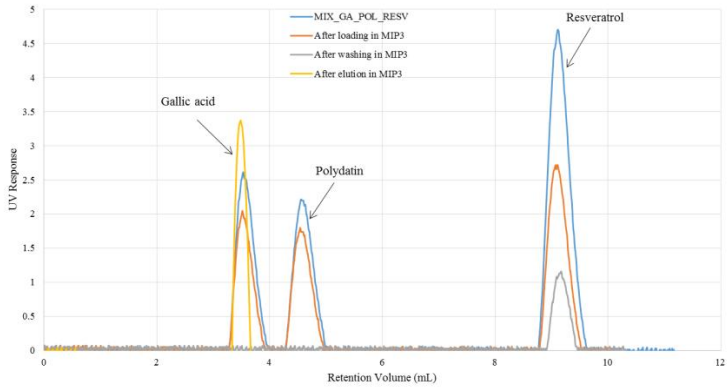


Figure 5.1: HPLC analysis of the competitive SPE adsorption and release of the mixture polydatin+galic acid+resveratrol in MIP3. ACN/H_2O 50/50 was use as first solvent.

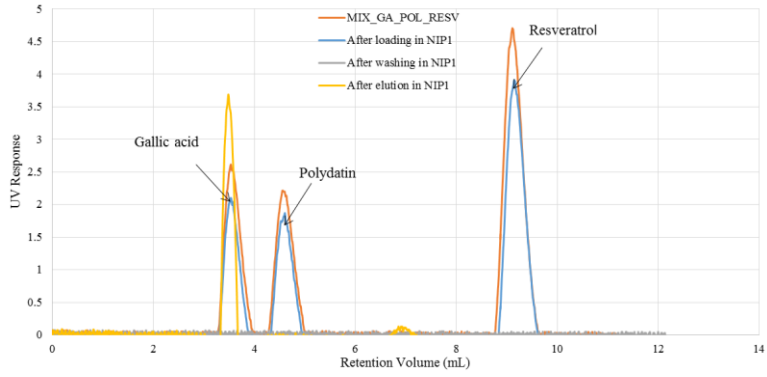


Figure 5.2: HPLC analysis of the competitive SPE adsorption and release of the mixture polydatin+galic acid+resveratrol in NIP1. ACN/H_2O 50/50 was use as first solvent.

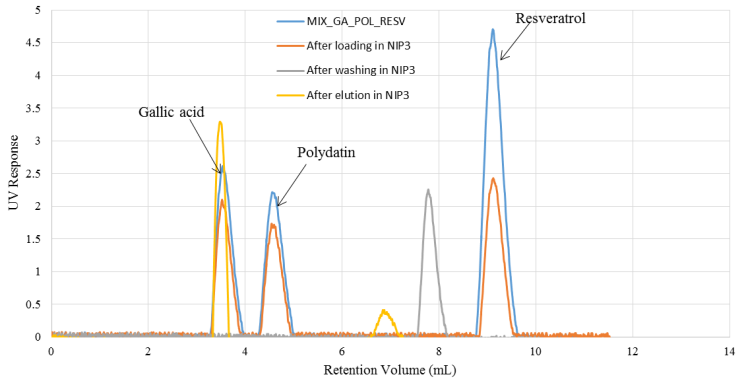


Figure 5.3: HPLC analysis of the competitive SPE adsorption and release of the mixture polydatin+galic acid+resveratrol in NIP3. ACN/H_2O 50/50 was use as first solvent.

ANNEX 6

FTIR spectra of the MIPs and NIPs with different functional monomers (AA, DMAEMA and NVP) and the crosslinker (TMTPA) used in the synthesis of the MIPs and NIPs.

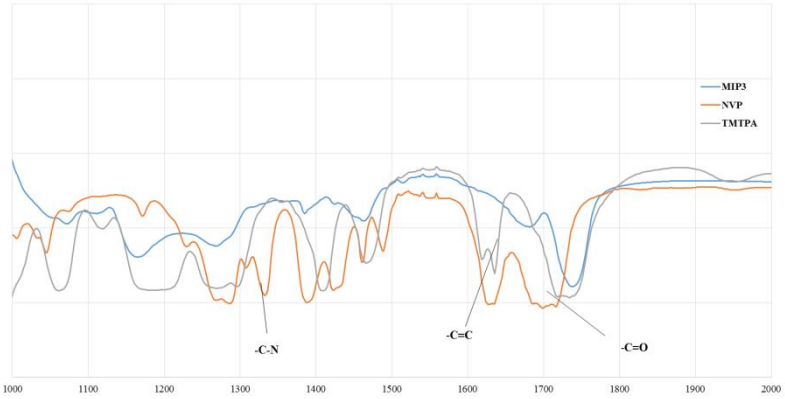


Figure 6.1: FTIR spectra of the MIP3, the functional monomer (NVP) and the crosslinker (TMTPA) used in the synthesis of the MIP3.

