

**Design and synthesis of hybrid
natural-synthetic polymers via
controlled radical polymerization for
the valorization of olive tree and vine
sub-products**

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A dissertation submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy in Chemical and Biological Engineering

At the Faculty of Engineering, University of Porto

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march 2026

*“Que nunca vos falte a coragem de acreditar que podem ser o que
quiserem.”*

Aos meus filhos, Constança & Afonso.

This work was funded by the **Foundation for Science and Technology (FCT, Portugal)** through the **PhD scholarship 2020.06057.BD**. We are grateful to the **Foundation for Science and Technology (FCT, Portugal)** for financial support through national funds: **FCT/MCTES (PIDDAC)** to **CIMO (UIDB/00690/2020 and UIDP/00690/2020)** and **SusTEC (LA/P/0007/2020 the support by LA/P/0045/2020 (ALiCE), UIDB/50020/2020, and UIDP/50020/2020 (LSRE-LCM)**, funded by national funds through FCT/MCTES (PIDDAC). We also acknowledge the support through to the project “**BacchusTech-Integrated Approach for the Valorization of Winemaking Residues**” (POCI-01-0247-FEDER-069583), supported by the **Competitiveness and Internationalization Operational Program (COMPETE 2020)**, under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF). and **OLEAF4VALUE** project This project has received funding from the Bio-Based Industries Joint Undertaking under the European Union’s Horizon 2020 research and innovation programme under **grant agreement n° 101023256**.



Acknowledgments

I would like to express my deepest gratitude to my supervisor, Professor Rolando Dias, for his invaluable guidance, encouragement, and continuous support throughout these years of work. His scientific expertise and critical insights were fundamental to the successful completion of this thesis. These were years of hard work, but also of camaraderie, which greatly contributed to the success of this project. Your guidance added depth and value to every step of this journey.

I also sincerely thank my co-supervisor, Professor Mário Rui Costa, for his availability, constructive discussions, and constant motivation.

I would like to sincerely thank LSRE-LCM and CIMO for providing the ideal conditions and unwavering support that enabled the successful development of this PhD work.

I am especially grateful to my friends and colleagues at CIMO, for their collaboration, stimulating conversations, moments of laughter, and for creating such a supportive and inspiring working environment. Thank you for being an integral part of this journey.

I would like to express my heartfelt gratitude to my parents, brothers, sisters in law and nephews for their unconditional love, constant encouragement, and endless patience. Your values, support, and belief in me have been the foundation of this achievement. Mum and Dad, without your sacrifices and guidance, this journey would not have been possible. This work belongs as much to you as it does to me.

Most importantly, I dedicate this work to my husband and my children. To my husband, thank you for your love, patience, and unwavering support through every step of this journey. To my children, you are my greatest source of strength and inspiration, giving true meaning to this achievement.

Finally, to my family and friends, thank you for believing in me and for standing by my side during this challenging yet rewarding path.

Abstract

The valorization of agricultural sub-products is essential for the development of sustainable biorefining processes within the circular bioeconomy. This thesis explores the design of novel hybrid polymeric materials via controlled radical polymerization (ATRP and RAFT), to be applied as adsorbents and delivery vehicles for the recovery of high-value compounds present in agricultural sub-products. In the first part, are described non-imprinted cellulose-synthetic hybrid adsorbents incorporating poly(4-vinylpyridine) prepared by ATRP, showing strong monomer-template interactions enabling the selective purification of anthocyanins, flavonoids, and secoiridoids from olive, wine, and almond extracts. The second part focused on enhancing material selectivity through surface molecular imprinting, yielding hybrid particles with quercetin specific recognition sites tailored for olive sub-products. These adsorbents displayed improved selectivity (imprinting factor ~ 2.6) and enabled the enrichment of luteolin-7-O-glucoside, apigenin-7-O-glucoside, and quercetin from olive leaf extracts.

The third part describes the extension of this strategy to vine sub-products, for which imprinted hybrid adsorbents demonstrated strong polyphenol binding (IF ~ 8), even in hydroalcoholic solvents, and enabled efficient fractionation of flavonols and anthocyanins, with enrichment factors of up to $5\times$ for quercetin and $12\times$ for quercetin-3-O-glucuronide. In parallel, a proof of concept for process scale-up was established using fully synthetic tailored adsorbents, confirming the feasibility of translating laboratory-scale developments into continuous and larger-scale operations.

Finally, the fourth part addressed the synthesis of amphiphilic copolymers via ATRP and RAFT, combining hydrophobic oleic acid-derived blocks p(GMA-OA) with hydrophilic poly(2-(dimethylamino)ethyl methacrylate) segments. These block copolymers exhibited pH and temperature responsive self-assembly, highlighting their potential in controlled drug release and smart functional materials.

Overall, the results demonstrate the feasibility of integrating advanced polymer engineering with sustainability principles, providing new strategies for the valorization of agricultural sub-products and the design of multifunctional hybrid materials.

Keywords: Controlled radical polymerization (ATRP, RAFT); Hybrid cellulose–synthetic materials; Molecular imprinting; Polyphenol recovery; Winemaking sub products; Olive oil sub products; Sorption/desorption processes; Pilot-scale biorefining; Amphiphilic block copolymers; Oleanolic acid delivery; Circular bioeconomy; Sustainable materials.

Resumo

A valorização de sub-produtos agrícolas é essencial para o desenvolvimento de processos de bio refinação sustentáveis no âmbito da bioeconomia circular. Esta tese explora o desenvolvimento de novos materiais poliméricos híbridos obtidos por polimerização radicalar controlada (ATRP e RAFT), para serem aplicados como adsorventes e veículos de liberação para a valorização de compostos de elevado valor acrescentado presentes nos resíduos agrícolas. Na primeira parte, descreve-se a preparação de adsorventes híbridos celulose-sintético não impressos, incorporando poli(4-vinilpiridina) via ATRP, que apresentaram fortes interações monómero-molécula modelo, permitindo a purificação seletiva de antocianinas, flavonoides e secoiridóides a partir de extratos de oliveira, vinha e amêndoa. A segunda parte centrou-se na descrição da melhoria da seletividade dos materiais através de impressão molecular superficial, originando partículas híbridas com sítios de reconhecimento específicos para a quercetina, desenvolvidas para subprodutos da oliveira. Estes adsorventes apresentaram boa seletividade (fator de impressão ~2,6) e permitiram o enriquecimento de luteolina-7-O-glicosídeo, apigenina-7-O-glicosídeo e quercetina a partir de extratos de folhas de oliveira. Na terceira parte mostra-se a aplicação desta estratégia aos subprodutos da vinha, onde adsorventes híbridos impressos demonstraram forte ligação a polifenóis (fator de impressão ~8), mesmo em solventes hidroalcoólicos, permitindo um eficiente fracionamento de flavonóis e antocianinas, com fatores de enriquecimento até 5× para quercetina e 12× para quercetina-3-O-glucuronídeo. Em paralelo, foi estabelecida uma prova de conceito para a ampliação do processo com adsorventes totalmente sintéticos, confirmando a sua viabilidade e a transposição dos desenvolvimentos laboratoriais para operações contínuas e em maior escala. Por último a quarta parte abordou a síntese de copolímeros anfifílicos via ATRP e RAFT, combinando blocos hidrofóbicos derivados do ácido oleico p(GMA-OA) com segmentos hidrofílicos de poli(2-(dimetilamino)etil metacrilato). Estes copolímeros em bloco mostraram autoagregação responsiva a pH e temperatura, evidenciando o seu potencial em sistemas de liberação controlada de fármacos e no desenvolvimento de materiais funcionais inteligentes. De forma geral, os resultados demonstram a viabilidade de integrar a engenharia polimérica avançada com princípios de sustentabilidade, oferecendo novas estratégias para a valorização de subprodutos agrícolas e para o design de materiais híbridos multifuncionais.

Palavra-chave: Polimerização radicalar controlada (ATRP, RAFT); Materiais híbridos celulose-sintético; Impressão molecular; polifenóis; subprodutos da vinificação; subprodutos da produção de azeite; Processos de sorção/dessorção; Biorrefinação em escala piloto; Copolímeros em bloco anfifílicos; liberação de ácido oleanólico; Bioeconomia circular; Materiais sustentáveis

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List of Abbreviations

2MI	– 2-Methylimidazole
4VP	– 4-Vinylpyridine
ACN	– Acetonitrile
AcOH	– Acetic Acid
AIBN	– 2,2-Azobis(2-methylpropionitrile)
AN	– Acrylonitrile
ATRP	– Atom Transfer Radical Polymerization
BET	– Brunauer–Emmett–Teller
BIBB	– α -Bromoisobutyryl Bromide
BPY	– 2,2-Bipyridine
BT	– Bath Temperature Sensor
C	– Catalyst
C18	– Octadecylsilane
CE	– Circular Economy
CPDT	– 2-Cyano-2-propyl Dodecyl Trithiocarbonate
CRP	– Controlled Radical Polymerization
CuBr	– Copper(I) Bromide
DAD	– Diode Array Detector
DBZT	– S,S-Dibenzyl Trithiocarbonate
DCC	– Dicyclohexylcarbodiimide
DE	– Diatomaceous Earth
DMAEMA	– 2-(Dimethylamino)ethyl Methacrylate
DMAP	– 4-(Dimethylamino)pyridine
DMF	– N,N-Dimethylformamide
DMSO	– Dimethyl Sulfoxide
DPPH	– 2,2-Diphenyl-1-picrylhydrazyl
DVB	– Divinylbenzene
EC50	– Half Maximal Effective Concentration
EF	– Enrichment Factor

EGDMA – Ethylene Glycol Dimethacrylate

EBrP – Ethyl 2-bromopropionate

ESI-MSn – Electrospray Ionization Tandem Mass Spectrometry

EtOAc – Ethyl Acetate

EtOH – Ethanol

FM – Functional Monomer

FRP – Free Radical Polymerization

FTIR – Fourier Transform Infrared Spectroscopy

GAE – Gallic Acid Equivalents

GMA – Glycidyl Methacrylate

GPC – Gel Permeation Chromatography

GP – Grape Pomace

HEMA – 2-Hydroxyethyl Methacrylate

HPLC – High-Performance Liquid Chromatography

HPLC-DAD – High-Performance Liquid Chromatography with Diode Array Detection

I – Initiator

ICP-MS – Inductively Coupled Plasma–Mass Spectrometry

IF – Imprinting Factor

INL – International Iberian Nanotechnology Laboratory

KBr – Potassium Bromide

L – Ligand

LALS – Low Angle Light Scattering

LabVIEW – Laboratory Virtual Instrument Engineering Workbench

LC-DAD-ESI-MSn – Liquid Chromatography with Diode Array Detection and Electrospray Ionization Tandem Mass Spectrometry

LC-MS – Liquid Chromatography–Mass Spectrometry

M – Monomer

MALLS – Multi-Angle Laser Light Scattering

MCC – Microcrystalline Cellulose

MCC-Br – Brominated Microcrystalline Cellulose (ATRP Macroinitiator)

MeOH – Methanol

MIP – Molecularly Imprinted Polymer

Mn – Number-Average Molecular Weight
 MOA – Methacrylated Oleic Acid
 mPEG – Methoxy Poly(ethylene glycol)
 mPEG-Br – Brominated Methoxy Poly(ethylene glycol)
 Mw – Weight-Average Molecular Weight
 MWCO – Molecular Weight Cut-Off
 MWD – Molecular Weight Distribution
 NIP – Non-Imprinted Polymer
 NMP – Nitroxide-Mediated Polymerization
 OA – Oleic Acid
 OAP – Oleic Acid-Modified Copolymer (p(GMA-OA)-b-p(DMAEMA))
 OAW – Organic Agricultural Waste
 ODS – Octadecylsilane
 P – Product
 PAN – Polyacrylonitrile
 PC – Phenolic Compounds
 P(4VP) – Poly(4-vinylpyridine)
 PDI – Polydispersity Index
 PMDETA – N,N,N',N'',N''-Pentamethyldiethylenetriamine
 RAFT – Reversible Addition–Fragmentation Chain Transfer (Polymerization)
 RALS – Right Angle Light Scattering
 RDRP – Reversible-Deactivation Radical Polymerization
 RI – Refractive Index
 RNS – Reactive Nitrogen Species
 ROS – Reactive Oxygen Species
 S – Solvent
 SEC – Size Exclusion Chromatography
 SEM – Scanning Electron Microscopy
 SI-ATRP – Surface-Initiated Atom Transfer Radical Polymerization
 SPE – Solid-Phase Extraction
 TBARS – Thiobarbituric Acid Reactive Substances
 TEA – Triethylamine

TEM – Transmission Electron Microscopy

THF – Tetrahydrofuran

TPC – Total Phenolic Content

UPLC – Ultra-Performance Liquid Chromatography

UV – Ultraviolet

UV-vis – Ultraviolet–Visible Spectroscopy

WT – Waste-to-Value

Chapter 1. General Information

1.1 Context and motivation

Growing concerns over environmental sustainability, climate change and resource depletion have intensified the search for renewable, eco-friendly alternatives to conventional, fossil-based materials. While agriculture is essential to global economies and food systems, it generates significant amounts of underutilized biomass, that can be called sub-products. In Mediterranean regions, two major crops, olive trees and vines are responsible for producing large quantities of agro-industrial sub-products, such as olive pomace, pits, leaves, grape marc, stalks and pruning residues. Often treated as waste or used in low-value applications such as animal feed or combustion, these materials are rich in valuable bio compounds such as polyphenols, lignin, cellulose, and antioxidants. Efficiently valorizing these sub-products could contribute to a more circular and sustainable agricultural economy.

In parallel, polymer science is undergoing a transformation toward greener materials and processes. The demand for bio-based, biodegradable and functional polymers that reduce environmental impact without compromising performance is increasing. Among the promising strategies is the development of hybrid natural-synthetic polymers, which combine the renewability of natural polymers with the functionality of synthetic polymers. However, designing such systems presents challenges in terms of compatibility, stability, and control over molecular architecture.

Controlled Radical Polymerization (CRP) techniques, especially Atom Transfer Radical Polymerization (ATRP) and Reversible Addition Fragmentation Chain Transfer (RAFT) have emerged as powerful tools to address these challenges. CRP allows precise control over polymer chain length, composition, and structure, enabling the synthesis of advanced polymeric architectures such as block, graft, and star copolymers. These methods are particularly suitable for incorporating natural compounds (e.g., modified lignin, polyphenols, or bio-based monomers) into well-defined polymer structures.

This PhD thesis aims to bridge the gap between natural molecules and synthetic polymers through the CRP, namely ATRP and RAFT based synthesis of hybrid materials, with the ultimate goal of valorizing (separate, concentrate, purify, encapsulate and release) bioactive compounds present in olive and vine residues. By integrating natural components into functionalized synthetic polymer, the aim is to develop novel, high-value materials with potential applications as adsorbents and delivery vehicles. This approach adds value to agricultural sub-products stream and contribute to the

development of sustainable material systems that align with the principles of the circular economy and environmental stewardship.

1.2 Bio circular economy

“Doing more and better with less” it’s how United Nations define the sustainable consumption and production to contribute for a sustainable development [1]. Indeed, in order to have sustainable production it is necessary to advocate a more sustainable consumption without a radical change of the consumer’s behavior. Thereby, the popular approach of Circular Economy (CE), plays an important role in creating more sustainable business.

Besides, by all the definitions of CE, the aim is to avoid and minimize product and resource consumption through multiple material loops [2]. The value of products and materials should be preserved for as long as possible, such as minimizing the waste or using them to create value-added products, according to the CE concept. Thus, following CE approach it is possible to create new and more sustainable processes, in order to meet the consumer’s needs[3].

In recent years, due to globalization and population growth, the consumption of food and vegetables has increased, generating an expansion in agricultural production. Consequently, this growth results in the expansion of the generation of organic agricultural waste (OAW). It is estimated that about 1300 million tons of agriculture waste is produced annually[4]. Examples of OAW include peels, fruit trimming, stalks, shells and forestall waste. Generally, this waste is accumulated in landfill, creating controversial consequences for the environment, instead of being reintroduced into the production chain with a novel purpose. To promote a CE approach in food sector, one aspect that deserves attention is the recovering and reusing of the OAW. The valorization of these wastes and sub-products of the food supply chain is becoming an outstanding sustainability issue in agriculture, as it yields multiple environmental, economic, and social benefits [5].

One of the challenges of agri-food research in this field is the focus on the reuse of waste and sub-products to increase the nutritional power of food or increase their durability [6]. These novel products are defined by Ascheman et al. [7] as waste-to-value (WT) products, to the circular approach that transforms wastes or surplus products, obtained during processing of others foods/products, into new value-added products [8].

Olive oil production illustrates this challenge well, with Spain alone generating an estimated 1.25 million tons of olive leaf waste each year, representing about 50% of global production [9]. This biomass contains high added value bioactive compounds (polyphenols, triterpenoids, essential oils, lipids, lignocellulose) with high market potential (food, feed, chemical, nutraceutical, cosmetic and pharmaceutical). However, it represents a problem for both the farmers and the whole olive oil industry, which needs to remove it from the fields and the olive oils mills. Nowadays, this biomass is still underexploited, being burnt in the fields, used as animals feed or burned to produce energy. Only a very reduced portion of olive leaves is used as starting material for the production of olive leaf extracts. Another example in this field is wine production. This latter is one of the most important agricultural activities around the world and consequently one that produces a huge number of sub products [10] (e.g. stalks, grape pomace, seeds, lees and wine pruning). However, some of this residue already has several uses and applications, such as feedstock, valuable products, fertilizer and compost as is shown in **figure 1-1**. This model is only an example how CE can be applied to industrial processes and how OAW can be recycled in a profitable way, while being respectful to the environment.

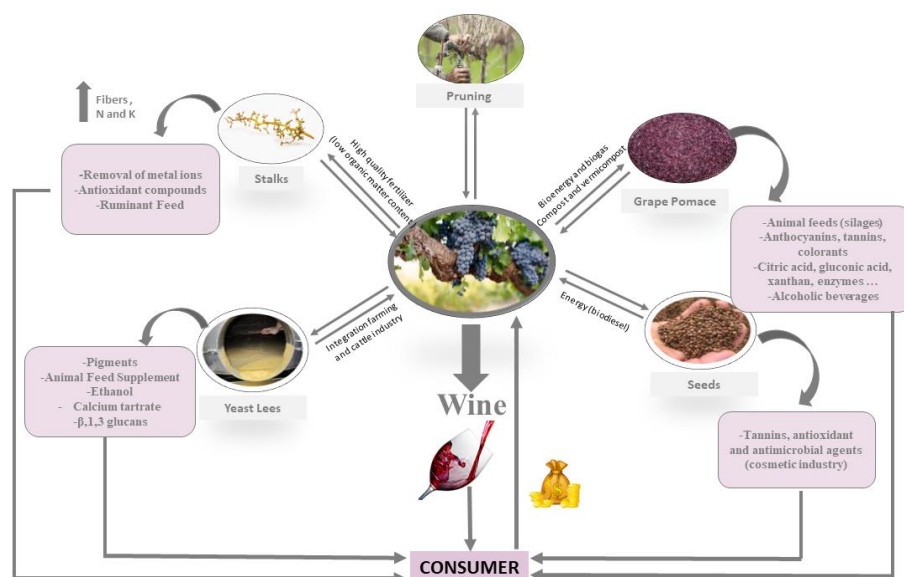


Figure 1-1: Diagram of the circular economy applied to wine industry sub-products. [Adapted from [11]]

In recent years, the implementation of regulations concerning the disposal of olive and wine agro-industrial (OWA) sub-products, combined with the rising consumer demand for sustainable and healthier products, has encouraged industries particularly in the food, cosmetic, and pharmaceutical sectors to transition towards more eco-friendly strategies.

These sub products are well recognized for their richness in phenolic compounds, making their valorization an attractive approach for biomass exploitation and the production of high value-added products.

1.3 Valorization of agriculture sub-products as a source of antioxidant phenolic compounds

Phenolic compounds (PC) are organic compounds produced by plants to provide them protection to different kinds of attacks such as UV radiation, parasites, insect pests and many other environmental threats. Close to 10.000 different polyphenols have already been identified. Their incidence changes among the different plant species, as well as the kind of vegetable parts (roots, stalks, barks, leaves, fruits, skin-fruits, shells, seeds, stones, etc.) inside the same species.

Phenolic compounds have considerable structural diversity, characterized by the hydroxyl groups on aromatic rings. In general, PC can be simply classified into five large groups: phenolic acids, flavonoids, tannins stilbenes and lignans[12], [13], as shown in **Figure 1-2**.

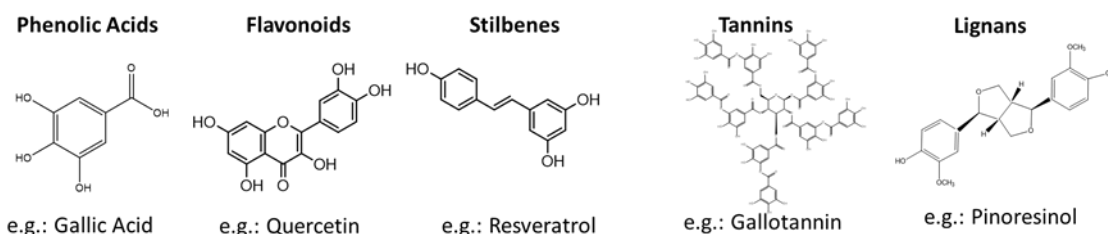


Figure 1-2: Chemical structures of phenolic acids and polyphenols representing the five main families.

Phenolic acids have a carboxylic acid functional group and can be divided into hydroxybenzoic acids and hydroxycinnamic acids, based on their C₁-C₆ and C₃-C₆ structures, respectively. This group includes compounds such as caffeic, ferulic, p-coumaric, chlorogenic, or synaptic acids, while hydroxybenzoic acids include, among others, p-hydroxybenzoic, protocatechuic, syringic, vanillic, and gallic acids, and related hydrolysable tannins[14], [15].

Flavonoids consist of two aromatic rings that are linked by three carbon atoms leading to an oxygenated heterocyclic ring. Depending on the type of heterocyclic involved, they can be classified into different subclasses: flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols [12], [15].

Stilbenes are a group of compounds derived from phenylpropanoids, characterized by the main chain of 1,2-diphenylethylene ($C_6-C_2-C_6$). They are present in the human diet in low quantities, and the main representative is resveratrol, detected in more than 70 species of plants, including grapes, berries, and peanuts [12], [15].

Lignans are produced by oxidative dimerization of two units of phenylpropane. These compounds are considered phytoestrogens, in addition to their anti-inflammatory, antioxidant or antitumor properties, or their beneficial effects in preventing cardiovascular diseases. Sesame and flaxseed are rich sources of lignans in the human diet [16]. Beyond the four major polyphenol families, several minor groups of related compounds can also be distinguished. Among these, the secoiridoids bicyclic monoterpenes (C_{10}) biosynthetically derived from geraniol are of special relevance. Olives are a notable source of secoiridoids, with oleuropein being the predominant compound. Additionally, phenylethyl alcohols, such as tyrosol and hydroxytyrosol, are also abundant in olives [13]. As polyphenols are plant metabolites, they are also present in agriculture waste, which can be revalued when the recovery of these bioactive compounds is considered. **Table 1-1** presents the main phenolic compounds identified in sub-products derived from olive oil and wine production

Table 1-1: Identified phenolic compounds present in sub products from olive oil and wine production.

Source	Sub-products	Main identified Phenolic Compounds	Ref.
Olive oil production	Olive leaves	Caffeic Acid, Verbascoside, Oleuropein, Luteolin 7-O-glucoside, Rutin, Apigenin 7-O-glucoside, Luteolin 4'-O-glucoside, oleanolic acid	[17], [18]
	Olive Pomaces	Hydroxytyrosol, Tyrosol, Oleuropein, 3,4 dihydroxybenzoic acid, Vanillic acid, Homovanillic acid, <i>p</i> -hydroxybenzoic acid, Luteolin, Rutin, Caffeic acid, Chlorogenic acid, Ferulic acid, Quercetin	[19], [20]
	Olive mill	Hydroxytyrosol, Tyrosol, Caffeic acid, Elenolic acid, Hydroxytyrosol 4-O-Glucoside, Ester of Caffeic, vanillic acid, oleuropein, verbascoside	[21]–[23]
Wine-production	Grape Pomaces	<i>p</i> -hydroxybenzoic acid, Protocatechuic acid, Gallic Acid, Ellagic acid, Vanillic acid, Syringic acid, <i>p</i> -coumaric acid, Chlorogenic acid, Caffeic acid, Resveratrol, Quercetin, Rutin, Kaemferol, Catechin	[24], [25]
	Grape stems	Gallic acid, (+)-Catechin, (+)-Epicatechin, Procyanidin B3, Procyanidin B2, Epicatechin Gallate, <i>trans</i> -Cafaric acid, <i>trans</i> -Resveratrol, 3-Viniferin	[26], [27]
	Wine lees	(+)-Catechin, (+)-Epicatechin, Procyanidin B1, Procyanidin B2, Myricetin, Quercetin, Gallic acid, <i>trans</i> -caftaric acid, <i>trans</i> -coutaric acid, Caffeic acid, <i>p</i> -coumaric acid, Ferulic acid	[28], [29]

1.3.1 Applications for Phenolic Compounds

It is well known that polyphenols present important antioxidant and anti-inflammatory activities, thus providing beneficial effects on human health, such as the protection of the cardiovascular system, anti-cancer effects or anti-aging actions.

Besides the preventive effects on human health, polyphenols are also being considered in the treatment of several diseases. Owing to these useful properties, a growing interest in these natural compounds is observed in different industries (**Figure 1-3**) namely by the pharmaceutical, biomedical, biotechnological and cosmetics industries [14]. Their exploitation is mainly due to their antioxidants, antimicrobial, and coloring properties,

among others, especially explored for food preservation, by the food and packing industries, and in the cosmetics and the textile industries.

Considering all these applications, the recovery of polyphenols from agriculture waste is an attractive route for biomass valorization by generating high value-added products. Therefore, it is necessary to find an efficient, green, and environmentally friendly method to targets these polyphenols.

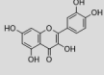
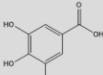
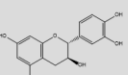
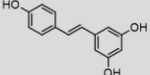
  Phenolic Compounds Applications  				
Food Industry ✓ Food additives: - Natural Preservatives - Antioxidant and antimicrobial agent - Natural Colorant ✓ Functional Ingredient	Textile Industry ✓ Natural dye ✓ UV protection ✓ Antimicrobial action ✓ Antifungal action	Cosmetic Industry ✓ Antimicrobial action ✓ Antioxidant action ✓ Anti-inflammatory action ✓ Tyrosinase inhibitor ✓ UV protection	Pharmaceutical Industry ✓ Therapeutic agent for: - Diabetes - Cancer - Neurological diseases - Cardiovascular disorders - Others	Packaging Industry ✓ Active Packaging: - Antimicrobial action - Antioxidant action ✓ Smarting packaging • Color indicator for deterioration

Figure 1-3: Some applications of phenolic compounds.

1.4 Sorption processes

The valorization of bioactive compounds from agricultural sub-products relies on efficient biotechnological strategies. Sorption processes are particularly effective for separating, concentrating, and purifying phenolic compounds [30]. Development of tailored adsorbents is an important issue for different application fields, ranging from analytical procedures to large scale industrial processes. For optimal efficiency, adsorbents must combine high selectivity and strong binding capacity toward the target molecules. Furthermore, the interplay among the adsorbent type, the physicochemical properties of the solute, and the solvent matrix ultimately determines the performance of adsorption and desorption processes.

Different kinds of adsorbents have been investigated, including activated carbons, minerals, lignocellulosic/polysaccharide-based materials, and synthetic resins. Among these, synthetic resins present important advantages, particularly in terms of stability and

ease of regeneration. The main mechanisms governing the sorption of bioactive compounds: hydrophobic interactions, hydrogen bonding, ionic interactions, and π - π stacking must be carefully considered when designing adsorption-desorption processes [31], [32].

Often to accomplish a fair amount of polyphenols retention, solvents with high water content should be considered. When the valorization of some underutilized effluents is sought, such as in olives debittering, artichoke washing, olive oil or natural juices production etc., this condition is met because water is mainly used in these processes. However, most agriculture sub-products extracts are produced using low-water content solvents to increase the polyphenols extraction efficiency and concentration (e.g., mixtures of solvents with more than 70% weight of ethanol). Thereafter, to perform the subsequent adsorption process in common synthetic resins, preparation steps are needed to increase the water content, such as solvents evaporation and re-suspension of dried extracts in aqueous systems. Besides the growth in process complexity and energy consumption, such operation leads to a drop of polyphenols concentration in the liquid because the solubility of many of these molecules is very low in aqueous systems[32]. To overcome these limitations, this research has focused on improving the specificity and capacity of adsorbents through the design of novel polymeric networks. Functionalization strategies and molecular imprinting techniques provide materials with enhanced selectivity and molecular recognition capabilities, thus offering more efficient and sustainable options for phenolic compound recovery.

1.5 Molecular Imprinted Polymers

Molecular Imprinted Polymers (MIPs) are special materials with specific recognition for a particular molecule. They are akin to artificial antibodies or enzymes as they mimic the “lock and key” principle inherent to antigen /antibody or substrate/enzyme binding. The imprinting process, that is present in **Figure 1-4**, consists in polymerization of the monomer and the crosslinker in the presence of the target molecule (also called template). Initially, the template interacts with the functional monomer to promote the interaction between them and after with addition of an appropriate initiator and crosslinker the polymerization occurs. In the end of polymerization and after removal of the template, the final product presents specific binding sites or empty cavities complementary to template molecules in size, shape and ligand.

This kind of material possesses many advantages like identifiability, predictability, and wide applicability that can yield important gain comparatively to alternative materials. Due to their excellent properties, MIPs have been widely used in many fields such as natural product separation, as sensors, adsorption process, solid phase extraction, environmental catalysis, and drug release.[33]–[38]. Some uses of MIPs as adsorption in a continuous separation processes have been employed by our research group to valorize phenolic compounds present in different agriculture residues (e.g., onion shell, chestnut shell, olive leaves...)[35], [36], [39].

The performance of MIPs is affected by several factors, namely in their preparation process (chemical composition, target /functional monomer interactions, final morphology, solvent...)[40]. Additionally, the specific molecular recognition capabilities of MIPs also depend on the final application, particularly the type of interaction between the monomer, the template and the solvents used.

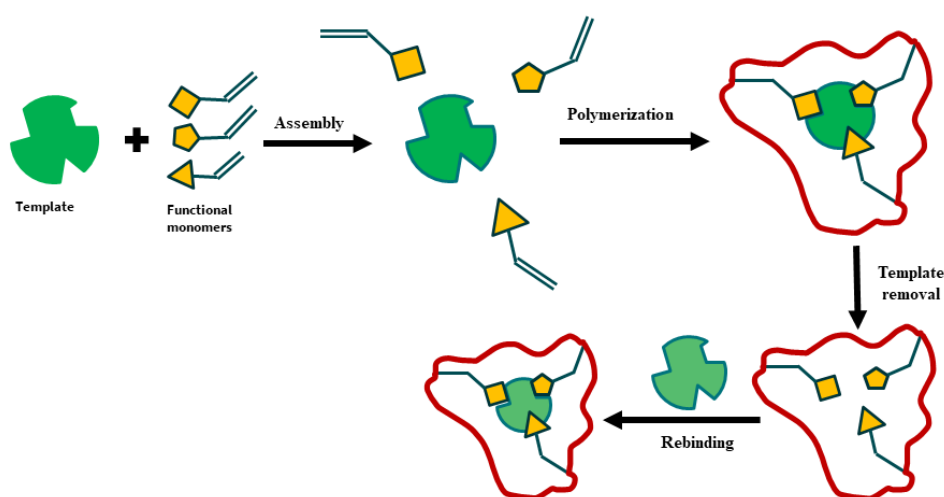


Figure 1-4: General scheme of molecular imprinting polymerization.

Computational methods have been considered in fabrication of MIPs to act as a predicting tool for pre-organized complex of template, functional monomer, and other parameters to performance screening toll for developed MIPs [41]–[43]. Adopting these tools, the wastage of reagents used in the laboratory during the synthesis can be reduced as trial-and-error step is carried out via computational software. Hence, they diminish the time-consuming selection of reagents and experimental work and reduce the cost and utilization of chemicals too. Methods, such as experimental design, quantum chemical calculations (focus on the interaction energies between the selected template and

functional monomer) are important and must be considered in molecular imprinting technique.

An improvement of specificity and capacity for the adsorption of selected compounds can be achieved through molecular imprinting technique and introducing functionalization in the final products. It is a potential advantage of MIPs compared with traditional adsorbents, justifying their application in polyphenols retention and subsequent recovery/fractionation.

Classical Free Radical Polymerization (FRP) and Controlled Radical Polymerization (CPR) play an important role for tailoring these kinds of adsorbents polymer networks. Furthermore, the production of hybrid materials, incorporating natural and synthetic polymers with molecular recognition, is also possible with an appropriate synthesis route, namely CRP, allowing the addressing of eco-sustainability requirements with the inclusion of the natural materials (in this case cellulose). The incorporation of natural polymers also allows us to control the final morphology/size of the final adsorbents making them easy to be used in continuous processes

1.6 Cellulose/synthetic hybrid materials

Modern technology requires the creation of a new generation of materials with improved functional characteristics. Hybrid materials are formed by the combination of natural polymers and inorganic components, providing a range of properties with great interest for different applications. The properties inherent to natural polymers, namely their biocompatibility, and biodegradability, open new prospects for these hybrid materials with special incidence in environmentally friendly materials (green materials).

The great interest in this research area is supported by the strong increase in the number of scientific publications, as showed in **Figure 1-5** according to the *web of science* database. Among various natural polymers, cellulose is one of the most abundant, renewable, biodegradable natural polymers on the earth. It's the main structural element of plants and makes up about third of all plant matter (half of wood is cellulose). For the first time, in 1838 the cellulose was described by the French chemist Anselme Payen [44]. Since then, cellulose many research works have dealt with cellulose, such as its biosynthesis [45], structure analysis [46], [47], chemical modification[48], regeneration of cellulosic materials [49] and in other fields[50].

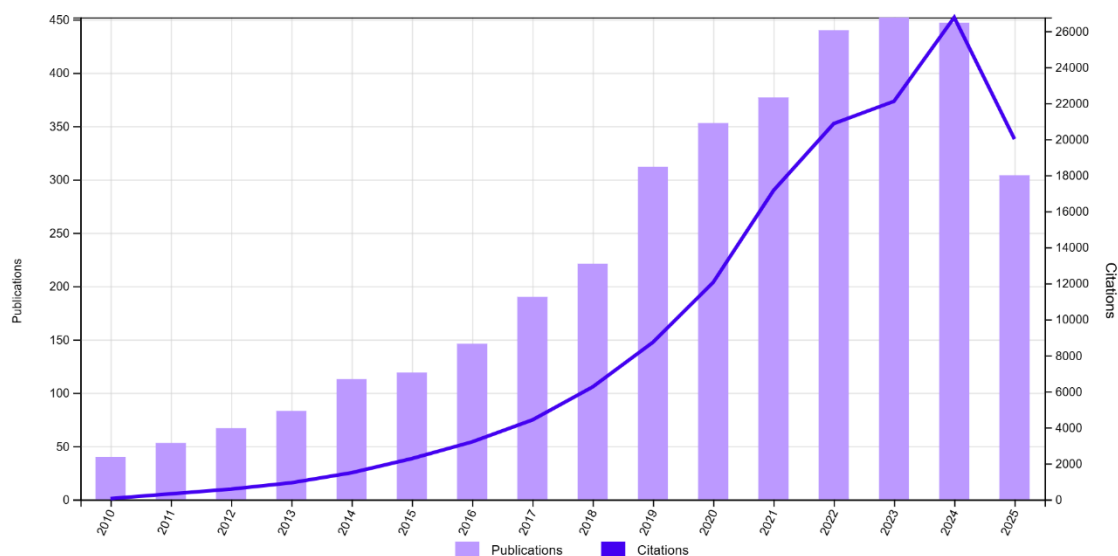


Figure 1-5: Publications and citations reported by entering the keyword “hybrid materials and cellulose”. Source: web of science data base September 2025.

Cellulose is a non-branched macromolecule that contains a chain of variable length of 1–4 linked β -D-anhydroglucopyranose units. Depending upon the source from which cellulose is extracted, the length of its chains varies. **Figure 1-6** shows the chemical structure of cellulose.

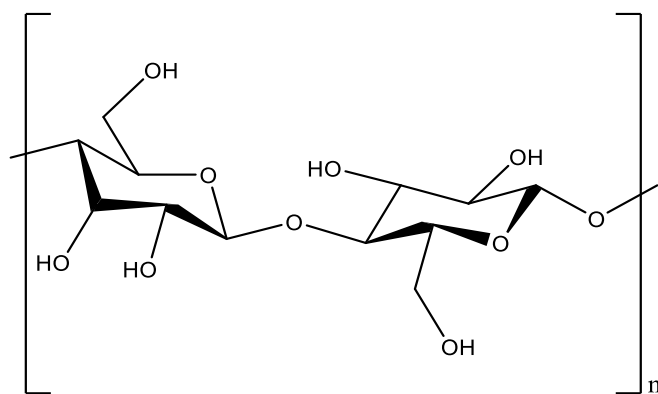


Figure 1-6: Chemical structure of cellulose.

Depending on solubility in alkaline conditions, cellulose is divided into three groups alpha, beta and gamma cellulose. Microcrystalline cellulose is synthesized from α -cellulose precursor obtained as a pulp from fibrous plant material using hydrochloric acid to reduce the degree of polymerization [51]. Reactive extrusion, enzyme mediated, steam

explosion, and acid hydrolysis are different processes that can be considered to obtain MCC. The MCC is a valuable additive in pharmaceutical, food, cosmetic, and other industries and will be considered in this word.

Due to their biodegradability, nontoxicity, biocompatibility, availability and functionality, MCC has been demonstrating a huge interest by scientific community. However, to fully exploit the potential of MCC and increase their applicability, it is necessary to modify their structure by introducing new functionalities. Various modification techniques have been reported to improve the physicochemical properties of natural polymers [52], [53] and among these, graft polymerization has received much attention during the last few decades [54], [55]. The grafting of many monomers into cellulose could be achieved through different approaches [56]. In this work, the functionalization of MCC backbone by esterification followed by Controlled Radical Polymerization (CRP), namely ATRP will be considered to develop novel advanced materials.

1.6.1 Grafting of synthetic polymers on MCC backbone

To modify cellulose in a controllable manner, it is necessary to well understand its structure. The molecular structure of cellulose is composed of linear chains of glucose units with three OH groups. These groups can form inter and intra hydrogen bonds, yielding a straight and crystalline chain structures[56]. This behavior makes it difficult to dissolve cellulose in water and in most of the organic solvents. Therefore, heterogeneous and homogeneous functionalization strategies are often performed to modify cellulose. However, OH groups provide functionality to the cellulose backbone. Among other methods, graft polymerization is an effective route to design environmentally conformable and biocompatible cellulose-based materials. It is known that it is hard to attach certain functional groups in cellulose backbone directly, so it is important to prepare cellulose precursor with special reactive groups. Graft copolymerization is a process in which side chain grafts are covalently attached to the main chain of polymer backbone to form branched copolymers, as presented in **Figure 1-7** [57]. The extent of polymerization grafting is referred to as grafting percentage and is calculated by the following formula [1]. The rate of grafting is dependent on the kind of polymerization, initiator concentration as well as the monomer, time of polymerization, and the nature of backbone of polysaccharide (cellulose)

$$\% \text{ Grafting} = \frac{\text{Weight of graft copolymers} - \text{Weight of cellulose}}{\text{Weight of cellulose}} \times 100 \quad [1]$$

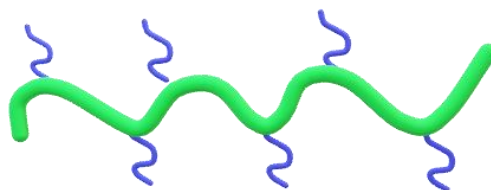


Figure 1-7: Schematic diagram of grafting.

Hence, the first step before synthesizing functional polymer-grafted cellulose is to activate the hydroxyl groups in cellulose backbones. These activations could be done by different methods, and in this work two main methods will be considered: immobilization of an ATRP initiator (macro-BIBB) and immobilization creation of macro chain transfer agents (macro-CTAS) on cellulose backbone by reacting OH groups as is represented in **Figure 1-8**. After this activation, the MCC are prepared to introduce new functionalities, by grafting process, considering surface-ATRP and RAFT polymerization.

1.6.1.1 Grafting by Controlled Radical Polymerization

Molecular weight is a defining parameter of polymers that influences their properties, including processing characteristics and mechanical characteristics. Polymers with high molecular weight possess better mechanical properties and polymers with low molecular weight have better processing performance. Therefore, to keep the balance between processability and mechanical performance, the molecular weight of polymers should be in a particular range.

Controlled Radical polymerization (CRP) is a versatile method for the preparation of well-defined polymers [58]. Unlike conventional free radical polymerization, CRP establishes a dynamic equilibrium between active propagating radicals and dormant species through reversible activation–deactivation processes, thereby minimizing irreversible radical termination and enabling improved control over molecular weight and architecture. [59]. In this process, active radicals are rapidly deactivated after the addition of one or a few monomer units, reverting to the dormant state and maintaining a low concentration of propagating species [60]. Therefore, considering this approach, it is possible to synthesize polymers with precise control over molecular weight (MW), molecular weight distribution (MWD), polymer composition, topology, and functionality.

1.6.1.1.1 Surface-Atom Transfer Radical Polymerization (SI-ATRP)

In 1995, Matyjaszewski and Sawamoto [61] discovered a versatile tool to yield polymers with low polydispersity and controlled molecular weight, called Atom Transfer Radical Polymerization (ATRP).

ATRP is one of the most robust and widely used CRP techniques for polymerization of a broad range of commercially available functional monomers [62], [63]. Their attraction is due to the simple experimental setup, the use of readily available initiators and catalysts that can be used in a range of solvents under a broad spectrum of reactions conditions, allowing precise control over the final polymer MW and architectures[64].

The process uses an alkyl halide as initiator, and a metal in its lower oxidation state with complexing ligands. The ATRP process occurs by the successive transfer of the halide from the dormant polymer chain to the ligated metal complex, establishing a dynamic equilibrium between active and dormant species, as is show in **Figure 1-8**.

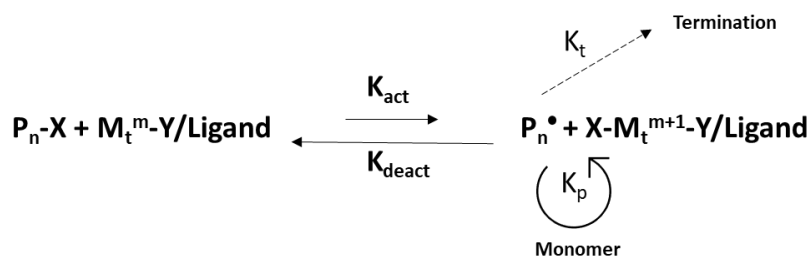


Figure 1-8: The Mechanism of ATRP[62].

Where P_n is the radical species, X and Y are the initiating sites, M_t is the transition metal, and m and m+1 are the valences of M_t .

Recently, ATRP has been widely employed as a very promising tool to graft polymers from different surfaces, and it's called SI-ATRP in that case. Considering "grafting from", SI- ATRP can be used to graft synthetic polymers on surfaces in a controlled manner. Using SI-ATRP, hybrid materials with well-defined polymer brush structure can be prepared precisely.

Typically, in SI-ATRP initiators are immobilized on the substrate by reacting with the superficial hydroxyl groups with an ATRP initiator precursor (usually α -bromoisobutyrylbromide, BIBB). Then the initiator-modified substrate is used to perform graft ATRP grafting as is described in chapter 2, **Figure 2-1** where the SI-ATRP of 4-vinyl pyridine (4VP) has been experimented to modify microcrystalline cellulose (MCC) surface properties, such us adsorption capabilities.

1.6.1.1.2 Surface Reversible Addition-Fragmentation Chain Transfer Polymerization (SI-RAFT)

The reversible Addition fragmentation chain transfer (RAFT) polymerization-based surface modification has arisen as a powerful tool for preparation of well-defined polymers grafted solid substrates. It is one of the most versatile and convenient methods of controlled radical polymerization[65].

Among all the controlled radical polymerization techniques, RAFT can be applied to the widest range of radically polymerizable monomers and consists of the addition of a small amount of thiocarbonyl thio-based (Chain Transfer Agent (CTA) also called “RAFT agent”) to a conventional free radical polymerization system. A representative accepted mechanism is shown in **Figure 1-9**. The reactivity and, hence, suitability of the RAFT agent for polymerizing different monomers is determined by the nature of the R and Z substituents. The Z- group influences both the activity of the thiocarbonyl group for radical addition and the stability of the resulting radical species, whereas R-group initiates the growth of new polymeric chains. The use of “RAFT agent” leads to polymers of narrow polydispersity and of predetermined narrowly distributed [65].

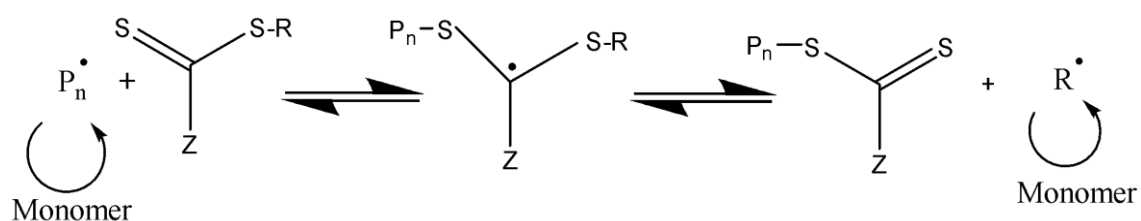
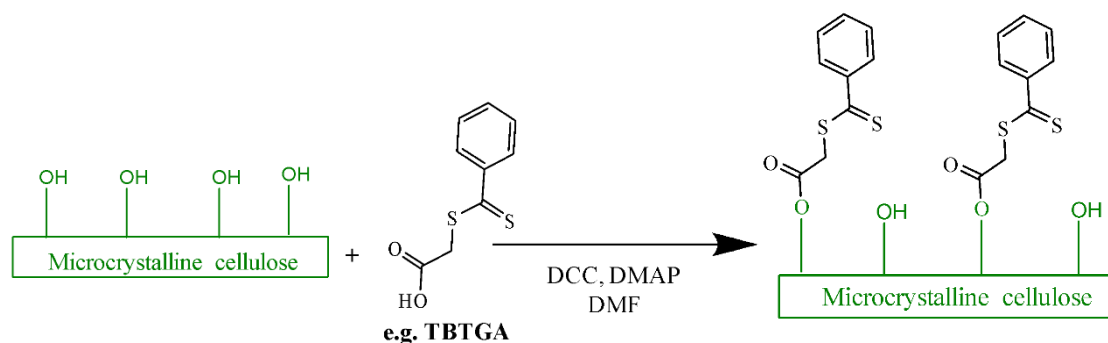


Figure 1-9: Generally accepted mechanism for RAFT polymerization. [65]

The process of monomer grafting on cellulose backbone through RAFT polymerization consists in attaching the CTA groups onto cellulose. CTAs with carboxyl groups are generally used for preparing cellulose-based CTA [66]. Depending on the location of the carboxyl group, CTAs can be attached via esterification through fragmenting covalent bond (R-group) [67] or through non-fragmentation covalent bond (Z-group) [68] corresponding to the grafting “from” or “grafting to” polymerization mechanism, respectively. The R-group initiates the growth of polymeric chains and the Z- group activates the thiocarbonyl bond toward radical addition and then stabilizes the resultant adduct radical. The propagating radical is added to the C=S moiety of RAFT agent to form an intermediate radical that will fragment back either to the original propagating radical or to a new carbon-centered radical. In the RAFT polymerization of cellulose,

CTA is firstly attached to cellulose. Then, the reaction completes with the binding of monomer assisted with an initiator such as AIBN. In **Figure1-10** an example of the detailed process of RAFT polymerization of styrene (STY) on microcrystalline cellulose (MCC) is represented.

a)



b)

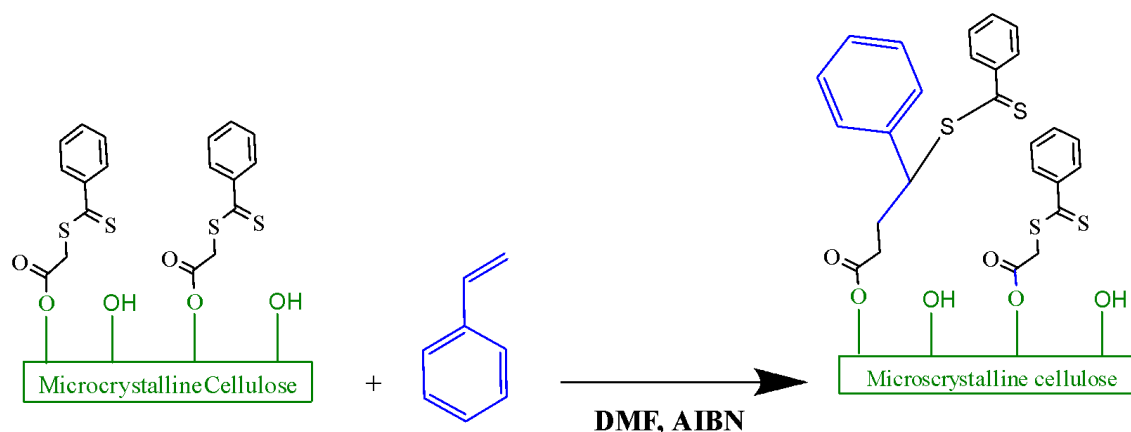


Figure 1-10: Depiction of a) immobilization of “RAFT agent” in microcrystalline cellulose (MCC) with production of the CTA macro-initiator MCC-CTA and b) grafting of Styrene polymer chains in MCC through surface RAFT polymerization.

The modification of cellulose can be proved by various analytical tools. The most used techniques are Fourier-Transform Infrared Spectroscopy, elemental composition, morphography and inductively coupled plasma mass spectrometry (ICP-MS).

1.6.2 Applications Cellulose grafted Synthetic Polymers

Nowadays, environmental issues, and the flow of the circulation of economy have become a major preoccupation, while innovative applications using natural materials may provide a solution. Cellulose has shown potential to be used as a basic material in several applications. Depending on the final applications, cellulose can be grafted with a wide variety of polymers. In the following table, are present some works reported in the last ten years about the modification of different kinds of cellulose, considering grafting via controlled radical polymerization.

Table 1-2: Summary of monomers grafting to cellulose including the type of CRP polymerization and the target applications, if its reported.

Kind of cellulose	Monomer	Kind of CRP polymerization	Applications	Ref.
Microcrystalline cellulose	methyl methacrylate	ATRP	-	[69]
Cellulose nanocrystal	4-vinyl pyridine	SI-ATRP	Catalysis	[70]
Cellulose nanocrystal	Styrene	SI-ATRP	Drug Delivery	[71]
Cellulose nanocrystal	Acrylic acid	RAFT	Stimuli responsive nanocomposites	[72]
Cellulose nanocrystal	Vinyl acetate	SI-RAFT	Nanocomposites	[73]
Cellulose nanocrystal	N-isopropylacrylamide and acrylic acid	RAFT	-	[74]
Cellulose membrane	Dimethylaminoethyl methacrylate	ATRP	Metals adsorbent	[75]
Hydroxypropyl-cellulose	2-acryloyloxyethyl ferrocenecarboxylate	ATRP	Biosensors	[76]
Cellulose nanocrystal	Methyl methacrylate	SI-ATRP	thermoplastic elastomer reinforcement	[77]
Cellulose nanocrystal	Styrene	(SI-ATRP)	nanocomposites	[78]
Cellulose nanocrystals	Poly (dimethylaminoethyl methacrylate)	RAFT	CO ₂ responsive colloids	[79]

The use of modified cellulose for different technological applications is being considered in several domains (see **Table 1-2** above and references therein) but the combination of

molecular imprinting with natural polymers scarcely was tried [80], [81]. As was mentioned above, part of this work was to develop hybrid materials (natural-synthetic polymers) with molecular recognition for valorization of polyphenols present in agriculture sub products.

1.7 Amphiphilic Block copolymers-based encapsulation /delivery systems

Amphiphilic block copolymers are macromolecules composed of chemically distinct blocks that contain both hydrophilic and hydrophobic properties. Their unique dual affinity allows them to self-assemble into nanostructures, such as micelles, vesicles, and lamellae, when dispersed in selective solvents [82]–[84]. This self-assembly behavior is the basis for their use in a variety of applications, ranging from nanomedicine and controlled drug delivery [85], [86] to emulsification, coatings, and stimuli-responsive materials [87].

The properties of block copolymers can be precisely adjusted by manipulating block length, composition, and functionalization [84],[88]. The introduction of bio-based hydrophobic blocks has gained particular attention, as it enhances the environmental sustainability of polymeric materials while expanding their potential for use in biomedical and food-related applications [89]. For instance, fatty acid-functionalized blocks can improve compatibility with hydrophobic active compounds such as antioxidants, lipophilic drugs, and nutraceuticals, enabling advanced delivery systems to be designed [90]

In this context, glycidyl methacrylate (GMA) is particularly important. Its dual reactivity provides a versatile platform for functionalization: the methacrylate group participates in radical polymerizations, while the pendant epoxide ring enables post-polymerization chemical modifications [91]. When combined with oleic acid (OA), a monounsaturated fatty acid derived from renewable sources, GMA-containing block copolymers can be transformed into amphiphilic systems with enhanced hydrophobic character and sustainable origins.

1.7.1 Glycidyl Methacrylate: Structure, Reactivity, and Applications

Glycidyl methacrylate (GMA) is a bifunctional monomer that contains both a vinyl group and an epoxide group as presented in **Figure 1-11**. The vinyl group enables its incorporation via conventional radical polymerization as well as via controlled radical

polymerization (CRP) techniques such as ATRP, RAFT, and nitroxide-mediated Polymerization (NMP) [62], [92]. With CRP well-defined copolymers with controlled molecular weights, narrow dispersity, and precise block structures could be produced [63]. The epoxide group is chemically versatile and can undergo nucleophilic ring-opening reactions with amines, thiols, alcohols, and carboxylic acids. This reactivity makes glycidyl methacrylate (GMA) an excellent “handle” for post-polymerization functionalization (PPF), enabling the attachment of diverse functional groups without disrupting the polymer backbone [91], [93].

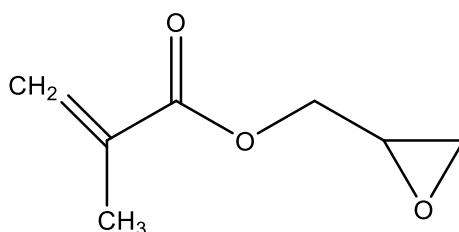


Figure 1-11: Chemical structure of Glycidyl methacrylate.

Due to its dual functionality combining a polymerizable methacrylate double bond and a reactive epoxide ring glycidyl methacrylate (GMA) has found wide application across diverse material systems. In the biomedical field, functionalized GMA copolymers have been developed for tissue engineering scaffolds, hydrogels, and drug delivery matrices [94]. In surface engineering, the pendant epoxy groups enable the covalent grafting of biomolecules, peptides, and enzymes onto polymer substrates, thereby enhancing biocompatibility and biological activity [95]; GMA-based copolymers have also been incorporated into coatings and adhesives, where they provide strong adhesion, chemical resistance, and tunable surface energy [96]. Furthermore, the chemical versatility of GMA facilitates the introduction of stimuli-responsive moieties (e.g., pH, redox, or temperature sensitive groups), making it a valuable component in the design of advanced smart materials [97]. Thus, GMA functions not only as a structural comonomer but also as a reactive platform for introducing bio-based moieties such as fatty acids.

1.7.2 Oleic Acid as a renewable hydrophobic moiety.

Oleic acid (OA) is a C₁₈ monounsaturated fatty acid (cis-9-octadecenoic acid), is one of the most abundant lipids in nature (see **Figure 1-12**). It is a major component of olive oil

and other vegetable oils [98]. From a materials science perspective, OA is attractive due to its renewable, biodegradable, biocompatible, and amphiphilic properties [99].

The carboxylic acid group of OA provide a reactive site for esterification, amidation, and epoxide ring-opening reactions. Meanwhile, its hydrophobic aliphatic chain imparts a non-polar character to OA, making it an excellent candidate for tuning the amphiphilicity of synthetic polymers.[100],[101]

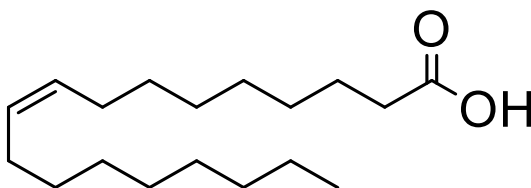


Figure 1-12: Chemical structure of Oleic Acid.

The incorporation of oleic acid (OA) into polymers can increase hydrophobicity and reduce water uptake [102], improve compatibility with lipophilic bioactive compounds thereby enhancing encapsulation efficiency [103] and ultimately enhance biodegradability and biocompatibility relative to petroleum-based hydrophobic blocks, while also providing opportunities for sustainable material design consistent with the principles of green chemistry and the circular bioeconomy [104].

Several studies have dealt with the covalent grafting of oleic acid (OA) onto various polymer backbones for instance, chitosan (a polysaccharide) works well following OA-grafting for nanomicelle formation [105], synthetic methacrylates such as PMMA gain improved surface and antifouling properties upon OA modification [106], epoxidized OA grafted onto hydroxyapatite particles yields amphiphilic, biocompatible filler materials [107] and OA-functionalized hyaluronan copolymers spontaneously form biocompatible, self-assembling nanocarriers for drug delivery [108]. These modifications enhance amphiphilicity, emulsification efficacy, and bioactivity in the resulting materials.

The most direct reaction route for combining GMA and OA is the epoxide ring-opening reaction. The carboxylic group of OA acts as a nucleophile, attacking the electrophilic carbon in the oxirane ring of GMA and leading to ester bond formation as is presented in **Figure 1-13** [109].

This reaction can proceed under both thermal and catalytic conditions. For instance, acid or base catalysts can enhance ring-opening efficiency, and enzymatic catalysis has also been investigated as a more environmentally friendly approach [32]. The extent of functionalization depends on reaction time, temperature, the molar ratio of the reactants, and solvent polarity [110].

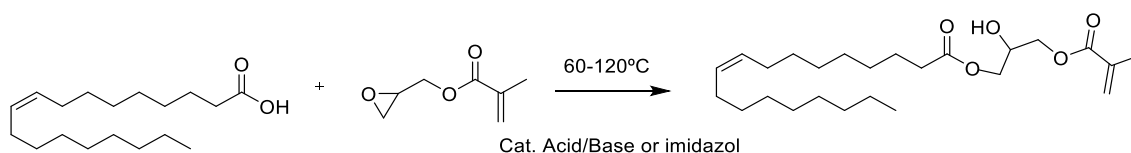


Figure 1-13: Expected epoxide ring-opening reaction between Oleic Acid (OA) and Glycidyl methacrylate (GMA).

Several synthetic strategies have been reported in the literature for the incorporation of oleic acid (OA) into glycidyl methacrylate (GMA)-based polymeric systems. These approaches differ in terms of reaction sequence, degree of architectural control, and potential limitations. The three main routes are summarized below: i) post-polymerization functionalization (PPF): GMA-containing (block) copolymers are first prepared by controlled radical polymerization (e.g., ATRP or RAFT). The pendant epoxide groups are then subjected to nucleophilic ring-opening with OA ($-\text{COOH}$), yielding β -hydroxy ester linkages and covalent OA grafts while retaining control over molecular architecture and enabling systematic variation of OA content [93], [111].

ii) Pre-functionalization (monomer first) approach: OA is first converted into a methacrylate monomer; this OA functional monomer is then copolymerized. The route is efficient and modular but can suffer from monomer instability and reduced polymerization, compromise molecular weight control a final conversion. [109], [112]

iii) One-pot strategies: in a few cases, GMA polymerization and OA (or fatty acid) functionalization are conducted in a single vessel. Although step-economical, these protocols frequently face competing reactions and diminished control over microstructure

and functionalization degree, so they are less prevalent for precision materials [113], [114].

Across all approaches, side reactions, including incomplete epoxide conversion, undesired crosslinking, and (trans)ester hydrolysis can alter physicochemical properties and narrow the process window for high fidelity OA incorporation [93].

After obtaining the hydrophobic block derived from the reaction between oleic acid (OA) and the epoxy groups of glycidyl methacrylate (GMA), a hydrophilic monomer must be polymerized to construct the desired hybrid synthetic natural block copolymers. Among the available candidates, 2-(dimethylamino)ethyl methacrylate (DMAEMA) is a particularly attractive choice. Its polymer, poly (DMAEMA), is well known for its stimuli-responsive behavior, as the pendant tertiary amine groups can undergo protonation deprotonation depending on the pH, and the polymer exhibits thermo responsive solubility changes in aqueous media. [115] The incorporation of a poly (DMAEMA) block alongside the OA–GMA hydrophobic segment facilitates the formation of amphiphilic block copolymers with the ability to undergo self-assembly and respond to environmental stimuli. Such hybrid systems broaden the scope of potential applications, particularly in drug delivery, nanostructured carrier design, and the development of smart functional materials [84], [116]. In this context, these copolymers are especially promising for the encapsulation and controlled release of bioactive polyphenols, such as oleanolic acid, offering enhanced stability, bioavailability, and biocompatibility, thereby addressing key challenges associated with the use of natural compounds in biomedical applications.

1.7.4 Amphiphilic Block copolymers (poly (DMAEMA)-b-poly (GMA)) functionalized with oleic acid.

The design of amphiphilic block copolymers combining poly(2-(dimethylamino)ethyl methacrylate) (poly (DMAEMA)) and (poly (GMA)) offers a versatile platform for functionalization with oleic acid (OA). In these systems, the poly (DMAEMA) block imparts hydrophilicity and pH-responsiveness due to the presence of tertiary amine groups, whereas the poly (GMA) block provides reactive epoxy moieties that can undergo nucleophilic ring-opening with the carboxyl group of OA. This reaction yields β -hydroxy ester linkages, covalently attaching OA chains to the polymer backbone. The resulting poly (DMAEMA)-b-poly (GMA/OA) copolymers acquire distinct amphiphilic character: the grafted OA segments aggregate into hydrophobic domains, while the poly

(DMAEMA) segments form hydrophilic, cationic coronas. Such structural organization promotes self-assembly into micelles or vesicle-like nanostructures in aqueous media [84]. Key potential applications of these systems have been reported in different fields. In drug delivery, the OA-modified hydrophobic domains can serve as reservoirs for lipophilic drugs, while the pH-responsive poly(DMAEMA) corona enables triggered release under acidic conditions such as those encountered in tumor tissues or endosomal compartments [117]. In addition, the OA-rich hydrophobic domains are capable of encapsulating plant-derived antioxidants, thereby protecting them from premature degradation and allowing a controlled release [84], [109], [118]. Beyond biomedical uses, the incorporation of bio-based fatty acids such as OA contributes to the development of green nanomaterials, as it reduces the reliance on petroleum-derived hydrophobic components and promotes more sustainable polymer design [109], [119]. Controlled radical polymerization (CRP) techniques such as ATRP and RAFT are particularly useful for synthesizing these block copolymers, as they provide well-defined architectures (diblock, triblock, or star-like) [84], [118]. The degree of OA incorporation can be finely tuned by adjusting the extent of epoxy conversion in the poly(GMA) block, thereby tailoring self-assembly behavior, nanostructure morphology, and encapsulation capacity [120]. In summary, the strategy developed in this work is based on the functionalization of glycidyl methacrylate (GMA) with oleic acid (OA) to generate hydrophobic segments, subsequently combined with a hydrophilic and stimuli-responsive block, such as poly(2-(dimethylamino)ethyl methacrylate) (poly (DMAEMA)). This design enables the construction of hybrid amphiphilic block copolymers that can self-assemble into nanostructured carriers. The ultimate objective is to exploit these systems for the encapsulation and controlled release of polyphenolic bioactive compounds, such as oleanolic acid. Through this approach, it is anticipated that the intrinsic instability and low solubility of such natural products can be mitigated, thereby improving their stability, bioavailability, and biocompatibility, and expanding their potential in biomedical and pharmaceutical applications.

1.8 Thesis structure and overview of its related publications

This thesis focuses on the development of hybrid polymeric materials that integrate natural and synthetic components through controlled radical polymerization. These materials are designed to function as adsorbents or delivery vehicles for the valorization

of bioactive compounds derived from agricultural residues. The research addresses four main challenges

1. Non-imprinted hybrid adsorbents by ATRP for the valorization of plant sub products.

Building on our group's previous work with poly(4-vinylpyridine) in polyphenol sorption, this first study focused on incorporating microcrystalline cellulose into poly(4VP) adsorbents via ATRP. The hybrid materials showed strong pyridyl–polyphenol interactions, enabling high retention and selective purification of anthocyanins, flavonoids, and secoiridoids from olive, wine, and almond extracts. Unexpected polymer populations observed during grafting suggest new opportunities for designing advanced materials with molecular recognition features.

2. Imprinted hybrid adsorbents by ATRP for the valorization of olive sub products:

To improve material selectivity, the second part of this work focused on developing hybrid materials with molecular recognition for application to olive by-products. Surface molecularly imprinted cellulose-synthetic hybrid particles were synthesized via ATRP using 4VP and EGDMA with quercetin as the template.

3. Imprinted hybrid adsorbents by ATRP for the valorization of vine sub products and proof of concept for scale-up of adsorption processes with fully synthetic tailored adsorbents.

The third part of this work explored imprinted hybrid adsorbents prepared via ATRP for the selective recovery of polyphenols from vine sub-products. Their effectiveness in valorizing winemaking residues was demonstrated, and a proof of concept with fully synthetic adsorbents confirmed the feasibility of scaling up adsorption processes to continuous and larger-scale operations.

4. Hybrid natural-synthetic amphiphilic copolymers by ATRP and RAFT for the encapsulation/release of bioactive compounds in plant sub products.

The last part of this work focused on the synthesis of copolymers comprising a hydrophobic block, obtained through the reaction between oleic acid and the epoxy groups of glycidyl methacrylate, and a hydrophilic poly(2-(dimethylamino)ethyl methacrylate) block. The pH and temperature-responsive behavior of these systems was investigated to promote self-assembly, underscoring their potential applications in drug delivery and the design of smart functional materials.

This cumulative dissertation comprises five articles that have been published in scientific journals without further modification. Together, the chapters present independent studies that contribute to the design, optimization and application of novel functionalized hybrid polymeric materials. In **Figure 1-14**, an overview of the thesis structure is illustrated.

Chapter 2 presents the synthesis of hybrid cellulose-poly(4-vinylpyridine) (MCC-P(4VP)) particles by ATRP and their application as adsorbents for polyphenols from winemaking, olive oil, and almond sub-products. The 4VP-based materials outperformed commercial resins in polyphenol retention, owing to the strong affinity of pyridyl groups. MCC-P(4VP) enabled efficient purification and fractionation of targeted compounds, including anthocyanins, flavonoids, and secoiridoids, highlighting their potential to enhance downstream recovery and biorefining efficiency. Unexpected heterogeneity in the ATRP grafting process was also observed, suggesting opportunities for the design of more selective hybrid materials.

Chapter 3 describes the development of surface molecularly imprinted cellulose–synthetic hybrid particles prepared via atom transfer radical polymerization (ATRP). The process involved the generation of cellulose-based macro-initiators, followed by the growth of an imprinted polymeric shell of 4-vinylpyridine (4VP) and ethylene glycol dimethacrylate (EGDMA) using quercetin as the template molecule. Ethanol was identified as the most suitable solvent, enabling the production of particles with adequate size and efficient incorporation of the co-monomers. Sorption/desorption assays demonstrated successful surface functionalization and effective molecular imprinting, with an imprinting factor of ~2.6. When tested with olive leaf extracts, the hybrid particles allowed the selective enrichment of flavonoids, yielding fractions enriched in luteolin-7-O-glucoside, apigenin-7-O-glucoside, and quercetin, with enrichment factors of up to 4.

These findings confirm the potential of molecularly imprinted natural-synthetic hybrid materials for the targeted recovery of high-value bioactive compounds, supporting sustainable strategies within the circular bioeconomy.

Chapter 4 presents the development of hybrid cellulose-synthetic particles bearing surface pyridyl groups and quercetin-imprinted cavities, synthesized via atom transfer radical polymerization (ATRP). Functionalization with pyridyl moieties was confirmed by FTIR, while SEM micrographs revealed clear surface modification compared with pristine cellulose. Competitive sorption/desorption assays demonstrated successful molecular imprinting with an imprinting factor of approximately 8, as well as high retention capacity for polyphenols, even in hydroalcoholic solvents with high ethanol content (ethanol/water 80/20 v/v). The materials were further applied to winemaking sub-products, enabling the selective fractionation and enrichment of flavonols and anthocyanins. Notably, enrichment factors of up to 5 were achieved for quercetin, 12 for quercetin-3-O-glucuronide in diatomaceous earth extracts, and 4 for flavonols such as myricetin and quercetin glucosides in grape pomace. These results demonstrate the feasibility and potential of engineered hybrid adsorbents for the sustainable valorization of agro-industrial sub-products into high-value bioactive compounds. **Chapter 5** presents the scale-up of a sorption process for the fractionation and enrichment of bioactive compounds from winemaking sub-products using synthetic molecularly imprinted adsorbents. The process was designed to operate with hydroalcoholic solvents and was experimentally validated through the development and operation of a pilot-scale sorption prototype, enabling automation of the fractionation method. Adsorbents were synthesized at the gram scale, and spent diatomaceous earth, a sub-product of wine filtration, was employed as a source of bioactive compounds. The resulting fractions were evaluated for antioxidant activity using radical scavenging, reducing power, and lipid peroxidation assays. The most enriched fraction exhibited a total phenolic content approximately 3.8 times higher than the original extract, with antioxidant activities enhanced up to 6.4-fold. These findings demonstrate not only the efficiency of the scaled sorption process but also its potential for biomass valorization, by combining materials development, process design, and application to real extracts within a circular bioeconomy framework. Furthermore, the applicability of the synthetic polymer-based adsorbents was validated in real conditions, paving the way for extending this strategy to hybrid materials with improved functionality and selectivity.

Chapter 6 investigates the synthesis of amphiphilic block copolymers composed of a hydrophobic p(GMA-OA) segment and a hydrophilic p(DMAEMA) segment, employing both ATRP and RAFT polymerization strategies. The most effective route involved the post-polymerization modification of glycidyl methacrylate units with oleic acid, particularly in DMSO with an excess of oleic acid, which reduced undesired branching and crosslinking. The successful preparation of the target p(GMA-OA)-b-p(DMAEMA) copolymers was confirmed by FTIR and GPC analyses. The resulting materials were applied to the encapsulation of oleanolic acid in both solid polymer dispersions and aqueous particle dispersions. SEM and TEM characterization revealed well-defined spherical aggregates of approximately 100 nm in size with an external shell of 5–8 nm. The copolymers exhibited high loading capacity (up to 0.17 mg/mL) and enabled sustained release of oleanolic acid, with release dynamics responsive to solvent composition, pH, and temperature. These findings demonstrate the potential of the synthesized block copolymers as versatile carriers for controlled delivery of bioactive compounds.

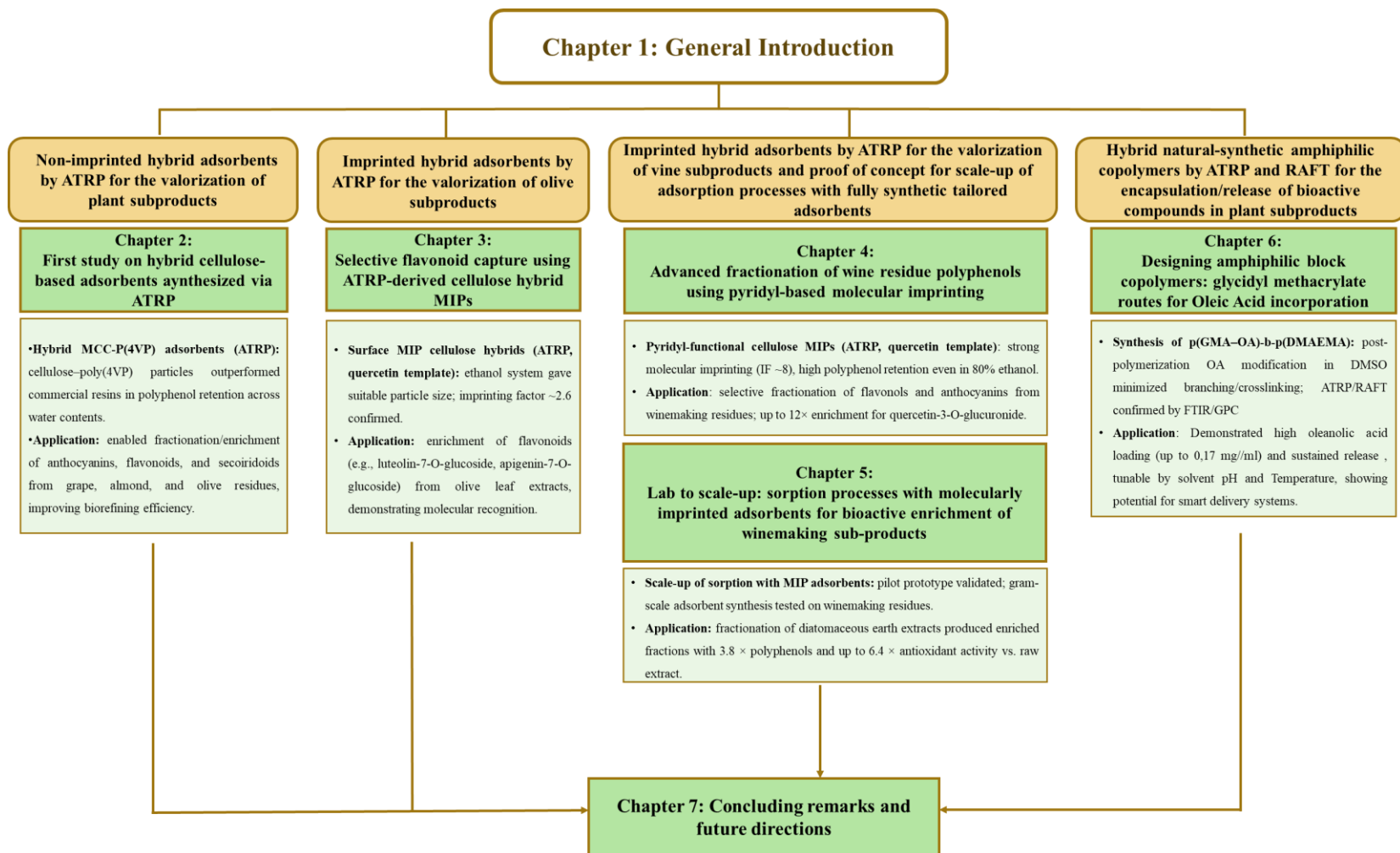


Figure 1-14: Overview of the thesis structure and complete research tasks.

1.9 References

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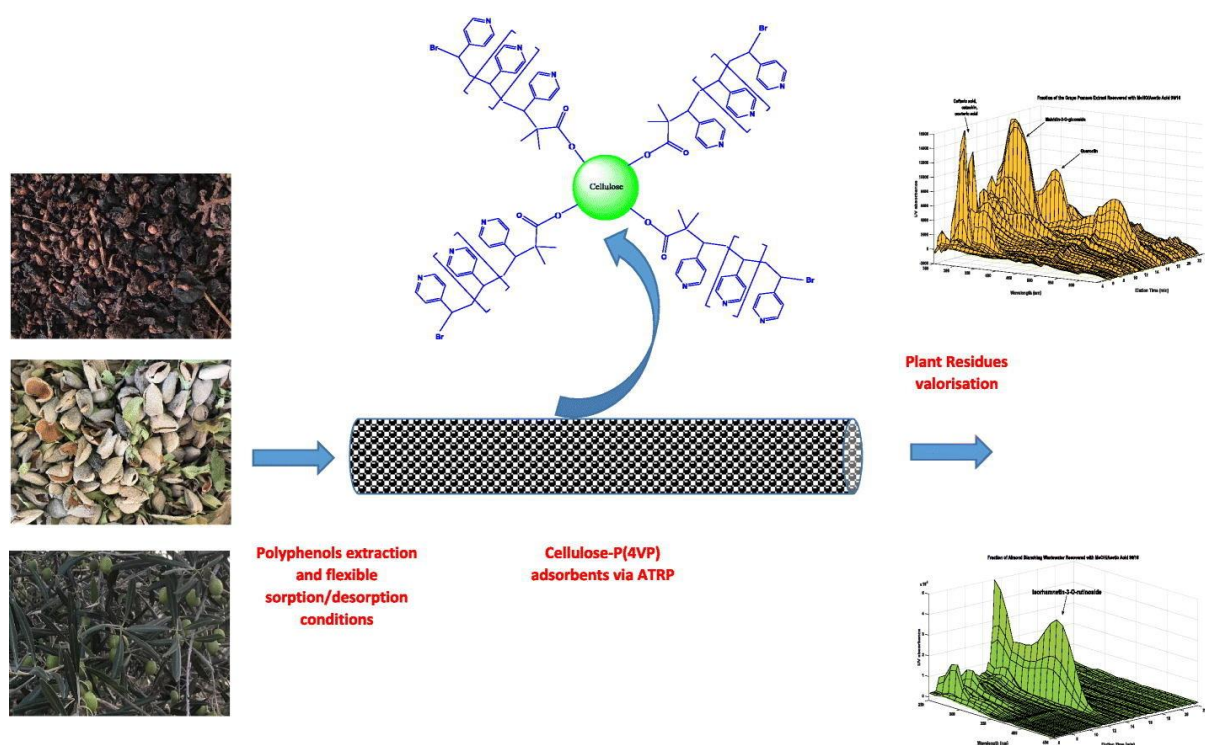
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Chapter 2. First study on hybrid cellulose-based adsorbents synthesized via ATRP



Hybrid cellulose-poly(4-vinylpyridine) (MCC-P(4VP)) particles were synthesized via ATRP and showed superior polyphenol retention compared to commercial resins, owing to strong pyridyl-polyphenol interactions. They enabled efficient purification of key compounds such as anthocyanins, flavonoids, and secoiridoids from agriculture sub-products, highlighting their value in downstream processing.

Hybrid cellulose-poly(4-vinylpyridine) adsorbents produced via ATRP and their application to target polyphenols in winemaking, olive oil production and almond processing residues

C..P. Gomes, R.C.S. Dias, M.R.P.F.N. Costa, Hybrid cellulose-poly(4-vinylpyridine) adsorbents produced via ATRP and their application to target polyphenols in winemaking, olive oil production and almond processing residues, *Reactive and Functional Polymers*, Volume 164, 2021, 104930, <https://doi.org/10.1016/j.reactfunctpolym.2021.104930>.

Abstract

Hybrid cellulose-poly(4-vinylpyridine) particles (MCC-P(4VP)) were synthesized via ATRP grafting and used as adsorbents to target polyphenols in residues derived from winemaking, olive oil production and almond processing. These hybrid particles preserve good conditions to be used in continuous sorption/desorption processes and were assessed for the retention of polyphenols in solvent mixtures ranging from 0 to 100% water content. Other seven adsorbents were used in these assays, including three commercial resins and further materials synthesized in this research with 4VP.

We show that the 4VP based adsorbents present a much higher average polyphenol retention in a broad range of working conditions, namely concerning the water content of the extract. This behavior stems from the strong binding capacity of the pyridyl functional group towards many polyphenols and excels with MCC-P(4VP) adsorbents when the hydrophobic interactions play a minor role. MCC-P(4VP) adsorbents were also used for purification and fractionation of polyphenols using simple sorption/desorption steps. A fraction enriched with the malvidin-3-O-glucoside anthocyanin was obtained from grape pomace. Fractions with high content of isorhamnetin-3-O-rutinoside were isolated from almond. Fractions enriched in secoiridoids and flavonoids were produced from olive mill wastewater while many phenolic alcohols and acids were removed. These results show the potential of the MCC-P(4VP) adsorbents to improve downstream processing of polyphenols and increase the biorefining efficiency, namely through the direct handling of organic extracts at high polyphenol concentration.

This research also has disclosed the formation of a polymer population deviating from the expected homogeneous process in ATRP grafting. The partition of ATRP reactants between two regions in the homogeneous phase could be a source of some potentially interesting issues relevant to monomers presenting strong H-bond capability, such as 4VP. Thus, many future developments are possible for the designing of the functional materials here addressed, including the introduction of molecular recognition features.

Keywords: Hybrid materials, Cellulose-Synthetic, ATRP grafting, Adsorbents, Downstream processin

2.1 Introduction

Development of tailored adsorbents is an important issue for different application fields, ranging from analytical procedures to large scale industrial processes. High specificity/selectivity towards target compounds, high retention capacities and easy of regeneration are key parameters to evaluate adsorbents' performance. At the end, the triangulation between the kind of adsorbent, target solute(s) and solvent matrices will determine the efficiency of the aimed sorption/desorption process. For relatively simple systems the interactions between adsorbent/adsorbate(s)/solvent(s) can be described/predicted by molecular simulation but real samples are difficult to handle with these tools due to the heterogeneity of common adsorbents and the complexity of the mixtures solute/solvent intervenient in the sorption/desorption processes. Therefore, some kind of semi-empirical approach is often used to design new adsorbents.

Among different kinds of adsorbents (e.g. activated carbons, minerals, lignocellulosic/polysaccharides), synthetic resins present important advantages, namely their durability, stability, ease of regeneration and the possibility to create tailored active materials. Indeed, some classes of polymeric synthetic resins (e.g. Amberlite[®], Supellite[™] or Reillex[™] resins) are produced at large scale to be used in a broad range of application fields (environmental protection, biotechnology, pharmaceuticals, etc.). Improvement in specificity and capacity for the adsorption of selected compounds can be achieved with synthetic polymeric resins using synthesis routes allowing the functionalization of the materials or even the introduction of molecular recognition capabilities through molecular imprinting techniques. Classical free radical polymerization (FRP) and controlled radical polymerization (CRP) mechanisms play a key role for the tailoring of new adsorbent polymer networks. Moreover, the production of hybrid materials, incorporating natural and synthetic polymers, is also possible with appropriated synthesis routes, namely CRP, allowing the addressing of eco-sustainability requirements with the inclusion of the natural materials (e.g. cellulose).

Due to the relatively simple operation requirements, low cost and prevention of the degradation of the target compounds, sorption/desorption is a commonly used method to target polyphenols in plant extracts.

In practice, two general classes of processes are found in this application field:

- i) The recovery of target polyphenols from plant extracts to be used as high-added value products in food, cosmetic, pharmaceutical or chemical industries.
- ii) The treatment of effluents generated in industries involving the processing of plant extracts (generally wastewater effluents). The treatment of olive mill wastewater is an important issue in this context.

A major difficulty to be addressed with the sorption/desorption of plant extracts is the high complexity of the mixtures inherent to these systems that include a plethora of different compounds. Moreover, extracts composition are strongly dependent on their production conditions, namely the kind(s) of solvent used. Therefore, the development of tailored adsorbents is a key point aiming at the design of efficient sorption/desorption processes to target polyphenols.

This research explores the tailoring of functional adsorbents considering the grafting of synthetic polymers in cellulose backbone through Atom Transfer Radical Polymerization (ATRP). Actually, the grafting of cellulose with synthetic polymers through the use of ATRP, other CRP techniques, or free radical polymerization is being considered in the scientific community as an effective route to generate new materials for many different applications. Ions removal from aqueous solutions, dyes degradation, stimuli-responsive hydrogels fabrication or aqueous aggregates generation [1-5], are just a few examples of such kinds of applications (ref. [1] contains a comprehensive list of cellulose modification techniques and applications). A significant change in the native cellulose properties can be achieved with the grafting of synthetic polymers in this abundant and biodegradable material, thus increasing sustainability.

Additionally, nitrogen containing polymers, especially those based on vinylpyridine monomers [6], present enhanced adsorption capabilities towards specific compounds, which justify their use for the tailoring of some kinds of advanced materials. Indeed, due to their chelating capability, 4-vinylpyridine based materials are being considered for heavy metals adsorption with applications in wastewater/industrial effluents treatment [7-10], enzymes/protein adsorption [11,12] and other applications involving biomedicine or catalysis [13]. Due to the potential strong interaction between 4-vinylpyridine polymers and many polyphenols, our research explores hybrid cellulose-poly(4-vinylpyridine) materials to target these classes of compounds. As far as is of our knowledge, this

approach is considered for the first time for the processing of real plant extracts. The materials here synthesized are assessed to target polyphenols in winemaking, olive oil and almond industries that are three important agricultural/industrial sectors with worldwide expression and particular joint relevance in the Mediterranean area. We show that hybrid cellulose-poly(4-vinylpyridine) adsorbents present a very good performance for polyphenols adsorption in a broad range of working conditions with extracts of these three different plants, namely considering diverse solvents (e.g. with high organic content mixtures and with wastewater effluents). Indeed, the synthesized materials include a microcrystalline cellulose core (20 μm) preserving good conditions to be used in continuous sorption/desorption processes and excels for polyphenols retention when hydrophobic effects play a minor role due to functionalization with the pyridyl group. Thus, more flexible and efficient downstream processing of polyphenols can be designed with these adsorbents.

2.2 Materials and Methods

2.2.1 Reagents

Microcrystalline cellulose (MCC, powder, 20 μm , cotton linters), α -bromoisobutyryl bromide (BIBB, 98% purity), triethylamine (TEA, 99.5% purity), 4-(dimethylamino) pyridine (DMAP, purity $\geq$ 99%), ethyl 2-bromopropionate (EBrP, 99% purity), CuBr (97% purity) and N,N,N',N'',N'''-pentamethyldiethylenetriamine (PMDETA, 99% purity) were purchased from Sigma-Aldrich. Acrylonitrile (AN, 99% purity) and 4-vinylpyridine (4VP, 95% purity) were provided by Alfa Aesar. Analytical reagent grades for acetonitrile (ACN), dimethylformamide (DMF), acetic acid (AcOH), and methanol (MeOH) were bought from Fisher Scientific and for ethanol (EtOH) from PanReac. Millipore water (Milli-Q quality) was used in all the experiments unless otherwise mentioned.

2.2.2 Immobilization of α -bromoisobutyryl bromide (BIBB) in microcrystalline cellulose (MCC)

The immobilization of BIBB in MCC was performed using the reported esterification procedure [14,15]. Typically, 1 g of MCC was dispersed in DMF (100 mL) using sonication. Then, triethylamine (8 mL) and DMAP (4 g) were added to the MCC

dispersion and, afterwards, BIBB (8 mL) was dropwise fed to the suspension at 0 °C. The reaction was allowed to proceed at room temperature during 24 h. At the end, ethanol was used to terminate the esterification. Products were washed with DMF until achievement of a colorless liquid phase and then ATRP macroinitiator MCC-Br was purified using dialysis with deionized water, followed by drying in vacuum oven at 40 °C.

2.2.3 Grafting of synthetic polymer chains in MCC through ATRP

CuBr (5 mg) was dissolved in DMF (2 mL) via sonication. Then, MCC-Br (50 mg), PMDETA (20 μ L), EBrP (40 μ L), 4VP (3.25 mL) were added to the CuBr solution. The resulting solution was purged with a flow of dry argon for 30 min and the polymerization took place during 24 h at 60 °C. Acrylonitrile polymer chains were grafted in MCC following a similar procedure using AN instead of 4VP. The final products (MCC-Br-4VP or MCC-Br-AN) were washed with DMF and MeOH and dried in vacuum oven. Through these purification procedures, hybrid MCC-synthetic particles (DMF insoluble) and free polymer (methanol insoluble) were isolated and the monomer conversion, as well as the grafting ratio were gravimetrically estimated.

2.2.4 Products characterization using FT-IR spectroscopy

Purified and dried products were characterized through FT-IR spectroscopy with a Perkin Elmer, model Spectrum TwoTM, instrument. These analyses were directly performed in ATR mode or, when needed, polymers were mixed with KBr and pressed into pellets in order to collect the correspondent IR spectra.

2.2.5 Polymer characterization using SEC/RI/LALS/RALS

SEC analysis was considered to get information concerning the structure of different soluble polymers synthesized in this research. The used SEC apparatus is composed of a Viscotek GPCmax VE 2001 pumping system with Refractive Index (RI), Low Angle Light Scattering (LALS) and Right Angle Light Scattering (RALS) detection. Polymers were fractionated in a train of two columns PEL GEL 5 μ m mixed – D + PEL GEL 3 μ m mixed – E with dimensions L×D= 300 mm × 7.5 mm. DMF with 20 mM LiBr was used as eluent and the measurements were performed at 50 °C and Q=0.5 mL/min.

2.2.6 Preparation of different kinds of almond residues and grape pomace extracts

Almond fruits, including the hull, shell and the nuts were collected in Torre de Moncorvo (Trás-os-Montes, Portugal) during the autumn of 2019. After fruit components separation and drying, hull and almond shell were milled and used in Soxhlet extraction considering different runs with EtOH/water 80/20, ACN/DMF 50/50 and EtAc/EtOH 50/50 (v/v) solvents. Soxhlet extractions were performed using a ratio of 30 g of dried mass per 200 mL of solvent.

Grape pomace was obtained in the autumn of 2019, directly from a red wine producer located at Vila Real (Douro region, Portugal). Grape pomace before and after distillation (for brandy production) were both considered as polyphenols sources. After drying, grape pomace was also milled and used in Soxhlet extraction following the same procedures above described for almond residues.

All the extracts produced were filtered through a 0.45 µm nylon filter before use in sorption/desorption assays.

2.2.7 Aqueous effluents derived from the almond blanching process and olive mill wastewater

The almond blanching process was performed boiling 230 g of kernel fruits with skin in 700 mL of deionized water during 1 min. The liquid extract was filtered to remove solid mass and then used in sorption/desorption assays.

Olive mill wastewater was collected in winter of 2019 from an olive oil producer in Moura (Alentejo, Portugal) and filtered to remove suspended solids before use in sorption/desorption processes.

2.2.8 Sorption/desorption experiments

For assessment of the sorption/desorption capabilities of the different adsorbents considered in this work, 200 mg of the dry materials were used in solid phase extraction (SPE) packing cartridges. Adsorbents were first cleaned (successive loading and elution steps using methanol/acetic acid 90/10 with UV monitoring) and afterwards conditioned with the desired testing solvent during at least 24 h before use. Cartridges were then loaded with 5 mL of the selected extract considering typically 15 min for total percolation time. The adsorbed polyphenol profile was quantified through HPLC-DAD analysis of the liquid collected at column outlet (UV/vis monitoring was also considered for global

absorption quantification). Adsorbent saturation runs were also performed by continuous feeding of the column with the selected extract, up to achievement of a constant UV response at column outlet. Recovery of the adsorbed compounds was assessed by collecting fractions with different elution solvents, ranging from washing with the same solvent used for loading, up to the use of stronger displacement mixtures (e.g. methanol/acetic acid 90/10). The effect of the temperature in polyphenols desorption was also assessed by changing the temperature of the elution solvents (e.g. room temperature up to 50 °C). Phenolic profile of the recovered fractions was measured using HPLC-DAD analysis.

2.2.9 Polyphenols identification/quantification through gradient HPLC-DAD

A Jasco MD-4010 photo diode array (PDA) detector was used to carry out the HPLC-DAD analyses. Nucleosil® C18 analytical column, L×I.D.=15 cm × 4.6 mm and 5 µm particle size, working at room temperature, have been chosen. The flow rate was 1 mL/min and the mobile phases used for chromatographic analysis were: (A) acetonitrile/water (10/90) at pH = 3 (adjusted with acetic acid) and (B) acetonitrile/water (90/10), also at pH = 3. The linear gradient method was used, starting with 100% of solvent A and ending with 100% of solvent B. Previous results of our research with plant extracts analysis through LC-DAD-ESI-MSⁿ [16-18] were also considered in the present work for polyphenols identification.

2.2.10 Measurement of the bromide content in the MCC immobilized with BIBB

These bromine determinations were performed by ICP-MS (Inductively Coupled Plasma – Mass Spectrometry) using a Thermo Scientific™ iCAP™ Q instrument at LAQV/REQUIMTE – Laboratory of Applied Chemistry (Trace Element Analysis unit), Faculty of Pharmacy of the University of Porto (FFUP). To perform these analyses, MCC-Br was first dissolved in dimethyl sulfoxide at 70 °C at a concentration of 0.675 mg/mL.

2.2.11 SEM analysis of the adsorbent particles

Scanning Electron Microscopy (SEM) characterization of the particles involved in this research was performed at the International Iberian Nanotechnology Laboratory (INL),

Braga, using the FIB/SEM system HELIOS Nanolab 450S. SEM imaging was obtained using electron beam of 3 keV, beam current 25 pA and field free lens model

2.2.12 Measurement of the surface area and pore volume of the adsorbents

The Brunauer–Emmett–Teller (BET) specific surface area and pore volume of the produced adsorbent particles were determined through N₂ adsorption/desorption isotherms at 77 K using a Quantachrome NOVA 4200e adsorption analyzer.

2.3 Results and Discussion

Figure 2-1 depicts the synthetic steps considered to get hybrid cellulose-synthetic materials via ATRP. Our approach follows the same methodology described in previous related works [5,14,15]. At a first stage (**Figure 2-1(a)**), BIBB was immobilized in microcrystalline cellulose (MCC) through the reaction with OH groups in a DMF mixture containing TAE and DMAP (see section 2.2.2 for the detailed experimental conditions used). Confirmation of the BIBB immobilization in MCC, generating the ATRP macro-initiator MCC-Br, was performed using FTIR, as shown in **Figure 2-2**. Comparison of the FTIR spectra for native MCC and purified MCC-Br shows clearly the emergence of a peak in MCC-Br (1740 cm^{-1}) correspondent to the stretching of the carbonyl group (C=O) resulting from the reaction between OH groups and BIBB (see also **Figure 2-1(a)**). The Br content in the macro-initiator MCC-Br was measured by ICP-MS and the concentration of 135 mg/g was obtained, which is in the same range of measurements reported in previous related works (see [15] and references therein).

The synthesized ATRP macro-initiator was afterwards used to graft synthetic polymer chains in MCC, namely P(4VP) and PAN chains, as depicted in **Figure 2-1(b)**, considering the ATRP of 4VP for illustration purposes. Besides the ATRP macro-initiator, this synthesis step included also the use of EBrP initiator aiming at inducing the formation of free synthetic polymer in addition to the grafted chains. This procedure is often exploited to get indirect information concerning the structure of the grafted polymer through the analysis of the free product [14,19].

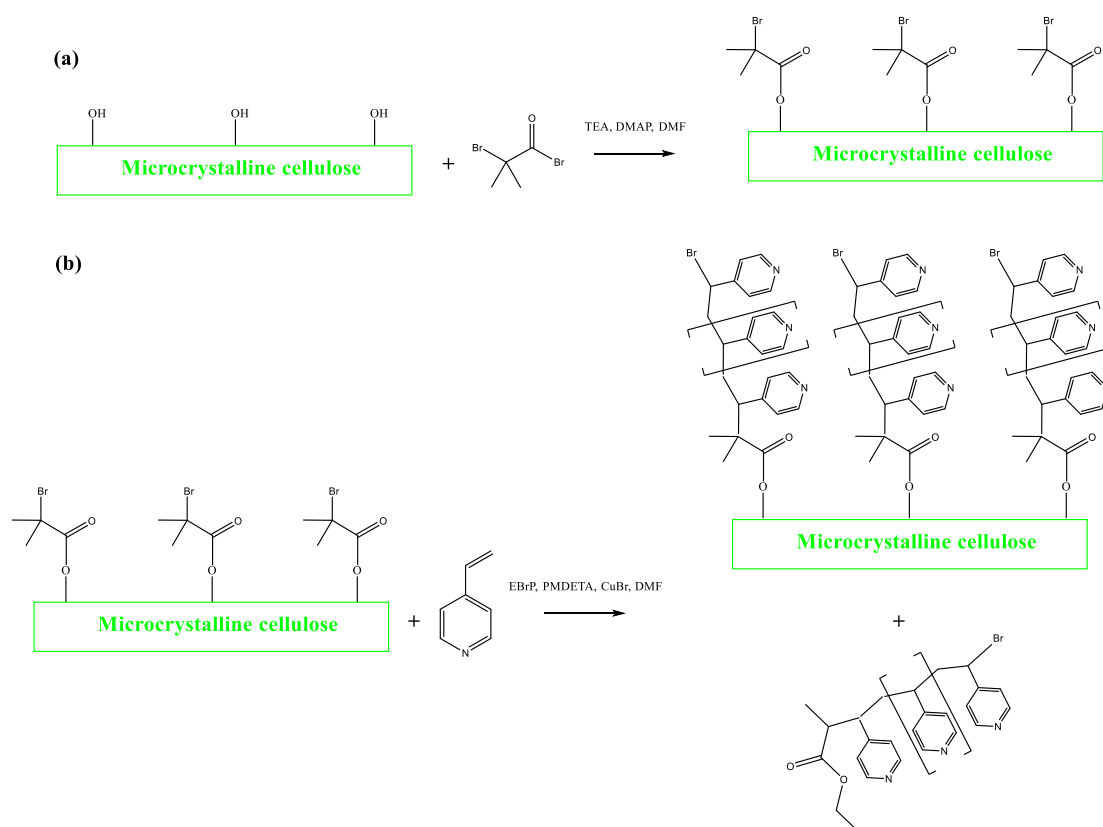


Figure 2-1: Depiction of (a) immobilization of α -bromoisobutyryl bromide (BIBB) in microcrystalline cellulose (MCC) with production of the ATRP macro-initiator MCC-Br and (b) grafting of 4VP polymer chains in MCC through ATRP. In the grafting step, besides the macro-initiator MCC-Br, the ATRP initiator EBrP was also included to generate the formation of free P(4VP) homopolymer

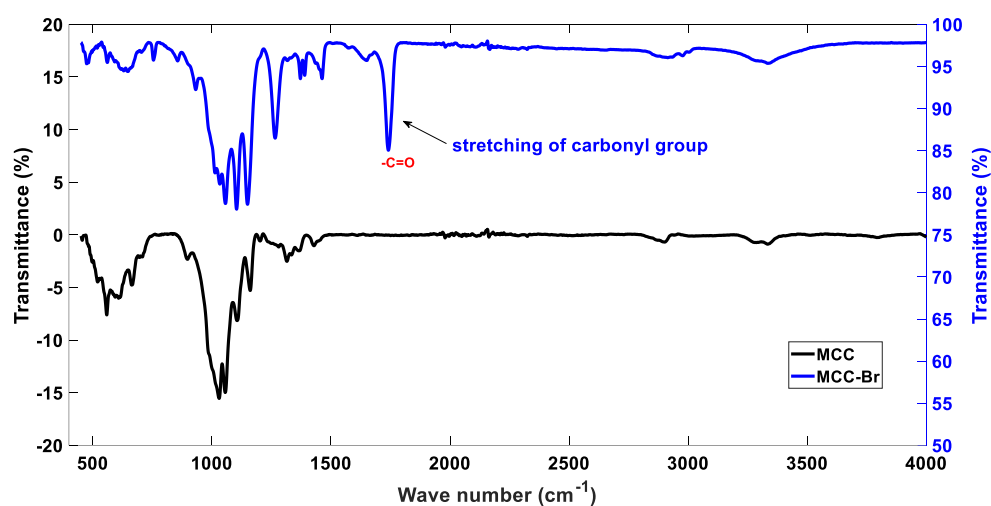


Figure 2-2 FTIR spectra for the native microcrystalline cellulose (MCC) and for the purified product resulting from the immobilization of α -bromoisobutyryl bromide in MCC (MCC-Br).

With the systems here considered, both free P(4VP) and PAN formed in these grafting ATRP runs are soluble in the DMF liquid phase while the cellulose containing products (starting with MCC-Br up to the MCC-P(4VP) or MCC-PAN hybrid materials) remain insoluble along the reaction (the heterogeneous approach was considered). **Table 2-1** contains detailed information concerning the synthesis conditions used to generate the MCC-P(4VP) and MCC-PAN hybrid adsorbents.

Table 2-1: Reaction conditions used to synthesize different ATRP and FRP products considered for the development of hybrid cellulose-poly(4-vinylpyridine) and cellulose-polyacrylonitrile adsorbents. All the polymerizations were performed with DMF as solvent.

Product	MCC-Br (g/L)	EBrP (mol/L)	Monomer (mol/L)	CuBr (mol/L)	PMDETA (mol/L)	AIBN (mol/L)
MCC-P(4VP)	9.511	5.749×10^{-2}	5.752	6.630×10^{-3}	1.804×10^{-2}	-
MCC-PAN	12.365	7.519×10^{-2}	7.521	8.569×10^{-3}	2.359×10^{-2}	-
P(4VP)	-	5.738×10^{-2}	5.709	7.114×10^{-3}	1.787×10^{-2}	-
PAN	-	7.522×10^{-2}	7.522	8.347×10^{-3}	2.257×10^{-2}	-
P(4VP) - FRP	-	-	3.478	-	-	1.427×10^{-2}
PAN - FRP	-	-	3.440	-	-	6.900×10^{-3}

Gravimetrically measured content of P(4VP) in the final MCC-P(4VP) product was 90%, while 64% of PAN was estimated for the MCC-PAN hybrid material. A high incorporation rate of the synthetic polymer in the hybrid materials was here explored in order to compare the adsorption capabilities of the modified materials relatively to cellulose. However, much lower synthetic content (e.g. lower than 10%) can be attained depending on the materials applications by changing the grafting conditions. Incorporation of the synthetic polymers in the final materials was also confirmed using FTIR analysis, as shown in **Figures 2-3** and **2-4**. Assignments corresponding to the $\text{C}=\text{N}$ (1415 cm^{-1}) and $\text{C}=\text{C}$ (1550 and 1600 cm^{-1}) pyridyl groups are clearly observable in the spectrum collected for the MCC-P(4VP) materials, while the nitrile group ($\text{C}\equiv\text{N}$) assignment is patent with the FTIR analysis for MCC-PAN (2245 cm^{-1}).

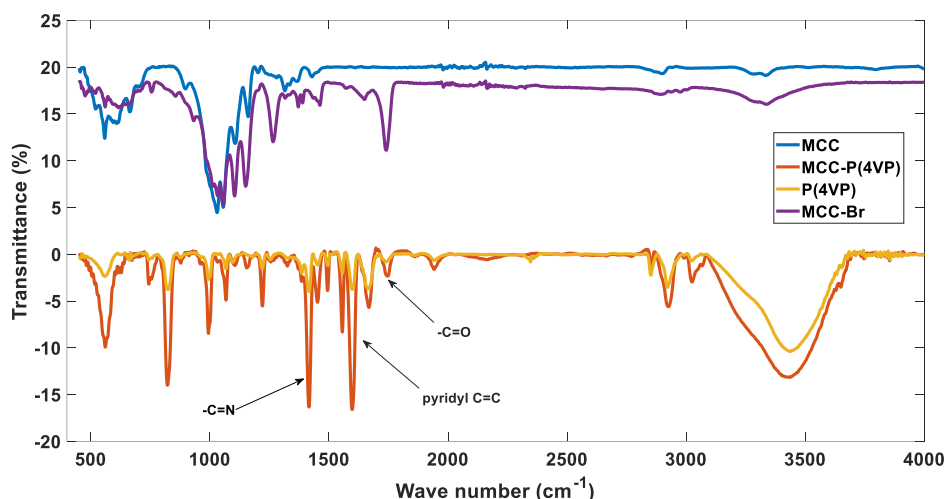


Figure 2-3: FTIR spectra for MCC, MCC-Br, ATRP synthesized hybrid cellulose-poly(4-vinylpyridine) (MCC-P(4VP)) and an ATRP synthesized P(4VP) homopolymer

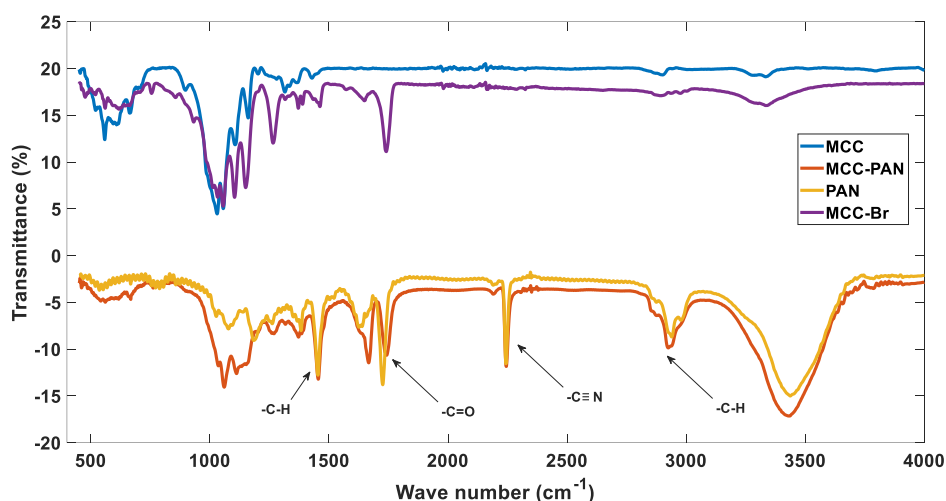


Figure 2-4: FTIR spectra for MCC, MCC-Br, ATRP synthesized hybrid cellulose-polyacrylonitrile (MCC-PAN) and an ATRP synthesized PAN homopolymer.