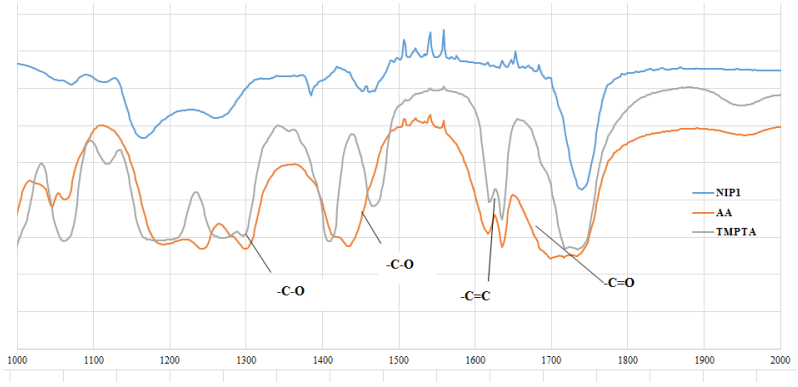


Figure 6.2: FTIR spectra of the NIP1, the functional monomer (AA) and the crosslinker (TMTPA) used in the synthesis of the NIP1.

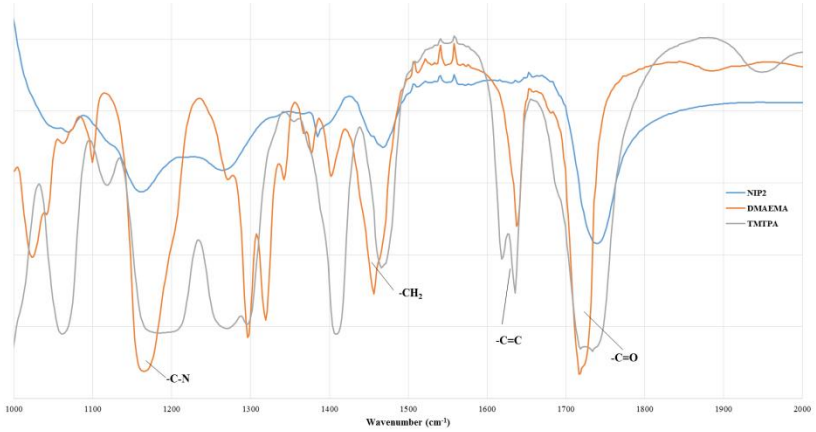


Figure 6.3: FTIR spectra of the NIP2, the functional monomer (DMAEMA) and the crosslinker (TMTPA) used in the synthesis of the NIP2.

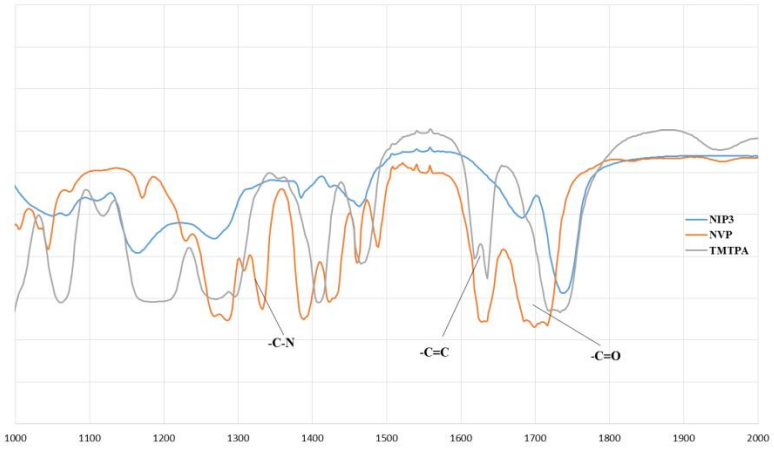


Figure 6.4: FTIR spectra of the NIP3, the functional monomer (NVP) and the crosslinker (TMTPA) used in the synthesis of the NIP3.