In **Figures 2-5** and **2-6** are presented the SEC/RI/LALS/RALS traces observed for the free P(4VP) and PAN polymers collected at the end of the grafting reactions leading to the formation of the MCC-P(4VP) and MCC-PAN hybrid materials, respectively. For comparison purposes, these plots include also the SEC traces for P(4VP) and PAN homopolymers synthesized via ATRP with EBrP initiation, as well as for samples of the same homopolymers produced by free radical polymerization (FRP) with AIBN initiation. The reaction conditions used in the synthesis of these four homopolymers can be found in **Table 2-1**. For the interpretation of the results in **Figures 2-5** and **2-6**, it should be noted that the chromatogram traces appearing after the system peak (starting at around 18 min elution time) are due to non-polymer species, namely 4VP monomer and

its possible metal coordination complexes (this effect is not pronounced with the AN monomer). Results presented in **Figure 2-5(a)** show the formation of a bimodal polymer population for the free polymer produced during the MCC-P(4VP) grafting process. Such bimodal polymer population is not observed for the P(4VP) homopolymer synthesized in a common ATRP process with EBrP/CuBr/PMDETA (see **Table 2-1**), as presented in **Figure 2-5(b)**. An unimodal polymer population is also formed when the P(4VP) homopolymer is synthesized via FRP, as shown in **Figure 2-5(c)**. The comparison between the bimodal polymer correspondent to the free chains formed in MCC-P(4VP) grafting and the unimodal polymers produced via ATRP or FRP homopolymerizations is highlighted in **Figure 2-5(d)**. This comparison shows that the free polymer formed during the grafting process is composed of a population close to that formed in a similar homogeneous ATRP process and another with very high molecular size, even higher than that found in FRP synthesis. Thus, a second free polymer population is formed during the grafting process in conditions different from those expected with the homogeneous conditions.

The formation of a bimodal polymer population is also observed for the free polymer with the ATRP grafting process involving the AN monomer, as presented in **Figure 2-6(a)**. Again, as expected, unimodal populations were measured for PAN homopolymers synthesized in a common ATRP process with EBrP/CuBr/PMDETA or via FRP, as shown in **Figures 2-6(b-c)**. The comparison in **Figure 2-6(d)** highlights again that the free polymer in grafting is composed of a population similar to that formed with an analogous homogeneous ATRP, plus another population with much higher molecular size. This second population is less pronounced in the grafting with AN than that observed with 4VP, as becomes evident through the comparison of **Figures 2-5(d)** and **2-6(d)**.

The formation of multimodal polymer populations can be found in non-linear polymerization systems due to variable degree of branching/crosslinking among the different species [20,21]. Following a different mechanism, the bimodal populations above described for the free polymer formed during MCC-P(4VP) and MCC-PAN synthesis should be a consequence of the coexistence of two regions with different reaction conditions in the liquid phase. In the bulk of the DMF liquid phase the reactant concentrations should be closer to that correspondent to a homogeneous ATRP, while in the vicinity of the solid phase (cellulose + grafted polymer) these concentrations can be substantially different.

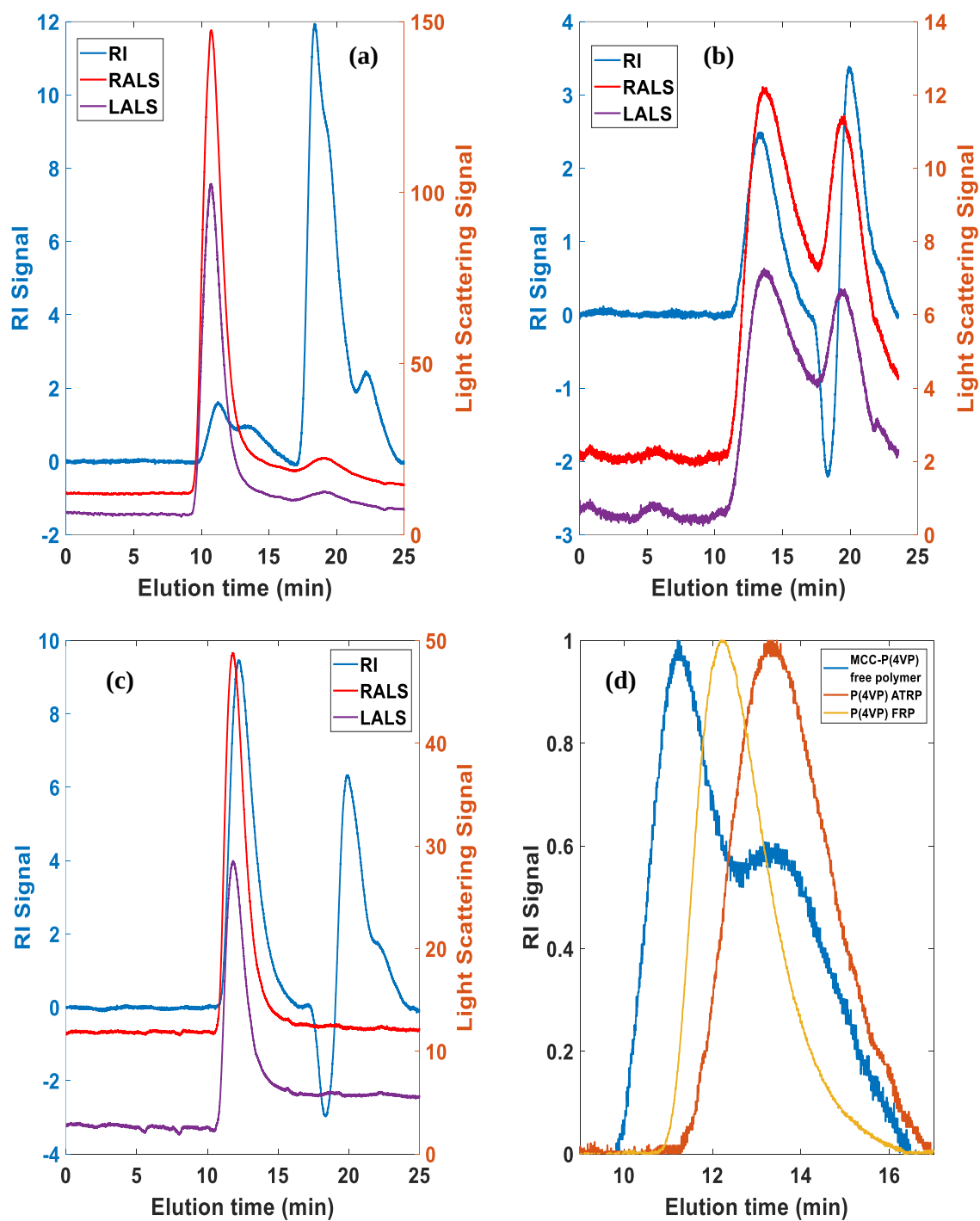


Figure 2-5: SEC/RI/LALS/RALS traces observed for different polymers involving the 4VP monomer. (a) Free polymer collected at the end of the ATRP grafting step involving MCC-Br, 4VP and EBrP. (b) P(4VP) synthesized through ATRP with EBrP initiator. (c) P(4VP) synthesized through FRP with AIBN initiator. (d) Comparison of the RI signals for these three samples. Details on the synthesis conditions of these polymers can be found in **Table 2-1**.

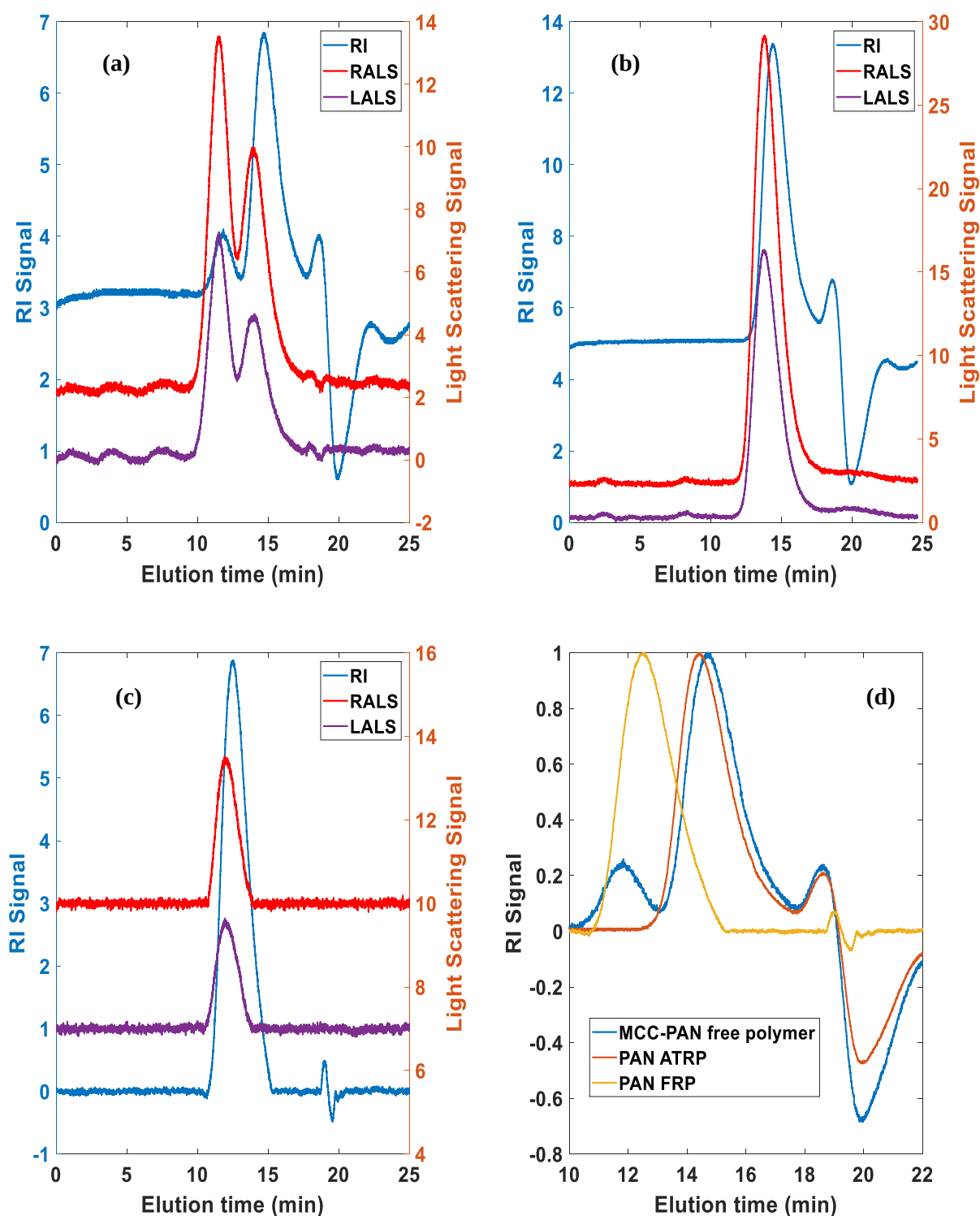


Figure 2-6: SEC/RI/LALS/RALS traces observed for different polymers involving the AN monomer. (a) Free polymer collected at the end of the ATRP grafting step involving MCC-Br, AN and EBrP. (b) PAN synthesized through ATRP with EBrP initiator. (c) PAN synthesized through FRP with AIBN initiator. (d) Comparison of the RI signals for these three samples. Details on the synthesis conditions of these polymers can be found in **Table 2-1**.

Dissimilar adsorption of the different ATRP reactants in the solid phase should lead to the formation of a polymer population not following the expected homogeneous process.

This issue is magnified when P(4VP) is involved due to the inherent strong H-bond capability and ability to generate metal coordination complexes. The issues just reported with the free polymer structure anticipate that the distribution of synthetic brushes grafted in cellulose should also deviate from the one in expected homogeneous ATRP products. In order to further investigate the formation of these bimodal polymer populations, other ATRP grafting process were performed with 4VP and AN, changing the concentration of MCC-Br macro-initiator, with the remaining conditions unchanged, as presented in **Table 2-1** and **appendix A (Table A-1)**. The SEC analysis of the free polymers formed in such different conditions (**Figure A-2**) show the decreasing of bimodality with the concentration of ATRP macro-initiator MCC-Br, supporting the possibility for the heterogeneity of the polymerization through the effect of the solid phase.

Thus, a thorough knowledge on the partition of the ATRP reactants among the phases involved should be used in order to design synthetic brushes with a target size. Multiphase polymer reaction engineering modeling tools, including also the plausible effect of radical termination in ATRP, are potentially helpful to design such synthesis conditions and also to discriminate the mechanisms leading to bimodality, but this subject is beyond the scope of the present paper.

SEM analysis illustrated in **Figure 2-7** (see also **appendix A, Figure A-1**) shows that the native MCC particles have characteristic dimension around 20 μm while the MCC-P(4VP) and MCC-PAN particles present correspondent dimensions in the order of 40 to 50 μm . The observed increase in particle size is a consequence of the ATRP grafting of these synthetic polymers in the native MCC.

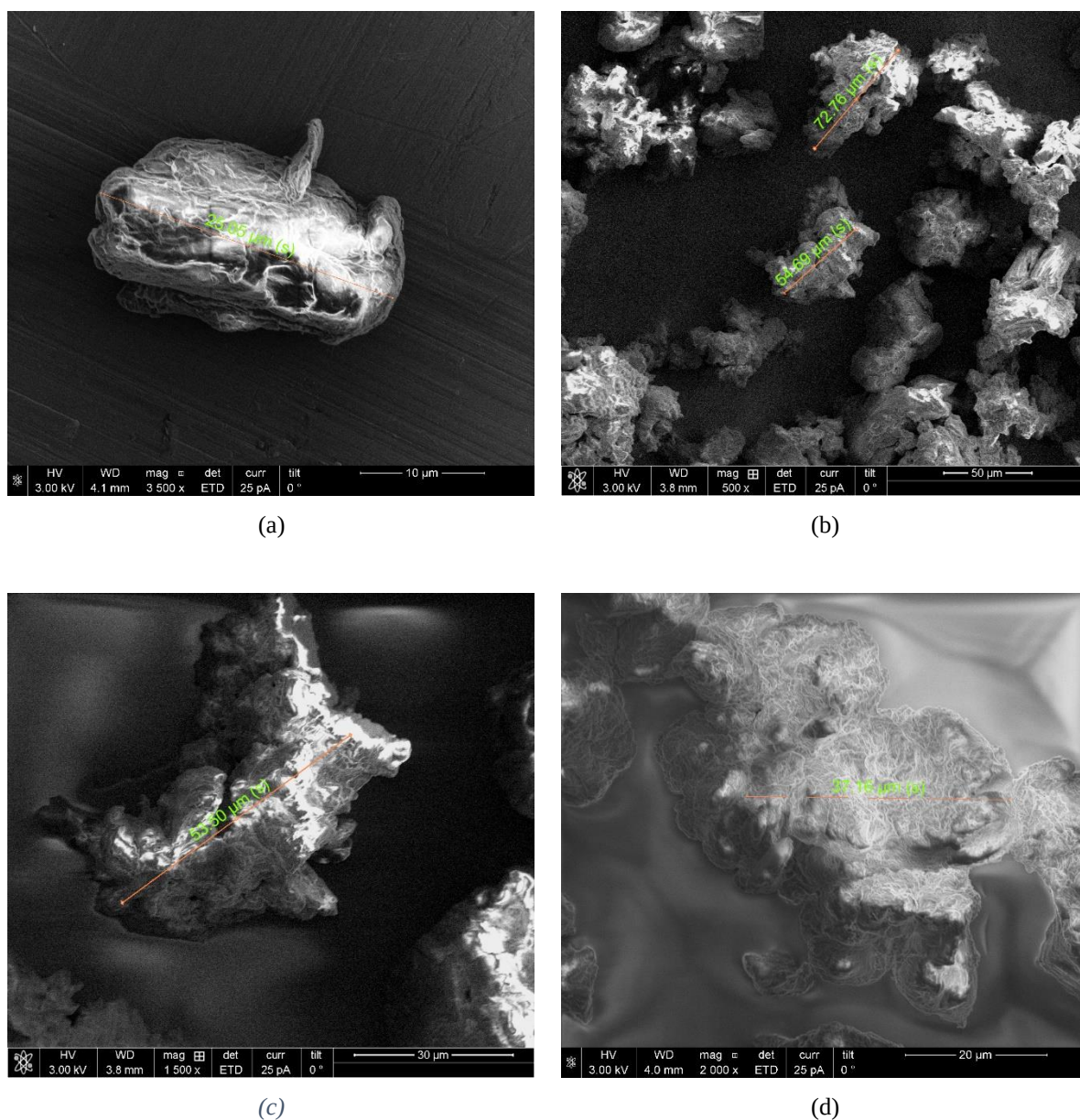


Figure 2-7: SEM images showing the morphology of some particles addressed in this research. (a) Native microcrystalline cellulose particles (MCC). (b) and (c) ATRP synthesized hybrid cellulose-poly(4-vinylpyridine) particles (MCC-P(4VP)). (d) ATRP synthesized hybrid cellulose-polyacrylonitrile particles (MCC-PAN).

The final goal of this research is the assessment of the hybrid MCC-P(4VP) and MCC-PAN materials to target polyphenols present in winemaking, olive oil and almond residues, namely when compared with a diverse range of alternative synthetic adsorbents. **Table 2-2** contains a brief description of the adsorbents considered within this purpose. Besides the MCC-P(4VP) and MCC-PAN synthesized adsorbents, the native MCC

particles (20 μm , cotton linters) and different commercial resins often used for polyphenols targeting were included in the testing runs. Moreover, 4VP and AN based adsorbent, synthesized via FRP in the framework of our previous researches [16-18], were also used with grape pomace extracts, olive mill wastewater and almond extracts/blanching wastewater.

Table 2-2: Description of the different adsorbents used to target polyphenols in winemaking, olive oil and almond residues.

Adsorbent	Product description
MCC-P(4VP)	Synthesized through BIBB immobilization in MCC followed by ATRP grafting of 4VP
MCC	Commercial microcrystalline cellulose
MCC-PAN	Synthesized through BIBB immobilization in MCC followed by ATRP grafting of AN
DAX-8	Commercial resin (Supelite™, polymethylmethacrylate resin of moderate polarity)
XAD4	Commercial resin (Amberlite®, hydrophobic polyaromatic resin based on styrene–divinylbenzene)
XAD7HP	Commercial resin (Amberlite®, moderately polar acrylic ester resin)
P(4VP-EGDMA)	Crosslinked polymer particles synthesized with 4VP/EGDMA 51/49 mol/mol through free radical polymerization with AIBN as initiator and ACN/DMF 30/70 v/v as precipitation solvent
P(4VP-AN-DVB)	Crosslinked polymer particles synthesized with 4VP/AN/DVB 45/45/10 mol/mol/mol through free radical polymerization with AIBN as initiator and ethanol/water 67/33 v/v as precipitation solvent

Figure 2-8 illustrates a relevant outcome of the present work considering the targeting of the polyphenols in an EtOH/water 80/20 grape pomace extract as a case study. Indeed, we show that a high polyphenol retention is possible in the MCC-P(4VP) adsorbent (c.a. 90% measured at 280 nm) while only a small fraction is adsorbed by the MCC or MCC-PAN materials (c.a. 10% at 280 nm). **Figure 2-8** is also used to illustrate the quantification method here considered for polyphenols retention by the different adsorbents. Our calculation procedure is based on the comparison of the area of the HPLC-DAD chromatogram of the original extract with that observed for the sample collected after the loading in the adsorbent. Because the UV absorbance of the different polyphenols changes with the wavelength, fixed values for λ were selected, namely 280, 320, 360 and

380 nm. The evaluation at 280 nm was mostly considered for the different adsorbents assessment, as discussed below, but this analysis can be extended to other fixed wavelength values or even considering the whole UV–vis spectra (see also the results concerning polyphenols recovery below presented). These complexities are also illustrated for the dynamics of adsorption in the Supplementary Material (appendix A, Figures A-3 to A-5). Besides the plethora of different compounds absorbing at very different wavelengths, the competitive displacement of specific molecules during the adsorption due to the effect of other species is other mechanism hardening the monitoring of such processes.

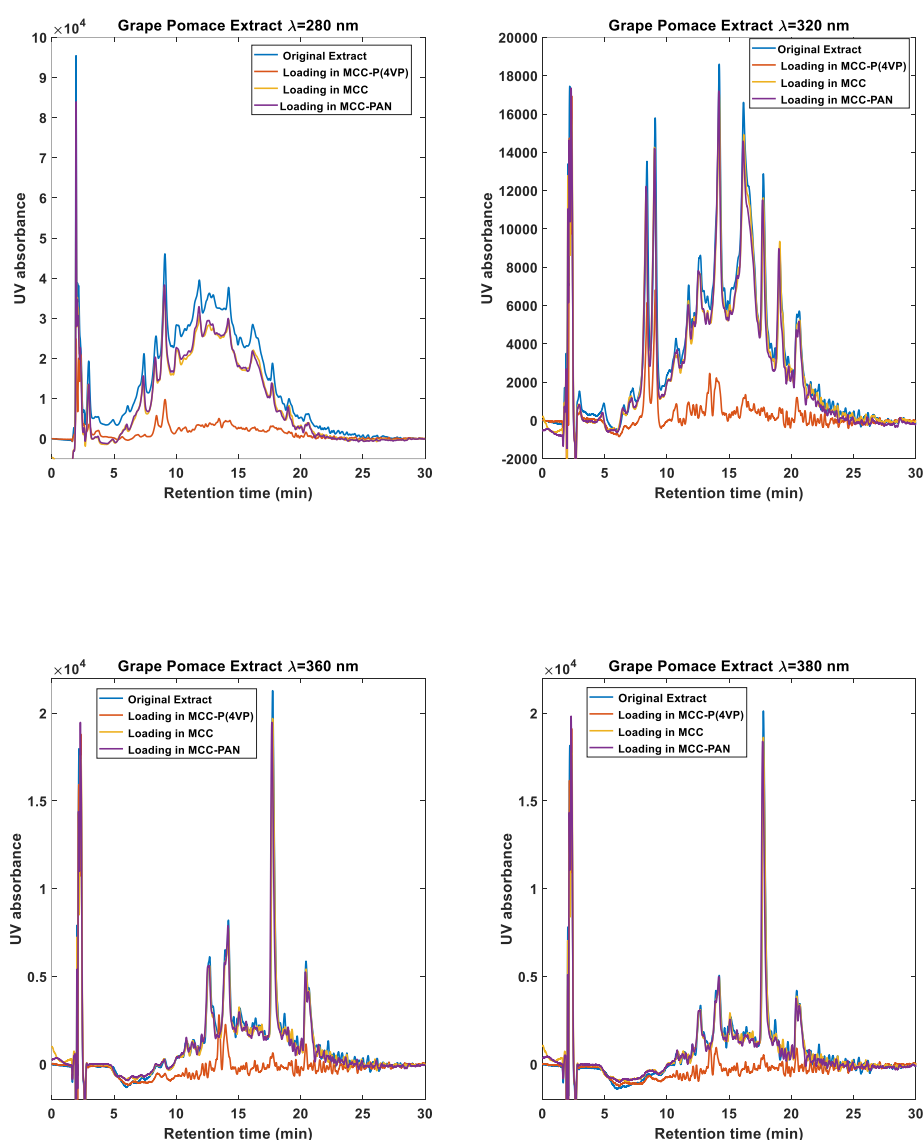


Figure 2-8: Comparison of the HPLC-DAD chromatograms for a grape pomace extract obtained in EtOH/water 80/20 and for the correspondent SPE processed solutions collected at column outlet after extract loading in different adsorbents. Loading in MCC-P(4VP), MCC and MCC-PAN is here compared and results at $\lambda=280$, 320, 360 and 380 nm are presented.

Figure 2-9 presents a concise view of results for the adsorption of different grape pomace extracts with all materials here considered. These results follow from the extension to other samples of the analysis described in **Figure 2-8** for the specific cases EtOH/water 80/20 with MCC-P(4VP), MCC and MCC-PAN adsorbents. Results in Figure 2-9 highlight the higher polyphenol retention that is achieved with the MCC-P(4VP) adsorbents comparatively not only to MCC or MCC-PAN but also to the commercial resins (DAX-8, XAD4 and XAD7HP) or other 4VP/PAN based materials (P(4VP-EGDMA) and P(4VP-AN-DVB)). Three different grape pomace extracts were considered in this analysis, namely those obtained with EtOH/water 80/20, ACN/DMF 50/50 and EtAc/EtOH 50/50, because the extraction solvent is an important designing parameter for real industrial applications (targeted polyphenols, process efficiency, ecological footprint, etc.). Considering the average of the polyphenols retention achieved for the three grape pomace extracts, MCC-P(4VP) scores 79% followed by P(4VP-AN-DVB) with 72% and P(4VP-EGDMA) with 54% (in spite of the almost nil retention observed with the ACN/DMF 50/50 extract). Much lower retention values are observed with the commercial resins or the MCC and MCC-PAN materials (in the range 10 to 30%).

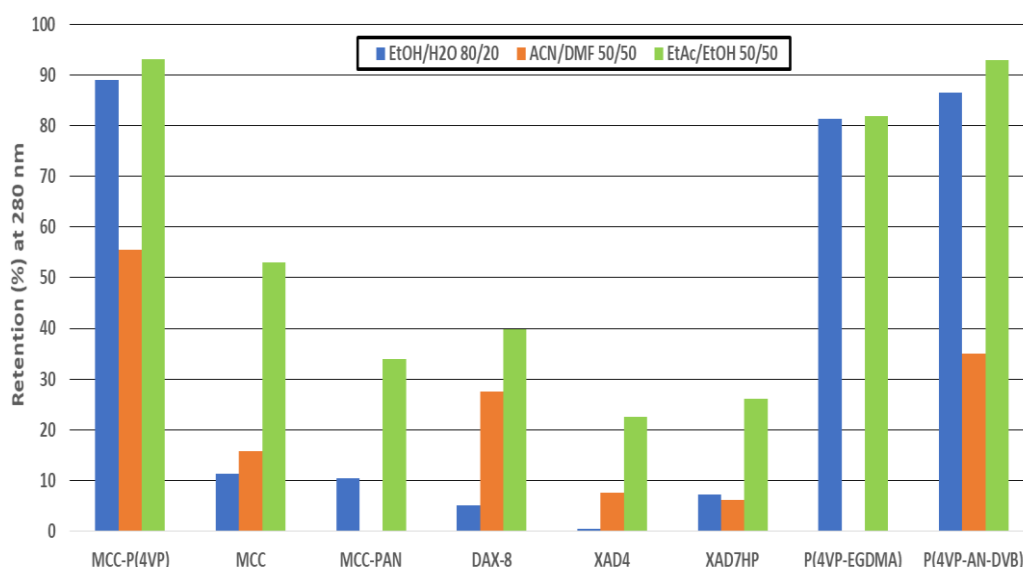


Figure 2-9: Comparison of the retention in different adsorbents observed with different grape pomace extracts (obtained with EtOH/water 80/20, ACN/DMF 50/50 and EtAc/EtOH 50/50 as solvents). Measurements were performed in SPE columns and retention was calculated using UV absorbance at $\lambda=280$ nm.

The outcomes above described are a consequence of the specific binding capabilities associated to adsorbents based on 4VP, as detailed in ref. [7]. The $-N=$ group in the pyridyl moiety contains one extra electron pair that is not involved in the ring conjugation system. This annular enclosed conjugated system is formed because each atom in the ring

contains one p orbital and each p orbital adds one electron to the total of six p electrons. The bonding between the carbon and nitrogen atoms in the pyridyl group is made through sp^2 hybrid orbitals and a sp^2 hybrid orbital is also present in the nitrogen atom that is occupied by a lone pair of electrons [7]. Therefore, this lone pair of electrons is able to participate in strong H-bonding mechanisms, as well as to bind with protons. Indeed, the pyridyl group behaves as a proton acceptor and is also a good ligand for multiple metal ions, giving rise to complexation [7]. Due to these specific features, adsorbents based on 4VP also present a strong binding capacity for many kinds of polyphenols, as shown in our previous works [16-18]. Comparison of the results here presented with MCC-P(4VP) and MCC-PAN adsorbents also confirm the stronger interaction of the pyridyl group with polyphenols comparatively to the nitrile group (the binding stabilization provided by the conjugated system in the pyridyl moiety plays an important role). Note that the N_2 adsorption/desorption results evidence a very low specific surface area and pore volume, not only for the native MCC, but also for the hybrid MCC-P(4VP) and MCC-PAN particles synthesized (see **appendix A, Table A-2**). These outcomes corroborate that the high polyphenol retention observed with the hybrid MCC-P(4VP) particles are mostly due to the functionalization of the external surface with the pyridyl functional groups. Results for the adsorption of hull and shell almond polyphenols contained in EtOH/water 80/20, ACN/DMF 50/50 and EtAc/EtOH 50/50 are presented in **Figure 2-10**. These measurements confirm the superior capacity of the MCC-P(4VP) adsorbent to retain polyphenols contained in almond residues also when solvents with high organic content are used. The strong interaction of the pyridyl group with many polyphenols, as above discussed, gives support to these outcomes. The effect of hydrophobic interactions, which is the driving force for the adsorption of polyphenols in many adsorbents used with plant extracts, has a lower impact in retention when working with 4VP based materials. Among all adsorbents tested, taking the average retention for the four almond extracts considered, MCC-P(4VP) scores with 76%, followed by P(4VP-AN-DVB) with 63% and P(4VP-EGDMA) with 55%. Again, a very small retention is observed with the commercial resins or with MCC and MCC-PAN materials.

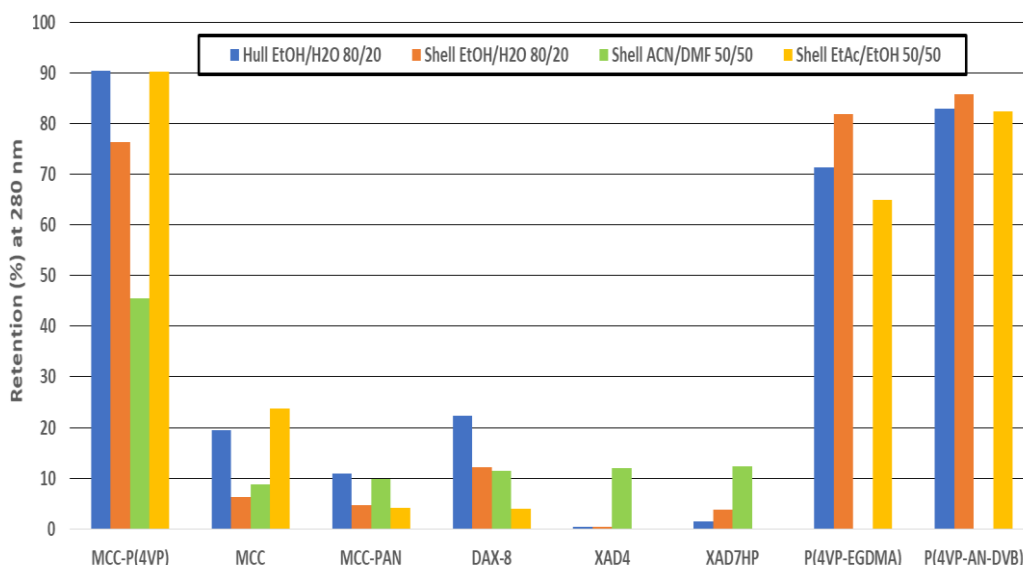


Figure 2-10: Comparison of the retention in different adsorbents observed with different almond hull and almond shell extracts (obtained with EtOH/water 80/20, ACN/DMF 50/50 and EtAc/EtOH 50/50 as solvents). Measurements were performed in SPE columns and retention was calculated using UV absorbance at $\lambda=280$ nm.

In **Figure 2-11** are presented the retained fractions measured with aqueous extracts containing polyphenols. Olive mill wastewater and almond blanching wastewater were processed with the different adsorbents and the results show an important contribution of hydrophobic interactions for adsorption. Commercial resins are now able to retain appreciable amounts of polyphenols (e.g. DAX-8 scores 74% for the average of the two extracts) but the best outcomes are observed with the two 4VP based adsorbents synthesized via FRP (89% for P(4VP-AN-DVB) and 83% for P(4VP-EGDMA)). The adsorbent MCC-P(4VP) scores now with 44% average retention and lower values are observed for MCC (13%) or MCC-PAN (21%). The combined effect between the adsorption capabilities of the pyridyl moieties and the hydrophobic interactions enhanced by the crosslinkers (EGDMA and DVB) reason the high polyphenol retention observed with the P(4VP-EGDMA) and P(4VP-AN-DVB) adsorbents. On the other hand, the pH conditions of the olive mill wastewater (pH=5) and of the almond blanching wastewater (pH=6.1) also contribute for their relatively lower adsorption capability as measured with the MCC-P(4VP) material (note the high P(4VP) content of this adsorbent). Indeed, as demonstrated before with P(4VP) based resins for heavy metals adsorption, the pH has an important effect on amounts and selectivity of the different cations retained [7]. When the pH is low enough, the pyridine ring becomes protonated and positively charged molecules cannot be adsorbed whereas anions can be retained by electrostatic

interactions. Similar pH effects are possible for phenolic acids in olive mill wastewater and almond blanching wastewater, namely by shifting the equilibrium for phenolic acids dissociation and P(4VP) protonation. However, the most important factor hindering polyphenols retention at acid conditions with MCC-P(4VP) materials should be the decrease of the pyridyl group capacity to establish hydrogen bonding with these compounds as a consequence of protonation. Adjustment of pH is therefore a tool that can be considered to increase adsorption rates with MCC-P(4VP) materials but our option here was the use of the simple processing conditions concerning the direct processing of the aqueous extracts.

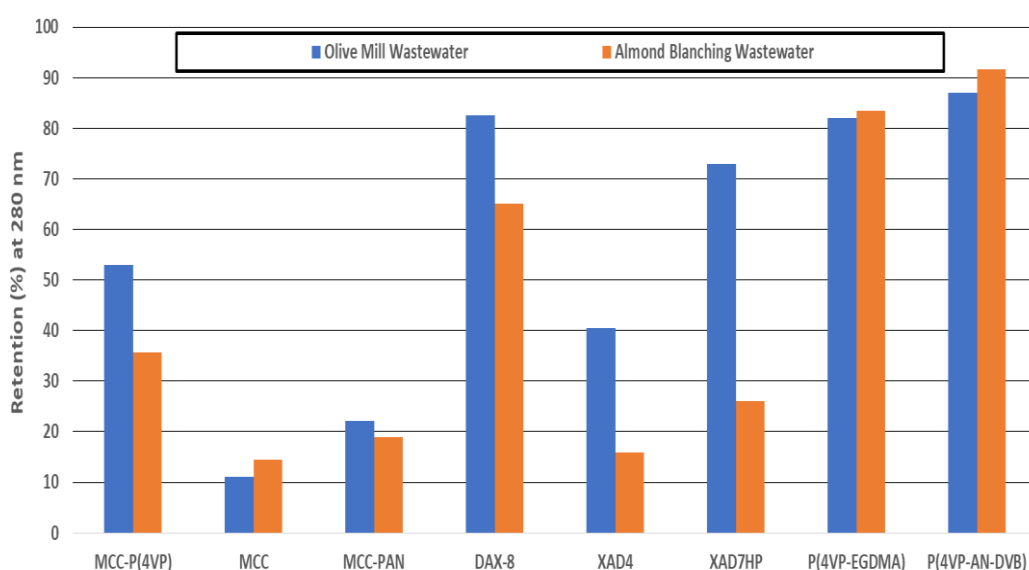


Figure 2-11: Comparison of the retention in different adsorbents observed with olive mill wastewater and almond blanching wastewater. Measurements were performed in SPE columns and retention was calculated using UV absorbance at $\lambda=280$ nm.

Overall, considering the nine different extracts described in **Figures 2-9 to 2-11**, the MCC-P(4VP) adsorbent presents an average retention capability of 70%, a slightly higher value (72%) results for P(4VP-AN-DVB), while 61% stands for P(4VP-EGDMA). These results confirm the good retention capability of 4VP based adsorbents for polyphenols considering a broad range of working conditions (much lower retention averages are observed with the other adsorbents). When only the organic or low water content extracts are considered (decreasing the hydrophobic effects and therefore enhancing adsorbent specificity), the MCC-P(4VP) scores with 77% while P(4VP-AN-DVB) and (4VP-EGDMA) with 67% and 54%, respectively.

Note that, as stated above, with complex mixtures like plant extracts, the polyphenols are adsorbed differently depending particular interactions with the polymer network moieties. Thus, when the simple global polyphenol retention is assessed, the value changes with the wavelength due to particular spectra of the species retained. This effect is highlighted in **Figure 2-12** by comparing the polyphenol retention measured at 280 and 380 nm for almond shell and grape pomace extracts in ACN/DMF 50/50 and considering the different adsorbents. These results also demonstrate the higher capacity of the MCC-P(4VP) adsorbent to retain polyphenols even when stronger organic solvents (ACN/DMF 50/50) are used. A more comprehensive view of polyphenols adsorption/desorption change with wavelength can be observed in **Figure 2-13** (see also appendix A).

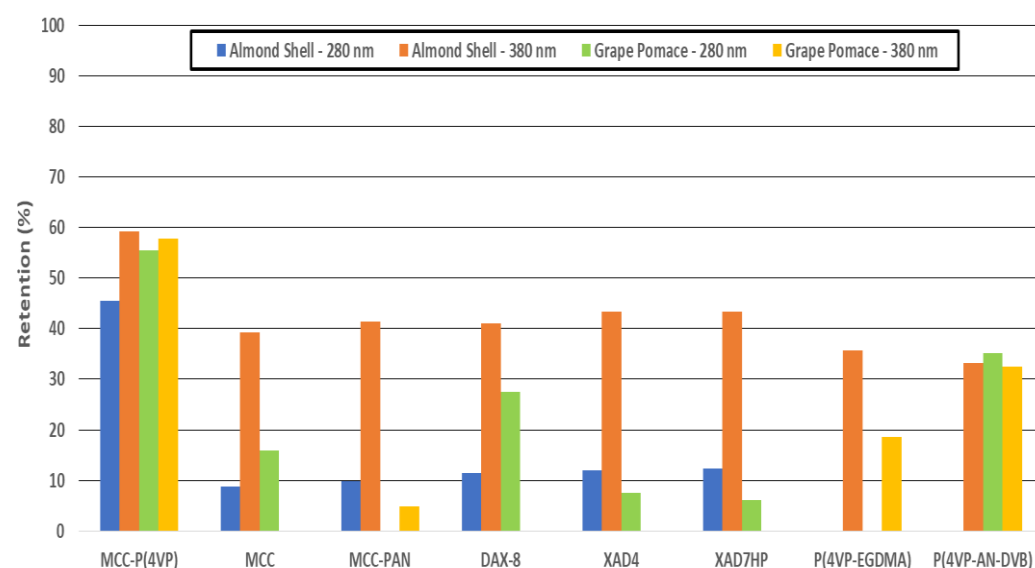


Figure 2-12: Comparison of the retention measured at $\lambda=280$ nm and $\lambda=380$ nm considering different adsorbents with almond shell and grape pomace extracts obtained in ACN/DMF 50/50.

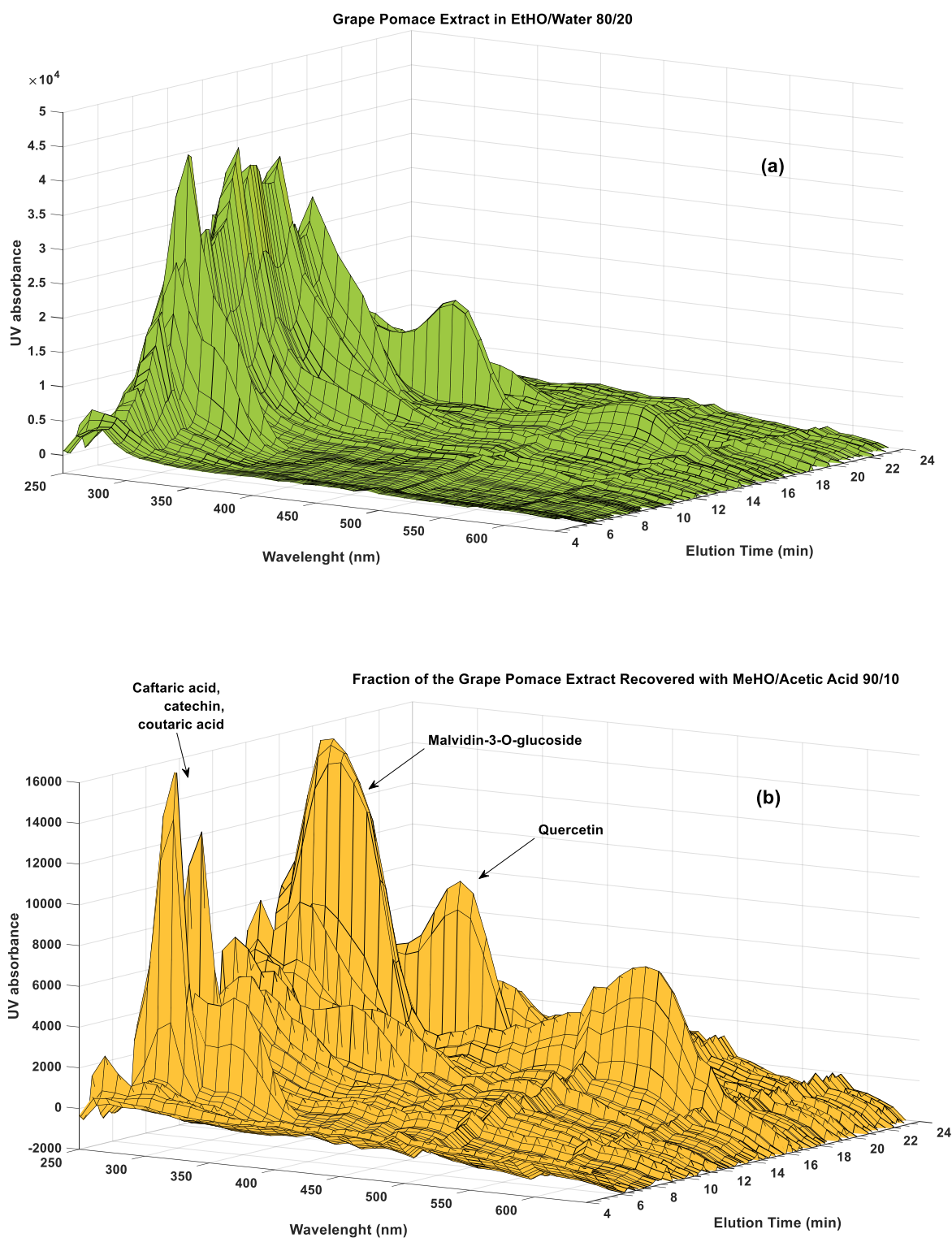


Figure 2-13: HPLC-DAD analysis of: (a) a grape pomace extract obtained in EtOH/water 80/20 and (b) a fraction of this extract recovered in MeOH/acetic acid 90/10 after loading in the MCC-P(4VP) adsorbent.

Results above presented demonstrate the good capability of the MCC-P(4VP) adsorbents to retain polyphenols in different plant extracts. This is an important issue for industrial processes dealing with very high liquid volumes containing polyphenols at low

concentration (e.g. food industry). In practice, the transport of such high volumes for secondary processing must be avoided and therefore efficient enrichment mechanisms should be considered. In the simplest case, this enrichment is just a volume reduction [22] and adsorbents with high retention capability play an important role in this context. These features were above illustrated with the 4VP-based materials synthesized in our research. Additionally, besides the concentration (enrichment) process, the recovery and fractionation of the polyphenols is also sought in many industrial applications. These purification and fractionation applications require the assessment of polyphenols desorption after their loading in the used materials. Selective desorption processes are often triggered by the use of a sequence of solvents with different elution strengths and/or temperature-swing mechanisms. The application of the MCC-P(4VP) adsorbents in the purification and fractionation of polyphenols in grape pomace, almond residues and olive mill wastewater was also addressed in the present work as illustrated in **Figure 2-13** (see also appendix A).

Figure 2-13(a) shows the HPLC-DAD chromatogram observed for a grape pomace extract obtained in EtOH/Water 80/20 and reveals a complex mixture of phenolic compounds that are known to be present in wine and vinery residues. Citric acid, caftaric acid, catechin, *p*-coumaric acid, quercetin glucuronide, resveratrol, quercetin and kaempferol are just a few examples of polyphenols present in such kinds of extracts [17,23-25], besides different sorts of anthocyanins and their 3-*O*-monoglucosides [23]. On the other hand, **Figure 2-13(b)** shows a fraction recovered after the loading of this grape pomace extract in the MCC-P(4VP) adsorbent. This specific fraction was recovered with MeOH/Acetic acid 90/10 (see section 2.2.8) and the HPLC-DAD analysis presented in **Figure 2-13(b)** highlights the purification and fractionation achieved with this simple sorption/desorption procedure. Indeed, the comparison of **Figures 2-12(a)-(b)** shows the production of fraction enriched with the *malvidin*-3-*O*-glucoside anthocyanin, as indicated by the increase of absorption intensity in the visible region of the spectrum. A set of well-defined peaks correspondent to other polyphenols are also now discernible in HPLC-DAD analysis as consequence of this simple sorption/desorption process. If needed, further sorption/desorption steps should be considered to achieve higher polyphenol purification/fractionation, depending on the application requirements. A similar analysis for an almond shell extract obtained in EtOH/water 80/20 and handled through a sorption/desorption process with the MCC-P(4VP) adsorbent is presented in the **appendix A, Figure A-6**. The initial extract exhibits a complex mixture of

polyphenols including catechin, procatechuic acid, epicatechin, vanillic acid, quercetin and quercetin-glucosides, kaempferol and kaempferol-glucosides, as well as isorhamnetin and isorhamnetin glucosides, among others [22,26,27]. Comparison of initial/processed extract demonstrates the purification of the almond shell extract achieved with this simple sorption/desorption process. The fraction recovered was enriched in isorhamnetin-3-O-rutinoside and also in quercetin-glucosides (different isomers, diglucosides or galactosides are possible [22,26,27]). These outcomes are useful for downstream processing of almond residues and also for analytic purposes, namely for sample preparation aiming at polyphenols identification and quantification.

The first application of the MCC-P(4VP) adsorbent with aqueous extracts is presented in Appendix A. Polyphenols present in almond blanching wastewater were adsorbed in the MCC-P(4VP) material and a fraction afterwards recovered with MeOH/Acetic acid 90/10. The purification of the initial almond blanching wastewater was achieved and an isorhamnetin-3-O-rutinoside rich fraction was obtained. Note also the difference between the initial phenolic profile for the almond shell extract in EtOH/water 80/20 and almond blanching wastewater (see **Appendix A, Figures A-7**) caused by the raw materials used and mainly by the extraction solvents (EtOH/water 80/20 and water). In both cases the MCC-P(4VP) adsorbent was usefully used for purification/fractionation of the contained polyphenols.

The processing of olive mill wastewater with the MCC-P(4VP) adsorbent is illustrated in **appendix A Figure A-8**. The main polyphenols in this kind of complex aqueous extracts belong to the classes of phenolic alcohols (e.g. tyrosol and hydroxytyrosol), phenolic acids (vanillic acid, caffeic acid, sinapic, vanillin acid, etc), secoiridoids and derivatives (oleuropein, elenolic acid, oleoside, etc) and flavonoids (apigenin, luteolin, luteolin-7-glucoside, etc) [28-30]. In Appendix A the phenolic profile is also presented for a fraction recovered with MeOH/water(pH=2) 90/10 after the loading of the olive mill wastewater in MCC-P(4VP). The fraction thus recovered was enriched in secoiridoids and flavonoids while other major components of the olive mill wastewater, namely many phenolic alcohols and acids, were successfully removed from the solution using this simple sorption/desorption process with the MCC-P(4VP) materials. These results show that the use of the adsorbents here developed deserves technological scrutiny for olive mill wastewater valorization, namely in integrated processes including crystallization [31] or membranes filtration [32]

2.4. Conclusions

Microcrystalline cellulose particles were grafted via ATRP with a high content of poly(4-vinylpyridine) brushes. The hybrid particles were used as adsorbents to target polyphenols in different kinds of plant extracts derived from grape pomace, almond residues and almond processing wastewater, as well as olive oil production effluents. It was observed that the morphology of the final hybrid particles keeps appropriated for use in continuous sorption/desorption processes. For comparison purposes, a hybrid adsorbent resulting from the ATRP grafting of polyacrylonitrile in cellulose was also synthesized.

The produced hybrid adsorbents were assessed for the retention of polyphenols in organic solvents (ACN/DMF 50/50 and EtAc/EtOH 50/50), organic/aqueous mixtures with low water content (EtOH/water 80/20) and completely aqueous extracts (almond and olive mill wastewater). These assays included also the use of other adsorbents, namely the native microcrystalline cellulose, commercial resins (DAX-8, XAD4 and XAD7HP) as well as other polymer networks synthesized via radical polymerization with 4-vinylpyridine as co-monomer. Considering the average polyphenol retention as comparative parameter and nine different extracts, the MCC-P(4VP) adsorbent and the 4VP based networks present a much higher performance (c.a. 70% retention) comparatively to MCC, MCC-PAN or commercial resins (retention in the range of 10 to 20% observed for many materials). Averaging with exclusion of the aqueous extracts leads to an even higher retention capability for MCC-P(4VP) (c.a. 77%) also when compared with the other 4VP based networks. These results are grounded by the strong binding capacity of the pyridyl group towards many polyphenols and show that the design of adsorbents working with a lower impact of the hydrophobic interactions is achieved increasing the 4VP content in the materials.

MCC-P(4VP) adsorbents were also used for purification and fractionation of polyphenols using simple sorption/desorption steps. With this straightforward approach, a fraction enriched with the malvidin-3-O-glucoside anthocyanin was obtained from a grape pomace extract; fractions containing high content of isorhamnetin-3-O-rutinoside were isolated from almond shell as well as from almond blanching wastewater and fractions enriched in secoiridoids and flavonoids were produced from olive mill wastewater while many phenolic alcohols and acids were removed. These results show the potentiality of

the MCC-P(4VP) adsorbents to improve downstream processing of polyphenols and increase the biorefining efficiency (e.g. allowing direct handling of organic extracts with high polyphenol concentration).

This research also shows that the use of controlled radical polymerization techniques, namely ATRP, offers many possibilities for the designing of natural-synthetic functional materials to be used as tailored adsorbents. The combination of molecular imprinting with ATRP functional grafting in cellulose to gain materials with molecular recognition features and the optimization of the cellulose/synthetic incorporation ratios in the final materials are issues to be addressed in future works. A comprehensive cost-benefit analysis on the use of the kind of adsorbents here addressed is also needed including sustainability factors, as well as, the potential increase on process efficiency and flexibility due to the use of tailored materials. The formation of a polymer population not following the expected homogeneous process here demonstrated through SEC analysis also deserves scrutiny in future works to gain precision in the designing of synthetic brushes with a target size. This is particularly important when monomers with strong H-bond capability, such as 4VP, are involved.

2.5. References

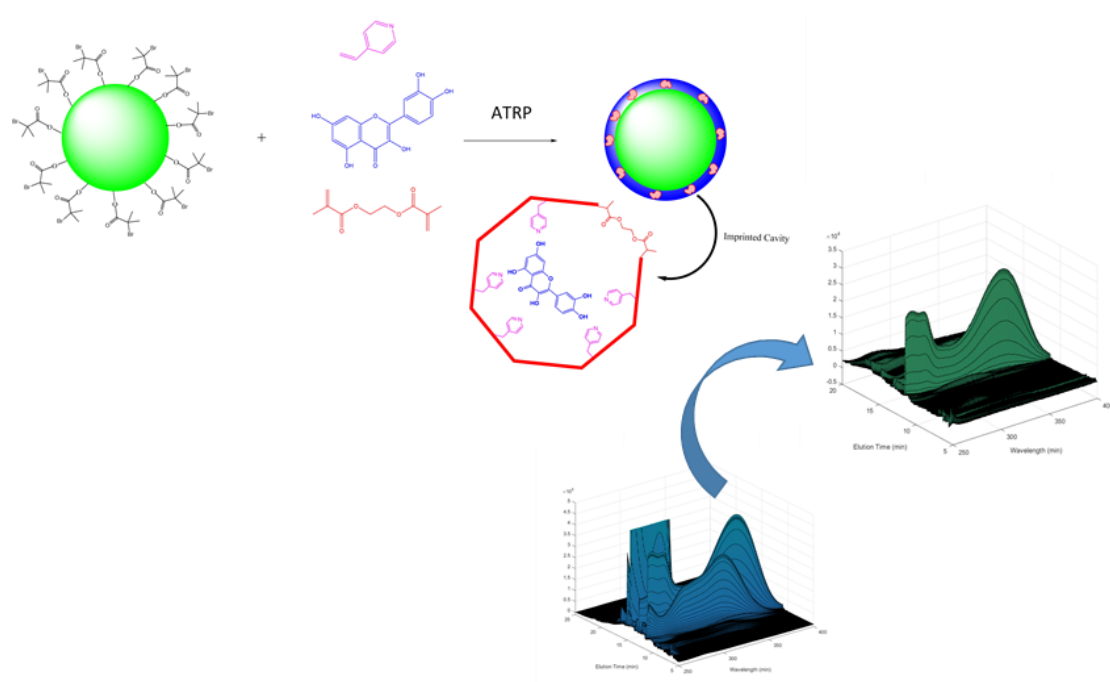
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Chapter 3. Selective flavonoid capture using ATRP-derived cellulose hybrid MIPs



Surface molecularly imprinted cellulose-synthetic hybrid particles were prepared via ATRP. Modification of the surface of the pristine cellulose and the achievement of molecular imprinting with the quercetin template were both observed. The developed particles were used for the enrichment of flavonoids in olive leaf extracts. Fractionation of these complex extracts was possible with the production of samples enriched with flavonoids

Surface Molecularly Imprinted Cellulose-Synthetic Hybrid Particles Prepared via ATRP for Enrichment of Flavonoids in Olive Leaf

Catarina P. Gomes, Rolando C. S. Dias, Mário Rui P.F.N. Costa, Surface Molecularly Imprinted Cellulose-Synthetic Hybrid Particles Prepared via ATRP for Enrichment of Flavonoids in Olive Leaf, *Macromolecular Reaction Engineering*, Volume 17, 2023, 2300011, <https://doi.org/10.1002/mren.202300011>

Abstract:

Surface molecularly imprinted cellulose-synthetic hybrid particles were prepared via atom transfer radical polymerization (ATRP). The two-step process involves the immobilization of α -bromoisobutyryl bromide in the pristine microcrystalline cellulose, to generate ATRP macro-initiator particles, and then the creation of a crosslinked-imprinted shell in the particles surface considering ATRP of co-monomers 4-vinylpyridine (4VP) and ethylene glycol dimethacrylate (EGDMA) with quercetin as imprinting template molecule. Among the different polymerization recipes tested, a system with ethanol as solvent preserves a final size of the hybrid particles suitable for their application as adsorbent, while also allowing the incorporation of the 4VP/EGDMA co-monomers. Testing of imprinted and non-imprinted particles with the sorption/desorption of standard phenolic compounds shows the modification of the surface of the pristine cellulose and also the achievement of molecular imprinting (imprinting factor ~ 2.6). The particles were used for the enrichment of flavonoids in olive leaf extracts and the special features of the developed molecularly imprinted adsorbents were again highlighted with this complex mixture of phenolic compounds. We show that production of fractions rich in luteolin-7-O-glucoside, apigenin-7-O-glucoside or quercetin, among other flavonoids, is possible (e.g. with estimated enrichment factors up to 4). These results point up for the usefulness of natural-synthetic materials with processes to get high-added value compounds in the framework of circular bio-economy.

Keywords: Surface molecular imprinting; Cellulose; Atom transfer radical polymerization; Natural-synthetic hybrid particles; Adsorbents; Flavonoids; Olive leaves

3.1. Introduction

Cyclic sorption/desorption processes are widely used in industry as well as in analytical procedures to make separation and purification of target compounds. Adsorbents for these applications range from natural materials and their modified counterparts (e.g. activated carbons, silica-based, other minerals, lignocellulosic/polysaccharides) to synthetic polymer resins (e.g. the industrially relevant Amberlite[®], Supellite[™] or Reillex[™] resins). Durability, stability, ease of regeneration and the possibility to create tailored active materials are important benefits associated to synthetic polymer resins. Environmental protection, biotechnology, pharmaceuticals and chemicals production are application fields with a large-scale use of polymer resins for separation and purification.

Nowadays, a growing effort is being observed for the valorization of agricultural residues and sub-products, namely through the enrichment and isolation of compounds with high-added value. This downstream processing approach is being considered with many systems in the framework of sustainability and circular bio-economy policies. A specific case with high potential economic relevance concerns the valorization of olive leaf. Indeed, around 4.5 million tons of leaves are generated each year as a by-product of the olive and olive-oil production. These olive leaves must be removed from the fields and olive-mills. Usually they are either burned, or simply used as fuels or for animal feed. However, olive leaf is extremely rich in bio-resources, being estimated to contain around 1 million tons of bioactive compounds, 1 million tons of cellulose and 1.5 million tons of lignin which are currently underexploited [1]. In particular, the extraction and isolation of high-added value bioactive compounds in olive leaf (e.g. different kinds of polyphenols and triterpenoids) is especially appealing due to their high-market potential in the food, feed, chemical, nutraceutical, cosmetic and pharmaceutical sectors [1].

Extraction, followed by sorption/desorption processes (to purify and concentrate liquid mixtures), are often considered to get target compounds from plant residues and sub-products. Actually, a key issue in this field concerns the processing of diluted streams with presence of many unwanted compounds. Solvent evaporation (with possible thermal effects on bioactive compounds) and/or crystallization (difficult with complex mixtures) are energetically demanding and challenging in view of the use of sustainable, efficient and economically feasible industrial applications. Harsh conditions requiring

considerable cost and energy expenditure are in general inherent to crystallization for separation, purification and downstream production (technology based on solvent evaporative methods). Therefore, the lower energy consumption, cyclability, preservation of temperature-sensitive compounds, operational flexibility, amenability to intensification and industrial scalability make sorption/desorption processes attractive in this domain. Nowadays, membrane-supported selective separation is also playing an important role in this field. In practice, the valorization of underutilized effluents derived from olives debittering, artichoke washing, olive oil or natural juices production, among many others, is being already made with sorption/desorption processes [2].

Here, we address the development of tailored adsorbents and their application in sorption/desorption processes for the enrichment of flavonoids in olive leaf extracts. Surface molecularly imprinted cellulose-synthetic hybrid particles, prepared via atom transfer radical polymerization (ATRP), were developed to assess the combination in the same material of high retention capability (selection of appropriated synthetic functional monomers), selectivity towards target compounds (molecular imprinting), enhancement of binding site accessibility and high mass transfer (particles with active surface). These features are joined in core-shell particles with cellulose core, highlighting the possibility for generation of advanced natural-synthetic materials with application in the development of sustainable processes in the framework of circular bio-economy.

The synthesis approach here considered for surface molecular imprinting (see a recent overview on the subject in [3]) includes: (i) the immobilization of α -bromoisobutyryl bromide in pristine microcrystalline cellulose (MCC) to generate ATRP macro-initiator particles (MCC-Br) and, (ii) the grafting of a crosslinked-imprinted shell in the surface of these particles through ATRP with 4-vinylpyridine (4VP) as functional monomer, ethylene glycol dimethacrylate (EGDMA) as crosslinker and quercetin as the flavonoid template. Selected imprinted particles are assessed with standard polyphenol compounds and also with an industrial olive leaf extract. The production of flavonoid-enriched fractions from the complex olive leaf extract, through sorption/desorption processes with developed imprinted particles, is highlighted.

3.2. Experimental Section

3.2.1 Materials

All chemicals were used as purchased, without further purification. Microcrystalline cellulose (MCC, powder, 20 μ m, cotton linters), α -bromoisobutyryl bromide (BIBB, 98% purity), triethylamine (TEA, 99.5% purity), 4-(dimethylamino) pyridine (DMAP, purity $\geq$ 99%), CuBr (97% purity) and N,N,N',N'',N'''-pentamethyldiethylenetriamine (PMDETA, 99% purity) were purchased from Sigma-Aldrich. 4-vinylpyridine (4VP, 95% purity) was provided by Alfa Aesar. Analytical reagent grades for acetonitrile (ACN), dimethylformamide (DMF), acetic acid (AcOH), and methanol (MeOH) were bought from Fisher Scientific and for ethanol (EtOH) from PanReac. Quercetin (hydrate, purity 95%) and rutin (purity 97%) were supplied by Acros Organics. Oleuropein (pure) was purchased from PanReac and vanillic acid (purity 97%) from Sigma-Aldrich. These standard polyphenols were used in the synthesis and testing of the materials here addressed. The water used in the experiments is ultrapure water supplied by the local laboratory.

3.2.2 Immobilization of α -bromoisobutyryl bromide (BIBB) in microcrystalline cellulose (MCC)

The immobilization of BIBB in MCC was performed using the reported esterification procedure, as described previously [4]. Briefly, in a typical experiment, 1 g of MCC was dispersed in DMF (100 mL) using sonication. Then, triethylamine (8 mL) and DMAP (4 g) were added to the MCC dispersion and, afterwards, BIBB (8 mL) was dropwise fed to the suspension at 0 $^{\circ}$ C. The reaction was allowed to proceed at room temperature during 24 h. At the end, ethanol was used to terminate the esterification. Products were washed with DMF until achievement of a colorless liquid phase and then ATRP macroinitiator MCC-Br was purified using dialysis (3.5k MWCO, ThermoFisher Scientific dialysis bag) with deionized water, followed by drying in vacuum oven at 40 $^{\circ}$ C.

3.2.3 Surface imprinting in MCC-Br macro-initiator particles via ATRP

The required amounts of dried ATRP macroinitiator MCC-Br, functional monomer 4VP and template quercetin were added to the polymerization solvent and the interaction functional monomer-template was promoted in an ultrasounds ice bath during 30 min.

Then, the remaining reactants (EGDMA, CuBr and PMDETA) were dissolved in that solution and a flow of dry argon was used to purge the mixture for 30 min. Afterwards, polymerization took place in a sealed vessel during 72 h at 70 °C. The final polymerization products were purified in 3.5k MWCO dialysis bags with successive changing of the methanol/acetic acid 90/10 cleaning solvent. In parallel to the above-described synthesis procedure for surface molecularly imprinted particles (MIPs), non-imprinted particles (NIPs) were prepared by avoiding the presence of quercetin. The purified MIPs and NIPs were dried in vacuum oven at 40 °C up to the measurement of a constant weight. Gravimetric analysis was considered to estimate the amount of synthetic polymer in the final hybrid MCC particles.

3.2.4 Products characterization using FT-IR spectroscopy

Purified and dried products were characterized through FT-IR spectroscopy with a Perkin Elmer, model Spectrum Two™, instrument. These analyses were directly performed in ATR mode or, when needed, polymers were mixed with KBr and pressed into pellets in order to collect the correspondent IR spectra.

3.2.5 HPLC-DAD Analysis

An HPLC system (KNAUER) consisting of a gradient pump (P6.1 L) equipped with a degasser, an autosampler (6.1 L), a column thermostat (CT2.1) and a diode array detector (DAD - 6.1 L) was used in this research. ClarityChrom® was the software allowing the control of the HPLC system. The chromatographic analysis was performed using an Ascentis® C18 (SUPELCO®) column with a particle size of 5 µm and dimensions 25 cm × 4.6 mm. A gradient of solvents was used as a mobile phase varying from 100% of water-ACN (9:1) to 100% water-ACN (1:9) for 45 min. The mobile phase water pH was adjusted to 3 using acetic acid. The flow rate of the chromatographic analyses was 1 mL /min, and the temperature of the column was set at 45 °C.

3.2.6 Identification and Quantification of Polyphenols in Olive Leaf Extracts using LC-DAD-ESI-MSⁿ

These analyses were carried out at the Centro de Investigação de Montanha (CIMO). The chromatographic analysis was performed using a Dionex UltiMate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) system equipped with a diode array detector coupled to

an electrospray ionization mass detector (LC-DAD-ESI/MSⁿ), a quaternary pump, an auto-sampler, a degasser and an automated thermostatic column compartment. Chromatographic separation was carried out with a Waters Spherisorb® S3 ODS2(C18), L×I.D. = 15 cm×4.6 mm and 3 µm particle column (Waters, Milford, MA, USA) working at 35 °C. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was isocratic 15% B (5 min), 15% B to 20% B (5 min), 20–25% B (10 min), 25–35% B (10 min), 35–50% B (10 min), and re-equilibration of the column, using a flow rate of 0.5 mL/min

3.2.7 UV/vis spectroscopy

A spectrophotometer P9 Double Beam UV-Visible, VWR, was used for fast assessment of cleaning steps, breakthrough curves and quantification of simple polyphenol solutions, namely with the loading, washing and elution steps for assessment of MIPs/NIPs sorption/desorption with polyphenol standards.

3.2.8 Sorption/desorption experiments with polyphenol standards

Assessment of the sorption/desorption capabilities of the MIPs/NIPs was performed with 25 mg of the dry materials in solid phase extraction (SPE) packing cartridges (Agilent Bond Elut reservoir, 1 mL capacity). Adsorbents were first cleaned (successive loading and elution steps using methanol/ acetic acid 90/10 with UV/vis monitoring) and afterwards conditioned with the desired testing solvent (e.g. ethanol/water 80/20), during at least 24 h before use. Cartridges were then loaded with 2.5 mL of the selected polyphenol solution at concentration 0.126 mM considering typically 15 min for total percolation time. The adsorbed polyphenol profile was quantified through UV/vis analysis of the liquid collected at column outlet. The washing desorption step was performed by percolating the same volume of clean solvent through the adsorbent particles and, afterwards, the elution desorption step was made with the same volume of methanol/acetic acid 90/10. Desorbed amounts of polyphenol were quantified also by UV/vis analysis of the collected liquid phases in the washing and elution desorption steps.

3.2.9 Sorption/desorption experiments with olive leaf extracts

An industrial olive leaf extract provided by NATAC (Alcorcón, Madrid, Spain) was used along these experiments. The required amount of solid olive leaf extract was dissolved in

the selected solvent (e.g. ethanol/water 80/20) to meet the target concentration (e.g. 5 mg/mL). Breakthrough curves were assessed with the adsorbent particles in SPE cartridges, as above described. Fixed volumes of the extract solution (e.g. 2.5 mL) were percolated through the particles and the liquid phase at column outlet was analyzed by UV/vis spectroscopy to evaluate the sorption process. After saturation, pre-defined desorption steps were performed with fixed volumes of successive different solvents (e.g. from pure water to pure ethanol). Samples collected at column outlet for each desorption step were analyzed by HPLC-DAD for evaluation of olive leaf extract fractionation and enrichment of target compound

3.3. Results and Discussion

3.3.1. Rationale for Materials Synthesis

The reaction steps considered for the synthesis of surface molecularly imprinted cellulose-synthetic hybrid particles are depicted in **Figure 3.1**. Our approach follows previous related works with ATRP surface grafting in cellulose [5-7]. The first step (Figure 3.1(a)) concerns immobilization of BIBB in microcrystalline cellulose (MCC) through the reaction with OH groups. Confirmation for the generating of the ATRP macro-initiator MCC-Br was performed using FTIR, as presented in **Figure 3.2**. Comparing the FTIR spectra for pristine MCC and for the purified MCC-Br product, a new peak at 1740 cm^{-1} is observed in MCC-Br as consequence of the stretching of the carbonyl group (-C=O) resulting from the reaction between OH groups in cellulose and BIBB (see also **Fig. 3.1(a)**). This is the same reaction step also reported in our previous work concerning the grafting of 4VP in MCC without molecular imprinting [4]. Using ICP-MS (Inductively Coupled Plasma – Mass Spectrometry), following our synthesis procedure, the bromide content in MCC-Br is estimated to be of 135 mg/g [4], which is in a similar range of values reported in related works [7].

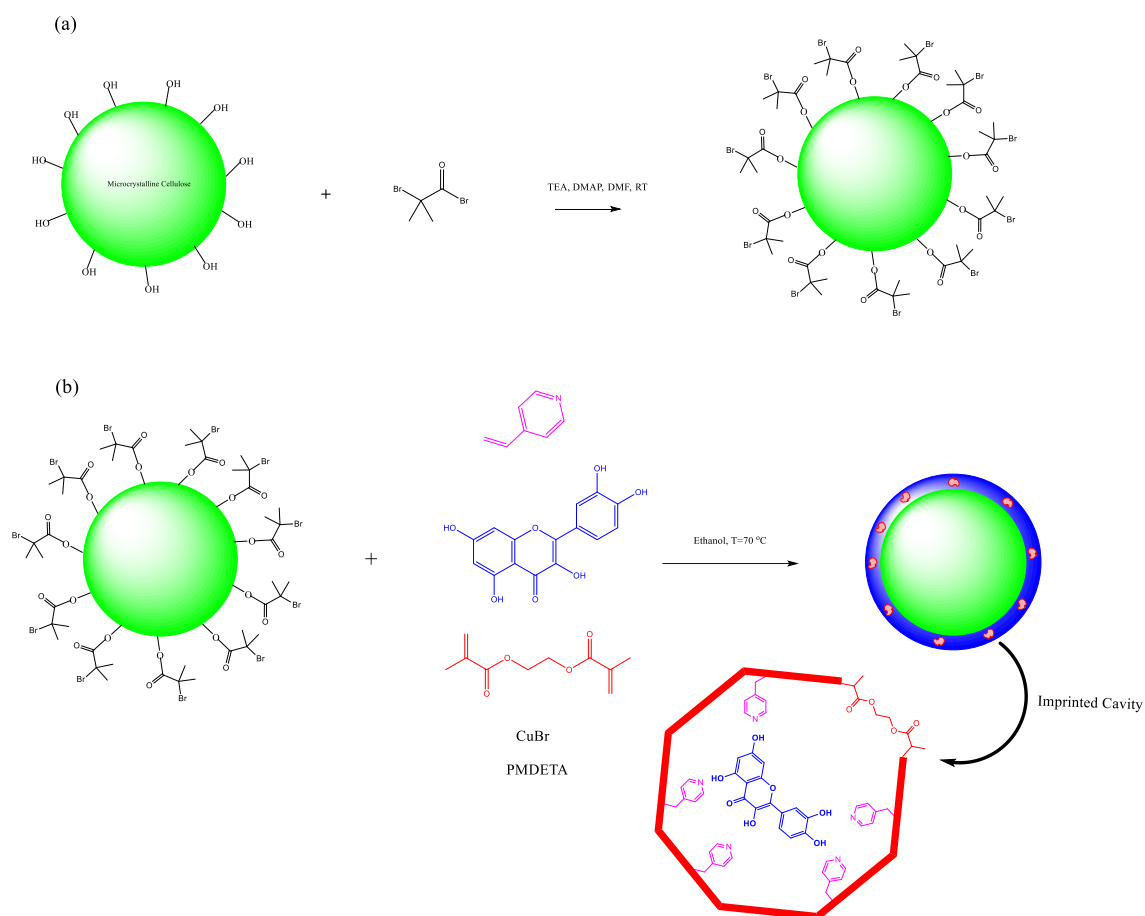


Figure 3-1: Depiction of the synthesis procedure considered for the development of surface molecularly imprinted cellulose-synthetic hybrid particles via ATRP. (a) immobilization of α -bromoisobutyryl bromide in pristine microcrystalline cellulose (MCC) to generate ATRP macro-initiator particles (MCC-Br). (b) grafting of a crosslinked-imprinted shell in the particles surface considering ATRP of co-monomers 4VP/EGDMA in the presence of quercetin.

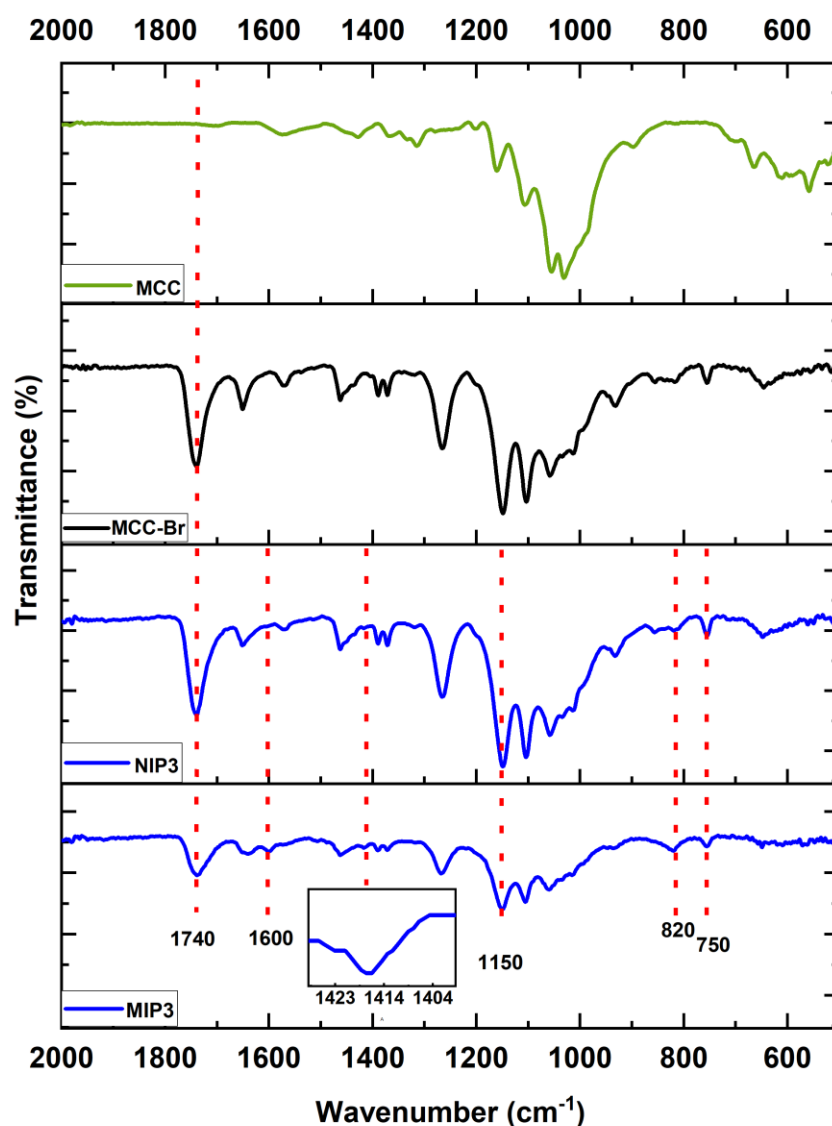


Figure 3-2: Comparative FTIR spectra for pristine microcrystalline cellulose (MCC), the product resulting from the immobilization of α -bromoisobutryl bromide in MCC (MCC-Br), NIP3 and MIP3.

The general aim of molecular imprinting is the creation in a polymer network of cavities with stereo-specificity for a selected target compound. For this purpose, functional monomers with potential strong interactions with a template molecule (usually non-covalent interactions such as hydrogen bonding, Van der Waals, π - π staking, etc) should be selected. Note that in molecular imprinting the template molecule is not necessarily the target compound, as often considered when using a surrogate molecule due to the high price of the target, solubility limitations, etc. After the promotion of the complexation functional monomers - template, a polymerization in the presence of a crosslinking is conducted for molecular cavities formation. The main role of the crosslinker is prompting

the creation of binding sites (cavities) with geometrical stability. In spite of merits of this approach, many factors can weaken the molecular imprinting efficiency, i.e. the formation of a high number of specific binding cavities in the polymer network. Also, polymerization conditions, namely the kind and concentrations of all initial components in the reaction mixture (functional monomer(s), template, crosslinker, initiator, solvent(s)), reaction temperature or initiation mechanism strongly affect the molecular imprinting process [8].

The purposes of molecular imprinting above described are depicted in **Figure 3.1(b)** for the surface phenomena here seek. A thin synthetic shell with imprinted cavities is intended, via ATRP polymerization, for core cellulose particles. The reaction is made using the macro-initiator MCC-Br particles and the functional monomer 4VP, the template quercetin, the crosslinker EGDMA and the pair CuBr/PMDETA for the ATRP activation/deactivation mechanism. The specific synthesis conditions considered in the molecular imprinting of the surface of the MCC-Br particles via ATRP with 4VP/EGDMA in the presence of quercetin are presented in **Table 3.1**.

Table 3-1: Synthesis conditions considered in the surface molecular imprinting of MCC-Br particles considering ATRP of co-monomers 4VP/EGDMA in the presence of quercetin. Polymerization at 70 °C during 72 h.

Material	4VP (mM)	EGDMA (mM)	Quercetin (mM)	MCC-Br (mg/mL)	CuBr (mM)	PMDETA (mM)	DMF (mM)	ACN (mM)	Ethanol (mM)	Synthetic shell (wt%)
MIP1	87.8	17.5	11.0	1.03	1.9	1.9	12796.9	-	-	N.A.
MIP2	73.2	14.6	9.2	0.87	1.6	1.6	10901.8	2839.9	-	19
MIP3	87.4	17.4	6.5	5.15	1.9	1.9	-	-	16898.0	14
MIP4	87.8	17.5	6.5	5.21	1.9	1.9	-	16056.5	2534.6	10

Our goal is the formation of a thin enough imprinted shell in order to enhance binding site accessibility and mass transfer, leading to improved performance of the adsorbent particles in sorption/desorption processes. For that, a low concentration of the monomers 4VP and EGDMA was used, as detailed in **Table 3.1**. Note that much higher monomers concentrations lead an eventual to high incorporation of the synthetic copolymer in the MCC particles in view of the present goal. For instance, in the previous work concerning the grafting of 4VP in MCC, with an initial monomer concentration of 5.75 mol/L, the estimate for the content of the synthetic shell in the final MCC-4VP particles was ~ 90 wt% [4]. Under the polymerization conditions presented in **Table 3.1**, synthetic shell contents in the range 10 to 20 % were estimated (the CuBr and PMDETA concentrations follow usual ATRP ratios after definition of monomers and MCC-Br concentrations).

A too high content of the synthetic shell (depending on the polymerization conditions) and intra-/inter-particle reaction mechanisms can lead to substantially different materials, namely concerning their final morphology. In **Figure 3-3** are depicted possible polymerization reaction mechanisms involved in the formation of cellulose-synthetic hybrid particles via ATRP. Besides the main intra-particle reaction steps for crosslinked shell formation (e.g. propagation of multi-functional active radical centers and reversible activation/deactivation with CuBr), intra- and inter-particle radical termination is also possible. The inter-particle radical termination can lead to particle connection and eventually to gelation, as observed with our experiments when too concentrated solutions are used.

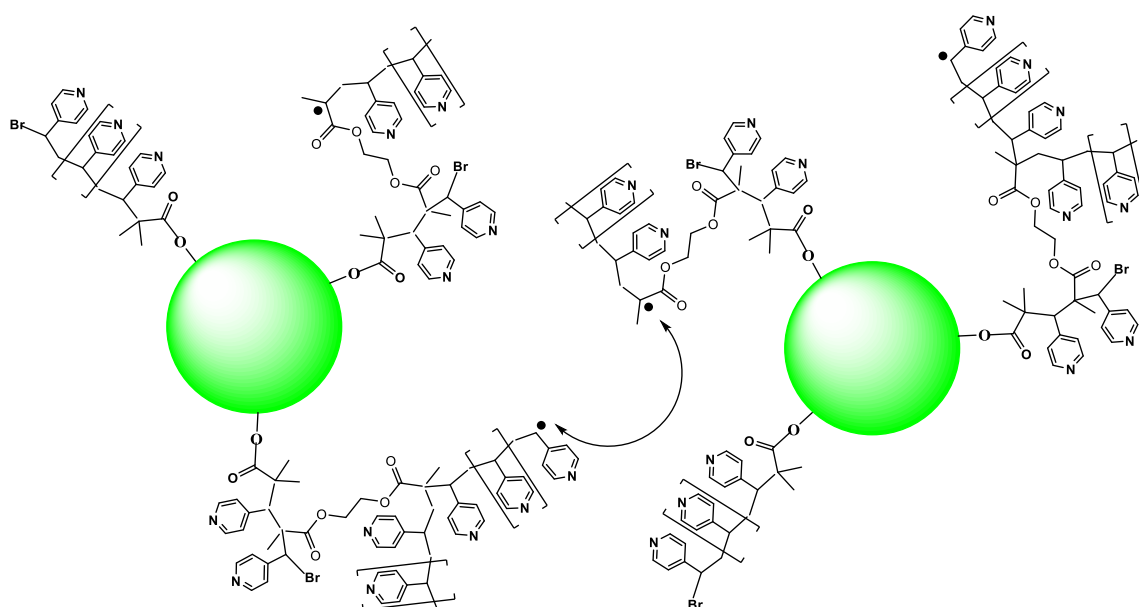


Figure 3-3: Depiction of possible polymerization reaction mechanisms involved in the surface molecular imprinting of cellulose-synthetic hybrid particles via ATRP. Besides the main intra-particle reaction steps for crosslinked shell formation (e.g. propagation of multi-functional active radical centers and reversible activation/deactivation with CuBr), intra/inter-particle radical termination is also possible. The inter-particle radical termination can lead to particle connection and eventually to gelation.

Due to the high number of different parameters involved in molecular imprinting recipes, although it is possible to use molecular simulation and/or of experimental design to help with designing tasks [8], some rules established by solubility constraints, expected thermodynamic effects, and so on, are important for the planning of the experimental runs. Obviously, the selection of the template depends on the application pursued. In this

work, quercetin was considered for the MIPs template because it is a flavonoid with a low price (e.g. as compared with luteolin or apigenin), it is reported to be present in olive leaf extracts and contains many interactions points common to other important flavonoids in olive leaf, namely rutin, luteolin, apigenin and other related glycosylated compounds. Therefore, quercetin was here considered as a kind of surrogate for the many flavonoids often found in olive leaf extracts. The ratio of functional monomer/template is known to play an important role in imprinting efficiency [8]. However, as pointed before, solubility constraints also define the maximum amount of template that can be used. The concentrations for quercetin in **Table 3-1** are a consequence of that kind of analysis for the present MIPs synthesis runs.

Hydrogen bonding capacity and polarity of the solvent have a strong influence in polymerization mechanisms and particular additional issues are observed with molecular imprinting polymerization systems. Potential interference with the interactions expected between functional monomer(s)-template and lack of initial solubility for all polymerization ingredients are factors constraining the selection of the working solvent. Our studies included the change of the polymerization solvent(s) as presented in **Table 3-1**, going from DMF (highly polar and allowing higher solubilization of ingredients), mixtures with ACN (acetonitrile presents a lower H-bonding capacity but cause solubility problems with reaction mixtures) and ethanol (presenting H-bonding effects but allowing a higher solubilization effect).

The selection of 4VP as functional monomer was driven by previous results concerning the development of MIPs for different kinds of polyphenols [8-12]. Indeed, 4VP can establish a strong binding with polyphenols, even at low extent of hydrophobic effects, due to the particular features for the $-N =$ group in the pyridyl moiety. Specifically, one extra electron pair that is not involved in the ring conjugation system that is able to participate in strong H-bonding mechanisms, as well as to bind with protons. The pyridyl group behaves as a proton acceptor (small nucleophilic molecule that can interact with exposed electrophilic residues), being also a good ligand for multiple metal ions, giving rise to complexation. Due to these particular properties, polymers based on 4VP are being considered for heavy metals ions adsorption and wastewater/industrial effluents treatment [13–17], enzymes/protein adsorption [18,19] and other applications involving electronics, biomedicine or catalysis [20].

Experimental runs reported in **Table 3-1** lead to products with different features concerning synthetic functionalization and morphology, impacting in their use with

sorption/desorption processes. With the product MIP1 a kind of solubilization of the macro-initiator MCC-Br particles was observed during polymerization. Under our working conditions, using DMF solvent makes the ATRP step close to a homogeneous process and the size of the final particles is too low to allow the practical use of the material as an adsorbent. A favorable morphology was observed with MIPS 2 to 4 but the FTIR analysis of the products indicates a clearer functionalization of the materials with 4VP for MIP3, as shown in **Figure 3-2** (see Supplementary Material appendix B, **figure B-1 to B-3** for FTIR analysis of MIPS/NIPs 1, 2, and 4). Characteristic peaks for 4VP monomer at 1600 cm^{-1} (C=C pyridyl), 1418 cm^{-1} (C=N), 820 cm^{-1} and 755 cm^{-1} (both correspond to the C-H bending in the pyridine ring) are all potential indicators for incorporation of this monomer, particularly the 1418 cm^{-1} assignment. Note that, in comparison with other works [8,12], a weaker signal for these 4VP peaks is here expected due to the lower incorporation of this monomer in the final products. The presence of EGDMA in polymer materials is usually confirmed by analyzing assignments at 1720 cm^{-1} and 1130 cm^{-1} (C=O and C-O groups, respectively). Peaks close to these vibrational regions are observed in all synthesized products but an important contribution of similar functional groups in MCC-Br is possible (note the relatively small concentration of EGDMA here used).

Overall, with ethanol as ATRP solvent, a solid/liquid heterogeneous process was observed along the reaction, preserving a final size of the hybrid particles suitable for their application as adsorbent. The incorporation of the 4VP/EGDMA co-monomers, apparent in FTIR analysis, indicated the MIP3 particles as good candidates for testing with selective sorption/desorption, as explored in the next sections.

3.3.2. Sorption/Desorption Assessment with Standard Polyphenols

To evaluate the surface modification achieved in the pristine MCC, specifically concerning sorption capacity for phenolic compounds due to synthetic shell formation and also to assess the benefits of molecular imprinting, SPE testing for MIP/NIP3 was performed with standard compounds, namely the flavonoid quercetin (the imprinting template), rutin (a glycoside of quercetin often found in olive leaf), vanillic acid (a phenolic acid also reported in olive leaf extracts) and the oleuropein secoiridoid, that is the most abundant compound in olive leaf. In order to approach practical industrial working conditions with olive leaf extracts, ethanol/water 80/20 was used as solvent in these studies

Results presented in **Figure3-4** put into evidence a very low retention capacity for quercetin of pristine cellulose while the amount retained by NIP3 and MIP3 in the loading step is much higher (more than 60% with the MIP). Clearly, these outcomes show the modification achieved for the cellulose surface and the active role of the 4VP/EGDMA shell in quercetin binding, namely due to the interaction with 4VP. Moreover, the quercetin retention observed with MIP3 (~62.7) is also much higher than with NIP3 (~24.5), highlighting the benefits of molecular imprinting. An imprinting factor IF~2.6 is estimated based on these measurements. Furthermore, the comparison of the results for NIP3 and MIP3 concerning the washing and elution steps show also the higher strength for the binding of quercetin with MIP3 comparatively to NIP3. These measurements are additional confirmations for the creation of specific binding sites in MIP3 due to molecular imprinting.

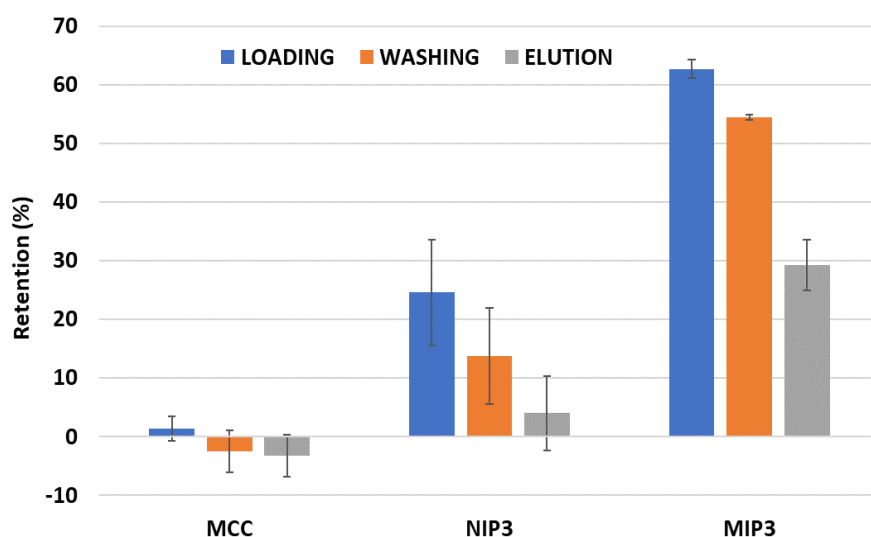


Figure 3-4: Fraction of the initial amount of quercetin retained in pristine MCC, NIP3 and MIP3 after the loading, washing and elution steps in SPE. Testing (n=2) using a quercetin solution in ethanol/water 80/20 with concentration 0.126 mM, 2.5 mL of volume of liquid phase in each step and 25 mg of adsorbent in the SPE cartridges. Estimates with UV measurements at 370 nm.

Measurements presented in **Figure 3-5** show a low retention of oleuropein in MIP3 comparatively to quercetin. This difference can be explored to make the separation between flavonoids and secoiridoids in olive leaf. Rutin and vanillic acid, especially the latter, are retained at a large extent in MIP3. This is a consequence of binding effects that are inevitable when the compounds bear functional groups similar to that we used to promote the interaction of the template with the functional monomer or other that are able to interact with polymer network. For instance, rutin contain phenolic OH groups that are

also able to interact with the 4VP/EGDMA network. Also, vanillic acid contains a phenolic OH and a carboxylic group that can bind with the pyridine moieties. Due to its smaller size, vanillic acid should also have a facilitated access to the binding sites. However, the later washing and elution steps clearly show the high binding strength of quercetin with MIP3. Note that vanillic acid is totally desorbed from the MIP in the elution step with methanol/ acetic acid 90/10 (see section 2.8 for experimental details). These results also highlight the possibility for the use of MIP3 to make the fractionation of complex olive leaf extracts through designed sorption/desorption processes.

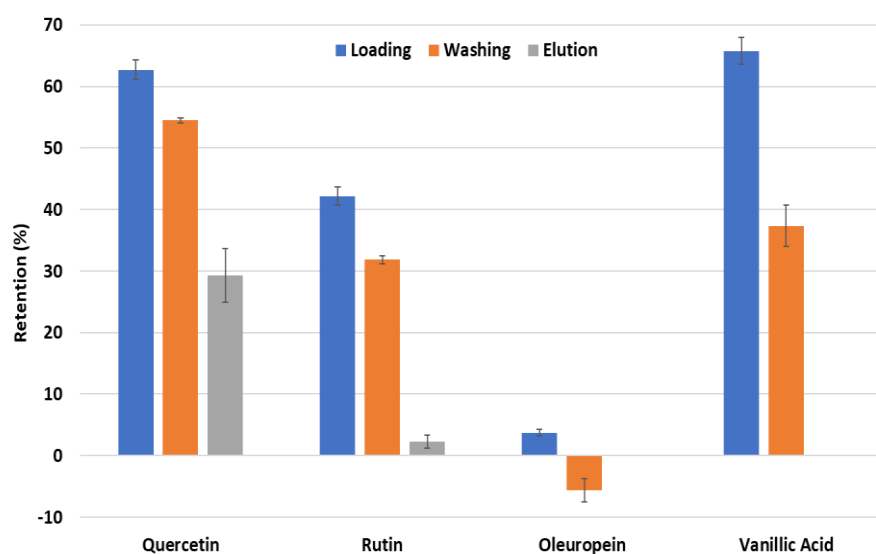


Figure 3-5: Fraction of the initial amount of standard compound's quercetin, rutin, oleuropein and vanillic acid retained in MIP3 after the loading, washing and elution steps in SPE. Testing (n=2) using solutions of the different standards in ethanol/water 80/20 with concentration 0.126 mM, 2.5 mL of volume of liquid phase in each step and 25 mg of adsorbent in the SPE cartridges. Estimates with UV measurements at 370 nm for quercetin and rutin and at 280 nm for vanillic acid and oleuropein

3.3.3. Enrichment of Flavonoids in Olive Leaf

Olive leaves are being considered with increasing interest as a source of bioactive compounds, including antioxidants, to be used in food (e.g. supplements with health-giving effects), natural preservatives, cosmetics, among other applications. Many of these bioactive compounds in olive leaf are phenolic molecules belonging to different classes, ranging from simple phenols and phenolic acids, secoiridoids, lignans and flavonoids. For instance, the simple phenyl alcohols tyrosol and hydroxytyrosol and the phenolic acids gallic acid and vanillic acid are commonly found in olive leaf. Oleuropein, a secoiridoid, is the main phenolic component in olive leaves but many other compounds belonging to this family are also observed, while pinoresinol is a lignan reported in this context.

Diverse kinds of flavonoids are present in olive leaf, namely the aglycones quercetin, luteolin, apigenin and diosmetin and the related glycosylated forms such as luteolin-7-O-glucoside, luteolin-5-O-glucoside, luteolin-7-O-rutinoside, quercetin-7-O-rutinoside, rutin or apigenin-4'-glucoside.

The relative distribution and amounts of these different phenolic compounds in olive leaf depend on the tree variety, geographical origin and collection season. Furthermore, the extraction technique used to get these molecules impacts on the composition of the final extract. Commonly used methods rely on solid/liquid extraction with hydroalcoholic solvent mixtures (e.g. ranging from pure water to pure ethanol or methanol), ethyl acetate, acetone or even dichloromethane (or similar solvents when the non-polar fraction is sought). Many studies addressed the effect of the extraction technique on phenolic profile and a dependence for the yield of the different phenolic compounds is known [21-32]. Anyway, at the end, extracts containing a huge diversity of compounds are obtained, thus demanding separation and purification steps to get individual molecules or mixtures suitable for practical applications (e.g. in food supplements or cosmetics). Here is highlighted the testing of the developed surface imprinted adsorbents for the fractionation of an industrial olive leaf extract. **Figure 3-6** shows the high-performance liquid chromatography with diode-array detection (HPLC-DAD) analysis of this extract showing a major peak for oleuropein (**Figure 3-6a**) but many other compounds are observed, including different kinds of flavonoids in the aglycone form (quercetin, luteolin) or glycosylated (e.g., luteolin-7-O-glucoside, as clearly shown in **Figure 3-6 b**). Verbascoside, phenolic acids, and phenyl alcohols are also identified, among a plethora of minority compounds (see the huge diversity in the magnified views, **Figure 3-6 c,d**). This tentative identification task was also aided by liquid chromatography-mass spectrometry (LC-MS) analysis, as described in the Experimental Section.

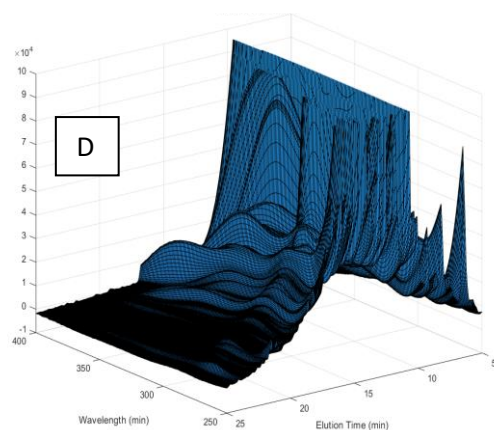
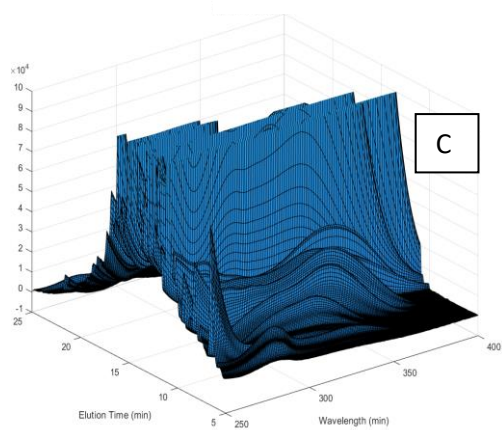
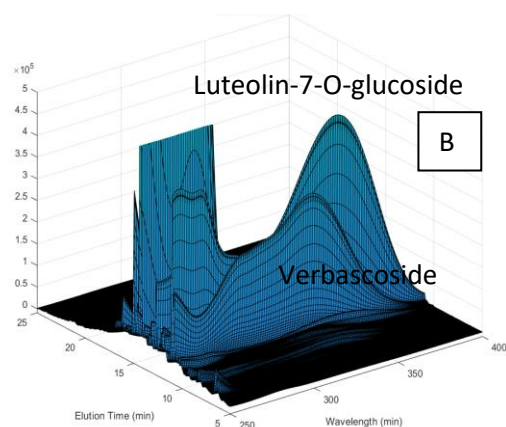
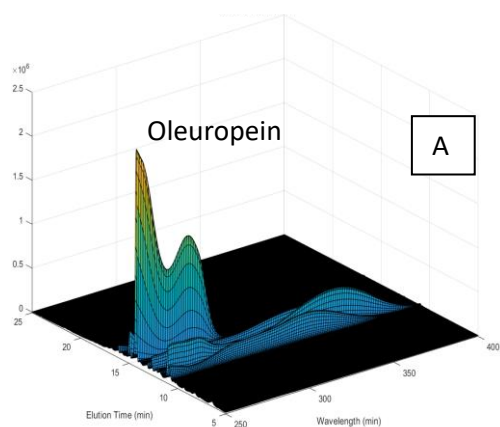


Figure 3-6: HPLC-DAD chromatogram for the olive leaf extract provided by NATAC that was used in this work. (A) full view of the chromatogram. (B), (C) and (D) magnified views of this chromatogram to highlight the compositional complexity of the extract.

Figure 3-7 shows the dynamics of sorption of this olive leaf industrial extract in the MIP3 particles for one of the runs performed here. Note that these breakthrough curves are affected by a competitive adsorption phenomenon involving the different compounds present in the olive leaf extract and the available binding sites. However, due to the 4VP functionalization of the adsorbent and imprinting with quercetin, a preferential sorption of flavonoids is expected. Therefore, for instance, the displacement of previously adsorbed secoiridoids by flavonoids is possible during this dynamic process. Hydroalcoholic mixtures with high alcohol content were considered in our loading runs. This is possible due to the strong binding capacity of 4VP for flavonoids even with low water content solvents, as shown previously. [7-12]. Therefore, a higher efficiency is possible due to the feasibility for the processing of industrial extracts produced in alcoholic solvents with a minimum of preparation steps.

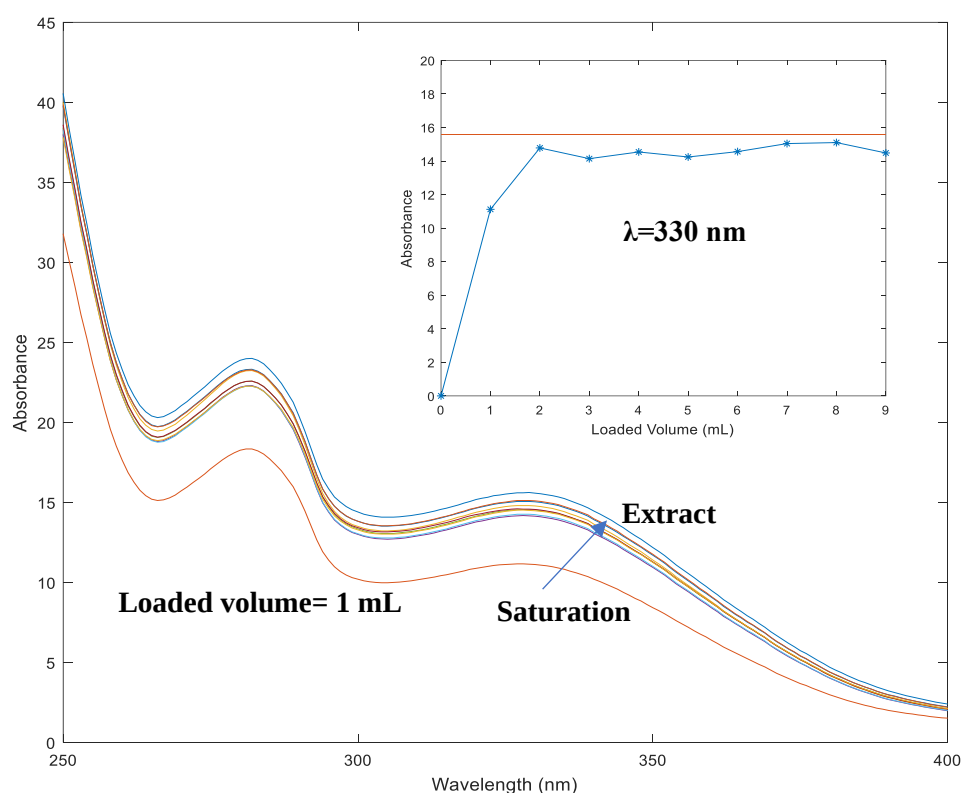


Figure 3-7: Dynamics for the SPE sorption of the olive leaf extract in ethanol/water 80/20 at $C=5$ mg/mL using 25 mg of MIP3. The UV-vis analysis of samples collected at SPE column outlet for different loading volumes show the dynamics observed with particles saturation (approaching the extract spectrum). The inset shows the sorption dynamics measured at $\lambda=330$ nm (breakthrough curve).

Figure 3-8 presents the 2D HPLC-DAD chromatograms for different fractions collected during the desorption of MIP3 particles preloaded with the olive leaf extract in ethanol/water 50/50 at a concentration of 5 mg mL⁻¹. Measurements at 280 and 330 nm are presented to highlight the fractionation of flavonoid and nonflavonoid compounds. A full view for selected fractions produced along this desorption process is presented in **Figure 3-9**. These results highlight the performance of the developed MIP particles for compounds separation due to the different binding strengths. Key compounds such as oleuropein, verbascoside, oleuroside, luteolin-7-O-glucoside, apigenin-7-O-glucoside, luteolin-4-O-glucoside, diosmetin-4-O-glucoside, and quercetin are considered to illustrate these aspects. The fraction recovered in water (**see Figures 3-8 and 3-9a**) is enriched with oleuropein, verbascoside, and oleuroside comparatively to the initial extract. Under these desorption conditions, flavonoids keep retained in the MIP particles. When the alcoholic content in the elution solvent is increased, some flavonoids such as luteolin-7-O-glucoside and apigenin-7-O-glucoside start to desorb and fractions with mixed composition flavonoid/nonflavonoid are obtained. Only when the elution strength of the solvent is substantially increased (e.g., water/ethanol 20/80 or methanol/acetic acid 90/10), the compounds with strong binding to the MIP sites can be recovered. This is observed with luteolin-4-O-glucoside, diosmetin-4-O-glucoside, and quercetin (the template of the MIP). As a consequence, fractions with high flavonoid content can be obtained, as presented in **Figures 3-8 and 3-9c, d**.

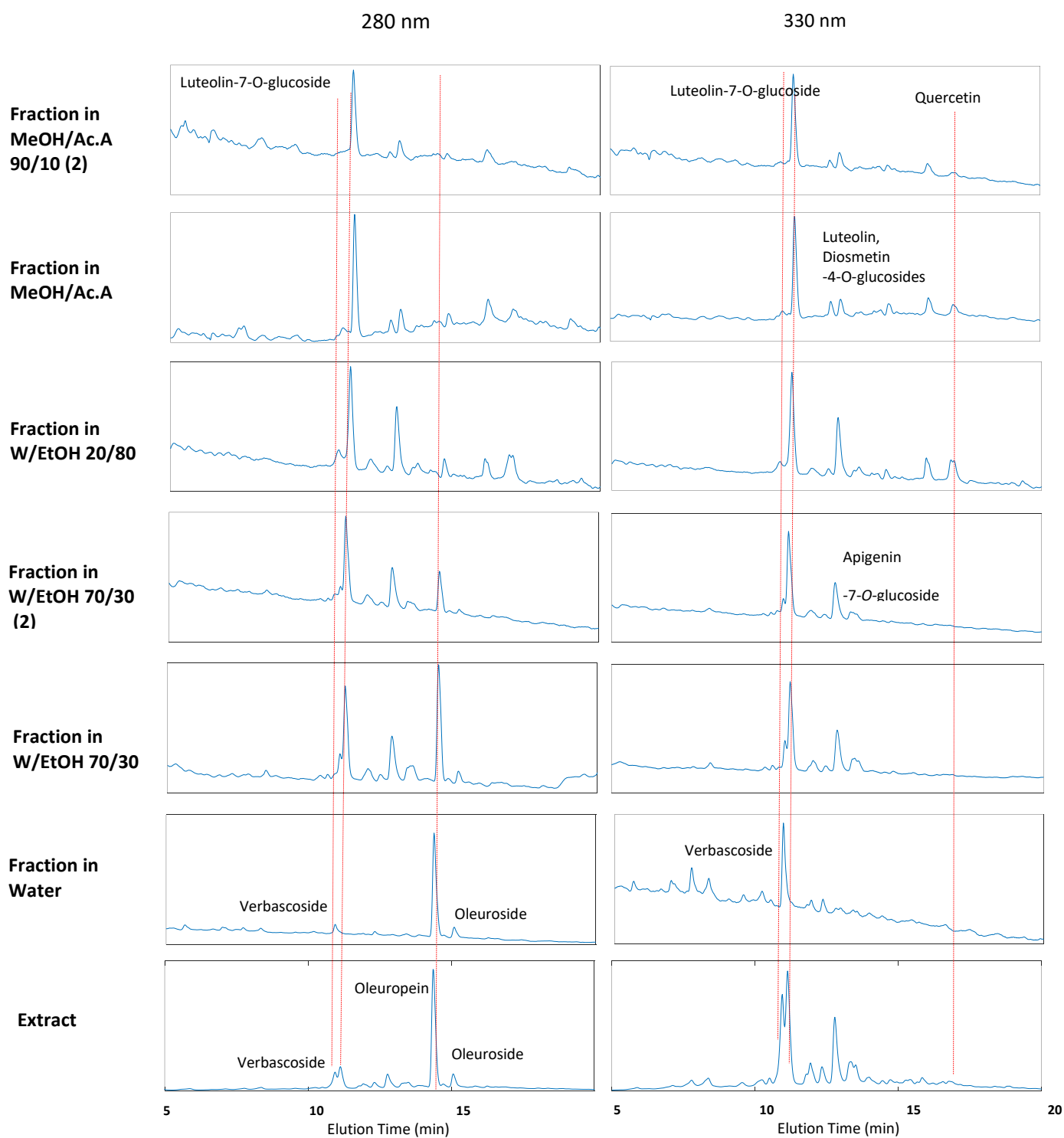


Figure 3-8: 2D HPLC-DAD chromatograms for different fractions collected during the desorption of MIP3 particles pre-loaded with the olive leaf extract in ethanol/water 50/50 at 5 mg/mL. Measurement at 280 and 330 nm are presented to highlight the fractionation of flavonoid and non-flavonoid compounds. For graphical simplicity, numerical scales in the plots were omitted.

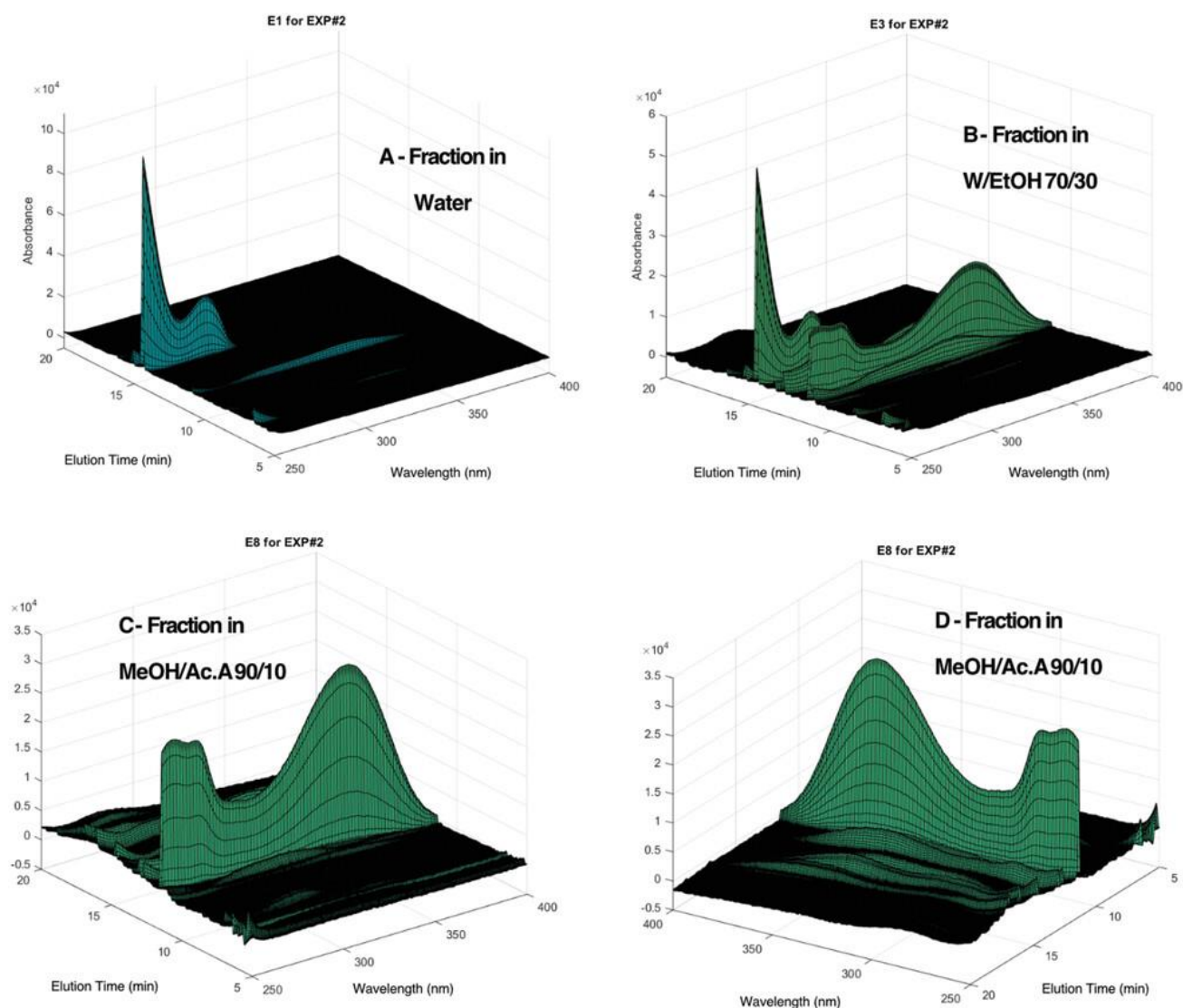


Figure 3-9: 3D HPLC-DAD chromatograms for different fractions collected during the desorption of MIP3 particles pre-loaded with the olive leaf extract in ethanol/water 50/50 at 5 mg/mL. A – Fraction recovered in water showing the enrichment of oleuropein. B – Fraction recovered in water/ethanol 70/30 showing the increase of flavonoids content, namely luteolin-7-O-glucoside. C - Fraction recovered in methanol/acetic acid 90/10 showing a high enrichment in flavonoids namely luteolin-7-O-glucoside and other minority related compounds. D – a different view of the same flavonoid enriched fraction.

HPLC-DAD results were used to estimate the enrichment factors achieved for the production fractions resulting from this sorption/desorption approach. The global enrichment factor for flavonoids was estimated from $EF = (A_{360}/A_{280})_F / (A_{360}/A_{280})_E$ with EF representing the enrichment factor and $(A_{360}/A_{280})_F$ the ratio of the chromatogram areas at 360 and 280 nm for the fraction in analysis and $(A_{360}/A_{280})_E$ the same ratio observed in the extract. Our measurements show to be possible to achieve

EF up to four times, as presented in **Table 3-2** and complemented with **Figure 3-9** and similar graphs presented in Supporting Information (**appendix B, figures B-4 to B-9**)

Table 3-2: Enrichment factors (EF) for total flavonoids and quercetin estimated for different fractions recovered with MIP3 particles after processing of an olive leaf extract considering different working conditions.

Processed Extract Solution	Solvent in the recovered fraction	<i>EF</i> for Flavonoids	<i>EF</i> for Quercetin
EtOH/W 50/50 at C=5 mg/L	Water	-	-
	EtOH/W 70/30	1.6	-
	MeOH/Ac.A 90/10	2.7	3.5
EtOH/W 80/20 at C=0.5 mg/L	MeOH/Ac.A 90/10	2.0	-
EtOH/W 80/20 at C=5 mg/L	Water	-	-
	EtOH/W 30/70	2.8	-
	EtOH/W 50/50	3.5	-
	EtOH/W 80/20	3.8	-
	MeOH/Ac.A 90/10	3.7	2.6

It is also worth to highlight the superior performance of MIP3 versus NIP3 concerning the enrichment of flavonoids in olive leaf extracts. **Figure 3-10** presents the comparison for 2D HPLC-DAD chromatograms for a fraction collected during the desorption of MIP3 and NIP3 particles preloaded with the olive leaf extract in ethanol/water 50/50 at 5 mg mL⁻¹. These results clearly show the higher selectivity of MIP3 toward flavonoids, namely, quercetin, in comparison with NIP3. These outcomes highlight the importance of the binding sites created through the presence of quercetin in the polymerization system, besides the strong interaction between the moieties of 4VP and flavonoid molecules

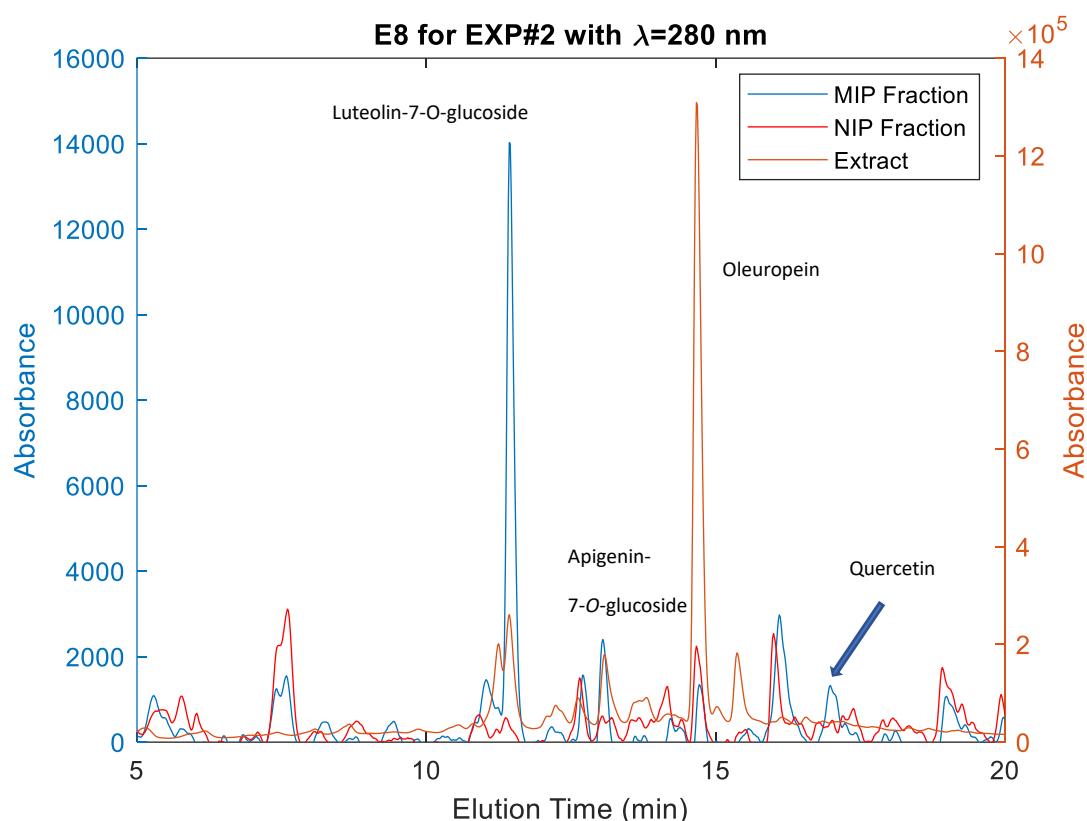


Figure 3-10: 2D HPLC-DAD chromatograms for a fraction collected during the desorption of MIP3 and NIP3 particles pre-loaded with the olive leaf extract in ethanol/water 50/50 at 5 mg/mL⁻¹. Fraction recovered in methanol/acetic acid 90/10 showing the high selectivity of MIP3 towards flavonoids, namely quercetin, in comparison with NIP3.

Additional information is presented in the Supplementary Material concerning the results for fractions resulting from the processing of the olive leaf extract in ethanol/water 80/20 at 0.5 mg/mL and ethanol/water 80/20 5 mg/mL. The mass of compounds estimated per gram of adsorbent achieved with these working conditions demonstrates the ability of the developed adsorbents to perform in a wide range of context concerning solvent composition and solute concentration. Previous studies addressing the development of MIP particles for the purification of olive leaf extracts consider the processing of ethyl acetate solutions with oleuropein as target compound [33] or considered operation with standard compounds [34]. Here we propose the use of the hybrid MIP particles for the fractionation of hydro-alcoholic industrial extracts with production of enriched flavonoids fractions but also oleuropein enriched mixtures, as above discussed.

In **Table 3-3** the mass of compound per mass of adsorbent values are estimated for different fractions resulting from the processing of olive leaf extracts with the hybrid cellulose-synthetic MIP3 particles. This parameter is defined as the amount of a specific

compound produced in each fraction per unit of mass of the MIP adsorbent particles. These values are estimated from the HPLC-DAD analysis of the fractions (see e.g. **Figures 3-3, 3-8, 3-9** and supplementary material) and the HPLC-DAD calibration data for different standard compounds (e.g. oleuropein, verbascoside, luteolin-7-O-glucoside, quercetin, etc).

Table 3-3: Mass of compound per mass of adsorbent values estimated for different fractions resulting from the processing of olive leaf extracts with the hybrid cellulose-synthetic MIP3 particles.

Sorption Conditions	Desorption Fraction	Mass of compound per mass of adsorbent (g/mg)					
		Oleuropein	Verbascoside	Oleuroside	Luteolin-7-O-glucoside	Apigenin-7-O-glucoside	Quercetin
EtOH/W 50/50 at C=5 mg/L	Water	3.01	0.15	0.32	-	-	-
EtOH/W 50/50 at C=5 mg/L	W/EtOH 70/30	1.62	0.07	0.15	0.18	0.10	-
EtOH/W 50/50 at C=5 mg/L	MeOH/Ac.A 90/10	0.02	0.01	0.02	0.20	0.05	0.05
EtOH/W 50/50 at C=5 mg/L	MeOH/Ac.A 90/10	-	-	-	0.06	0.02	-
EtOH/W 80/20 at C=5 mg/L	W/EtOH 50/50	0.01	-	-	0.20	0.01	0.02
EtOH/W 80/20 at C=5 mg/L	MeOH/Ac.A 90/10	-	-	-	0.13	0.02	-

Complementary to results in **Table 3-2**, the data in **Table 3-3** confirm the usefulness of the developed MIP particles for compounds fractionation. Indeed, higher amounts of secoiridoids and related compounds are produced in the aqueous fractions while higher flavonoids productivity is observed for organic fractions (e.g. MeOH/Ac.A 90/10). These outcomes indicate the developed natural-synthetic materials as promising adsorbents to be considered with cyclic sorption/desorption processes aiming at to get high-added value compounds in olive leaf.

3.4. Conclusions

Surface molecularly imprinted cellulose-synthetic hybrid particles were prepared via ATRP with quercetin as template and used in sorption/desorption processes involving standard phenolic compounds as well as olive leaf extracts. The later are complex

mixtures of flavonoid and non-flavonoid compounds (e.g. oleuropein). These testing runs evidenced the higher performance of the molecularly imprinted particles, not only in comparison with the native cellulose, but also in contrast with the non-imprinted hybrid materials. The quercetin-shaped imprinted cavities and the strong interaction between 4VP moieties and flavonoids was explored for the enrichment of olive leaf extracts through sorption/desorption processes with the developed particles. We show that hydro-alcoholic solvent mixtures with high alcoholic content can be considered for the processing of the olive leaf extracts with the new MIP particles. The production of rich fractions containing luteolin-7-O-glucoside, apigenin-7-O-glucoside or quercetin, among other flavonoids was demonstrated through this approach. Enrichment factors up to four times were estimated for some fractions produced.

Next steps in this research line include the mass-production of these kind of particles, intending the approaching the industrial scale for the efficient valorization of target compounds biomass contained, in the framework of circular bio-economy.

3.5 References

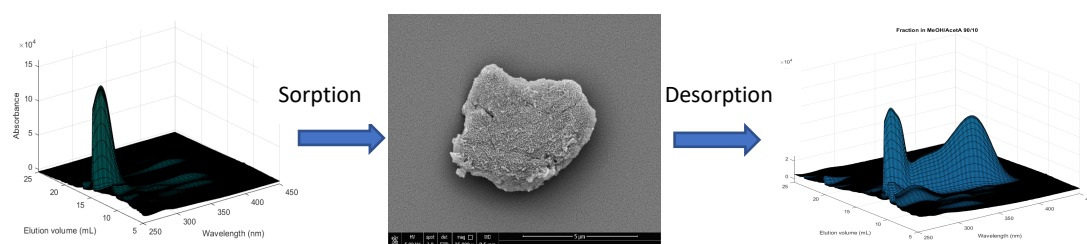
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Chapter 4. Advanced fractionation of wine residues polyphenols using pyridyl-based molecular imprinting



Hybrid cellulose-synthetic particles with surface active pyridyl moieties and molecularly imprinted cavities were prepared via atom transfer radical polymerization. High retention of polyphenols was observed with hydroalcoholic solvents of high ethanol content. These features were explored with the fractionation of flavonols and anthocyanins in winemaking residues. Enrichment was achieved for high-added value compounds using diatomaceous earth and grape pomace extracts.

Fractionation of Flavonols and Anthocyanins in Winemaking Residues using Molecularly Imprinted Cellulose-Synthetic Hybrid Particles with Pyridyl Active Surface

C. P. Gomes, R. C. S. Dias, M. R. P. F. N. Costa, "Fractionation of flavonols and anthocyanins in winemaking residues using molecularly imprinted cellulose-synthetic hybrid particles with pyridyl active surface, Nano Select, Volume 5, 2024, 2300153. <https://doi.org/10.1002/nano.202300153>

Abstract

Hybrid cellulose-synthetic particles with surface active pyridyl moieties and molecularly imprinted cavities for quercetin were prepared via atom transfer radical polymerization. The functionalization of the materials with pyridyl groups was confirmed by FTIR and the SEM micrographs of the hybrid particles demonstrate a clear surface modification comparatively with the pristine cellulose. Competitive sorption/desorption testing of the imprinted and non-imprinted particles with standard polyphenols show the achievement of an imprinting factor IF ~ 8 . Moreover, it was also confirmed the high retention capability of the hybrid materials for polyphenols, due to their strong binding with the surface pyridyl moieties, even when using hydroalcoholic solvents of high ethanol content (e.g. ethanol/water 80/20 v/v). The sorption capabilities of the synthesized materials for polyphenols were explored with the fractionation of flavonols and anthocyanins in winemaking residues. High concentration and enrichment factors were achieved for high-added value compounds, namely 5 times for quercetin and 12 for quercetin-3-o-glucuronide with a diatomaceous earth extract and up to 4 for flavonols in a grape pomace (e.g. myricetin and quercetin glucosides). This research demonstrates the feasibility for the combination between the development of engineered materials addressing sustainability with their application to the valorization of agro-industrial wastes.

KeyWords: Surface molecular imprinting; Cellulose; Atom transfer radical polymerization; Natural-synthetic hybrid particles; Pyridyl groups; Winemaking residues; Flavonols; Anthocyanins

4.1. Introduction

Pyridine-based polymers find special applications in science and engineering due to the particular features of the pyridyl moieties in their backbones. The physical and chemical properties of pyridine heterocycle outstand the application of this small molecule in chemical industries for a plethora of applications (pharma, food supplements,

insecticides, advanced materials, etc) while the pyridyl group excels in the correspondent macromolecular materials (often stemming from the monomers 2-vinyl pyridine, 4-vinyl pyridine or 2-methyl-5-vinyl pyridine). In brief, many particular features of pyridine/pyridyl groups, namely as compared with benzene/benzyl groups, stem from the -N= group in these rings, namely owing to the ability of the extra electron pair that is not involved in the ring conjugation system to participate in strong H-bonding mechanisms or to bind with protons. Indeed, pyridine and the pyridyl group behave as proton acceptors, being weak bases ($\text{pK}_a=5.22$ for pyridine) and also as a good ligand for multiple metal ions, giving rise to complexation [1]. Actually, pyridine-based polymers are used in practice as advanced materials in a wide range of domains, including the treatment of wastewater and industrial effluents (e.g. copper, nickel, chromium and mercury adsorption), noble metals recovery (e.g. gold adsorption from aqueous solutions or uranium capture in nuclear plants), enzymes/protein adsorption, electronics (e.g. light-emitting devices, sensors and electrochemical applications), biomedicine, or even catalysis [1-8]. The sorption of phenolic contaminants with pyridine-based polymers was also considered some decades ago [9-11] and the outperformance of the pyridine-based adsorbents, namely in comparison with styrene-DVB, copolymers was observed [1].

Notably, adsorbents with pyridyl moieties present also a strong binding with phenolic compounds in different kinds of plant extracts and are being considered for valorization of these classes of bioactive compounds [12-18]. Actually, multivalent hydrogen bonding of the phenolic groups in polyphenols (e.g. quercetin and other related flavonoids) with the pyridyl groups in the polymer networks is possible at a relatively high binding strength. Phenolic groups have a higher acidity as compared to other kinds of hydroxyls, namely the OH groups in the sugar moieties, because with the ionization of phenolic hydroxyls the negative charge and a set of the lone pair electrons in the phenoxide oxygen are delocalized by resonance to the carbons on the aromatic ring (the same is not possible with OH groups in sugars). At the end, the favorable interaction of the nucleophilic pyridyl group (weak base) with the phenolic hydroxyls (acidic groups) grounds the observed good performance of the related polymer networks for the retention of polyphenols, even at low extent of hydrophobic effects [12-18].

Targeting of specific compounds and/or functional groups with polymers arises from the particular selection of the functional moieties in the macromolecular chains. Besides this polymer functionalization level, an additional gain for materials tailoring can be conceived through the molecular imprinting technique that ideally leads to the formation

of stereospecific cavities inside a polymer network. The special features of molecularly imprinted polymers (MIPs) make these materials to perform in the separation, enrichment, and purification of plant active products and many promising developments are expected in this area for the next years [19-21].

The present work fits in this line of thought and tries to join in the same material three main features: i) functionalization with pyridyl groups, (ii) molecular imprinting with quercetin, iii) core-shell architecture for cellulose-synthetic hybrid particles.

The latter characteristic of the materials explores the creation of an active surface with pyridyl groups and molecular imprinted cavities in cellulose-synthetic hybrid particles. This architecture is achieved through atom transfer radical polymerization (ATRP), involving cellulose particles acting as a macroinitiator (an ATRP initiator is previously immobilized at cellulose particles surface), the functional monomer 4-vinylpyridine, a crosslinker for network formation and quercetin as imprinting template. The addressing of hybrid materials with cellulose and natural polysaccharides in general is being actively considered in polymer science and industry in view of improvement of sustainability, e.g. in adsorption [22], surface modification [23], electronics [24] including actuators [25] or even CO₂ capture [26], among many other routes and applications involving cellulose adaptation [27-29]. Our approach, with inclusion of cellulose, also attempts to fit on the ongoing research lines pursuing the “greenification” of MIP materials for advanced applications [30,31]. Overall, the particles developed in this work enclose a pyridyl active shell surface, enhancing mass transfer rates and binding site accessibility (important issues in sorption/desorption phenomena), while the core is made of cellulose. At the end, a substantial weight fraction of the hybrid particles is a natural material (cellulose core) while the relevant synthetic pyridyl active shell, aimed to target polyphenols, has a minor contribution for material weight composition.

The exploitation of agro-industry generated waste and by-products to obtain high added-value products, namely drugs, nutraceuticals, cosmeceuticals and platform chemicals in general is part of the United Nations 2030 Agenda for Sustainable Development. In this context, the developed materials are here applied to the valorization of winemaking residues through the targeting of the flavonols and anthocyanins there contained, thus endeavoring contributing for sustainability and circular bio-economy. Actually, it is well-known that grapevine and winemaking residues are a valuable feedstock for biorefinery because contain relevant amounts of cellulose, hemicellulose and lignin that can be used to produce biofuels (e.g. bioethanol, biobutanol) and many platform chemicals [36].

These residues also include a plethora of phenolic compounds, such as flavonoids, phenolic acids, stilbenes and macromolecules (e.g. tannins) that are suitable for economical exploitation in pharmaceutical, nutraceutical and cosmetic industries, after their purification. According to statistics of 2019, a global grape production of 77.1 million tones was observed, with 57% of this amount applied to wine production (29 million tons of wine estimated to be produced in 2018). Vineshoots (6.9–13.8 million tons produced per year), stalks (1.3–1.8 million tons/year), grape pomace (5.7–8.8 million tons/year) and wine lees (0.7–2.6 million tons/years) are the major classes of grapevine and winemaking residues being generated by this agricultural/industrial activity [36]. The approaches nowadays being considered for the valorization of polyphenols in winemaking residues aim at stable and economically feasible processes. Issues related with the variability of the biomass geographical origin, collection season and grape varieties, etc, motivate the use of efficient extraction and purification methods for polyphenols, allowing their steady supply. A complex mixture of compounds is inevitably obtained when the winemaking residues are extracted, independently of the method used in the range of techniques often considered (hydroalcoholic maceration, ultrasounds assisted, supercritical fluids, etc). In this context, it is mandatory the development of sustainable separation and purification methods to get individual polyphenol molecules or mixtures suitable for practical applications in the downstream industries (mainly the pharma, feed, food and cosmetic industries). A lower energy consumption, cyclability, preservation of temperature-sensitive compounds, operational flexibility, amenability to intensification, and industrial scalability make the purification of polyphenols by sorption/desorption highly competitive in comparison with the alternative crystallization or membrane filtration approaches.

Specifically, in this work, the residual diatomaceous earth used for wine filtration and grape pomace resulting from the vinification process are considered as feedstocks to get the enrichment of high added value compounds through sorption/desorption with the developed particles. Winemaking industry generate large amounts of residual diatomaceous earth (an amorphous silica network), used for wine filtration, which is, as well, a rich and currently underexploited source of phenolic compounds [16]. On the other hand, grape pomace is the major by-product resulting from the grapes pressing (stalks/stems are in general previously separated in the destemming step) and is estimated to constitute 15-30% (depending on grape varieties, etc) of the total weight for the bunch. Grape pomace is mainly composed of processed skins and seeds. Besides stalks/stems (5

to 7%) and grape pomace, winemaking also generates lees (2-6%) that result from the fermentation and sedimentation steps, thus containing dead yeast cells. [36,42] Grape pomace can be used for ethanol production, composting, production of fertilizers or as livestock feed but is often disposed of in landfills with important environmental impacts. [36] However, the great potential of grape pomace as lignocellulosic biomass and/or to produce extracts rich in phenolic compounds (e.g. phenolic acids, anthocyanins and flavonols) is gaining room via recent studies in the framework of circular bio-economy [36]. In a simple estimate, considering the recent years annual production of 60 to 80 million tons of grapes (with c.a. 60% used for winemaking) and estimating the generation of 130 to 200 kg of grape pomace per ton of grapes [36], a rough range of 5 to 10 million tons of grape pomace is available per year as biomass for valorization.

4.2 Experimental Section

4.2.1 Materials: All chemicals were used as purchased, without further purification. Microcrystalline cellulose (MCC, powder, 20 μ m, cotton linters), BIBB (α -bromoisobutyryl bromide, 98% purity), triethylamine (TEA, 99.5% purity), 4-(dimethylamino)pyridine (DMAP, purity $\geq$ 99%), CuBr (copper(I) bromide, 97% purity), and PMDETA (N',N''-pentamethyldiethylenetriamine, 99% purity) were purchased from Sigma-Aldrich. 4-vinylpyridine (4VP, 95% purity) was provided by Alfa Aesar. Analytical reagent grades for ACN (acetonitrile), DMF (dimethylformamide), acetic acid (AcOH), and methanol (MeOH) were bought from Fisher Scientific and for ethanol (EtOH) from PanReac. Quercetin (hydrate, purity 95%), rutin (purity 97%) and ferulic acid (purity 99%) were supplied by Acros Organics. These standard polyphenols were used in the synthesis and testing of the materials here addressed. The water used in the experiments was ultrapure water supplied by the local laboratory.

4.2.2 Surface Imprinting in MCC-Br Macroinitiator Particles via ATRP: The reaction path considered was previously detailed presented [17] and includes: i) the immobilization of BIBB in MCC, ii) the surface imprinting in MCC-Br macroinitiator particles via ATRP. The first step involves the MCC dispersion in DMF using sonication and then the reaction of BIBB with MCC hydroxyls in the presence of triethylamine and DMAP. The second step includes the dried ATRP macroinitiator MCC-Br produced in step i), the functional monomer 4VP, the template quercetin and the crosslinker EGDMA as well as the ATRP pair CuBr/PMDETA. Ethanol was considered in step ii) for the

synthesis of the particles here used and the polymerization took place in a sealed vessel during 72 h at 70 °C [17].

4.2.3 Products Characterization using FTIR Spectroscopy: Purified and dried MIP was characterized through FTIR spectroscopy with a Perkin Elmer, model Spectrum Two, instrument. These analyses were directly performed in ATR mode and also with the particles mixed with KBr and pressed into pellets in order to collect the correspondent IR spectra.

4.2.4 SEM analysis for the pristine cellulose, MIP and NIP hybrid cellulose-synthetic particles: Scanning Electron Microscopy (SEM) characterization of the particles involved in this research was performed at the International Iberian Nanotechnology Laboratory (INL), Braga, using the FIB/SEM system HELIOS Nanolab 450S. SEM imaging was obtained using an electron beam of 3 keV, beam current 25 pA and field free lens mode.

4.2.5 Measurement of the surface area and pore volume of the adsorbents: The Brunauer–Emmett–Teller (BET) specific surface area and pore volume of the produced adsorbent particles were determined through N₂ adsorption/desorption isotherms at 77 K using a Quantachrome NOVA 4200e adsorption analyzer.

4.2.6 HPLC-DAD Analysis: An HPLC system (KNAUER) consisting of a gradient pump (P6.1 L) equipped with a degasser, an autosampler (6.1 L), a column thermostat (CT2.1), and a DAD (photodiode-array detector, 6.1 L) was used in this research. ClarityChrom was the software allowing the control of the HPLC system. The chromatographic analysis was performed using an Ascentis C18 (SUPELCO) column with a particle size of 5 µm and dimensions 25 cm × 4.6 mm. A gradient of solvents was used as a mobile phase varying from 100% of water-ACN (9:1) to 100% water-ACN (1:9) for 45 min. The mobile phase water pH was adjusted to 3 using acetic acid. The flow rate of the chromatographic analyses was 1 mLmin⁻¹, and the temperature of the column was set at 45 °C.

4.2.7 Competitive sorption/desorption experiments with polyphenol standards: Assessment of the competitive sorption/desorption capabilities of the MIPs/NIPs was performed with 25 mg of the dry materials in SPE packing cartridges (Agilent Bond Elut reservoir, 1 mL capacity). Adsorbents were first cleaned (successive loading and elution steps using methanol/acetic acid 90/10 with UV/vis monitoring) and afterward conditioned with the desired testing solvent (e.g., ethanol/water 80/20), during at least 24 h before use. Cartridges were then loaded with 2.5 mL of the mixture of polyphenol

standards ferulic acid/rutin/quercetin solution in ethanol/water 80/20 with concentration 0.1 mM for each compound, considering typically 15 min for total percolation time. The adsorbed amounts for each polyphenol were quantified through HPLC-DAD analysis of the liquid collected at column outlet. The washing desorption step was performed by percolating the same volume of clean solvent (ethanol/water 80/20) through the adsorbent particles and, afterward, the elution desorption step was made with the same volume of methanol/acetic acid 90/10. Desorbed amounts for each polyphenol were quantified also by HPLC-DAD analysis of the collected liquid phases in the washing and elution desorption steps.

4.2.8 Preparation of diatomaceous earth and grape pomace hydroalcoholic extracts:

Diatomaceous earth and grape pomace winemaking by-products were supplied by Caves Campelo S.A. (Portugal) during the harvest season in September 2021. Extraction of phenolic compounds was performed using ethanol/water 80:20 (v/v) as solvent in a ratio 20/1 solvent/solid-residue. Sonication followed by agitation were used as extraction methods. At the end, the supernatants were collected and evaporated under vacuum on a rotary evaporator. The final dry residues were used to prepare solutions at the selected conditions (solvent, concentration) to be used in sorption/desorption runs with the HPLC-packed hybrid MIP particles.

4.2.9 Sorption/desorption experiments with winemaking residues extracts: Hybrid MIP particles (2400 mg) with active pyridyl groups were packed in a HPLC column (L=125 mm and D=8 mm) using the slurry method. A Knauer HPLC pump (model Azura P 4. 1S, titanium head) with maximum delivery pressure of 40 MPa and flow rate in the range 0.001 to 10 mL min⁻¹ was used to make the flow of the feeding solutions and desorption solvents through the hybrid MIP-packed column (flow-rate = 1 mL/min). A column oven was used to define the temperature of the sorption/desorption steps and the temperature of the feeding solution or desorption solvents was controlled using a thermostatic bath.

4.3 Results and Discussion

4.3.1. Materials design and characterization

Figure 4-1 depicts the reaction steps considered for the synthesis of molecularly imprinted cellulose-synthetic hybrid particles with pyridyl active surface. In brief, the

first step deals with the immobilization of α -bromoisobutyryl bromide (BIBB) in microcrystalline cellulose (MCC), as schematized in **Figure 1(a)**, with generation of an ATRP macro-initiator MCC-Br particles. Afterwards, as schematized in **Figure 1(b)**, a thin synthetic shell with imprinted cavities is created at the surface of the cellulose particles, via ATRP polymerization. This second reaction step involves the macro-initiator MCC-Br particles, the functional monomer 4VP, the crosslinker EGDMA and the template quercetin, with the presence of the pair CuBr/PMDETA for the ATRP activation/deactivation mechanism. Non-imprinted material synthesis follows the same procedure with the absence of the quercetin template (see experimental details in [17]). Concerning cellulose modification, this approach follows the reported approaches for synthetic surface grafting using ATRP [32-34]. Hybrid particles generated by surface initiated ATRP (SI-ATRP) can be conceived with different kinds of core materials such as silica, metal particles or cellulose [48-50]. This research considered cellulose for this purpose due to its abundance, biodegradability, biocompatibility and the framework of both, the developed adsorbents and application, in the agro-industrial field. Furthermore, our aim is the fabrication of molecularly imprinted cavities with quercetin as template, and for this purpose, this polyphenol was included in the second step reaction mixture. A thin enough synthetic layer is sought in this technique, to enhance mass transfer and binding site accessibility, and therefore the proper reaction conditions should be addressed. It was previously observed [17] the formation of cellulose-synthetic hybrid particles with a 14 wt% 4VP/EGDMA shell, when working with ethanol as solvent in the second reaction step (**Figure 1(b)**) and [4VP]=87.4, [EGDMA]=17.4, [Quercetin]=6.5, [CuBr]=1.9, [PMDETA]=1.9, [Ethanol]= 16898 mM, [MCC-Br]=5.15 mg/mL (see ref. [17] for other synthesis conditions tested). Moreover, it was demonstrated that these specific particles, obtained through a solid/liquid heterogeneous ATRP process in ethanol, preserve a final size suitable for their application as adsorbent [17] and therefore will be here also considered. Other approaches are being considered in the literature for the synthesis of hybrid cellulose-synthetic particles with molecular imprinting of polyphenols considering the free radical route instead of the controlled polymerization here explored. For instance, porous cellulose-based molecular imprinted particles for enrichment of resveratrol were prepared through the silanization of porous cellulose particles with 3-methacryloxypropyltrimethoxysilane followed by the formation of the synthetic part by reacting the C=C in the cellulose in the presence of EGDMA, 4VP and resveratrol (the template for imprinting) with AIBN as initiator [35]. A potential

advantage of the route here considered is the higher control on the synthetic shell formation achieved with controlled radical polymerization as compared to the FRP mechanisms.

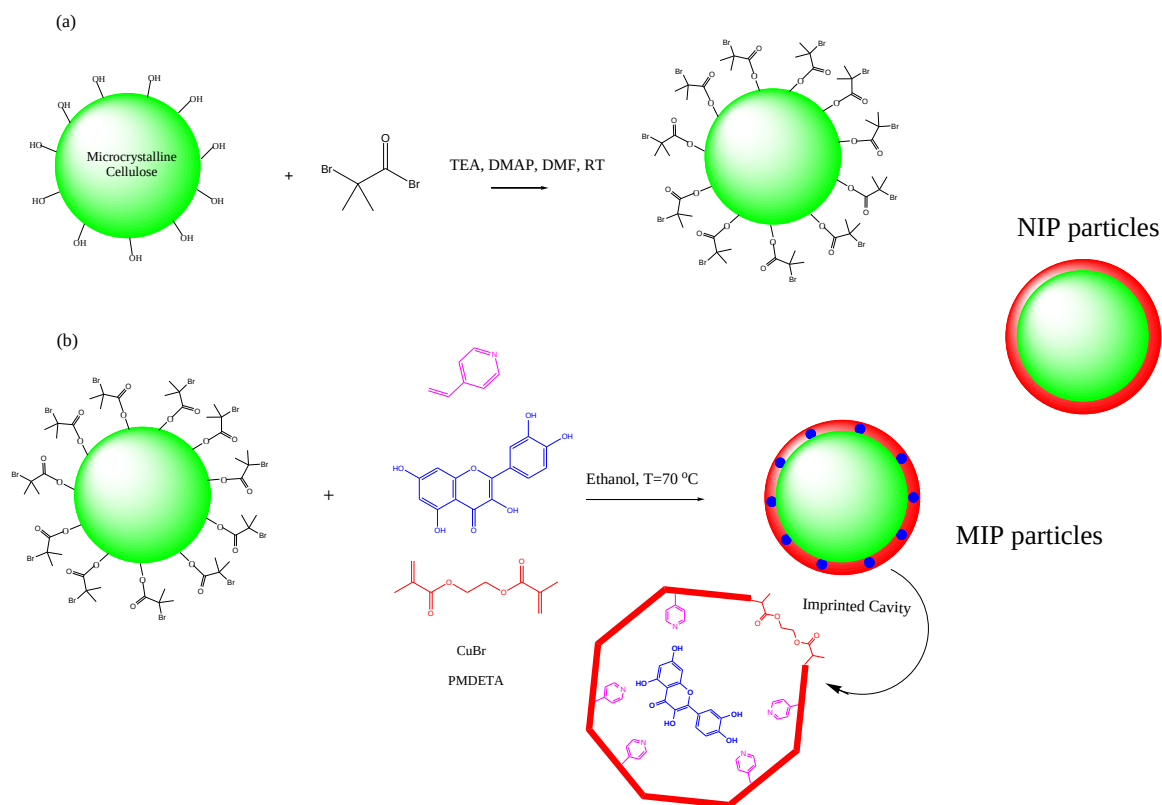


Figure 4-1: Depiction of the synthesis procedure considered for the development of surface molecularly imprinted cellulose-synthetic hybrid particles via ATRP. a) Immobilization of α -bromoisobutyryl bromide in pristine microcrystalline cellulose (MCC) to generate ATRP macroinitiator particles (MCC-Br). b) Grafting of a crosslinked-imprinted shell in the particles surface considering ATRP of co-monomers 4VP/EGDMA in the presence of quercetin [17]. The synthesis of non-imprinted polymer (NIP) particles (absence of the template molecule) is also depicted.

The FTIR (Fourier-transform infrared spectroscopy) analysis of the intermediate products (MCC-Br particles) and final cellulose-synthetic hybrid materials highlight the achievements on the native cellulose modification and creation adsorbents with a pyridyl active surface. In brief, (see **Figure C-1** in the supplementary material appendix C and ref. [17]): (i) a new peak at 1740 cm^{-1} is observed in MCC-Br as a consequence of the stretching of the carbonyl group ($-\text{C}=\text{O}$) resulting from the reaction between OH groups

in cellulose and BIBB, (ii) The presence of EGDMA in the final cellulose-synthetic hybrid particle is confirmed through the vibrational assignments at 1720 cm^{-1} and 1130 cm^{-1} (C=O and C–O groups, respectively), (iii) the functionalization of the final materials with pyridyl groups is demonstrated via the characteristic peaks at 1600 cm^{-1} (C=C pyridyl), 1418 cm^{-1} (C=N), 820 cm^{-1} and 755 cm^{-1} (both correspond to the C–H bending in the pyridine ring). A thin enough imprinted shell is here attempted in order to enhance binding site accessibility and mass transfer. For that, a low concentration of the monomers 4VP and EGDMA was used, and therefore, a weaker FTIR response of 4VP characteristic groups is expected (see refs. [12]-[18] dealing with different amounts of 4VP in the polymerization recipes).

Notably, the SEM analysis of the native MCC particles and of the molecularly imprinted and non-imprinted cellulose-synthetic hybrid materials highlight the modification of the surface achieved through the incorporation of the synthetic shell. In **Figure 4-2** relevant SEM micrographs are presented that put into evidence these outcomes. Figures 4-2(a) and (b) show the morphology of the pristine MCC particles used in this work (microcrystalline cellulose powder, $20\text{ }\mu\text{m}$, cotton linters, as designed by the supplier) with a wide dispersion in size (mostly in the range $5\text{ to }50\text{ }\mu\text{m}$) and the irregular format is discernible for these cotton linters. **Figures 4-2(c-d)** and **4-2(e-f)** show similar micrographs for the NIP (non-imprinted polymer) and MIP cellulose-synthetic hybrid particles, respectively. Overall, a slight global increase of the particles size seems to occur as a consequence of the grafting of the synthetic layer (characteristic size $< 50\text{ }\mu\text{m}$), both for NIP and MIP particles (see comparison of **Figures 4-2(c)** and **4-2(e)** with **Figure 4-2(a)**). Remarkably, Figures (d) and (f), in comparison with Figures (b), clearly evidence the surface modification achieved for the native MCC particles through the creation of a 4VP/EGDMA synthetic layer, both in NIP and MIP developed materials. Pictures added in the SEM micrographs for the surface of cellulose, NIP and MIP highlight these differences in articulation with the schemes presented in **Figure 4-1**.

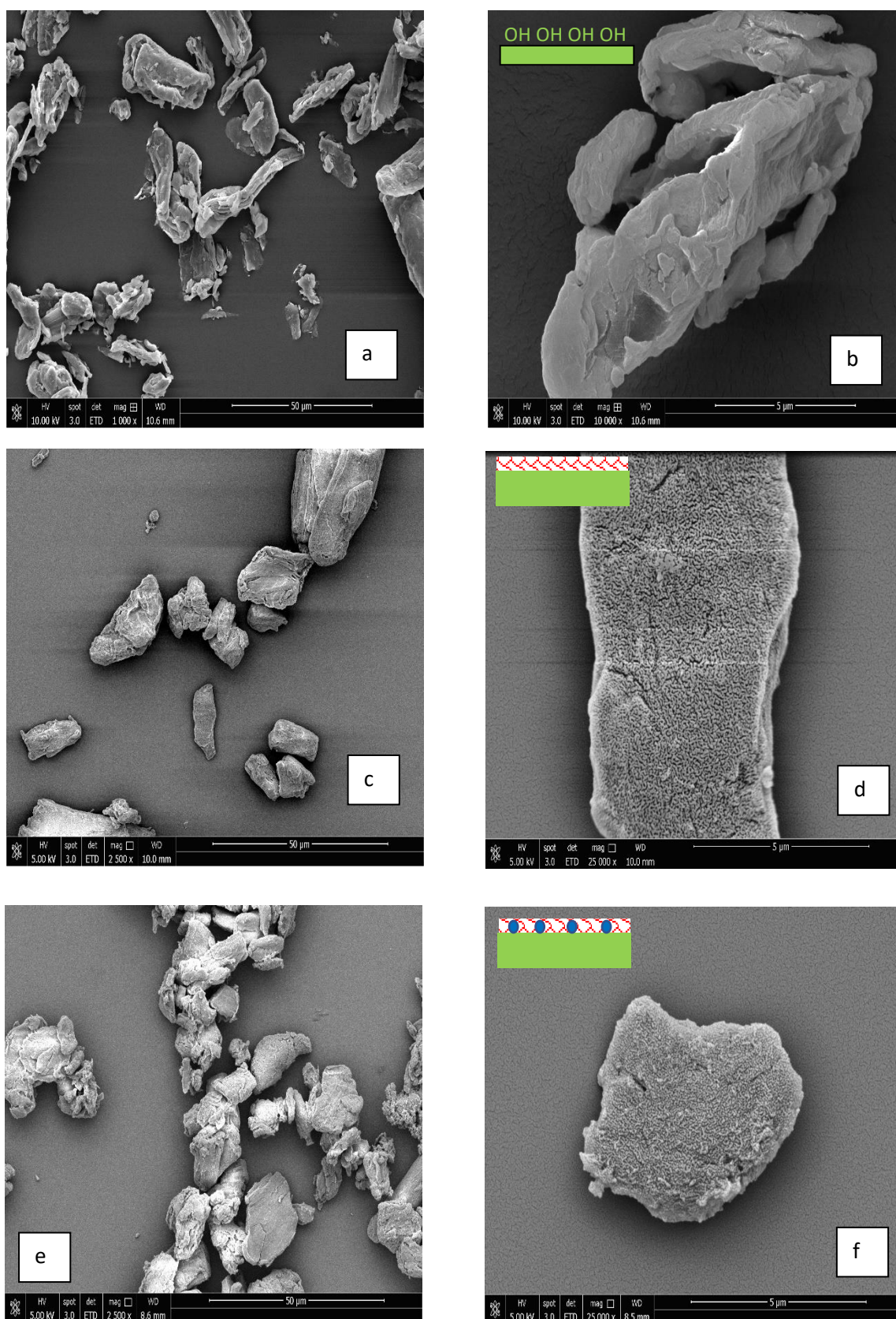


Figure 4-2: SEM images for the pristine cellulose particles (a and b), NIP hybrid cellulose-synthetic particles with pyridyl active surface (c and d) and MIP hybrid cellulose-synthetic particles with pyridyl active surface (e and f).

Overall, the nitrogen adsorption/desorption measurements with the native MCC particles and the cellulose-synthetic hybrid materials show very similar low specific surface area ($9.3 \text{ m}^2/\text{g}$ for the MIP particles and $8.6 \text{ m}^2/\text{g}$ for the native MCC), which is consistent with previous observations for totally synthetic 4VP/EGDMA particles ($2\text{--}4 \text{ m}^2/\text{g}$) indicating a low porogenic effect for such polymer networks formation [15]. Despite these low values for specific surface area (e.g. $135 \text{ m}^2/\text{g}$ estimated for the commercial resin DAX8) very high capabilities of the 4VP/EGDMA materials for adsorption of polyphenols are found as compared with several kinds of alternative adsorbents [12-18]. The functionalization of these materials with pyridyl groups is at the source of these outcomes, as discussed in the introduction section and also explored in the forthcoming analysis.

4.3.2. Competitive sorption/desorption in imprinted and non-imprinted particles with standard polyphenols

Having above put into evidence the surface modification achieved both for imprinted and non-imprinted particles, as compared to the native MCC, the effects of molecular imprinting were assessed through competitive sorption/desorption runs in the MIP and NIP hybrid materials. For that purpose, a mixture of the standard phenolic compounds ferulic acid, rutin and quercetin in ethanol/water 80/20 was loaded to the particles in SPE (solid-phase extraction) columns, followed by the washing (ethanol/water 80/20) and elution (methanol/acetic acid 90/10) steps. The composition of the columns outlet for these three steps was determined by HPLC-DAD. The selection of ferulic acid, rutin and quercetin was motivated by their representation of three major classes of phenolic compounds found in winemaking residues, namely the phenolic acids, glycosylated flavonoids and aglycone flavonoids, respectively. Additionally, quercetin was used as an imprinting template and therefore these competitive sorption/desorption runs with NIP/MIP particles can provide insights on the potential benefits of molecular imprinting. The selection of quercetin as imprinting template was driven by its reference structure for many flavonoid molecules, large availability (present in many different kinds of plants and related residues) and lower price. Actually, quercetin is a less expensive molecule and consequently can be used as surrogate for flavonoids imprinting.

In **Figure 4-3** are presented results for the HPLC-DAD analyses of the solutions collected at MIP and NIP SPE columns outlet after the competitive loading step of the mixture of

polyphenols. A clear high retention of quercetin in the quercetin-MIP (ca 62.1%) is observed as compared with the NIP (ca 7.4%) indicating a favorable impact of molecular imprinting for the retention of the template molecule under competitive sorption conditions. Taking in consideration the retention amounts determined by HPLC-DAD an imprinting factor $IF=8$ is estimated under these competitive sorption conditions. Note that for non-competitive sorption, an imprinting factor $IF=2.6$ was estimated for these same particles [17]. In reference [17] were also presented results for individual sorption/desorption assays with rutin, oleuropein and vanillic acid and demonstrated the relevant impact of NIP/MIP functionalization with pyridyl groups to enhance adsorption of polyphenols comparatively to the native cellulose.

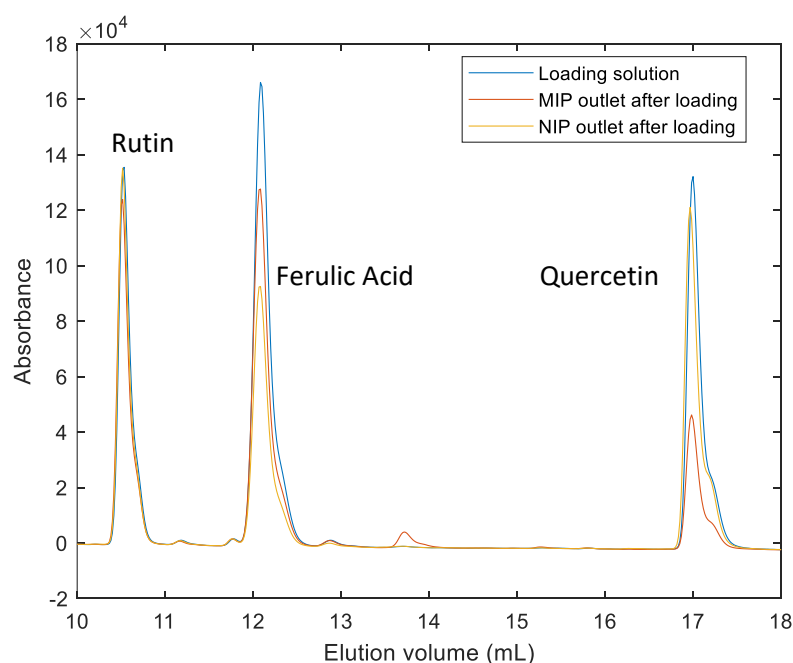


Figure 4-3: HPLC-DAD analyses for the solutions collected at MIP and NIP SPE columns outlet after de loading step. Competitive sorption/desorption assessment using a ferulic acid/rutin/quercetin solution in ethanol/water 80/20 with concentration 0.1×10^{-3} M for each compound, 2.5 mL of volume of liquid phase and 25 mg of adsorbent in the SPE cartridges. UV measurement at 280 nm are here presented.

Additional insights on the strength of the competitive binding of the three polyphenols in MIP and NIP particles is obtained analyzing the washing and elution, steps as presented in the Supplementary Material appendix C for the correspondent HPLC-DAD analyses (**Figures C-2 and C-3**) and summarized in **Figure 4-4**. A very strong binding of quercetin in the MIP is observed via the analysis of the high amounts of this molecule retained even after washing and elution of the particles. In opposition, rutin and ferulic acid are totally removed from the particles along the washing and elution steps, indicating a much lower

binding affinity. These outcomes clearly demonstrate the achievements obtained with molecular imprinting through the formation of binding sites enhancing the retention of quercetin.

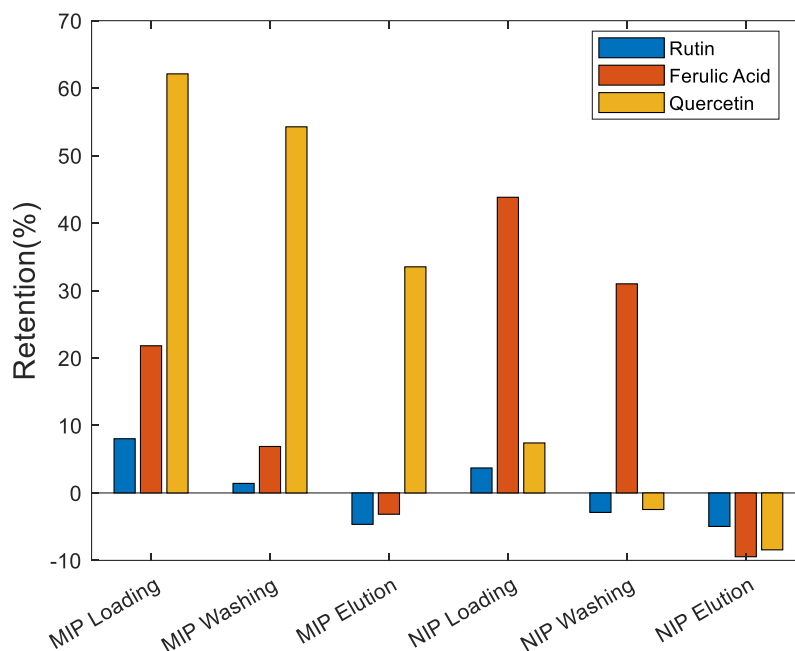


Figure 4-4: Fraction of the initial amount of standard compound's rutin, ferulic acid and quercetin retained in MIP and the NIP hybrid particles after the competitive loading, washing, and elution steps in SPE, according to the conditions specified in Figure 4-3. The retained amounts were estimated through the HPLC-DAD analysis of the samples collected at column outlet.

Interestingly, with the NIP particles, under the same competitive conditions, a stronger binding is observed for ferulic acid as compared with rutin and even quercetin (see **Figures 4-3 and 4-4** and also Figures C-2 and C-3 in the Supplementary material). To help with the interpretation of such results, in **Figure 4-5**, it is presented a simple sketch for the plausible interactions of the three standard polyphenols with the imprinted pyridyl active surface giving foundations for their different adsorption. For quercetin, a multivalent hydrogen bonding of the phenolic groups with the pyridyl moieties, enhanced due to stereospecificity for MIP cavities, is possible as represented in **Figure 4-5(a)**. A lower number of interactions of such kind is expected with rutin or ferulic acid. Note that, phenolic groups have an increased acidity as compared to -OH in the sugar moieties (see rutin molecular structure in **Figure 4-5**). Actually, with ionization of phenolic hydroxyls the negative charge and a set of the lone pair electrons in the phenoxide oxygen are delocalized by resonance to the carbons on the aromatic ring. Thereafter, the nucleophilic pyridyl group (a weak base) have a stronger binding with phenolic hydroxyls as compared

with the -OH groups in the sugar rings of rutin (charge delocalization not possible). Also, for rutin, steric hinderance is possible for binding due to the higher size of the molecule. These issues explain the low binding capacity observed for rutin, both with the MIP and NIP particles.

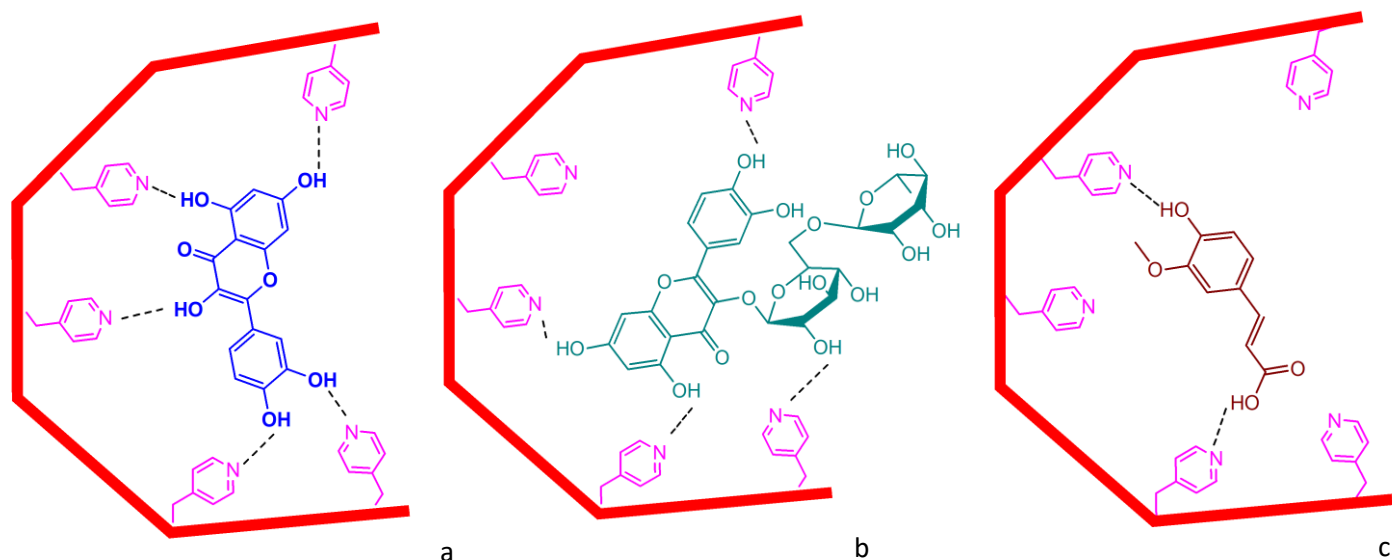


Figure 4-5: Simple sketch for some plausible intermolecular interactions of (a) quercetin, (b) rutin and (c) ferulic acid with the pyridyl active surface of the molecularly imprinted cellulose-synthetic hybrid particles synthesized in this work. A multivalent hydrogen bonding of the phenolic groups in quercetin with the pyridyl groups in the surface is possible. Lower number of pyridyl-phenolic hydrogen bonding interactions are expected for rutin and ferulic acid and steric hinderance is possible for rutin. Hydrophobic effects, competitive interaction of pyridyl groups with solvent molecules are other issues influencing solutes sorption/desorption processes. In spite of the expected effect of the stereospecificity of the surface imprinted cavities in the MIP particles, similar interactions are possible with the surface of the non-imprinted particles (NIP).

For ferulic acid, in spite of the low number of phenolic hydroxyls (**Figure 4-5(c)**), the higher acidity of the carboxylic group ($pK_a=4.61$ for ferulic acid) should contribute to increase the interaction with the weakly basic pyridyl moieties ($pK_a=5.22$ for pyridine base) in the active surface, both in MIP and NIP, in spite of the complex ionization effects with aqueous solvent mixtures. This explains the high retention for ferulic acid observed with the NIP and the low displacement observed for this molecule during the washing step with ethanol/water 80/20 (see e.g. **Figure 4-4**). However, in the elution step with methanol/acetic acid 90/10, the ferulic acid is totally desorbed (**Figure 4-4**), and this is enhanced by the presence of acetic acid and concomitant competition for binding with the basic pyridyl moieties. Note that the same situation was not observed for elution of quercetin in the MIP, giving support for the importance of the imprinted cavities for the

very high binding strength of this molecule in this material. These imprinting cavities should also play a central role with quercetin preferential competitive sorption in the MIP comparatively to the favorable retention of ferulic acid in the NIP under similar competitive conditions.

Hydrophobic effects and competitive interaction of the pyridyl groups with solvent molecules (water, ethanol, acetic acid, etc) are additional issues influencing the sorption/desorption processes. A “non-ideal” interferent solvent for MIPs performance is here being used (ethanol/water mixtures) to avoid other toxic and less biocompatible solvents (e.g. acetonitrile).

The knowledge acquired with the competitive sorption analysis above presented is applied below for the enrichment of polyphenols in winemaking residues, namely using the special features of the developed MIP particles to target flavonoids and related kinds of polyphenols.

4.3.3. Application with diatomaceous earth extracts

Here it is addressed the performance of the developed molecularly imprinted cellulose-synthetic hybrid particles with pyridyl active surface as adsorbent for the fractionation and enrichment of flavonoids in residual diatomaceous earth. Within this purpose, an hydroalcoholic extract of this material was considered as polyphenols feedstock (diatomaceous earth is used in winemaking for wine filtration and often discarded after saturation). Industrial and analytical applications already consider sorption/desorption processes in purification steps for a long time and their use with plant extracts and effluents from agro-industrial activities is also well-known [37]. The adsorbents play a central role in the efficiency of such processes, namely concerning specificity/selectivity, retention capacity and easiness for their regeneration. Many synthetic polymer networks (e.g. the Amberlite, Supellite and Reillex resins) excel in such kind of applications due to the possibility for their tailoring besides the often-observed durability and stability (e.g. as compared with lignocellulosic/polysaccharide, activated carbon or silica adsorbents). An overview for different kinds of adsorbents with practical use in the valorization of agro-industrial residues, namely through the concentration of polyphenols is presented in reference [37] and recent specific cases involving sorption/desorption for the valorization of winemaking residues can be found in [38,39] and refs. therein.

Figure 4-6 presents the phenolic profile of this extract, obtained through HPLC-DAD analysis, and a rich composition in such kinds of compounds is observed. Among the

plethora of different molecules detected, evidencing the complexity of these extracts, relevant flavonoid compounds can be identified, namely myricetin-O-hexoside (peak 13), quercetin-O-hexuronoside (14), myricetin (18), quercetin (21) and kaempferol (24). In the supplementary material, additional information is presented helping with chemical species identification in these chromatograms (Figures C-4-C-8), namely concerning the ones close to the analytic system peak (peak 1 in **Figure 4-6**), such as gallic acid and other phenolic acids (peak 3 and subsequent peaks up to ~ 8 min elution time) as well as anthocyanins (see also de next section). Detailed information on the identification of phenolic compounds in the winemaking residues extracts here considered can be found in refs. [16,33]. These previous works allow to conclude that peak 20 in **Figure 4-6** is not relative to a polyphenol molecule but another compound used during the vinification process.

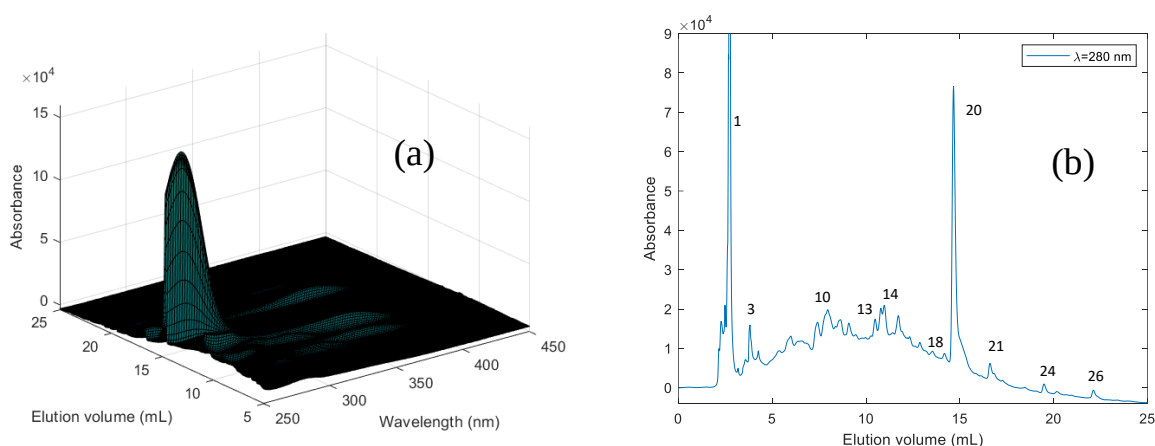


Figure 4-6: (a) 3D HPLC-DAD chromatogram and (b) correspondent 2D view at 280 nm for the diatomaceous earth extract used in this work. The following relevant identification for flavonol compounds in the extract is possible: (13) myricetin-O-hexoside, (14) quercetin-O-hexuronoside, (18) myricetin, (21) quercetin, (24) kaempferol.

Pyridyl groups have a strong binding towards phenolic compounds, as above discussed and demonstrated, even when hydroalcoholic solvents with low water content are used (e.g. ethanol/water mixtures with volume fraction of ethanol > 50 %). This is especially appealing with the processing of plant extracts containing polyphenols because allows working with high concentration (avoiding precipitation issues of such complex mixtures in water-rich solvents) and preserving a high retention in the sorption step. Having in

mind the industrial application, the volume to be processed decreases (lowering energetic costs with pumping) with enhancement of process intensification and sustainability. These features were previously demonstrated with other 4VP-based adsorbents and are here explored with the hybrid cellulose-4VP MIPs for the fractionation of diatomaceous earth and grape pomace contained polyphenols. An example for the ability to make the fractionation of such polyphenols mixtures is presented in **Figure 4-7**. In this case study, the particles were previously loaded at $T=15\text{ }^{\circ}\text{C}$ using a diatomaceous earth extract (**Figure 4-6**) in EtOH/water 80/20 and $C=3.75\text{ mg/mL}$. A low temperature was considered in this run aiming at to further enhance the adsorption of phenolic compounds, namely through H-bonding interactions with the adsorbent functional groups. After that, the desorption process includes the increase of the temperature to $45\text{ }^{\circ}\text{C}$ and the percolation through the column of a sequence of solvents, starting with water, and going towards the increase of ethanol content, up to a final stage with methanol/acetic acid 90/10. This solvent-gradient process is considered to produce fractions with different polyphenols composition, exploring the variable strength of their binding with the surface of the adsorbent containing active pyridyl groups, similarly to that above demonstrated with the mixture of rutin, ferulic acid and quercetin. A first important outcome that can be observed in **Figure 4-7** is the elimination in this fraction of the non-polyphenol compound in the diatomaceous earth extract (peak 20 in **Figure 4-6**). Notably, results in **Figure 4-7** also demonstrate the production of a fraction in ethanol/water 80/20 highly enriched in flavonols, namely quercetin (peak 21 in **Figure 4-6**) and quercetin-o-hexuronoside (peak 14 in **Figure 4-6**). The designation quercetin-o-hexuronoside is considered for identification because quercetin-3-glucuronide and quercetin-3-galacturonide are both possible via the LC-MS analysis [40]. However, the presence of quercetin-3-glucuronide in plant extracts, namely in vine and winemaking residues is well documented [41] and therefore is the most plausible identification for peak 14. **Figure 4-8** presents a 3D view for the HPLC-DAD analysis of the same fraction giving further insights on the fractionation and enrichment achieved. Actually, the comparison between **Figure 4-6** (the starting diatomaceous earth extract) and **Figures 4-7** and **4-8** clearly demonstrates the good performance of the particles developed for enrichment and fractionation of polyphenols. Considering the chromatogram areas at 280 nm, enrichment factors of 5 and 10 are estimated for quercetin and quercetin-o-hexuronoside, respectively, in this fraction comparatively to the initial extract.

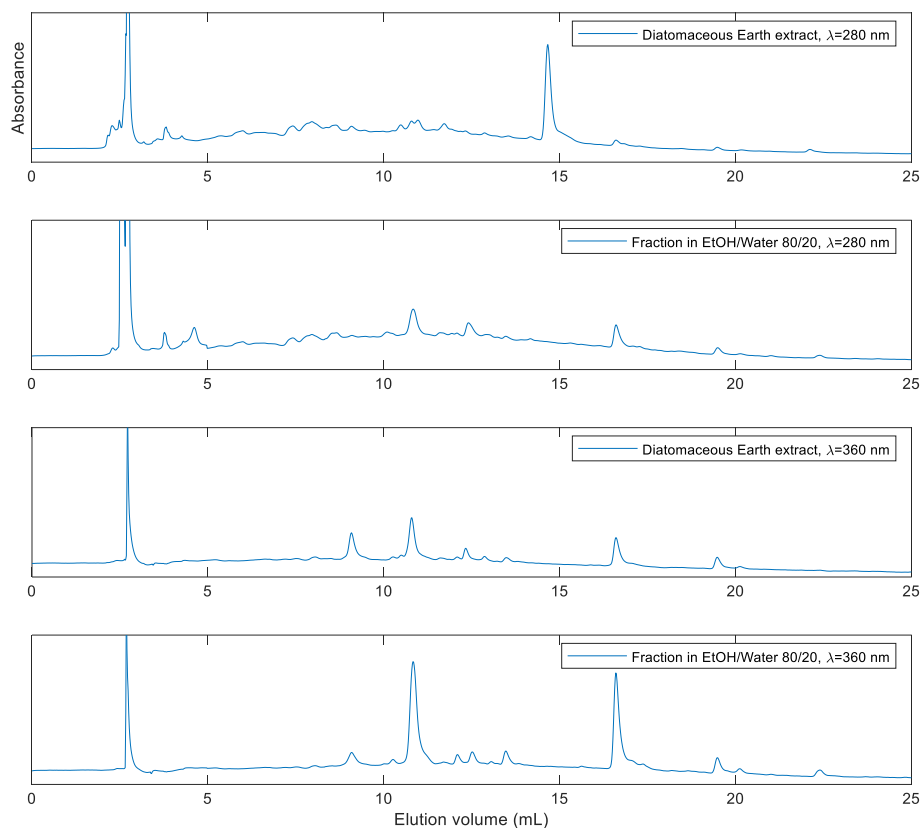


Figure 4-7: HPLC-DAD analyses for the diatomaceous earth extract and a fraction desorbed from the pyridyl active MIP hybrid particles at $T=45$ °C with EtOH/water 80/20 as eluent. The previous sorption step was made at $T=15$ °C using a diatomaceous earth extract in EtOH/water 80/20 and $C=3.75$ mg/mL. The wavelengths $\lambda=280$ and 360 nm were selected (see insets) to highlight the enrichment of flavonols in this fraction.

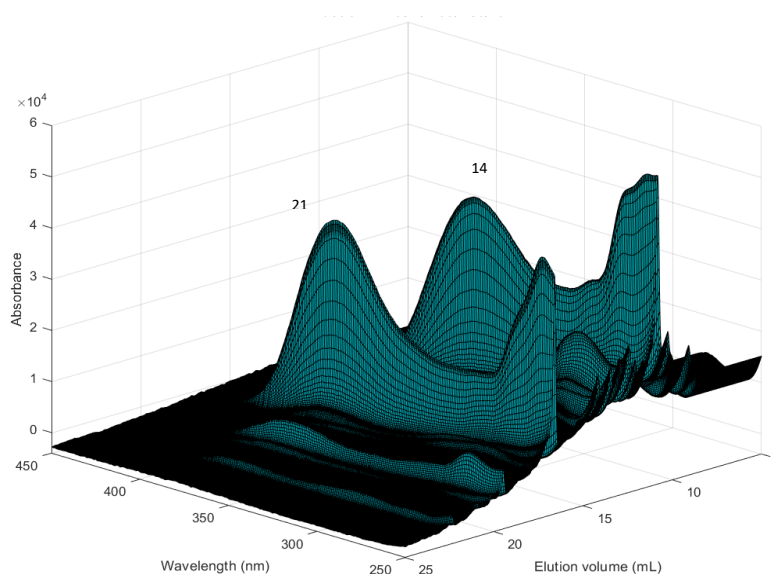


Figure 4-8: 3D view for the HPLC-DAD analysis for the fraction desorbed from the pyridyl active MIP hybrid particles at $T=45$ °C with EtOH/water 80/20 as eluent. The previous sorption step was made at $T=15$ °C using a diatomaceous earth extract in EtOH/water 80/20 and $C=3.75$ mg/mL. The huge enrichment with the flavonols kaempferol, quercetin and quercetin-o-hexuronoside achieved in this fraction is here highlighted.

In **Figures 4-9** and **4-10** is presented a similar analysis for a fraction desorbed with methanol/acetic acid 90/10. Note again the important outcome related with the elimination of the non-phenolic compound correspondent to peak 20 in the produced fraction. Moreover, a huge purification and concentration effect is observed in this fraction for quercetin-o-hexuronoside (peak 14) as compared with the initial fraction. This achievement becomes clear through comparison of **Figures 4-6**, **4-9** and **4-10**. Considering the chromatogram areas at 280 nm, the enrichment factor of 12 is estimated for quercetin-o-hexuronoside in this fraction comparatively to the initial extract. The extra acidic group in quercetin-o-hexuronoside (quercetin-3-glucuronide) and its higher binding strength with the basic pyridyl groups at the adsorbent surface should ground this particular separation and concentration outcome. Globally, a very high binding effect seems to be observed for this compound due the multiple interaction points with pyridyl moieties, namely the phenolic -OHs and the extra -COOH in the -glucuronide group (see section 2.2).

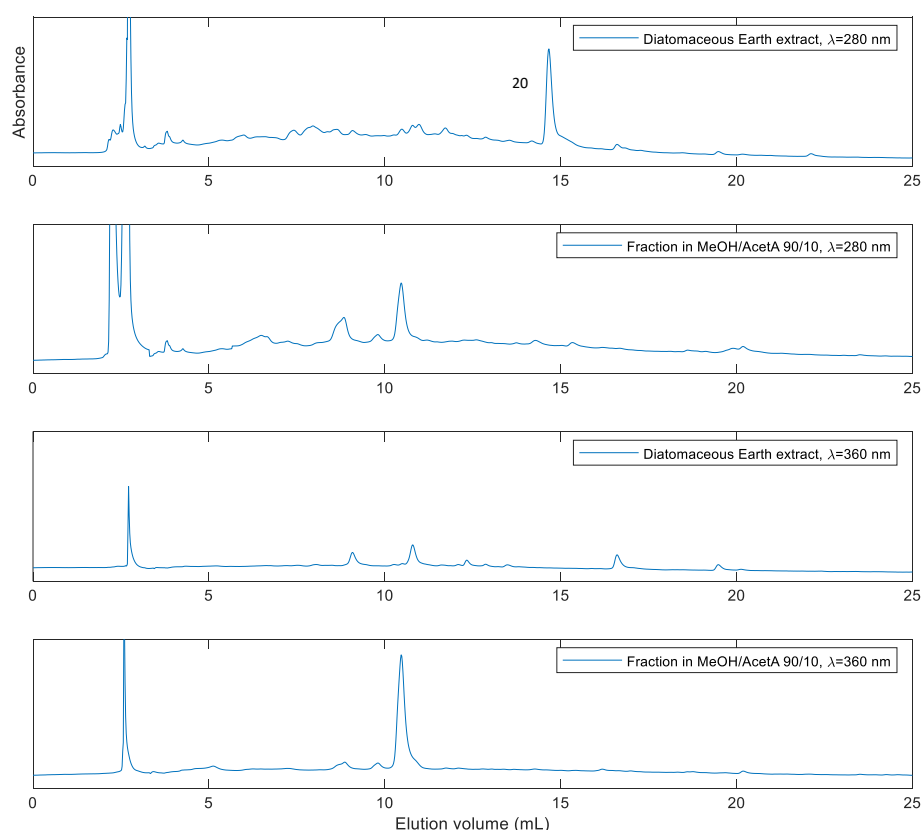


Figure 4-9: HPLC-DAD analyses for the diatomaceous earth extract and a fraction desorbed from the pyridyl active MIP hybrid particles at $T=45$ °C with MeOH/AcetA 90/10 as eluent. The previous sorption step was made at $T=15$ °C using a diatomaceous earth extract in EtOH/water 80/20 and $C=3.75$ mg/mL. The wavelengths $\lambda=280$ and 360 nm were selected (see insets) to highlight the enrichment of flavonols in this fraction.

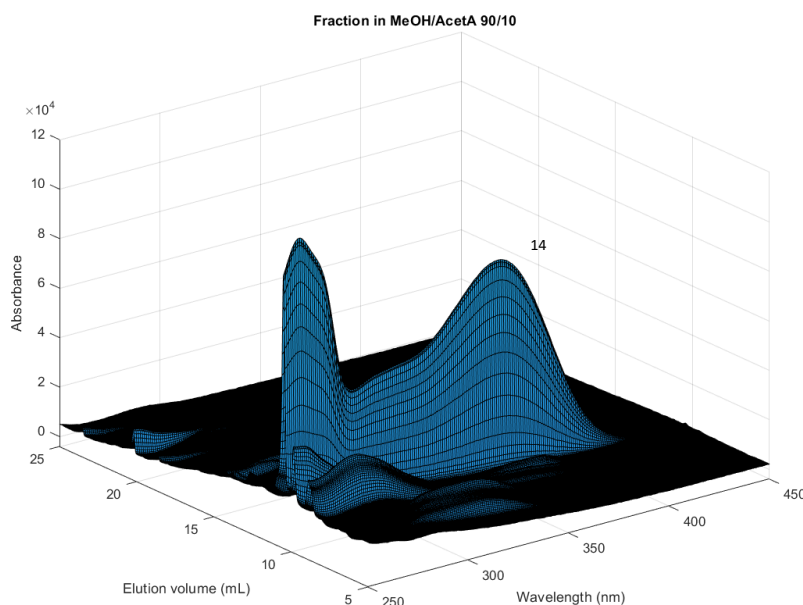


Figure 4-10: 3D view for the HPLC-DAD analysis for the fraction desorbed from the pyridyl active MIP hybrid particles at $T=45\text{ }^{\circ}\text{C}$ with MeOH/AcetA 90/10 as eluent. The previous sorption step was made at $T=15\text{ }^{\circ}\text{C}$ using a diatomaceous earth extract in EtOH/water 80/20 and $C=3.75\text{ mg/mL}$. The huge enrichment with the flavonol quercetin-o-hexuronoside achieved in this fraction is here highlighted.

4.3.4. Application with grape pomace extracts

Several studies showed the rich phenolic profile of the grape pomace biomass, including phenolic acids anthocyanins and many flavonols (see e.g. [40] and references therein). It is well-known that many of these phenolic compounds present important antioxidant and anti-inflammatory properties, able to alleviate neurodegenerative and cardiovascular diseases, thus making them very useful in the pharmaceutical, cosmetic and food industries [40]. Often, the classification of phenolic compounds considers flavonoids as a specific major class that can be subdivided into subgroups considering a three-ring structure as reference (see e.g. the quercetin structure in **Figure 4-5a**) and analyzing the linkages relatively to the ring containing the -O- group and the degree of unsaturation and oxidation of this same ring [43,44]. When the simple linkage of other phenolic ring to that containing the -O- group occurs in position 2 a set of subgroups of compounds is identified, namely flavones, flavonols (see quercetin structure), flavanones, flavanonols, flavanols (catechins), anthocyanins and chalcones [43]. It was demonstrated that, among the phenolic compounds in found in grape pomace, two flavonoid classes are predominant, namely the flavonols and the anthocyanins. The aglycones quercetin, myricetin or kaempferol and the related glycosylated analogues (rutin, myricetin-O-hexoside, quercetin-o-hexuronoside, etc) are documented to be present in grape pomace.

On the other hand, anthocyanins are glycosylated counterparts of anthocyanidins, belonging also to the flavonoid family, that are widely found in grapes, wine and winemaking residues/sub-products. The anthocyanidin malvidin and the related anthocyanin malvidin 3-o-glucoside (also named oenin), delphinidin/delphinidin 3-glucoside (myrtillin), peonidin/peonidin 3-glucoside are pairs anthocyanidin/anthocyanin with high relevance in different kinds of grape and wine varieties (e.g. defining the red, bluish, purple, etc, tonality of wine). Note that many other glycosylated counterparts of anthocyanidins are found in nature [45], namely in grapes and wine (e.g. malvidin, delphinidin, peonidin -3,5-O-diglucosides or the -arabinose, -galactose, -rhamnose, -xylose related anthocyanins). Besides other applications, anthocyanins (synthesized by plants via the flavonoid pathway [46]) can be used as natural dyes. Their colors depend on the pH and on the methylation/acylation at the hydroxyl groups belonging to the constituent phenolic rings [43]. Actually, the equilibrium between different molecular structures (e.g. flavylium cation and quinoidal bases), explains the shift between colors observed in practice (e.g. red for the flavylium cation and blue for the quinoidal base, as observed with cyanidin-3-glucoside [47]).

Such a complex mixture of phenolic compounds in grape pomace, namely concerning the contained flavonols and anthocyanins subclasses of flavonoids, is illustrated in **Figure 4-11** below for the extract of this biomass here considered. Our aim is to demonstrate the usefulness of the developed hybrid particles for the fractionation of these families of molecules. In **Figure 4-12** are presented results for the fractionation of a grape pomace extract attained with the hybrid MIP particles and using a similar process to that above described with the diatomaceous earth extract. At a first stage the particles were loaded at $T=15\text{ }^{\circ}\text{C}$ using an extract in EtOH/water 54/46 and $C=1.1\text{ mg/mL}$. After that, the desorption process included the increase of the temperature to $45\text{ }^{\circ}\text{C}$ and the running of a solvent-gradient process, starting with water, afterwards increasing the ethanol content in hydroalcoholic mixtures, up to a final stage with methanol/acetic acid 90/10. **Figure 4-12** compares the HPLC-DAD chromatogram of the initial grape pomace extract with those correspondents to some fractions obtained with this solvent-gradient process. In this comparison, the wavelength $\lambda=360\text{ nm}$ was selected to highlight the combined change of flavonols and anthocyanins along the different fractions produced

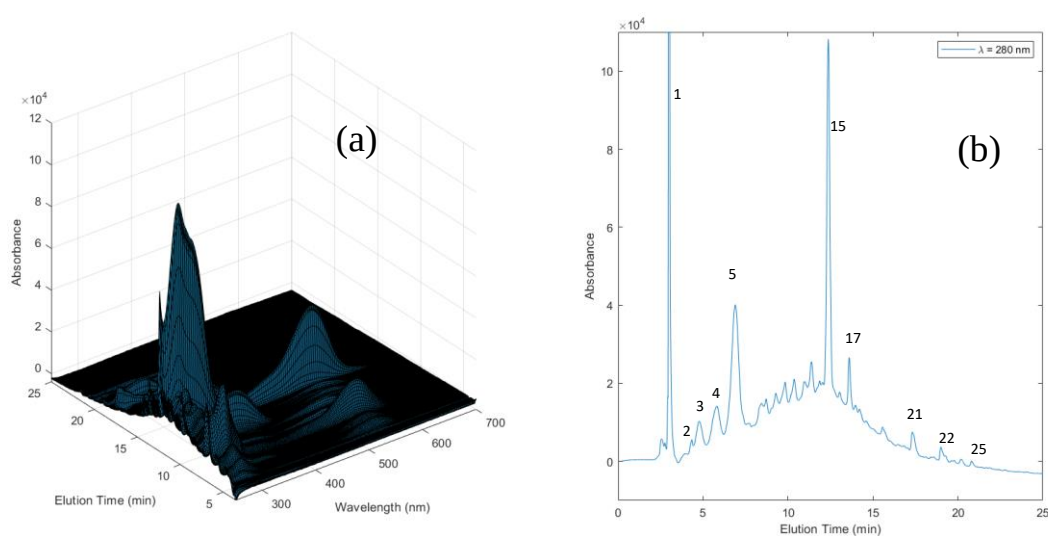


Figure 4-11: 3D HPLC-DAD chromatogram (a) and correspondent 2D view at 280 nm (b) for a grape pomace extract used in this work. The following relevant identification for anthocyanin and flavonol compounds in the extract is possible: (5) malvidin 3-O-glucoside (oenin), (15) malvidin-3-O-acetylglucoside (21) quercetin.

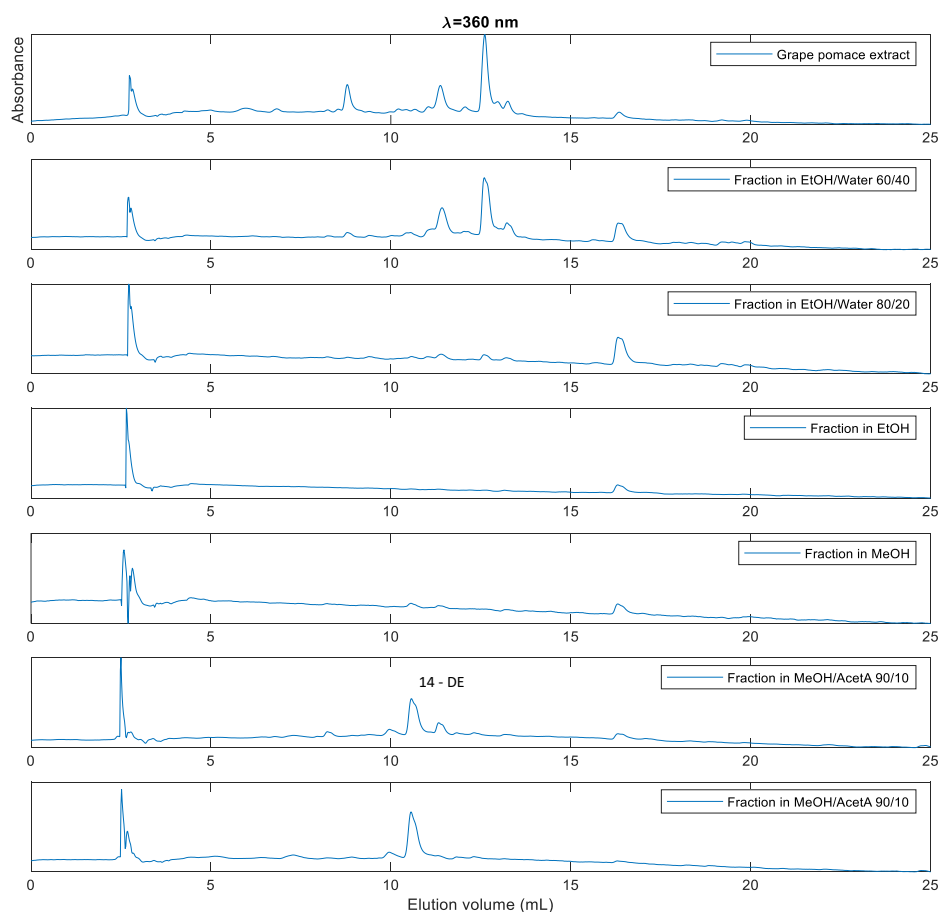


Figure 4-12: HPLC-DAD analyses for different fractions desorbed from the pyridyl active MIP hybrid particles at $T=45\text{ }^{\circ}\text{C}$ with diverse solvents as eluents (see inset legend for each fraction). The previous sorption step was made at $T=15\text{ }^{\circ}\text{C}$ using a grape pomace extract in EtOH/water 54/46 and $C=1.1\text{ mg/mL}$. The wavelength $\lambda=360\text{ nm}$ was selected to highlight the combined change of flavonols and anthocyanins composition along the different fractions.

These results show that, when compared with the initial grape pomace extract, the fractions with solvent composition up to ethanol/water 60/40 are rich with anthocyanins, specially the abundant malvidin-3-o-acetylglucoside, (peak 15 in **Figure 4-11**). The fractions obtained with ethanol/water 80/20, ethanol and methanol are highly enriched with quercetin (the template used for imprinting of hybrid particles surface), while the fractions with methanol/acetic acid 90/10 present a very high content in a molecule potentially matching with quercetin-3-glucuronide, as above analyzed with the diatomaceous earth extract. As before, besides the -OHs groups in quercetin-3-glucuronide, the extra -COOH in this molecule should ground a strong binding with pyridyl basic moieties, leading to its displacement only with a stronger elution solvent, namely with the inclusion of acetic acid. Considering the chromatogram areas at 280 nm, enrichment factors of 4.2 and 8 are estimated for quercetin and quercetin-3-glucuronide, in fractions EtOH/water 80/20 and 2nd MeOH/AcetA 90/20, respectively,

Figure 4-13 compares the same HPLC-DAD chromatograms at $\lambda=550$ nm to highlight the particular change of anthocyanins along the different fractions produced. Note again that their presence is magnified in the first stages of desorption (initial water richer fractions up to ethanol/water 60/40) and minimized in the ethanol/water 80/20, ethanol and methanol fractions. Interestingly, the presence of three well defined anthocyanins is observed in the first fraction obtained with methanol/acetic acid, showing the importance of acidification for the elution of traces of these compounds still bind to the adsorbent. Globally, results in **Figures 4-12 and 4-13** show the usefulness of the developed adsorbents for the fractionation of flavonols and anthocyanins in grape pomace extracts. Note that a different designing of the temperature-swing/solvent-gradient processes can be considered to achieve an additional tailoring of the composition of the phenolic compounds in the produced fractions.

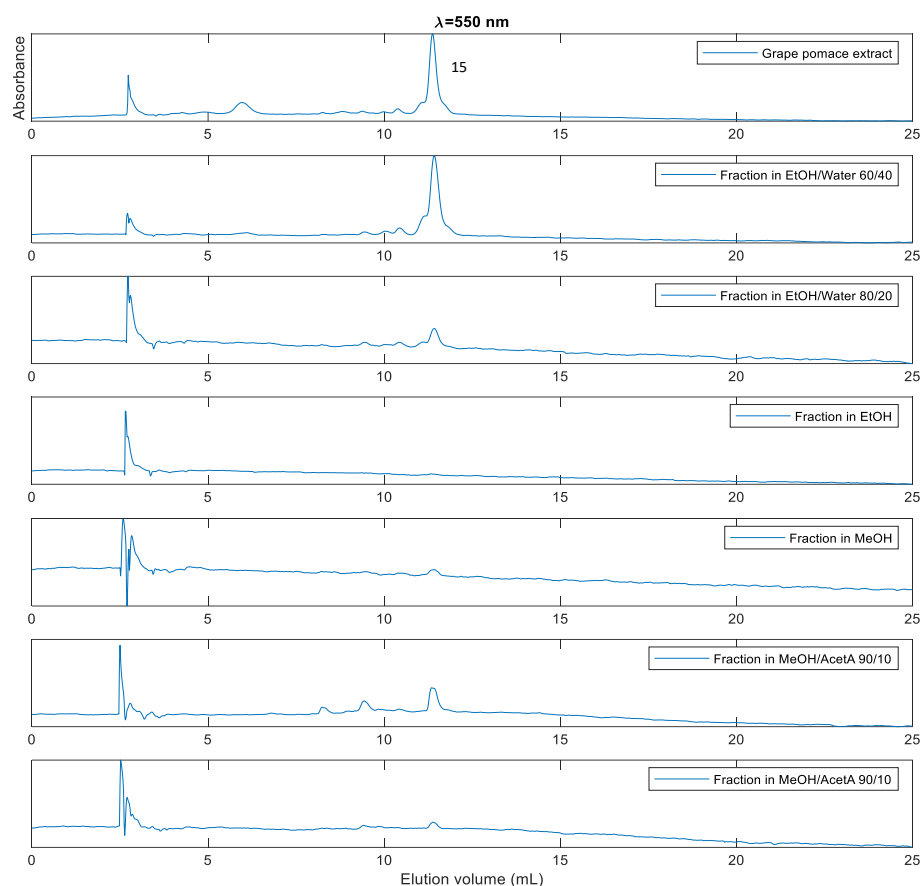


Figure 4-13: HPLC-DAD analyses for different fractions desorbed from the pyridyl active MIP hybrid particles at $T=45\text{ }^{\circ}\text{C}$ with diverse solvents as eluents (see inset legend for each fraction). The previous sorption step was made at $T=15\text{ }^{\circ}\text{C}$ using a grape pomace extract in EtOH/water 54/46 and $C=1.1\text{ mg/mL}$. The wavelength $\lambda=550\text{ nm}$ was selected to highlight the change of anthocyanins composition along the different fractions.

A different set of operation conditions concerning the grape pomace extract used and loading conditions were considered in other experimental runs and some relevant outcomes are presented in **Figures 4-14 and 4-15**. In this run, the sorption step was made at $T=20\text{ }^{\circ}\text{C}$ using a grape pomace extract in EtOH/water 80/20 and $C=1\text{ mg/mL}$. A different grape pomace extract was here used in order to simulate the likely variability of the starting biomass (changing of the grapes variety, geographical origin, etc) and the eventual impact in the fractionation process. Results in **Figures 4-14 and 4-15** highlights again the good performance of the developed hybrid MIP particles with the enrichment of different classes of polyphenols in grape pomace. Specifically, **Figure 4-14** shows a fraction enriched for two flavanols which identification is compatible with myricetin and quercetin glucosides previously found in the diatomaceous earth extract (see previous section). Enrichment factors up to 4 in these flavonols were estimated in the fractions produced. As mentioned above, non-specific binding of other molecules than the MIP

template is unavoidable with such complex mixtures and working with interfering solvents as ethanol/water mixtures. However, the results here presented demonstrate the usefulness of using quercetin as a kind of surrogate for materials textural improvement and enrichment of other flavonols in the mixture. On the other hand, **Figure 4-15** shows the production of anthocyanins rich fractions when manipulating through sorption/desorption the same extract. Note the lower incidence for flavonols observed for fraction in **Figure 4-15** and the clear presence of anthocyanins such as oenin (malvidin 3-o-glucoside), malvidin-3-o-acetylglucoside and other minority related compounds derived from malvidin, delphinidin, cyanidin, petunidin and peonidin [40] which become apparent in the visible region of the spectrum.

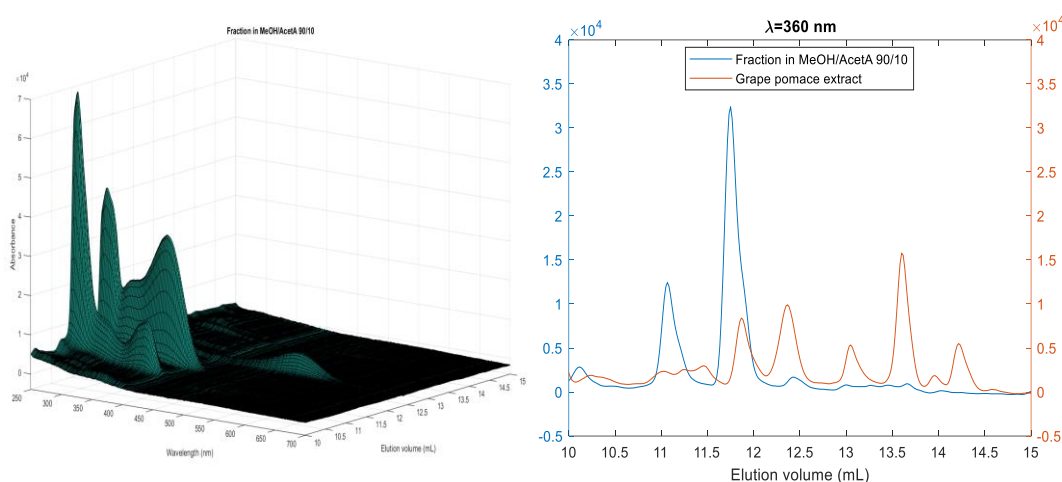


Figure 4-14: HPLC-DAD analysis for a fraction desorbed from the pyridyl active MIP hybrid particles at $T=45\text{ }^{\circ}\text{C}$ with MeOH/AcetA 90/10 as eluent. The previous sorption step was made at $T=20\text{ }^{\circ}\text{C}$ using a grape pomace extract in EtOH/water 80/20 and $C=1\text{ mg/mL}$. A fraction enriched with flavonols is here showed.

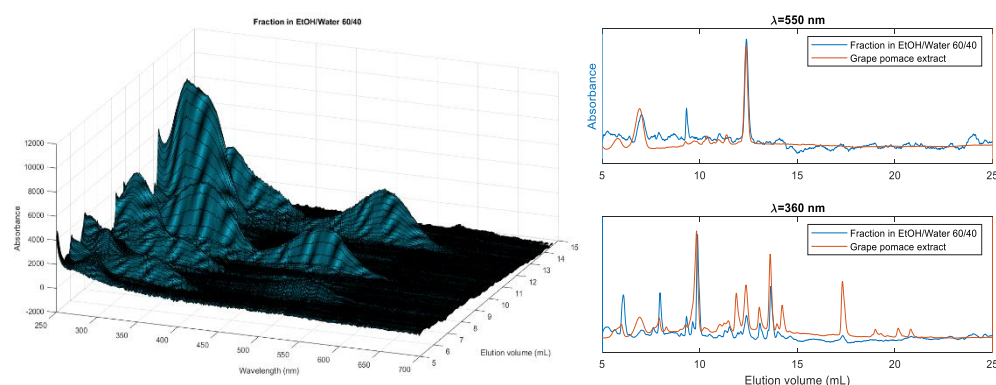


Figure 4-15: HPLC-DAD analysis for a fraction desorbed from the pyridyl active MIP hybrid particles at $T=45\text{ }^{\circ}\text{C}$ with EtOH/water 60/40 as eluent. The previous sorption step was made at $T=20\text{ }^{\circ}\text{C}$ using a grape pomace extract in EtOH/water 80/20 and $C=1\text{ mg/mL}$. A fraction enriched with anthocyanins is here showed.

4.4 Conclusions

Hybrid cellulose-synthetic particles with surface active pyridyl groups and molecularly imprinted binding sites generated with quercetin as template were prepared via ATRP. The functionalization of the materials with pyridyl moieties was confirmed and the SEM analysis of the hybrid particles clearly demonstrate their surface modification as compared with the native cellulose. An imprinting factor $IF \sim 8$ was estimated through competitive sorption/desorption of a mixture of standard polyphenols quercetin/ferulic acid/rutin in the imprinted and non-imprinted developed particles. Furthermore, a high retention of polyphenols in the hybrid materials was observed even when using hydroalcoholic solvents with high ethanol content (e.g. ethanol/water 80/20 v/v). This important outcome is possible due to the strong binding of polyphenols with the surface active pyridyl moieties.

The particular features of the synthesized materials were scrutinized in view of their use as adsorbents for the efficient fractionation of flavonols and anthocyanins in winemaking residues. Agro-industrial diatomaceous earth and grape pomace residues were used as a source of these bioactive compounds and high concentration and enrichment factors were achieved with the designed sorption/desorption processes. For instance, fractions with enrichment factor 5 for quercetin and 12 for quercetin-3-o-glucuronide were observed with the processing of the diatomaceous earth extract. On the other hand, the production of fractions with separated enrichment of anthocyanins and flavonols was demonstrated for grape pomace extracts. Enrichment factors up to 4 for flavonols (e.g. myricetin and quercetin glucosides) were obtained with grape pomace extracts.

The present work considers the possible addressing of sustainability concerns combining three action lines: i) the incorporation of cellulose in the developed adsorbents, ii) the designing of sorption/desorption methods contributing for process intensification and iii) the valorization of winemaking residues in framework of circular bio-economy. The efficient exploitation of agro-industry generated waste and by-products to obtain high added-value products, namely drugs, nutraceuticals, cosmeceuticals, a working line in the United Nations 2030 Agenda for Sustainable Development, drives the research line here explored.

4.5 References

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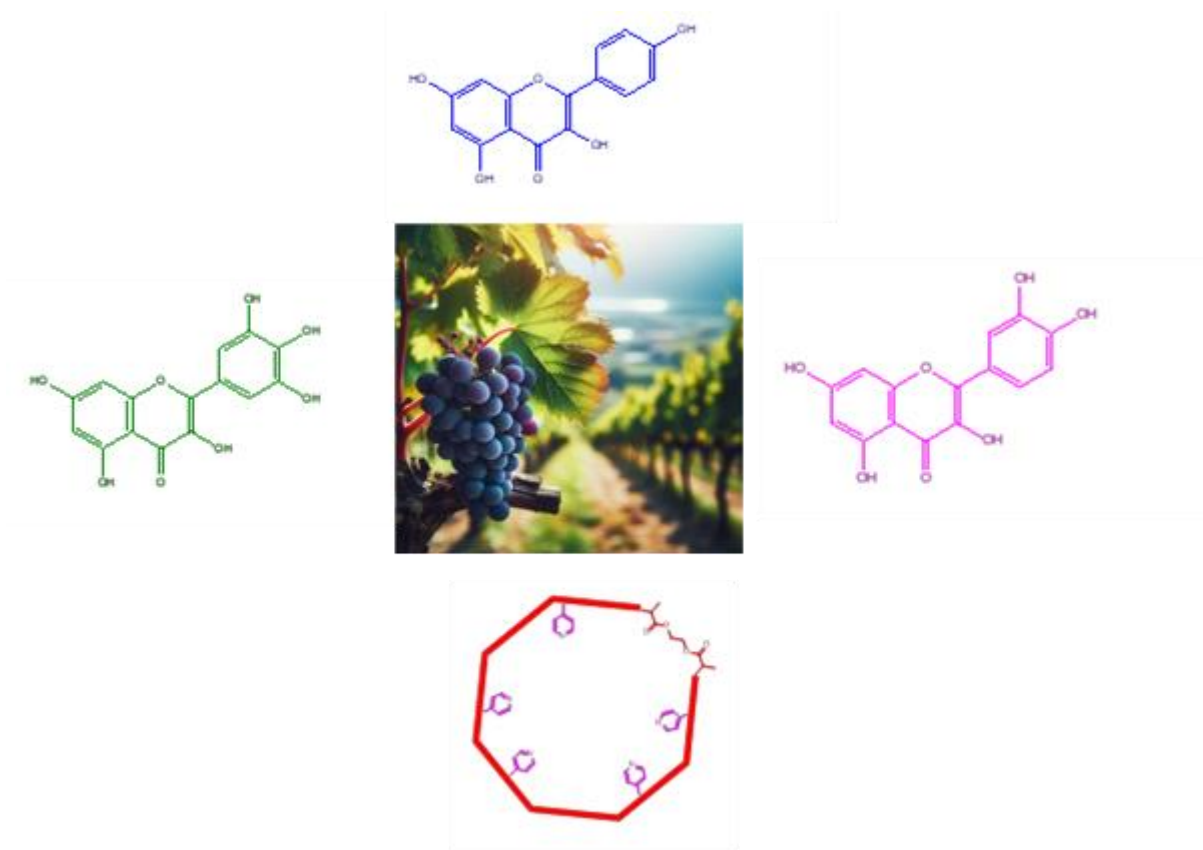
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**Chapter 5. From lab to scale-up: sorption processes with
Molecularly Imprinted adsorbents for bioactive enrichment of
winemaking sub-products**



The enrichment of winemaking residues was addressed by sorption-desorption using an adsorbent with molecular imprinting architecture. The process was scaled up in a prototype working with hydroalcoholic solvents. The purified products obtained showed improved bioactivity due to the presence of many flavonols.

Scale-Up of a Sorption Process Working with Molecularly Imprinted Adsorbents for Enrichment of Winemaking Residues and Improvement of Bioactivity

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Abstract

This work presents the scale-up of a sorption process for the fractionation and enrichment of bioactive compounds in winemaking residues using molecularly imprinted adsorbents. The process works with hydroalcoholic solvents and the improvement of the bioactivity of the produced fractions, comparatively to the raw extracts, is demonstrated. The proposed approach was experimentally validated through the designing and running of a pilot size sorption prototype for the automation of the fractionation method. The synthesis of the molecularly imprinted adsorbents was made at the gram-scale and spent diatomaceous earth, used for wine filtration, was considered as a possible source of bioactive compounds in winemaking residues.

The different fractions produced were evaluated for their antioxidant activity through three different assays, namely radical scavenging activity, reducing power and inhibition of lipid peroxidation. The results obtained show the improvement of the bioactivity of most of the fractions comparatively to the original diatomaceous earth extract. The most enriched fraction is estimated to have a total phenolic content c.a. 3.8 times higher than the original extract. The radical scavenging activity, the reducing power and the inhibition of lipid peroxidation for this fraction were measured to be 6.4, 4.2 and 4.5 times higher, respectively, than the initial diatomaceous earth extract.

This work provides new insights on biomass valorisation and circular bioeconomy by combining in the same research materials development, process design and application to real extracts with proved improvement of the bioactivity of purified products.

Keywords: Diatomaceous Earth Residues, Molecular Imprinting, Phenolic compounds, Fractionation, Antioxidant Activity, Sustainability, Circular Bioeconomy.

5.1. Introduction

Nowadays, the potential reuse and valorization of residues/by-products from agri-food industries has gained prominence as it contributes to the bio-circular economy [1].

The winemaking industry is one of the sectors that generates a significant number of by-products, with grape pomace, which includes mostly grape skins and seeds, being the major and most known. Nevertheless, other undervalued residues like wine lees (sediment consisting of yeast, grape solids, and other substances) and diatomaceous earth (used as a filtration agent) are also produced. These different winemaking residues have been reported to contain relevant amounts of added-value bioactive compounds, mainly polyphenols, with high market potential to be used by food, chemical, nutraceutical, cosmetic and pharmaceutical industries [2,3]. These compounds are well-known for their potent antioxidant properties with several showing additional activities such as antimicrobial and anti-inflammatory. Their bioactivity may vary with the molecules configuration and the position and number of hydroxyl groups [4], but mainly stems from their ability to scavenge free radicals and reactive oxygen/nitrogen species (ROS/RNS), reduce oxidized intermediates, induce metal chelation, and inhibiting enzymes [5]. By showing these beneficial properties, they have been positively associated to the prevention of certain diseases, including cancer, diabetes, cardiovascular issues, and degenerative disorders [6,7]

Within polyphenols, flavonoids are particularly significant and are further categorized into various classes based on their chemical structure [7]. Extracts from winemaking residues, especially grape pomace (GP) and diatomaceous earth (DE), have been shown to be rich sources of these polyphenolic compounds, offering various health benefits, as aforementioned. Moreover, numerous studies have further investigated the use of the natural extracts obtained from these residues to replace synthetic additives in various industries such as pharmaceutical, cosmetic, and food [6], [8–11]. To this aim, El-Zainy et al. [12] extended the shelf-life of raw beef sausages by adding grape seed polyphenols, offering a healthier alternative to synthetic additives. Ferreira et al. [13] explored replacing synthetic antioxidants with grape pomace extracts in cosmetics, and Maluf et al. [11] demonstrated the efficacy and safety of grape pomace as an antioxidant in cosmetic formulations. Recently the potential cosmeceutical activity of extracts obtained from diatomaceous earth has been described by Duarte et al. (2023)[3].

These studies underscore the potential of substituting synthetic additives with natural extracts from agricultural waste, with the dual goal of promoting consumer preference for natural products and aligning with the principles of a bio-circular economy.

Overall, the interesting properties exhibited by polyphenols have ignited a growing interest in their application across the food, pharmaceutical, and cosmetic industries, thus contributing to the rise of their market value. According to the Grand View Research in 2022, the global market size for polyphenols was estimated at 1.6 billion US dollars, with an expected compound annual growth rate of 7.4% between 2022 and 2030 [14]. The market value of phenolic compounds experiences a substantial increase with the purity of the product. To achieve this, among various techniques, sorption and desorption processes are employed, making use of efficient and selective adsorbents. These processes are utilized to enrich, concentrate, or even purify phenolic compounds present in agricultural waste [15]. An extensive overview of various adsorbents that find practical applications in the valorization of agro-industrial residues, particularly in the concentration of polyphenols has been reviewed by [16]. Indeed, sorption/desorption processes are an attractive possibility for complex extract handling because of the inherent lower energy consumption, cyclability, preservation of temperature-sensitive compounds, operational flexibility, amenability to intensification, and industrial scalability. The successful use of sorption and desorption processes related to the valorization of winemaking residues has also been reported but is dependent on the availability of suitable adsorbents [17,18]. One possibility for working with such kinds of biomass is the consideration of Molecularly Imprinted Polymer (MIP) sorbents, which are synthesized using the molecular imprinting technique. Often, MIPs make use of synthetic monomers with the capability to grant to the final adsorbent the capability to recognize and bind to specific molecular structures. This selectivity arises from the distinct cavities integrated into the polymeric network of the material, which are formed during the polymerization process. Our research group has already demonstrated the potential application of MIP adsorbents in continuous processes for valorizing polyphenolic compounds from agricultural residues [19–26]. Specifically, MIPs containing pyridyl moieties have been proposed because of their remarkable efficacy in adsorbing phenolic contaminants, outperforming other materials like styrene-DVB copolymers [27]. The strong binding between the hydroxyl groups and pyridyl groups makes them highly effective for retaining phenolic compounds [19–26].

The objective of the present work is to scale up the synthesis of a pyridyl-based molecular imprinted polymer (using quercetin as template) considering an inverse suspension

polymerization towards its use as an adsorbent at a pre-industrial scale, and also the designing and built-up of a sorption/desorption process prototype to work with the packed MIP particles. This approach will ultimately lead to the production of enriched polyphenol fractions from an initial hydroethanolic extract, obtained with winemaking residues as a potential source to be valorized, namely the diatomaceous earth used to filtrate the wine, which is a less explored residue of this huge industry.

5.2. Materials and Methods

5.2.1 Winemaking by-products

The winemaking by-products were provided by Caves Campelo S.A. (Portugal) during the harvest and production season in September 2022. The samples of diatomaceous earth were arranged on separated trays and dried in an oven (Scientific, Series 9000, Scientific Engineering, Johannesburg, South Africa) at 40 °C for 3 days, and then ground into powder in a mill (IKA, A11 basic) to pass a sieve of 20 mesh. The samples were stored at 20 °C inside a container and protected from light.

5.2.2. Extraction method

Phenolic compounds were extracted using ethanol/water 80:20 (v/v). Fifteen grams of diatomaceous earth was extracted with 100 mL of solvent by sonication (SONO SWISS, sw1) during 5 min followed by stirring (Lbx instruments, S03 series) for 1 hour protected from light at room temperature. The samples were centrifuged (Eppendorf, Centrifuge 5810 R) at $7871 \times g$ for 5 min, and the pellet was re-extracted. After collecting the supernatants, they were gathered and the ethanol (Fisher Scientific, Loughborough, UK) was evaporated under vacuum in a rotary evaporator (Julabo, TW12, Julabo Labortechnik, Seelbach, Germany) at 40 °C. The solution obtained for the diatomaceous earth extract was freeze dried at -20 °C and submitted to lyophilisation. The same extraction procedure was follow considering a Red Grape Pomace (GP) as polyphenols source.

5.2.3 Characterization of phenolic compounds by HPLC-DAD

Samples were analysed using a high-performance liquid chromatography (HPLC) system, KNAUER, comprised of a binary pump (P6.1L) with a degasser, an autosampler (6.1L), a column thermostat (CT2.1) operating at 45 °C, and a diode array detector (6.1L). For chromatographic analysis, an Ascentis C18 (Supelco) packed column with 5 µm particle size and dimensions of 25 cm × 4.6 mm was employed. The HPLC analyses were performed using the following solvent-gradient method: starting with 100% solvent A, which consisted of H₂O (pH=3)/acetonitrile (ACN) in a ratio 90/10 (v/v), and ending with 100% solvent B, composed of H₂O (pH=3)/ACN in a ratio of 10/90 (v/v). The pH of water was adjusted with acetic acid. The gradient from solvent A to solvent B took 45 minutes, with a flow rate of 1 mL/min. The ClarityChrom software was used to control the system and process the obtained data.

5.2.4. Molecularly Imprinted Adsorbent

5.2.4.1 Reagents

Quercetin (hydrate, purity 95%) was supplied by Acros Organics and was used as template in the synthesis of molecularly imprinted polymers. The functional monomer 4-vinylpyridine (4VP, 95% purity) was provided by Alfa Aesar. The crosslinker ethylene glycol dimethacrylate (EGDMA, 98% purity) and the initiator 2,2'-azobis(2-methylpropionitrile) (AIBN, 98% purity) were supplied from Sigma-Aldrich. The surfactant Span 80 (sorbitan oleate) and the *n*-heptane were provided from Sigma-Aldrich. The solvent dimethylformamide (DMF, 99% extra pure) was purchased from Acros Organics, acetonitrile (ACN, 99.8% HPLC grade), ethanol (99.8% purity, analytic grade), acetic acid glacial (AcOH, ≥ 99.7% analytical reagent grade) and methanol (99.8% purity, HPLC grade) were provided from Fisher Chemical. The used water was ultrapure grade and supplied locally.

5.2.4.2 Synthesis of Quercetin-Molecularly Imprinted Sorbent at gram-scale

The quercetin-molecularly imprinted sorbent was obtained by inverse suspension polymerization following a similar procedure as described previously [20, 21]. *n*-Heptane was chosen for the continuous phase in the inverse suspension polymerization, with span

80 as stabilizer. Firstly, Span 80 was dispersed in *n*-heptane under vigorous stirring at room temperature. In parallel, the chosen template, quercetin, was mixed with the selected functional monomer (4VP) and the solvent ACN/DMF in a ratio 85/15 (v/v) until the formation of a homogeneous solution. During this step, the interaction between the template and the functional monomer (T-FM) was promoted for 30 minutes in an ultrasound bath. Subsequently, the remaining reagents, including the crosslinker (EGDMA) and the initiator (AIBN) were dissolved in the solution and added to the continuous phase. The final solution was transferred to a Parr 5100 pressurized glass reactor with maximum capacity of 1 L. The reactor was pressurized with argon at 2 bar and the temperature set-point was 60 °C. A total polymerization volume of 600 mL was considered. The reactor is equipped with a magnetic drive internal stirrer including double turbine type six-blade impellers at 45° angle. The polymerization was carried out with a stirring speed of 400 rpm. Other details on the synthesis of related 4-vinylpyridine adsorbents can be found in [20-26, 38-39].

5.2.5 Designing and Running of a Pilot Size Sorption/Desorption Prototype

Sorption/desorption experiments were conducted using a continuous process prototype designed to perform the efficient recovery of the high value products from winemaking residues. Initially, the experiments involved packing the synthesized adsorbent particles (via a slurry process) into an empty column (approximately 17 g of adsorbent in a column with dimensions $L \times D = 250 \text{ mm} \times 20 \text{ mm}$). Subsequently, the packed column was placed in the continuous process prototype. The column was first cleaned and conditioned using the same solvent employed during the loading process. Subsequently, the chosen winemaking residue extract was percolated through the column, followed by the necessary steps for recovery and cleaning.

The prototype was designed to simulate sorption/desorption processes at an industrial scale. It included tanks for storing the hydroalcoholic extracts obtained from the winemaking residues such as diatomaceous earth and grape pomace extracts. These extracts were added to the tank to initiate the sorption process. From the feeding tank the extracts were filtered through a material with a sufficiently low pore size (e.g., in this case 1 μm) to remove particles that could obstruct the adsorption columns. After this, the stream was pumped into the core of separation processes, which consisted of a column packed with the molecularly imprinted sorbent. Four-way valves were employed to pipe

the extract and the desorption solvents to the column during the sorption/desorption cycles. The temperature of the sorption/desorption columns was controlled within a typical operating range of 15-25 °C for sorption and 45-60 °C for desorption processes. At the outlet, another four-way valve drives the eluent through a UV-vis sensor, whose response was considered to monitor the process. The prototype also includes a binary pump to produce the change of the solvent composition along the desorption process. Two reservoirs, each containing solvents with predefined reference compositions, were used to provide the necessary supply for this gradient pump. All parameters, including flow rate, temperature, valves positions, experiments execution and UV data acquisition were controlled using LabVIEW software. A schematic diagram of the continuous sorption/desorption prototype developed and used for these experiments is presented in **Figure 5-1**.

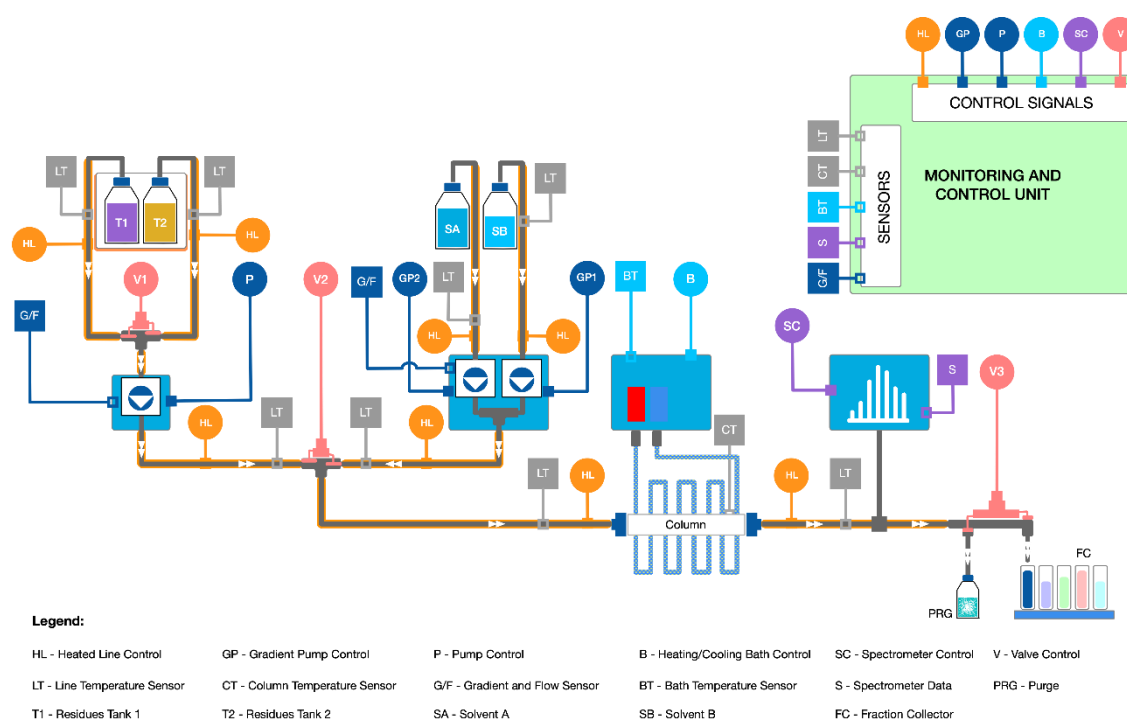


Figure 5-1: Schematic diagram of the pilot size sorption/desorption prototype used in this work.

5.2.6. Total phenolic content

The total phenolic content of the hydro-ethanolic extracts prepared from diatomaceous earth and of the corresponding obtained fractions was determined by spectrophotometry in a microplate reader (Epoch 2, Biotek), using the Folin-Ciocalteu reagent. Different concentrations (0.005; 0.01; 0.05; 0.1; 0.25 and 0.5 mg/mL) of an ethanolic solution of gallic acid, used as standard, were used to obtain the calibration curve. For the analysis,

the extracts and corresponding fractions were evaporated under vacuum (Stuart RE 300 rotary evaporator), freeze-dried and lyophilized. Then, the lyophilizates were redissolved in ethanol 80:20 to known concentrations, using serial dilutions (1:2) if necessary. In a test tube, 250 μ L of extract from each sample or standard were mixed with 1.25 mL of Folin-Ciocalteu reagent (1:10 v/v in water) and 1 mL of sodium carbonate (75 g/L). The tubes were vortexed for 15 seconds and placed to rest in the dark for 30 minutes at 40 °C for colour development. At the same time, a negative control using ethanol was prepared. After, the tubes were centrifuged at 12000 rpm for 2 minutes and the solutions from each test tube were then transferred to a 96 wells microplate with the help of a micropipette. Finally, the absorbance was measured at 765 nm in a microplate reader (Epoch 2, BioTek Instruments, USA). The results were expressed in mg of gallic acid equivalents per gram of extract (mg GAE/g extract). Similarly, an analogous assessment was conducted for the total phenolic content of the GP extract.

5.2.7. Antioxidant activity

Stock solutions with known concentration were prepared from the lyophilized extracts (DE extract, GP extract and the eluted fractions) by re-dissolution in ethanol/water (80:20, v/v). After serial dilution (1:10) of the stock solutions, the antioxidant activity was measured following three different assays, namely DPPH radical scavenging activity, reducing power and inhibition of lipid peroxidation in porcine brain tissue. Briefly, DPPH radical-scavenging activity was evaluated as previously described [3] using an Epoch 2 microplate reader (BioTek Instruments, USA) and calculated as a percentage of DPPH discoloration using the formula:

$$EC_{50} = \frac{A_{DPPH} - A_S}{A_{DPPH}} \times 100 \quad (1)$$

where A_S is the absorbance of sample solution, and A_{DPPH} is the absorbance of the DPPH solution at 515 nm. Reducing power evaluated the capacity to convert Fe^{3+} into Fe^{2+} , by measuring the absorbance at 690 nm as described by Silva et al. [28]. The lipid peroxidation inhibition was evaluated based in the formation of thiobarbituric acid reactive species (TBARS) in a suspension of lipid-rich porcine brain as described by Spréa et al. [37]. The absorbance was measured at 532 nm. The results of the in vitro assays were expressed as EC_{50} values, corresponding to the sample concentration providing 50% of antioxidant activity in the DPPH and TBARS assays, or 0.5 of absorbance in the reducing power assay. Trolox was used as positive control.

5.3. Results and Discussion

5.3.1 Rationale of MIP synthesis

When the synthesis of molecularly imprinted adsorbents is considered, it is meaningful a preliminary study on the interactions between the chosen template molecule and the functional monomer, as well as on the working conditions of the final material. Pyridine-based polymers are extensively used in various fields, serving as advanced materials with a broad range of applications encompassing wastewater and industrial effluent treatment, enzyme and protein adsorption, electronics biomedicine, and catalysis, among others [27, 29–31]. Considering that adsorbents containing pyridyl moieties have shown a strong affinity for phenolic compounds present in diverse plant extracts, they hold promising features for the valorisation of this class of bioactive compounds [19–26]. Particularly, the design of polymer networks capable of adsorbing a specific compound in a selective way and that can be applied in continuous processes is highly advantageous. In previous works [20–22], we successfully demonstrated the strong interaction between quercetin (the template molecule) and 4-vinylpyridine (4VP, the functional monomer) resulting in materials with molecular imprinting effect and improved selectivity. Therefore, these synthesis conditions were used in the present work to scale up the production and apply this type of adsorbents at a pre-industrial scale.

Quercetin is one of the flavonoids more abundant in plant matrices, it is composed by three aromatic rings, five hydroxyl groups and a carbonyl function. The predicted interaction of quercetin with 4VP is presented in **Figure 5-2**, where the strong hydrogen bonds formed between the pyridyl group of 4VP and the hydroxyl groups of quercetin are highlighted.

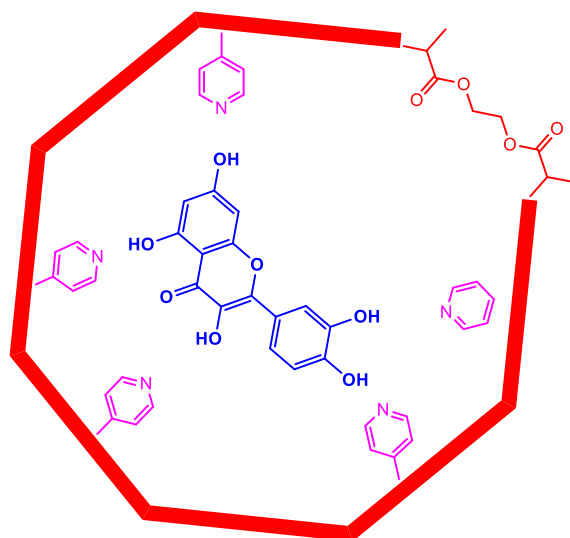


Figure 5-2: Plausible interactions between functional monomer (4VP) and the template (quercetin).

Considering that quercetin is relatively affordable and this aglycone and its derivatives are abundant in most plant matrices, including winemaking residues (as observed in this study), this molecule was selected as a template for the synthesis of the MIP adsorbent. Furthermore, quercetin can also be regarded as a kind of surrogate molecule for other flavonols, a practice often employed in the pharmaceutical industry with other chemicals. Our approach considers the structural similarities between quercetin and other target polyphenols for the introduction of a broader degree of specificity for flavonols into the designed adsorbents.

Nonetheless, the molecular imprinting effect is not only dependent on the interactions between template and functional monomer. Other parameters such as the solvent(s) used and system composition considered in the copolymerization also affect the molecular imprinting efficiency. For example, the use of protic solvents, such as water, can disrupt the hydrogen bonds between the functional monomer and the template. In contrast, aprotic solvents like acetonitrile (ACN) are commonly used to enhance hydrogen bond interactions.

Recently, our research group was devoted at finding improved synthesis conditions to valorise the bioactive compounds present in plant matrices. Different experiments, considering diverse templates and functional monomers were carried out [19–26] using extracts from different raw biomass such as onion skin, winemaking residues, olive leaves and almond hull/shell/skin. Often, the materials were synthesised at small scale (e.g. final quantity of produced material around 200 mg). The performance of materials was studied in solid phase extraction cartridges and in small columns with dimensions between

$L(\text{mm}) \times D(\text{mm}) = 10 \times 4.6$ and $L(\text{mm}) \times D(\text{mm}) = 100 \times 4.6$. In this work we proceeded to the scale up of the synthesis of the molecularly imprinted adsorbent with subsequent application in a pilot size sorption/desorption prototype.

When considering the final application of such kinds of materials as adsorbents into continuous process, the morphology of the particles becomes a critical parameter. Too small particles can lead to high pressures, rendering them unsuitable for use in continuous processes. Inverse suspension polymerization is a technique enabling the production of particles with a desirable morphology for efficient packing and utilization in continuous processes. However, it is important to note that the final morphology is also dependent on the specific synthesis conditions. **Table 5-1** summarizes the recipe employed to produce the material considered as adsorbent in this work. With these polymerization conditions, particles with size suitable for use as adsorbent in continuous processes working at high pressure were successfully obtained.

Table 5-1: Polymerization conditions used to synthesize the molecularly imprinted adsorbent used in this work.

Template	Functional Monomer	Solvent (v/v)	Y_M	Y_I	Y_{CL}	$Y_{FM/T}$	$Y_{C/D}$	Yield (%)
Quercetin	4VP	ACN/DMF (85/15)	30.43	3.97	51.48	15.69	3.62	~100

The polymerization was performed using the inverse suspension polymerization technique with the AIBN initiator and the EGDMA crosslinker, at 60 °C for 24 hours.

The parameters used in **Table 5-1** to describe the initial state of the polymerization system and are defined as follows:

- Y_M - mass fraction of functional monomer and crosslinker in the solution (%);
- Y_I - mole fraction of initiator relative to the FM and the crosslinker (%);
- Y_{CL} - mole fraction of the crosslinker in the mixture FMs + crosslinker (%);
- $Y_{FM/T}$ - mole ratio of the FM to the template molecule;
- $Y_{C/D}$ - mass ratio between the oil phase and the monomer phase in inverse suspension polymerization.

5.3.2 FTIR and SEM characterization

FTIR analysis provides key information on the formation of the molecularly imprinted sorbent, specifically regarding the incorporation of the functional monomer into the polymeric network. **Figure 5-3** presents the FTIR spectra obtained for the MIP

synthesized and used in this work. Taking into consideration the 4VP monomer, characteristic peaks at 1600 cm^{-1} corresponding to C=C pyridyl, 1418 cm^{-1} (C=N), 820 cm^{-1} and 755 cm^{-1} corresponding to the C-H bending in the pyridine ring, these assignments provide evidence of the successful incorporation of 4VP into the synthesized material. The peaks at 1720 cm^{-1} and 1130 cm^{-1} are correspondent to C=O and C-O, respectively, characteristic of EGDMA (crosslinker). The observation of these peaks also confirms the incorporation of the crosslinker in the final materials.

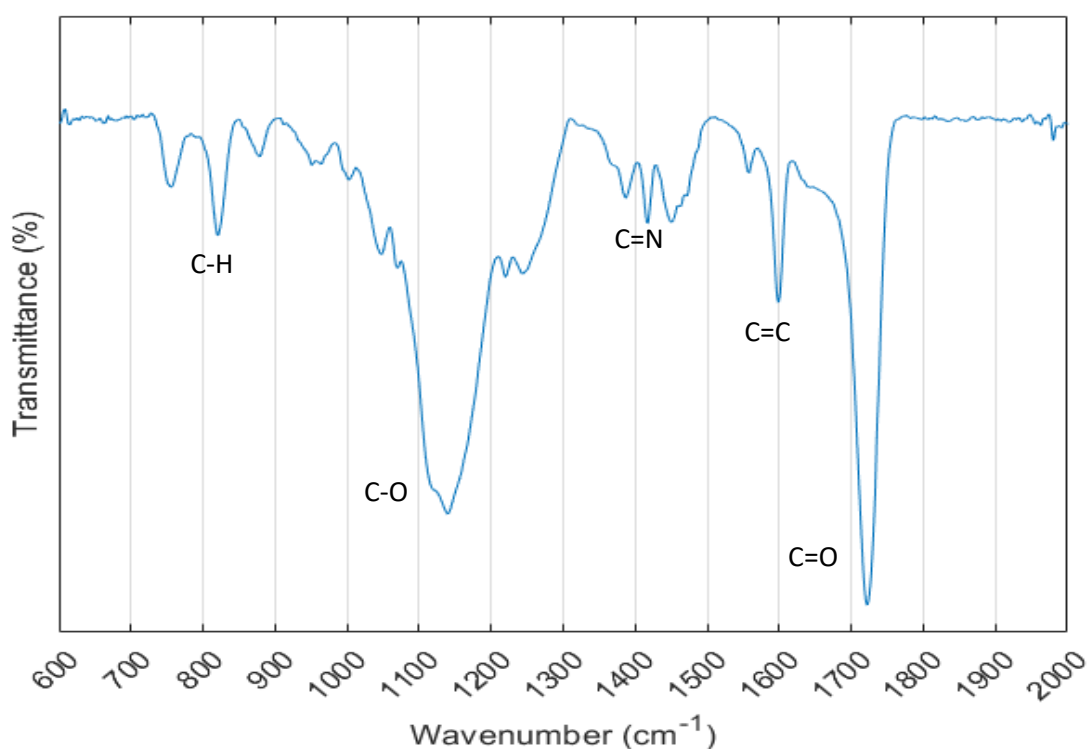


Figure 5-3: FTIR spectra collected for the molecularly imprinted material synthesized with the functional monomer 4VP, the crosslinker EGDMA and the template molecule quercetin.

Figure 5-4 shows the SEM micrographs for the synthesized MIP sorbent. Particles agglomerates with sizes in the rough range 5 to 100 μm were formed. It is worth to mention that the agglomeration observed is due to the selected inverse suspension polymerization technique involving the 4VP functional monomer, the EGDMA crosslinker and the ACN/DMF solvent in the dispersed phase. These aggregates connect particles with diameters $<1\text{ }\mu\text{m}$ (precipitated at early stages of the polymerization) in final particle assemblies that are suitable for application as packing materials for continuous sorption/desorption processes, as explored in this research. The too low size of polymer particles hindering their application due to the possible backpressure problem is circumvented through this synthesis approach. Actually, in addition to the reactant's concentrations and solvent composition, affecting particle agglomeration, other factors

such as the thermodynamics of phase separation and stirring conditions also change the size and morphology of the final particles. The synthesis method above described for the MIP particles attempts the addressing of these issues and look for the feasibility of the extension to the sorbent production at the gram-scale.

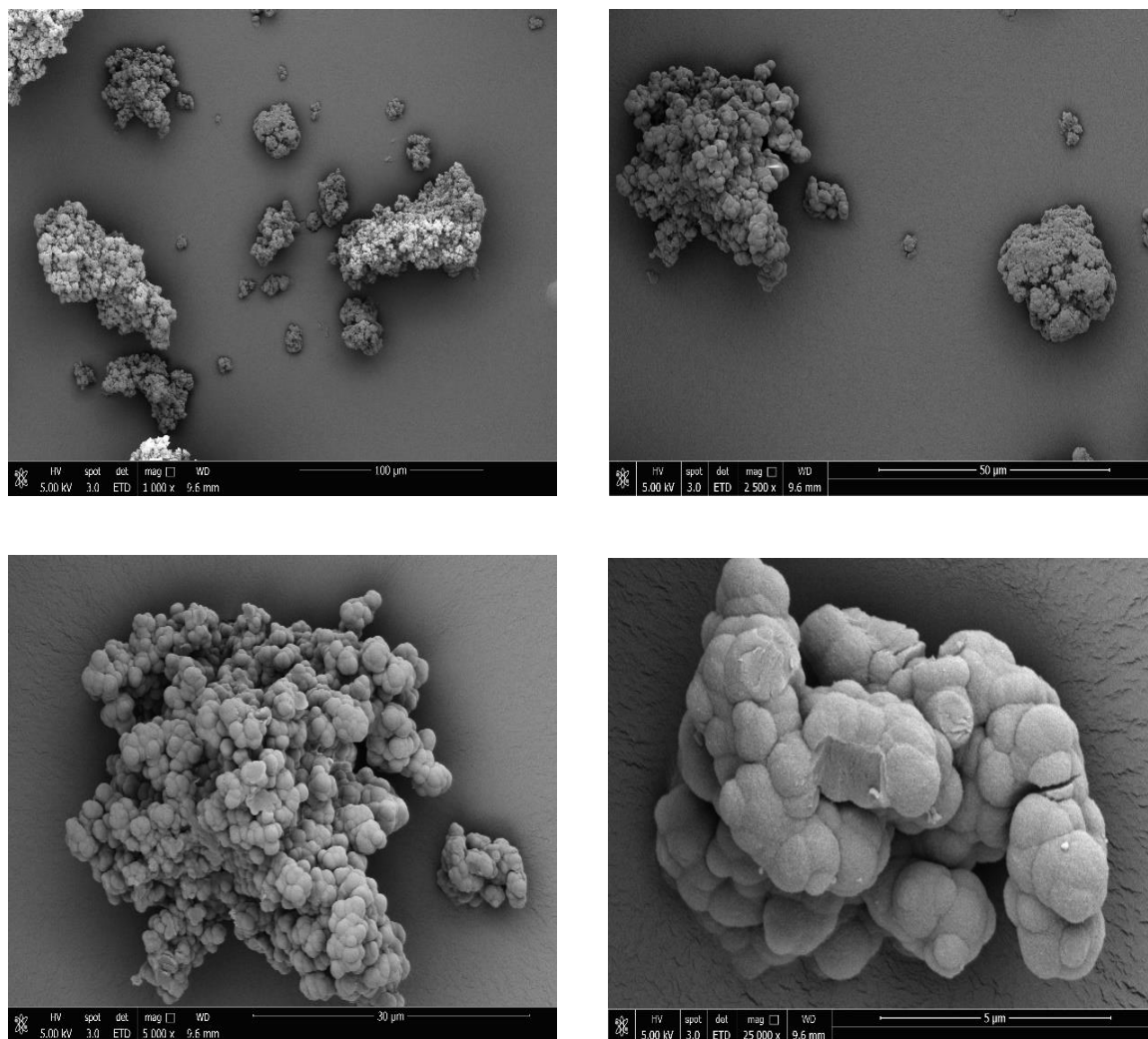


Figure 5-4: SEM images for the molecularly imprinted adsorbent synthesized in this work through an inverse-suspension approach.

5.3 Processing of the diatomaceous earth extract with the developed MIP sorbent

Recently, Duarte et al. [3] reported for the first time the phenolic composition of an hydroethanolic extract obtained from diatomaceous earth used for wine filtration. The study found that the diatomaceous earth extract is a promising and unexplored source of phenolic compounds, notably flavonols and anthocyanins. As expected, the extract in the current research also exhibited a complex mixture of these phenolic compounds. **Figure 5.5** illustrates both the 3D HPLC-DAD chromatogram and the corresponding 2D HPLC-DAD chromatogram measured at $\lambda=280$ nm for the DE extract used in this study. Tentative identification of the contained compounds was accomplished by comparison with the HPLC-DAD analysis for representative polyphenol standards. Considering the retention time and UV-vis spectrum, the peak eluting at 16.6 minutes was identified to be quercetin (peak 6 in **Figure 5(b)**). Moreover, based on data from previous works by our research group involving HPLC-DAD and LC-DAD-MS/MS analyses for the identification of compounds present in winemaking residues [3,23,38], some additional peaks in **Figure 5-5** were also identified. The outcomes for this tentative identification analysis are summarized in **Table 5-2**. Note that peak 5 in **Figure 5-5** is not associated with a polyphenol molecule but rather with another compound used during the vinification process [3,23,38]. Interestingly, the flavonol aglycones quercetin, myricetin and kaempferol as well as some correspondent glycosylated molecules are identified in the diatomaceous earth extract, indicating that a relevant amount of such bioactive compounds are lost during the wine filtration process.

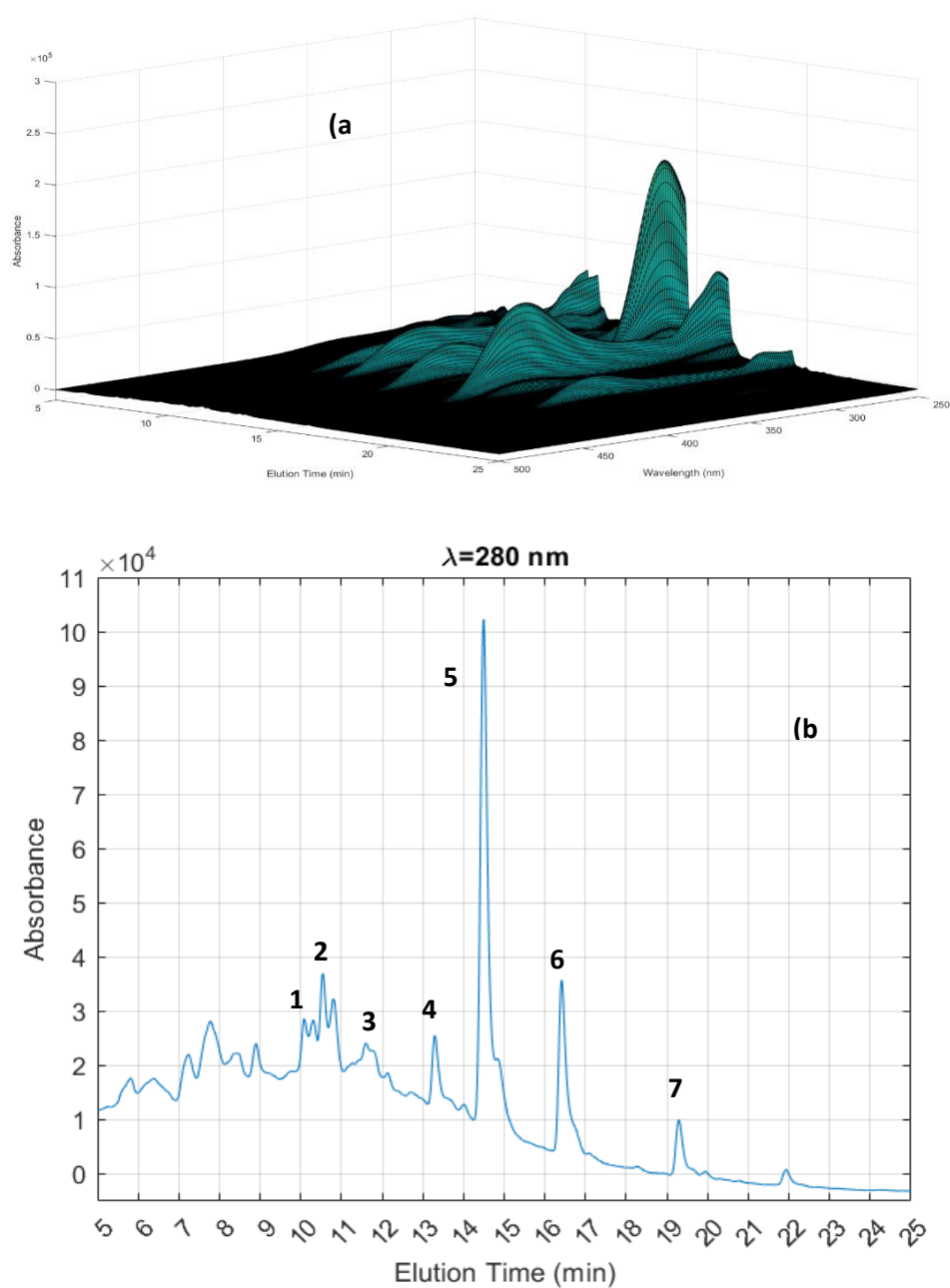


Figure 5-5: a) 3D HPLC-DAD chromatogram and correspondent b) 2D chromatogram measured at $\lambda=280$ nm for the diatomaceous earth extract processed with the developed molecularly imprinted polymer particles.

Table 5-2: Tentative identification for relevant phenolic compounds in the raw diatomaceous earth extract (see **Figure 5-5**).

Peak	Elution Time (min)	Tentative Identification
1	10.2	Myricetin- <i>O</i> -hexoside
2	10.7	Quercetin- <i>O</i> -hexuronoside
3	11.7	Malvidin-3- <i>O</i> -acetylglucoside
4	13.4	Myricetin
6	16.6	Quercetin
7	19.4	Kaempferol

The extract prepared from the DE residue was used with the prototype illustrated in **Figure 5-1** to highlight the efficacy of the synthesized molecularly imprinted adsorbent with the fractionation of the target classes of molecules. For that purpose, initially, the material was loaded with diatomaceous earth extract by circulating approximately 890 ml of the extract at $T=25\text{ }^{\circ}\text{C}$ with a flow rate of 2 mL/min, for 24 hours, considering a recycling mechanism. The solvent considered was a mixture of ethanol and water in an 80/20 (v/v) ratio and extract concentration of 3.75 mg/mL. The adsorption process was monitored by collecting fractions at specific intervals, which were subsequently analyzed in a UV-vis spectrophotometer. **Figure 5-6** shows the UV spectra from the collected fractions during the loading process and the normalized breakthrough curve measured at $\lambda=280\text{ nm}$. Since the prototype was used with a recycling mechanism, it is crucial to note that the breakthrough curve may be influenced by the competitive adsorption phenomenon, which involves various compounds present in the DE extract and the availability of binding sites.

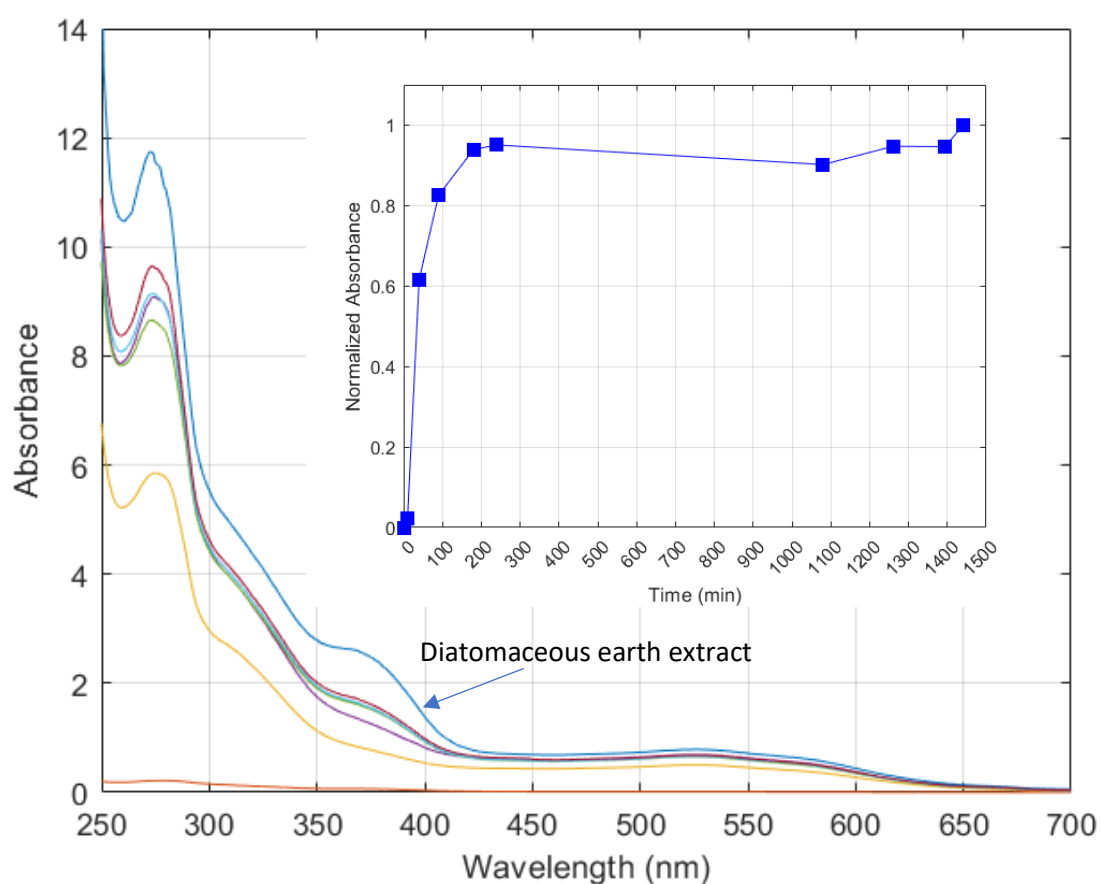


Figure 5-6: UV-vis spectra for the samples collected during the saturation process of the molecularly imprinted adsorbent with a diatomaceous earth extract in ethanol/water 80/20 (v/v) at concentration of 3.75 mg/mL. Column outlet was sampled at different time instants for quantification of the dynamics of adsorption. The inset in the plot shows the normalized breakthrough curve measured at $\lambda=280$ nm.

The driving force for a possible separation of different polyphenols in plant extracts by sorption/desorption is quantified by the particular isotherms describing the change of the adsorbed amounts with concentration for a specific sorbent and working solvent. These features were recently demonstrated with standard polyphenols and using an adsorbent similar to that here considered, with practical application to the purification of olive leaf extracts [39]. However, when addressing real plant extracts, the determination of the isotherms quantifying these concentration effects for all compounds is not practical due to the plethora of chemicals there contained. Therefore, for productivity improvement and simplicity of the sorption/desorption process, working with an as higher as possible extract concentration is advantageous. The upper limiting value is defined by the global solubility of the extract in the selected solvent if precipitation of less soluble compounds is not desired. A key feature of the 4-vinylpyridine sorbents here explored, namely in comparison with common commercial alternatives, is the possibility to work with

hydroalcoholic mixtures with high alcoholic content and having also a high retention capability, as demonstrated before [20-26,38-39]. Thus, in previous works, the running of adsorption processes with different winemaking residues extracts concentrations as higher as 1 mg/mL in EtOH/water 80/20, 1.1 mg/mL in EtOH/water 54/46 and 3.75 mg/mL in EtOH/water 80/20 were demonstrated to be possible [38]. The same is observed in the present work with the consideration of an extract concentration of 3.75 mg/mL in EtOH/water 80/20, as highlighted in **Figure 5-6**.

After the extract loading step in the MIP particles, the fractionation (elution) process was carried out at T=45 °C. The subsequent stage involved the use of eight distinct elution solvents, which included pure water, mixtures of ethanol/water, pure ethanol, pure methanol (MeOH) and MeOH/AcOH (90/10 v/v). **Table 5-5** presents the solvents used and the corresponding compositions for each elution fraction. By changing the eluting solvent, it became possible to obtain fractions with a simplified composition compared to the initial DE extract. **Figure 5-7** shows the HPLC-DAD 2D chromatogram of the diatomaceous earth extract in ethanol/water 80/20, used with the load of the MIP particles, and the corresponding fractions collected after elution with the different solvents described in **Table 5-3**. Measurements at 280, 360 and 550 nm are presented to emphasize the fractionation and consequent purification/concentration of different families of flavonoids (such as flavonols and anthocyanins).

Table 5-3: Solvent and their compositions used in each elution fraction.

Fractions	Solvent	Composition (v/v)
F1	Water	100
F2	Water	100
F3	Ethanol/water	20/80
F4	Ethanol/water	40/60
F5	Ethanol/water	60/40
F6	Ethanol/water	80/20
F7	Ethanol	100
F8	MeOH	100
F9	MeOH/AcOH	90/10

A more comprehensive analysis of selected fractions collected during this desorption process is illustrated in **Figure5-8**, featuring HPLC-DAD-3D chromatograms. These results underscore the efficacy of the synthesized MIP adsorbent for fractionate and concentrate compounds based on their distinct binding strengths. One significant

outcome, also observed in **Figure 5-7**, is the removal of the non-polyphenolic compound (peak 5) when increasing the percentage of organic solvents (ethanol and methanol). Indeed, fractions F8 and F9 do not contain this peak.

Another important result in **Figures 5-7 and 5-8** is the production of fractions (F7, F8, and F9) that are highly enriched with flavonols, specifically quercetin (peak 6) and kaempferol (peak 7). The fractionation of one identified anthocyanin (peak 3) from the flavonols group, specifically quercetin-O-hexuronoside, quercetin, and kaempferol, is also achievable in some fractions such as F3, F4, and F5.

Based on the 2D HPLC-DAD chromatograms illustrated in **Figure 5-7**, was performed the estimation of the enrichment factors (EF) for the fractions generated during the sorption/desorption process. **Figure 5-9** displays these outcomes considering the flavonols as reference compounds and the enrichment achieved for such kind of polyphenols in some fractions is evidenced (e.g. $EF > 2$ for F7 and F8). It is worth to mention that the enrichment factor for these fractions has the potential for further enhancement by implementing an alternative solvent gradient during the elution step. This approach would entail using a specific solvent composition until all the compounds are fully extracted, potentially leading to higher levels of enrichment.

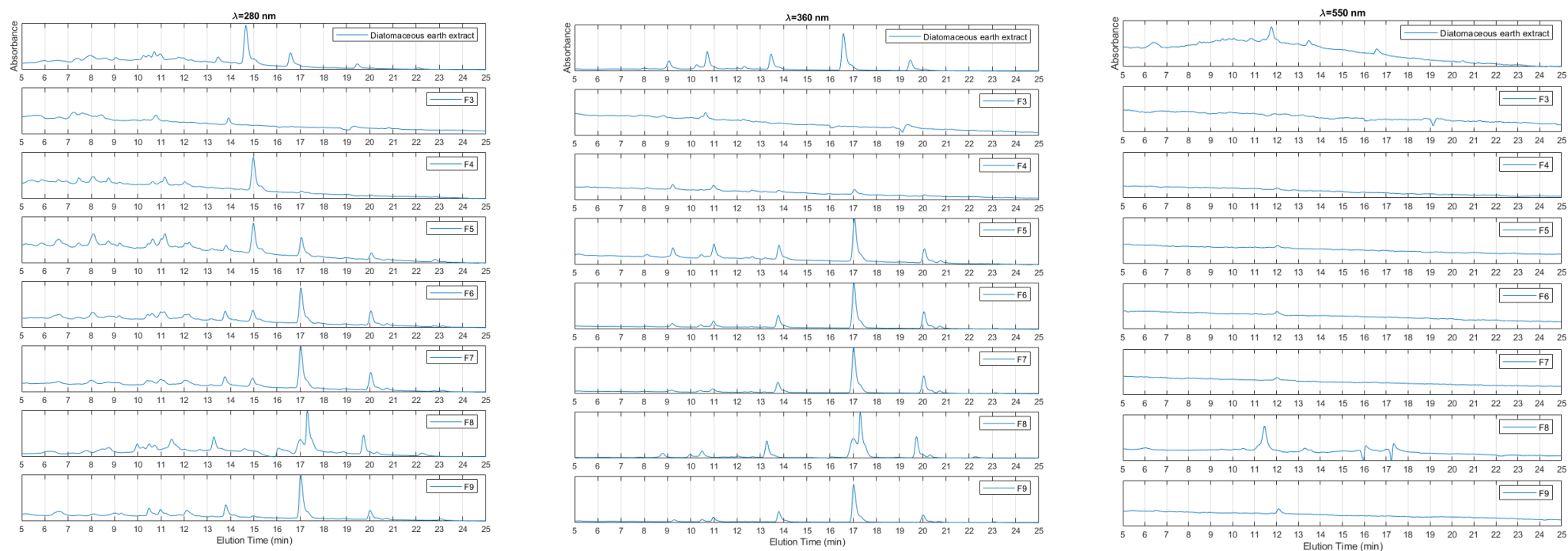


Figure 5-7: 2D HPLC-DAD chromatograms for the initial DE extract and for different fractions collected during the desorption process of the compounds retained in the molecularly imprinted adsorbent. Previous loading step was performed with a DE extract in ethanol/water 80/20 (v/v) at a concentration of 3.75 mg/mL. Measurements at 280, 360 and 550 nm are here presented to highlight the fractionation of different families of flavonoids (flavonols and anthocyanins).

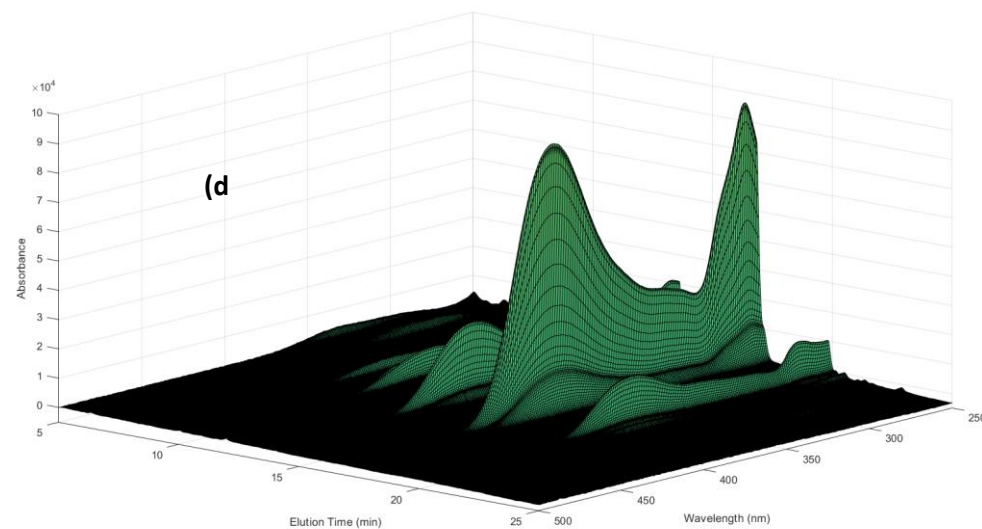
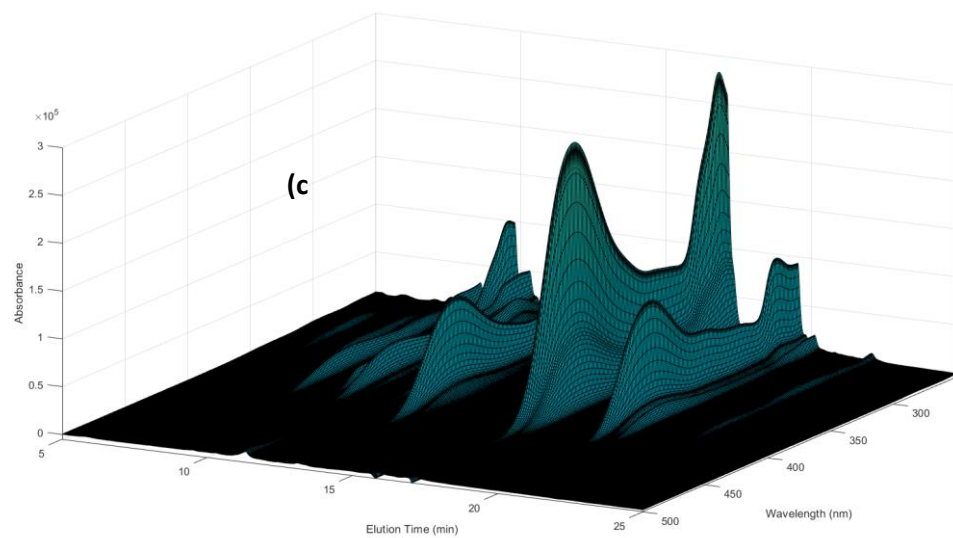
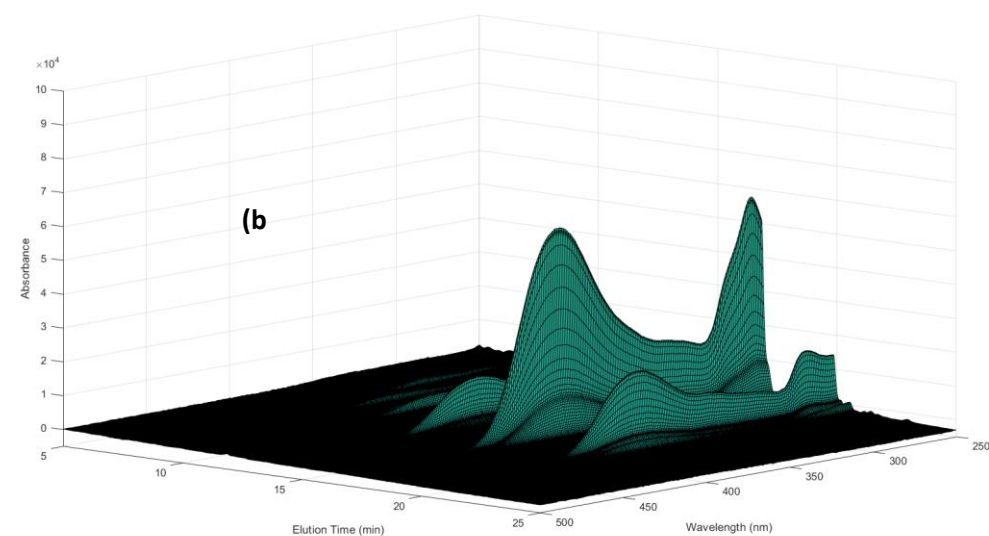
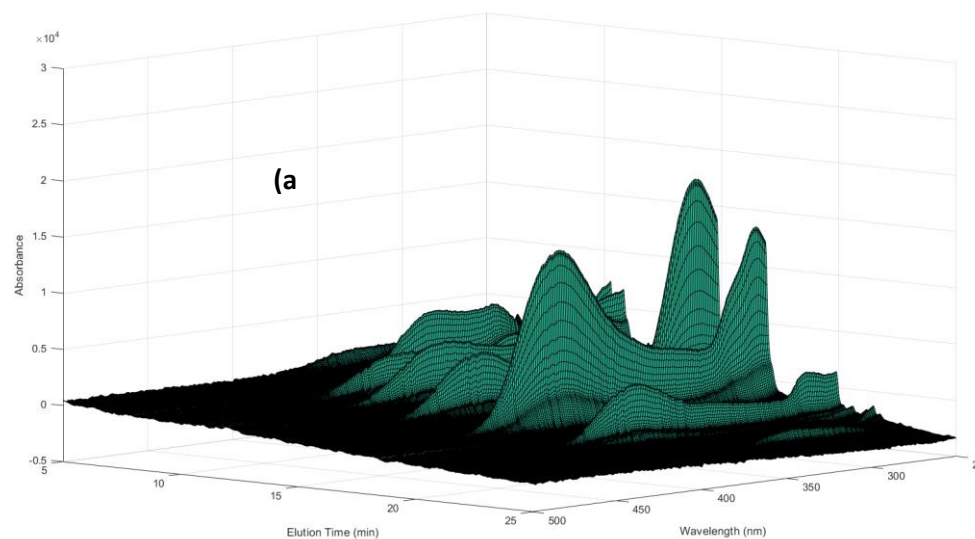


Figure 5-8: 3D HPLC-DAD chromatograms for different fractions collected during the desorption of the molecularly imprinted adsorbent. Previous loading step was performed with a DE extract in ethanol/water 80/20 (v/v) at a concentration of 3.75 mg/mL. a) fraction recovered in ethanol/water 60/40 (F5) b) fraction recovered in ethanol (F7) c) fraction recovered in methanol (F8) d) fraction recovered in MeOH/AcOH 90/10 (F9).

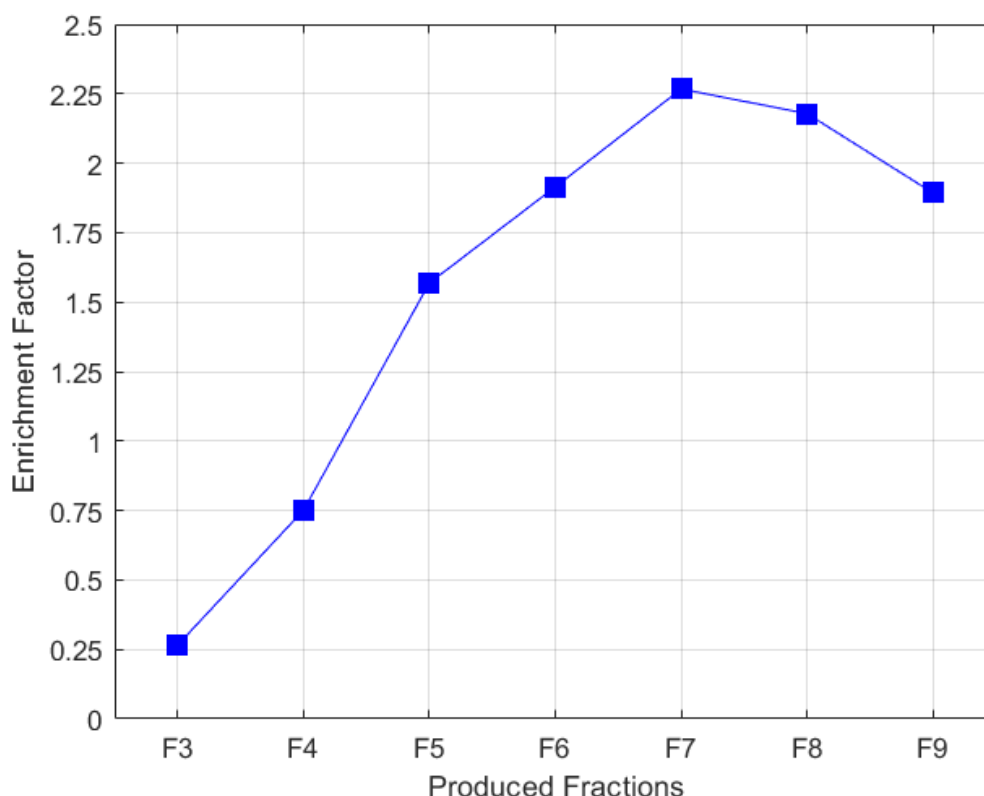


Figure 5-9: Enrichment factors calculated from the HPLC-DAD chromatogram areas of the fractions obtained following the sorption/desorption processes with the diatomaceous earth extract. These calculations were performed considering the flavonol compounds as reference.

5.3.4 Total phenolic content (TPC) of the purified fractions

Numerous studies have focused on the phenolic composition of grape pomace and other residues from the winemaking production, either by determining their overall content in phenolic compounds or by identifying and quantifying specific individual compounds [32–34]. However, when it comes to the utilization of diatomaceous earth extracts, limited scientific literature is available [3]. Although chromatographic analysis provides precise information on individual compounds, the determination of total phenolic content (TPC) by the Folin-Ciocalteu reaction has great potential to be used as a screening assay by the winemaking industry. By estimating the TPC in the initial diatomaceous earth extracts and fractions, this simple assay would easily allow to select the most promising purified products. The TPC determined for the initial DE extract and for selected fractions obtained with the MIP particles are shown in **Figure 5-10**.

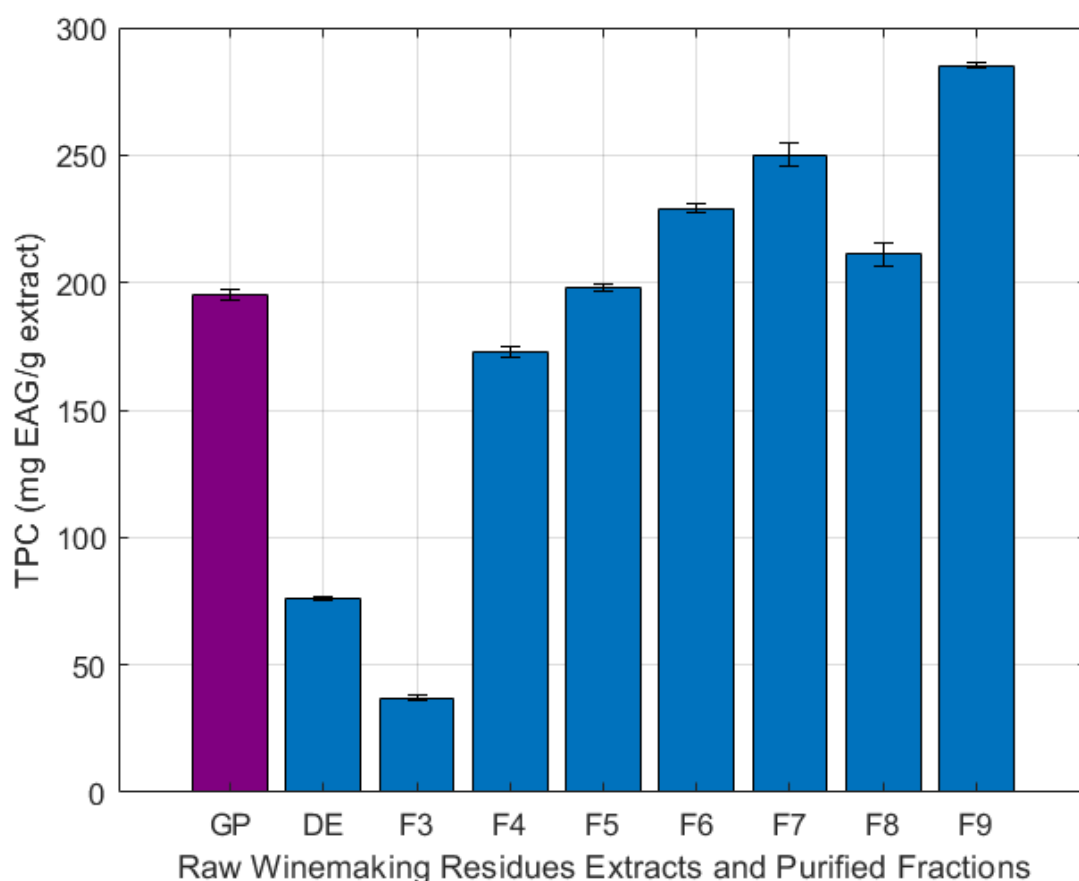


Figure 5-10: Total phenolic content (TPC, expressed in mg GAE/g extract) for a GP extract, a DE extract and for the fractions purified through the processing of the GP extract by sorption/desorption with the MIP particles.

The TPC value determined for the initial DE extract (75.58 ± 1.0 mg GAE/g of extract) is lower than that previously reported by Duarte et al. [3] (126.92 ± 0.9 mg EAG/g extract). In this case, it is noteworthy to highlight that, following the sorption/desorption experiments with the developed MIP, except for fraction F3, all obtained fractions exhibited significantly higher TPC values as compared to the initial extract, demonstrating the effectiveness of the molecularly imprinted adsorbent in producing enriched fractions with higher concentrations of total phenolic compounds (see **Figure 5-10**). Furthermore, drawing an analogy with the TPC of fractions obtained from the DE extract and a raw GP extract, known for its notable TPC values [3] and their direct application in some industries [12,13,35], it becomes apparent that the TPC values of the fractions from the DE extract consistently exceed those from the GP extract. This highlights the significance of the valorisation achieved with the proposed sorption/desorption process.

5.3.5. Antioxidant activity of the purified fractions

Numerous pathologies, degenerative processes, and health conditions, including diabetes, arterial hypertension, aging, and cancer are intricately linked to oxidative stress. Oxidative stress induces significant cellular damage due to the elevated production of reactive oxygen species (ROS) that target vital macromolecules such as proteins, DNA and lipids. The antioxidant potency of phenolic compounds is related to their reducing properties, as they function as hydrogen or electron donors. This characteristic predicts their potential as free-radical scavengers, essentially acting as antioxidants. Additionally, phenolic compounds exhibit the capacity for metal chelation, particularly with iron and copper ions, effectively suppressing the formation of free radicals catalysed by these metals. Numerous methods have been devoted to assess antioxidant properties, with commonly used assays, including DPPH scavenging activity, reducing power, and TBARS inhibition. The results obtained by applying these assays for the DE extract, a GP extract and for selected fractions obtained after sorption/desorption processes are shown in **Table 5-4** and **Figure 5-11**.

The EC₅₀ values for the DE extract in various assays were determined as follows: 0.373 ± 0.008 mg/mL in the DPPH assay, 0.250 ± 0.004 mg/mL in the reducing power assay, and 0.036 ± 0.0007 mg/mL in TBARS inhibition. It is important to note that the DE extract has not been extensively studied in terms of its phenolic composition or antioxidant activity, making direct comparisons with reported results challenging. However, when considering the findings published by Cristina et al. [3] for the same extract, obtained under identical conditions to ours, we observe lower EC₅₀ values, specifically 0.17 ± 0.01 mg/ml in the DPPH assay.

In evaluating the outcomes of the sorption/desorption processes using the MIP particles as adsorbent, it is crucial to emphasize the noteworthy findings. It was observed that all fractions, except for F3, exhibited superior results, indicated by lower EC₅₀ values, signifying heightened antioxidant potency compared to the initial DE extract (see **Table 5-4** and **Figure 5-11**).

Table 5-4: Antioxidant activity of DE extract and of selected fractions obtained after the sorption /desorption process with the molecularly imprinted particles

Extract/Fractions	Antioxidant activity EC ₅₀ values (mg/mL)		
	DPPH scavenging activity	Reducing Power	TBARS inhibition
GP extract	0.142±0.002	0.134±0.006	0.008±0.0002
DE extract	0.373±0.008	0.250±0.004	0.036±0.0007
F3	0.310±0.010	0.693±0.015	0.418±0.0050
F4	0.286±0.009	0.164±0.004	0.017±0.0002
F5	0.212±0.016	0.120±0.002	0.014±0.0008
F6	0.302±0.014	0.110±0.002	0.006±0.0009
F7	0.077±0.006	0.060±0.004	0.009±0.0005
F8	0.108±0.008	0.060±0.008	0.017±0.0014
F9	0.058±0.009	0.060±0.003	0.008±0.0004

EC₅₀ values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. Trolox EC₅₀ values: 0.0536 mg/mL (DDPH), 0.0451 mg/mL (reducing power), and 0.00251 mg/mL (TBARS)

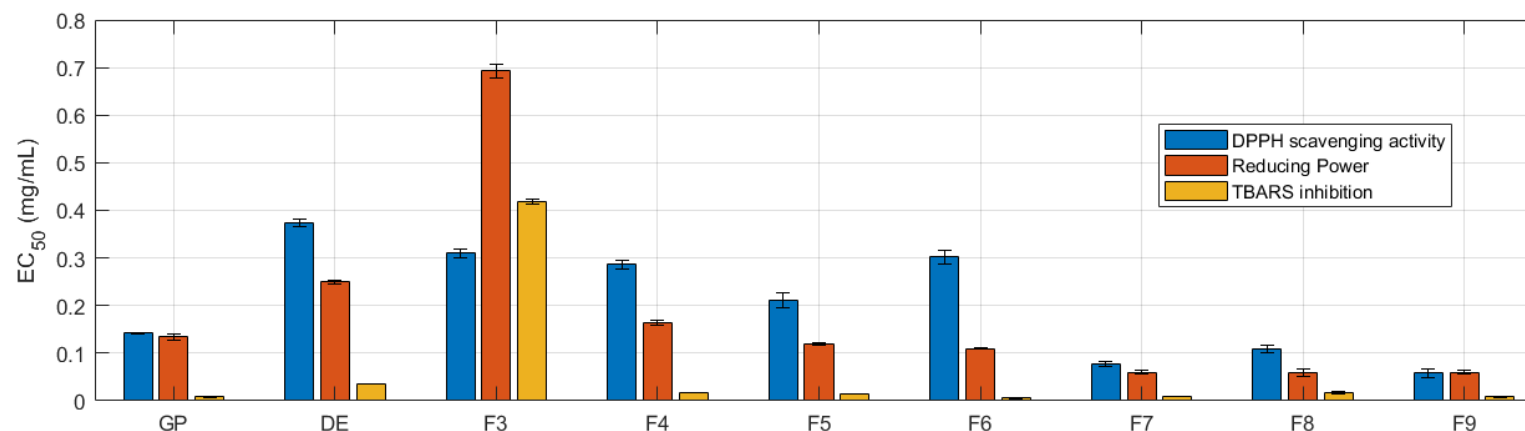


Figure 5-11 Antioxidant activity for a GP extract, a DE extract and fractions obtained by sorption/desorption of the DE extract with the molecularly imprinted particles.

A plausible interpretation of these findings is rooted in **Figures 5-9 and 5-10**, depicting the estimated enrichment factors for the fractions obtained after treatment with the MIP sorbent. From that data, a distinct trend emerges as the organic solvent content increases, because there is a noticeable enhancement in the presence of flavonols across nearly all fractions. These results demonstrate the effectiveness of the molecularly imprinted sorbent for the concentration and enrichment of flavonoids, namely flavonols. The enriched fractions exhibit remarkably attractive EC_{50} values, indicating their potential utility across various industries.

Particularly, for fraction F9 was measured a total phenolic content c.a. 3.8 times higher than the original extract (see **Figure 5-10**). Moreover, the DPPH scavenging activity, the reducing power and the TBARS inhibition of this fraction are estimated to be 6.4, 4.2 and 4.5 times higher, respectively, than the initial diatomaceous earth extract (see **Table 5-4**). Notably, as presented in **Table 5-4** and **Figure 5-11**, many fractions purified exhibited superior results as compared to a raw grape pomace extract, which is a product currently employed in some industries as a natural antioxidant.

It is worth noting that flavonoids have both antioxidant properties and radical scavenging activity, but the extent of their activity can vary depending on their specific chemical structure and the conditions of the assay. Generally, it is difficult to make a broad statement about which group shows more radical scavenging activity because it can depend on factors such as the specific compound within each class and the type of free radicals being targeted [36]. Flavonols, such as quercetin and kaempferol, are known for their strong antioxidant properties due to the presence of multiple hydroxyl groups on their structure and they are effective scavengers of various types of free radicals [36].

5.4 Conclusions

This paper focuses on the valorisation of phenolic compounds found in winemaking by-products, specifically in spent diatomaceous earth, used in industry for wine filtration. The study involved the synthesis at the gram-scale of a custom-designed adsorbent through the inverse suspension polymerization technique. Special attention was given to particular polymerization conditions, trying to avoid solvents weakening the interaction between pyridyl groups of the functional monomer (4-vinylpyridine) and the template molecule (quercetin). The selected conditions were specifically designed to produce a material with high performance, making it well-suited for utilization in a large-scale

continuous process (e.g. avoiding high backpressure through control of the size of the molecularly imprinted polymer particles). The synthesized adsorbent was subsequently used to work in a designed pilot size sorption/desorption prototype running fractionation processes based on hydroalcoholic solvents.

The different fractions produced with the sorption/desorption prototype were evaluated for their total phenolic content and antioxidant activity with three different assays (radical scavenging activity, reducing power and inhibition of lipid peroxidation). Fractions with a higher TPC than the initial extract were obtained, emphasizing the successful enrichment of phenolic compounds. Interestingly, the rise in phenolic content correlated with increased antioxidant activity of these fractions. Indeed, the results obtained show the improvement of the bioactivity of most of the fractions comparatively to the original diatomaceous earth extract. For the most enriched fraction was measured a total phenolic content c.a. 3.8 times higher than the original extract. The DPPH scavenging activity, the reducing power and the TBARS inhibition of this fraction were estimated to be 6.4, 4.2 and 4.5 times higher, respectively, than the initial diatomaceous earth extract. Notably, many fractions purified exhibited superior results as compared to a raw grape pomace extract, which is a product currently employed in some industries as a natural antioxidant. This work combines in the same research materials development, process design and application to real extracts with demonstration of improvement of the bioactivity of purified products. The proof of concept here presented can be extended to the purification of other kinds of winemaking residues or to the valorisation of other sorts of biomass with contribution for sustainability and circular bioeconomy.

5.5 References

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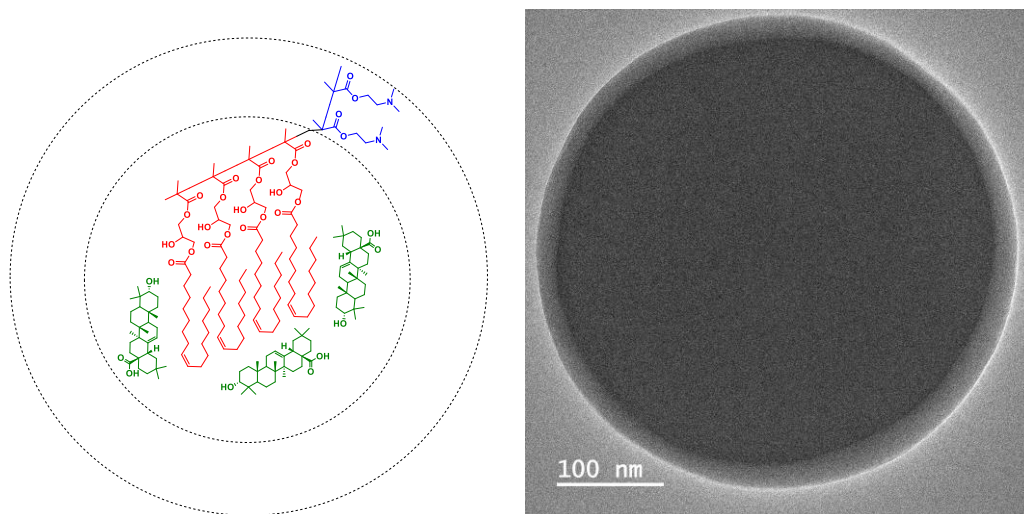
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Chapter 6. Designing of amphiphilic block copolymers: Glycidyl Methacrylate routes for oleic acid incorporation



Amphiphilic block copolymers with a hydrophobic part built up from glycidyl methacrylate and oleic acid, and a hydrophilic segment made from 2-(dimethylamino)ethyl methacrylate, were synthesized using ATRP and RAFT polymerization. The proposed reaction routes suppress most of branching and/or cross-linking, making the material synthesis simpler and more easily scalable. The ability of the copolymers to self-assemble in an aqueous environment and form well-defined spherical nanoaggregates was demonstrated, with oleanolic acid encapsulated. The sustained release of oleanolic acid and the transitions induced by pH/temperature changes are highlighted.

Reaction Routes with Glycidyl Methacrylate for the Incorporation of Oleic acid in Amphiphilic Block Copolymers

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Abstract

This research focused on assessing different reaction routes for synthesizing amphiphilic block copolymers, with the hydrophobic part built using glycidyl methacrylate and oleic acid and the hydrophilic segment made from 2-(dimethylamino)ethyl methacrylate. The main objectives of this study were to identify reaction routes leading to low-impact branching and/or crosslinking mechanisms involving epoxides and oleanolic acid, and to synthesize p(GMA-OA)-*b*-p(DMAEMA) copolymers in a simple and scalable manner. Block copolymer synthesis was considered using both ATRP and RAFT polymerization. The reaction route involving the synthesis of methacrylated oleic acid proved problematic, whereas higher synthesis efficiency was achieved when the glycidyl methacrylate moieties were modified with oleic acid after polymerization. It was found that working with DMSO solvent and a stoichiometric excess of oleic acid was particularly important for reducing branching/crosslinking. FTIR and GPC analyses were used to assess the progress of the chemical composition and molecular architecture of the intermediate homo- and co-polymers, and to demonstrate the successful production of the intended p(GMA-OA)-*b*-p(DMAEMA) materials.

The synthesized block copolymers were used for encapsulating and delivering oleanolic acid considering solid polymer dispersions and aqueous particle dispersions. SEM and TEM analysis demonstrate the ability of the p(GMA-OA)-*b*-p(DMAEMA) copolymers to self-assemble in an aqueous environment and well-defined spherical aggregates were observed, for example with size up to 100 nm in size with an external shell around 5–8 nm thick. A high loading capacity for oleanolic acid was measured, with a range of up to 0.17 mg/mL in aqueous particle dispersions. The dynamics of oleanolic acid release was measured under different conditions, including changes in solvent composition, pH and temperature. The results demonstrate sustained release of the encapsulated oleanolic acid and transitions induced by pH/temperature changes.

Key Words: Glycidyl Methacrylate; Oleic Acid; Amphiphilic Block Copolymers; Self-Assembly; Encapsulation

6.1. Introduction

The presence of both vinyl and epoxy reactive groups makes the glycidyl methacrylate (GMA) monomer a versatile starting chemical for synthesizing other tailored monomers, or for producing GMA-based polymers that can be further functionalized through post-polymerization modification [1]. Nowadays, this reactive chemical system has gained significant importance in the production of epoxy-functional methacrylic resins for use as coatings and adhesives, due to the potential incorporation of vegetable oil macromonomers and/or fatty acids (FA) into the final products [2-7]. Indeed, the demand for the reducing or replacing of petroleum-derived raw materials in these industries lead to solutions considering for instance the creation of methacrylated derivatives of fatty acids through the catalytic addition of the epoxy group of GMA to the carboxylic acid group of the FA (epoxy ring opening reaction). This approach was used to synthesize vinyl monomers incorporating oleic acid (OA), lauric acid, myristic acid, stearic acid and others [2-7]. These monomers were then polymerized through free radical mechanisms to generate different products with a range of applications [8-11]. The epoxy ring-opening mechanism involving the moieties of poly(glycidyl methacrylate)-based polymers with multiple functional groups, such as amine, thiol, azide and carboxylic acid groups, as well as hydrolysis reactions, has also been considered in post-polymerization modification approaches for synthesizing a variety of customized functional polymers [1,12]. The development of chromatographic stationary phases is an important application field for GMA-based polymers of this sort. Chemical modification of the epoxy groups enables amino, hydroxyl or thiol groups, among others, to be introduced into polymer particles, providing the selectivity and stability required for the analytical separation of many different compounds [13-16].

Interestingly, the widespread adoption of reversible-deactivation radical polymerizations techniques (RDRP) such as atom transfer radical polymerization (ATRP) and reversible addition–fragmentation chain transfer (RAFT) polymerization has resulted in the creation of GMA-based block copolymers for use in encapsulation, drug delivery, surface modification and other areas of materials science [17,18]. In this context, well-defined amphiphilic poly (glycidyl methacrylate)-*b*-poly (poly(ethylene glycol)methacrylate) diblock copolymers were recently synthesized using a recyclable photoRDRP mechanism [19]. Block copolymers involving GMA are also possible through living ionic

polymerization [20], but controlled radical polymerization permits the use of less stringent reaction conditions to produce a variety of tailored molecular architectures.

It is well established that a multitude of competitive mechanisms are possible for the reactions of glycidyl/epoxy groups with alcohols or carboxylic acids, amongst other reagents [21]. In the context of the reaction between glycidyl methacrylate and fatty acid carboxylic groups, there is the possibility of a secondary reaction involving epoxides with the formed hydroxyl groups. This secondary reaction is more likely to occur as the carboxylic acid groups are depleted [9,21]. Therefore, species with a high number of reactive centers can be formed in processes involving the transformation of epoxy groups, with the aim of functionalizing GMA monomers or post-modifying polymers containing GMA moieties. Consequently, branching and/or crosslinking mechanisms are possible in such synthesis routes leading to the potential formation of an insoluble fraction in the resulting materials [9]. While a moderate level of branching/crosslinking is acceptable for some applications, such as the production of coatings and adhesives, it can have a detrimental effect on the properties of materials used in other fields, such as those involving well-defined block copolymers.

This study focuses on analyzing various reaction pathways involving glycidyl methacrylate, with the aim of incorporating oleic acid into amphiphilic block copolymers. The ultimate objective is to synthesize hybrid synthetic-natural block copolymers comprising a hydrophobic block resulting from the reaction between OA and the epoxy groups of GMA and a hydrophilic poly(2-(dimethylamino)ethyl methacrylate) block that can be stimulated by changes in pH and/or temperature. The synthesis routes that lead to more straightforward and scalable production of p(GMA-OA)-*b*-p(DMAEMA) copolymers are analyzed, as is the use of these materials for encapsulating and delivering hydrophobic molecules. This research also examines reaction routes that lead to a low enough impact of branching and/or crosslinking mechanisms involving epoxides, in order to prevent unwanted effects on the molecular architecture and end-use properties of the desired materials.

Nowadays, the use of many different drug delivery vehicles is being considered for various applications with the aim of enhancing biological activity against numerous diseases [22-27], including utilization in plants [28-31]. The present work uses oleanolic acid, a pentacyclic triterpenoid found in many plants including the olive tree, where it occurs alongside oleic acid, as a model hydrophobic drug to examine the encapsulation

and delivery properties of the p(GMA-OA)-*b*-p(DMAEMA) copolymers that have been developed.

6.2. Materials and methods

6.2.1. Materials

All chemicals were used as purchased, without further purification. CuBr (97% purity), 4-(dimethylamino)pyridine (DMAP, purity $\geq$ 99%), 2-(dimethylamino)ethyl methacrylate (DMAEMA, 98% purity), poly(ethylene glycol) methyl ether of MW 2000 (mPEG 2000), (hydroxyethyl)methacrylate (HEMA, 99% purity), 2-cyano-2-propyl dodecyl trithiocarbonate (CPDT), ethyl 2-bromopropionate (EBrP, 99% purity), 9-(diethylamino)-5H-benzo[a]phenoxazin-5-one (Nile Red), N,N,N',N'',N'''-pentamethyldiethylenetriamine (PMDETA, 99% purity) and styrene (purity $\geq$ 99%) were purchased from Sigma-Aldrich. α -bromoisobutyryl bromide (BIBB, 98% purity), 2,2'-bipyridine (BPY, 99% purity), dicyclohexylcarbodiimide (DCC, 99% purity), 2-methylimidazole (2MI, 99% purity) were purchased from Thermo Scientific Chemicals. 3.5k MWCO cellulose dialysis bag was purchased from Thermo Fisher Scientific. Triethylamine (TEA, 99.5% purity), glycidyl methacrylate (GMA, 97% purity), oleanolic acid (97% purity) were purchased from Acros Organics. N,N-dimethylformamide (DMF), acetic acid (AcOH), methanol (MeOH), ethanol (EtOH), diethyl ether, hexane and tetrahydrofuran (THF) solvents were purchased from Fisher Chemical while, dimethyl sulfoxide (DMSO) was purchased from Fisher BioReagents. Anisole and 2,2'-azobis(2-methylpropionitrile) (AIBN), were purchased from Fluka Analytical, S,S-dibenzyltrithiocarbonate (DBZT, 97% purity) was purchased from Strem Chemicals and oleic acid (OA, 99% purity), was purchased from Alfa Aesar. Unless otherwise mentioned below, all the reactants and solvents were used as received. The water used in the experiments is ultrapure water supplied by the local laboratory (Millipore water, Milli-Q quality).

6.2.2. Synthesis of methacrylated oleic acid through reaction with glycidyl methacrylate

The synthesis of methacrylated oleic acid (MOA) through reaction with glycidyl methacrylate was performed in accordance with the principles reported in previous

related works on this subject [2-11]. As detailed below, the set of reactions performed included changes in temperature (between 40 and 100 °C), composition of the initial mixture (e.g. molar ratio of GMA/OA between 0.5 and 1.1 as detailed in **Table 6-1**, presence or absence of catalyst and solvent), and reaction time (e.g. between 1 and 24 hours). Briefly, the prescribed amounts of OA, GMA, the 2MI catalyst and the solvent were mixed and allowed to react with stirring for the defined time period. FTIR analysis was used to measure the consumption of epoxy groups of GMA for the different products, using the 910 cm⁻¹ assignment as a reference.

6.2.3. Synthesis of methacrylated oleic acid via Steglich esterification with HEMA

To enable comparison with the method involving the reaction of glycidyl methacrylate, this work also considered the synthesis of methacrylated oleic acid via Steglich esterification with HEMA. For this purpose, the process described in the literature was considered [10]. Briefly, defined amounts of oleic acid, DMAP and HEMA were mixed in THF in a two-necked flask at room temperature with stirring, and then placed in an ice-water bath purged with N₂ gas. In a separate beaker, DCC was dissolved in the minimum necessary amount of THF and added dropwise to the mixture. The ice-water bath was then removed once the addition of the DCC was complete. After 24 hours reaction time, the precipitated urea was filtered off and the THF was removed using a rotary evaporator. The resulting ester was purified by column chromatography using silica gel as the stationary phase and a cyclohexane-EtOAc (95:5, v/v) mixture as the eluent, affording the pure monomer.

6.2.4. Synthesis of the ATRP macroinitiator mPEG-Br

In order to consider mPEG as a potential hydrophilic block in copolymers involving oleic acid-based hydrophobic blocks, the ATRP macroinitiator mPEG-Br was synthesized using the methodology reported for reactions involving hydroxyl groups and BIBB [32-34]. In brief, a defined amount of mPEG was dispersed in DMF using sonication. Then, triethylamine and DMAP were added to the mPEG dispersion, after which BIBB was added dropwise to the suspension at 0 °C. The reaction was then allowed to proceed at room temperature for 24 hours, after which ethanol was used to terminate the esterification. The products were washed with DMF until a colorless liquid phase was achieved, after which the ATRP macroinitiator mPEG-Br was purified with deionized water and dried in a vacuum oven at 40 °C. The resulting ATRP macroinitiator, mPEG-

Br, was then used to form block copolymers, considering ATRP processes involving methacrylated oleic acid monomers described in **Sections 6.2.2 and 6.2.3**.

6.2.5. Synthesis of p(GMA) and p(DMAEMA) homopolymers via RAFT polymerization

The synthesis of the homopolymers p(GMA) and p(DMAEMA) via RAFT polymerization was performed under conditions similar to those reported in the literature [35-38]. These polymerizations were carried out in bulk by dissolving defined amounts of the RAFT agent (CPDT or DBZT) and the initiator (AIBN) in the monomer, after which the mixture was purged with a flow of dry argon for 30 minutes. The vessel was then sealed, after which polymerization was performed at 60 °C for the selected time. Typical molar ratios used were M/I/RAFT=7.5/0.02/0.06 (see **Table 6-2** for additional information). The resulting polymers were isolated and purified by successive precipitation in non-solvents (e.g. diethyl ether for p(GMA)) and dialysis using 3.5k MWCO cellulose bags. For the purification of p(DMAEMA), the method involving the solubilization of the crude polymer in water at pH ~4, followed by increasing the pH to ~12 and the temperature to ~65 °C [39], was adopted here too. The purified RAFT homopolymers were dried at 40 °C in a vacuum oven, after which they were characterized in terms of gravimetric yield, FTIR and SEC analysis.

6.2.6. Synthesis of p(GMA) and p(DMAEMA) homopolymers via ATRP

The synthesis of the homopolymers p(GMA) and p(DMAEMA) via ATRP polymerization was performed under conditions similar to those reported in the literature [35-45]. These polymerizations were carried out in anisole solvent solution. Briefly, defined amounts of CuBr and ligand (BPY or PMDETA) were dissolved in anisole, then the required amounts of monomer and initiator (EBrP) were added. The mixture was then purged with a flow of dry argon for 30 minutes. After sealing the vessel, polymerization was performed at 60 °C for the selected time. Typical molar ratios used were M/I/C/L=2.6/0.1/0.1/0.27 (see **Table 6-2** for additional information). As previously described, the resulting polymers were isolated and purified by successive precipitation in non-solvents and dialysis using 3.5k MWCO cellulose bags. As above, the purification of p(DMAEMA) also included solubilizing the polymer in water at pH 4, followed by increasing the pH to 12 and the temperature to 65 °C [39]. The purified ATRP

homopolymers were dried at 40 °C in a vacuum oven and then characterized in terms of gravimetric yield, FTIR and SEC analysis.

6.2.7. Synthesis of p(GMA)-*b*-p(DMAEMA) copolymers via RAFT polymerization

The synthesis of copolymers p(GMA)-*b*-p(DMAEMA) via RAFT polymerization was performed using macro-RAFT agents which synthesis and purification was described in **Section 6.2.5**. The p(GMA) or p(DMAEMA) macro-RAFT homopolymers were dissolved in DMSO at the desired concentration in the presence of the required amounts of the correspondent comonomer and AIBN initiator. The mixture was purged with a flow of dry argon for 30 minutes after which polymerization was performed in the sealed vessel at 60 °C for the selected time. **Table 6-3** provides information about the initial composition of the polymerization mixture. As before, the resulting polymers were isolated and purified by successive precipitation in non-solvents and dialysis. The purified p(GMA)-*b*-p(DMAEMA) copolymers were dried at 40 °C in a vacuum oven, after which they were also characterized in terms of gravimetric yield, FTIR and SEC analysis.

6.2.8. Synthesis of p(GMA)-*b*-p(DMAEMA) copolymers via ATRP

The synthesis of the copolymer p(GMA)-*b*-p(DMAEMA) via ATRP polymerization was performed using ATRP macroinitiators, the synthesis and purification of which are described in **Section 6.2.6**. These polymerizations were also carried out in a DMSO solvent solution. Defined amounts of CuBr and ligand (BPY or PMDETA) were dissolved in the solvent, and then the required amounts of comonomer and macro ATRP initiator were added. The mixture was purged with a flow of dry argon for 30 minutes, after which polymerization was performed in a sealed vessel at 60 °C for the selected time. **Table 6-3** provides information about the initial composition of the polymerization mixture. The resulting polymers were isolated and purified by successive precipitation in non-solvents and dialysis. The purified p(GMA)-*b*-p(DMAEMA) copolymers obtained by ATRP were dried at 40 °C in a vacuum oven. They were then characterized in terms of gravimetric yield, FTIR and SEC analysis.

6.2.9. Incorporation of oleic acid in p(GMA) homopolymers

The incorporation of oleic acid into p(GMA) homopolymers synthesized by ATRP or RAFT polymerization (see **Sections 6.2.5 and 6.2.6**) was carried out in DMSO solution

at 60 °C. In brief, the prescribed amounts of oleic acid (OA), p(GMA), the 2MI catalyst and the solvent were mixed and allowed to react for the defined time period. **Table 6-4** provides information about the initial composition of the reaction mixture. The modified p(GMA) product was then isolated and purified by precipitation and dialysis. This was followed by drying at 40 °C in a vacuum oven and characterization in terms of gravimetric yield, FTIR and SEC analysis. This was followed by drying at 40 °C in a vacuum oven and characterization in terms of gravimetric yield, FTIR and SEC analysis.

6.2.10. Incorporation of oleic acid in p(GMA)-b-p(DMAEMA) copolymers

The incorporation of oleic acid into p(GMA)-b-p(DMAEMA) copolymers, synthesized by ATRP or RAFT polymerization (see **Sections 6.2.7 and 6.2.8**), was also carried out in DMSO solution at 60 °C, in a manner analogous to that described in **Section 6.2.9** for p(GMA) homopolymers (see **Table 6-5** for information about the initial composition of the reaction mixture).

6.2.11. One-pot synthesis of p(GMA)-b-p(DMAEMA) copolymers with incorporated OA through ATRP with p(GMA) macroinitiator

The one-pot synthesis of p(GMA)-b-p(DMAEMA) copolymers with incorporated OA was performed through the ATRP technique. This process involved the dissolution of the specified amounts of p(GMA) ATRP macroinitiator (see **Section 6.2.6**), CuBr, ligand (BPY or PMDETA) and DMAEMA monomer, along with the defined amounts of OA and 2MI catalyst, in DMSO. The mixture was purged with a flow of dry argon for a period of 30 minutes. This was followed by the promotion of polymerization and reaction of OA with epoxy groups of p(GMA) in a sealed vessel at a temperature of 60 °C for the designated time. **Table 6-5** provides information about the initial composition of the reaction mixture. The resulting polymer product was then isolated and purified by precipitation and dialysis. Subsequent to this, the samples were subjected to drying at a temperature of 40 °C within a vacuum oven. This was followed by characterization in terms of gravimetric yield, FTIR and SEC analysis.

6.2.12. Polymer characterization using SEC/RI/LALS/RALS with DMF solvent

SEC analysis was considered to get information concerning the structure of different DMF soluble homopolymers and copolymers synthesized in this research. The used SEC apparatus is composed of a Viscotek GPCmax VE 2001 pumping system with Refractive

Index (RI), Low Angle Light Scattering (LALS) and Right Angle Light Scattering (RALS) detection. Polymers were fractionated in a train of 2 columns Phenogel columns: 1) 500 Å (molecular weight 1 k to 15 k) + 2) 1000 Å (molecular weight 1 k to 75 k) with dimensions $L \times D = 300 \text{ mm} \times 7.5 \text{ mm}$. DMF with 20 mM LiBr was used as eluent and the measurements were performed at 50 °C and $Q = 1 \text{ mL/min}$.

6.2.13. Polymer characterization using SEC/RI/MALLS with chloroform solvent

Chloroform soluble homopolymers and copolymers were analyzed using a different SEC apparatus that is composed of a Polymer Laboratories PL-GPC-50 integrated SEC system with differential refractometer working at $950 \pm 30 \text{ nm}$, attached to a Wyatt Technology DAWN8⁺ HELEOS 658 nm MALLS detector. The polymer samples were fractionated by molecular size using a train of 3 GPC columns PLgel ($300 \text{ mm} \times 7.5 \text{ mm}$) with nominal particle size 10 µm and pore type MIXEDB-LS, maintained at constant temperature of 30 °C and using chloroform as the eluent at a flow rate of 1 mL/min.

6.2.14. FTIR analysis of the polymers

Purified and dried products were characterized through FT-IR spectroscopy with a Perkin Elmer, model Spectrum TwoTM, instrument. These analyses were directly performed in ATR mode or, when needed, polymers were mixed with KBr and pressed into pellets in order to collect the correspondent IR spectra.

6.2.15. Encapsulation and release of oleanolic acid with the synthesized amphiphilic copolymers

Block amphiphilic copolymers were first dissolved in THF in the presence of oleanolic acid and afterwards the solvent composition was dropwise changed up to reach THF/water 50/50. The final solution was dialyzed against water in a cellulose bag membrane (3.5k molecular weight cut off). The estimation of the encapsulated oleanolic acid was obtained through HPLC-DAD analysis of the liquid phase after filtration, considering both the THF/water 50/50 system and the final aqueous dispersion. Final aqueous dispersions were subjected to the analysis of oleanolic release by dropping in water with defined values of pH and temperature. Released oleanolic acid was also quantified through HPLC-DAD analysis of the liquid phase after filtration. The suitability of block amphiphilic copolymers for the formation of aggregates and encapsulation of hydrophobic compounds was also assessed using Nile Red and following visually the

color change along the self-assembly process. Encapsulation of oleanolic acid in a solid dispersion was also performed using p(GMA-OA)-*b*-p(DMAEMA) materials acting as a polymeric carrier and a solvent evaporation method with THF.

6.2.16. SEM and TEM analysis

Dried polymer particles were analyzed by scanning electron microscopy (SEM) with an FEI Quanta 650 FEG scanning electron microscope under a high vacuum. Secondary electrons were detected by an Everhardt Thornley SED (secondary electron detector). The images were acquired at 10 kV, spot size 3 and with a working distance of 10 mm. The samples were sputtered with gold, using the following setup: sputtering current = 30 mA and duration = 30 s. Stable aqueous dispersions of aggregates encapsulating oleanolic acid was analyzed by TEM with a probe-corrected FEI Titan G2 80-200 kV ChemiSTEM set-up. Such aggregates were formed by self-assembling of the synthesized p(GMA-OA)-*b*-p(DMAEMA) copolymers. These SEM and TEM analyses were performed at the national Iberian Nanotechnology Laboratory (INL), Braga, Portugal.

6.2.17. HPLC-DAD analysis for quantification of encapsulation and release of oleanolic acid with the synthesized amphiphilic copolymers

An HPLC system (KNAUER) consisting of a gradient pump (P6.1 L) equipped with a degasser, an autosampler (6.1 L), a column thermostat (CT2.1), and a DAD (6.1 L) was used in this research. ClarityChrom was the software allowing the control of the HPLC-DAD (High-Performance Liquid Chromatography with Diode Array Detector) system. The chromatographic analysis was performed using an Ascentis C18 (SUPELCO) column with a particle size of 5 μ m and dimensions 25 cm $\times$ 4.6 mm. An isocratic analysis was performed, using ACN/water 85/15 with 0.05 % formic acid as eluent, for 25 min at T = 25 °C.

6.2.18. UV/Vis spectroscopy and HPLC-DAD for monitoring purification processes

A spectrophotometer P9 Double Beam UV–Visible, VWR, was used for fast assessment of cleaning and purification steps described above. The HPLC-DAD system outlined in **Section 6.2.17** was also used to assess the purification processes by analyzing the liquid phases involved, as well as to quantify GMA conversion in the different reaction routes detailed above (liquid phase analysis).

6.3. Results and Discussion

The synthesis of methacrylated derivatives of natural fatty acids via the catalytic reaction between epoxy and carboxylic acid groups is a widely considered topic in current research and industrial applications. Among compounds with vinyl and epoxy reactive groups, glycidyl methacrylate is often preferred due to its simplicity of preparation from methacrylic acid and glycidol, and its peculiar reactivity enables it to undergo many polymerization reactions, as well as post-polymerization modification mechanisms. This approach is used to prepare vinyl monomers that incorporate oleic acid, among others, with the aim of developing more sustainable coatings and adhesives [2-7]. Interestingly, the unique reactivity and post-polymerization modification possibilities of GMA have also been explored in the preparation of advanced block copolymers, namely using controlled radical polymerization techniques [17-20,35-37]. This research combines the two working lines to synthesize hybrid synthetic-natural block copolymers comprising a hydrophobic block resulting from the reaction between oleic acid and the epoxy groups of GMA, and a hydrophilic part based on p(DMAEMA). To this end, various reaction routes are assessed here, including the methacrylation of oleic acid and post-polymerization modifications, with the aim of minimizing side reactions that could have a detrimental effect on the properties of the desired amphiphilic block copolymers.

6.3.1 Experiments with methacrylated oleic acid monomer (MOA)

Table 6-1 provides an overview of the various operating conditions considered in this study for synthesizing methacrylated oleic acid monomer (MOA) through a reaction with glycidyl methacrylate. The synthesis described by runs 1 to 5 follows operating conditions like those reported in the literature for the reaction between OA and GMA, with the aim of preparing monomers for use in a subsequent free radical process for applications in the coating industry or for polymer networks [4,7,8-9]. FTIR analysis of the products shows that the epoxy groups of GMA are consumed in these reactions involving the methacrylation of oleic acid (the disappearance of the 910 cm^{-1} peak was followed), with total depletion observed in Run 1 and partial conversion in the others. The products of runs 1 to 5 were not fully soluble in ethanol, methanol, acetonitrile, DMF, DMSO, THF or chloroform at concentrations of 1 mg/mL or lower and temperatures up to 70 °C. The MOA products were also insoluble in the DMAEMA monomer, which was intended for use as the second building block in the production of the desired amphiphilic

copolymers. These insolubility observations are consistent with the formation of branched species due to secondary reactions as depicted in **Figure 6-1** and reported in the literature [9,21]. However, it was possible to carry out their free radical polymerization with styrene at 70 °C using an AIBN initiator, as demonstrated by the analysis of the products (see appendix D, **Figure D-3** in Supplementary Information), in accordance with the previously described procedure [9]. This confirms that using these synthesis recipes to generate MOA monomers is an effective approach for the subsequent production of branched/cross-linked materials. The ATRP and RAFT homopolymerization of the soluble part of the MOA monomers obtained in runs 1 to 5 produced gel-like products that were also unsuitable for the intended block copolymer synthesis.

Runs 6 to 13 in **Table 6-1** were designed to minimize secondary reactions leading to branching/crosslinking (see **Figure 6-1**). To this end, a molar excess of OA compared to GMA was employed, along with the absence of, or an alternative to, the catalyst (KOH attempted in these runs, as reported in Ref. [21]), and dilution with a solvent or decreased temperature. Homogeneous solutions were observed after the reaction in the specified solvents, and the conversion of GMA was estimated to be around 50% by HPLC-DAD. FTIR analysis of the solutions also demonstrates incomplete epoxide conversion. These results indicate the formation of MOA and the limitation of branching to an acceptable level. However, difficulties were encountered in product separation, particularly regarding the removal of unreacted OA (a saponification process was attempted) and the isolation of MOA resulted in a very low final yield (<10%). Note that laborious procedures are also needed to make the separation and purification of methacrylated OA when alternative methods, such as the esterification with HEMA, as described in **Section 6.2.3**, are considered. A final yield of around 70 % was observed when this approach was adopted. The use of the MOA monomers obtained via OA reaction with GMA or Steglich esterification with HEMA for block copolymers generation using ATRP and the *m*PEG-Br macroinitiator (see **Section 6.2.4** and the possible reaction scheme in appendix D. **Figure D-1**) was demonstrated to be possible (see appendix **Figures D-4 and D-5** in SI) but at very low conversion yield (<5%). Furthermore, attempts to generate homopolymers via ATRP/RAFT using MOA monomers produced via OA/GMA reaction or OA/HEMA esterification also resulted in very low polymerisation yields (<10%), hindering the subsequent step of generating amphiphilic block copolymers with DMAEMA. This is likely the result of the reactivity of the MOA monomers being too low within the ATRP/RAFT processes. Difficulties observed with the simultaneous dissolution of the

MOA monomers and RAFT/ATRP components also contributes to this shortcoming. Overall, the production of the intended p(GMA-OA)-*b*-p(DMAEMA) copolymers using MOA monomer was found to be challenging in terms of scalability.

Table 1-1: Different working conditions considered for the synthesis of methacrylated oleic acid through reaction with glycidyl methacrylate. The catalyst amount is presented as the mass fraction relative to sum of GMA and OA content while the solvent amount is expressed as the mass ratio considering also the sum of GMA and OA amount as reference.

Run	Molar ratio GMA/OA	Catalyst (%)	Solvent and mass ratio to GMA		T (°C)	Reaction time (h)
1	1.1	-	-	-	100	72
2	1.1	0.5 to 2	-	-	70	18
3	1.1	-	-	-	70	18
4	1.1	0.5 to 2	-	-	70	5
5	1.1	-	-	-	70	5
6	0.5	-	-	-	45	24
7	0.5	0.5 to 2	-	-	45	24
8	0.5	1	-	-	50	24
9	0.5	1	THF	2 to 12	50	24
10	0.5	1	DMSO	2 to 12	50	24
11	0.5	1	DMF	2 to 12	50	24
12	0.5	1	Anisole	2 to 12	50	24
13	0.5	1	Toluene	2 to 12	50	24

6.3.2 Experiments with post-polymerization modification of GMA moieties

Given the shortcomings described above in the synthesis of MOA monomers and their potential subsequent use in producing ATRP/RAFT amphiphilic block copolymers, alternative post-polymerization routes with GMA modification were explored. Interestingly, these results demonstrate the potential to control the branching degree in the OA/GMA reaction, particularly when excess OA and diluted conditions are considered. Therefore, post-polymerization modification of homo and copolymers containing GMA moieties was adopted under these reaction conditions.

Figures 6-2 to 6-6 depict the various reaction pathways involved in this post-polymerization modification approach. **Figure 6-2** shows the formation of the p(GMA-OA) homopolymer through the transformation of the p(GMA) polymer with OA.

Figure 6-3 illustrates the production of the p(GMA-OA)-*b*-p(DMAEMA) copolymer using pre-formed p(GMA-OA) in ATRP or RAFT processes. **Figure 6-4** shows the synthesis of the block copolymer p(GMA)-*b*-p(DMAEMA) by RAFT or ATRP, while **Figure 6-5** shows its transformation into a p(GMA-OA)-*b*-p(DMAEMA) copolymer via reaction with OA. Finally, **Figure 6-6** shows a diagram of the one-pot synthesis of a p(GMA-OA)-*b*-p(DMAEMA) copolymer, which involves the simultaneous post-polymerization modification of a p(GMA) homopolymer and DMAEMA polymerization. **Table 6-2** provides information on the experimental conditions for synthesizing p(GMA) and p(DMAEMA) homopolymers using ATRP and RAFT polymerization techniques. This is the first step in any post-polymerization transformation route aimed at producing p(GMA-OA)-*b*-p(DMAEMA) amphiphilic block copolymers. These recipes follow general principles reported for the ATRP and RAFT homo-polymerization of GMA or DMAEMA [35-45]. Indeed, the synthesis of various types of homo- and block copolymers or grafted materials, including the GMA and DMAEMA monomers, based on ATRP and RAFT polymerization techniques, has been reported in recent years [35-45]. These studies explore the versatility of GMA moieties for subsequent functionalization [30,41] and the excellent performance of DMAEMA materials for adsorption [38] and gene delivery due to their biocompatibility [45], among other applications. The reaction time was adjusted to prevent the formation of bulky homopolymers that would hinder their solubilization in subsequent steps, with the aim of using them as macroinitiators or macro-RAFT agents for block copolymers formation. The yield shown in **Table 6-2** was calculated by dividing the mass of the isolated polymer by the initial mass of the monomer.

The recipes in **Tables 6-3 to 6-5** describe the various experimental conditions that were investigated in this study to produce p(GMA)-*b*-p(DMAEMA) and p(GMA-OA)-*b*-p(DMAEMA) copolymers. In this context, it is particularly relevant that DMSO was selected as the solvent in an attempt to reduce the incidence of branching reactions due to the high reactivity of the oxirane ring and the derived hydroxyl group [1]. Indeed, it has been reported that using DMSO as a solvent result in polymers maintaining linearity when different nucleophilic agents are used (e.g. secondary amines, thiols, aromatic alcohols and the azide anion) in the post-polymerization treatment of p(GMA) [1]. The proposed treatment conditions in DMSO require a short reaction time to avoid the formation of micro- and macro-gels, which are often observed with alternative, longer-time p(GMA) modification processes that also aim for full conversion of the epoxy groups [1]. These

outcomes were adapted for the reaction between oleic acid and the epoxide groups in p(GMA) homopolymer or p(GMA)-*b*-p(DMAEMA) copolymer moieties, as detailed in **Tables 6-4 and 6-5**. For compatibility purposes, DMSO was also selected as the solvent for the synthesis of p(GMA)-*b*-p(DMAEMA) copolymers, as described in **Table 6-3**. The reaction time was optimized to achieve total conversion of the epoxide groups (see FTIR characterization below), and the recipes for incorporating oleic acid were designed to use a stoichiometric excess of OA compared to the epoxides to try the minimization of secondary reactions involving the oxirane ring. The mass increase values reported in **Tables 6-3 to 6-5** are calculated by comparing the mass recovered for the final product with the initial mass of the polymer used in the reaction, $100 \times (m_{product} - m_{polymer})/m_{polymer}$.

6.3.3 FTIR analysis

FTIR analysis was performed to evaluate the incorporation of different building units into various homopolymers and copolymers, including p(GMA), p(DMAEMA), p(GMA)-*b*-p(DMAEMA), and p(GMA-OA)-*b*-p(DMAEMA). **Figure 6-7** shows the results of the FTIR analysis of representative products, and further related information is provided in appendix D, in Supplementary Information (SI). The specific assignment at 910 cm^{-1} , corresponding to the stretching vibration of the epoxy ring, is observed in p(GMA) and p(GMA)-*b*-p(DMAEMA), but not in p(GMA-OA)-*b*-p(DMAEMA). This indicates the successful formation of p(GMA)-*b*-p(DMAEMA), and the latter reaction of the contained epoxy groups with oleic acid. Note also that the expected specific vibrational assignments due to the DMAEMA moiety, corresponding to the $-\text{N}(\text{CH}_3)_2$ groups at around 2770 cm^{-1} , are observed in p(DMAEMA), as well as in p(GMA)-*b*-p(DMAEMA). Combined with the 910 cm^{-1} assignment, this demonstrates the presence of both the GMA and DMAEMA moieties in the copolymer. Notably, in the p(GMA-OA)-*b*-p(DMAEMA) product, the increased prominence of the band in the $2800\text{--}3000\text{ cm}^{-1}$ region (CH_2 and CH_3 stretching) and the enhanced vibrational assignment at 1466 cm^{-1} (OH in plane bending) both indicate successful oleic acid incorporation into the final p(GMA-OA)-*b*-p(DMAEMA) product.

Figures D-6-D-12 in appendix D, provide additional evidences obtained by FTIR for the achievement of the compositional features designed for intermediate and final materials described in **Tables 6-2 to 6-5**.

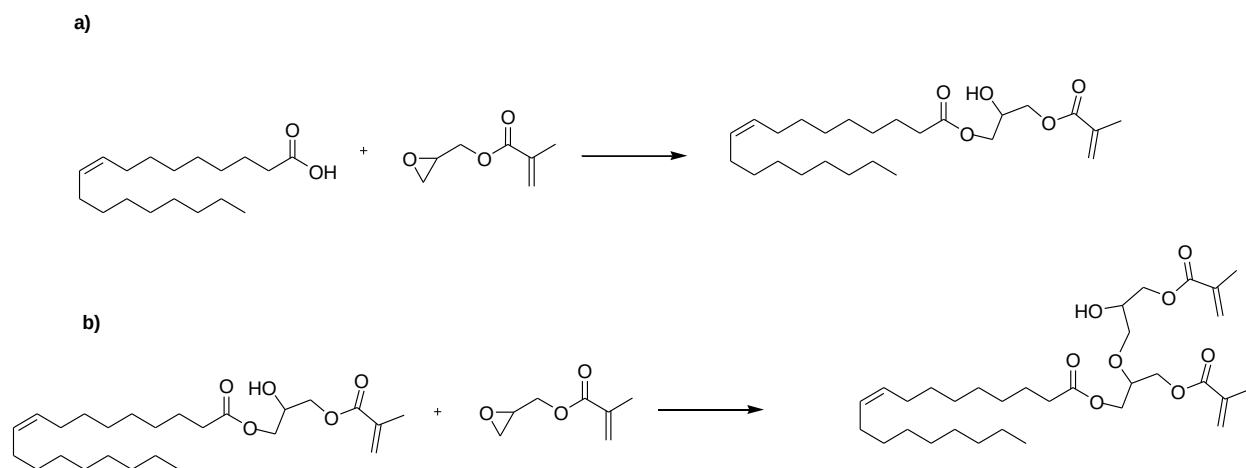


Figure 6-1: a) A schematic representation of the formation of methacrylated oleic acid through a reaction with glycidyl methacrylate. b) A secondary reaction involving epoxides and the hydroxyl groups formed in the main route, which leads to the formation of multifunctional species. These species can react in later stages to form high-molecular-weight branched/crosslinking molecules.

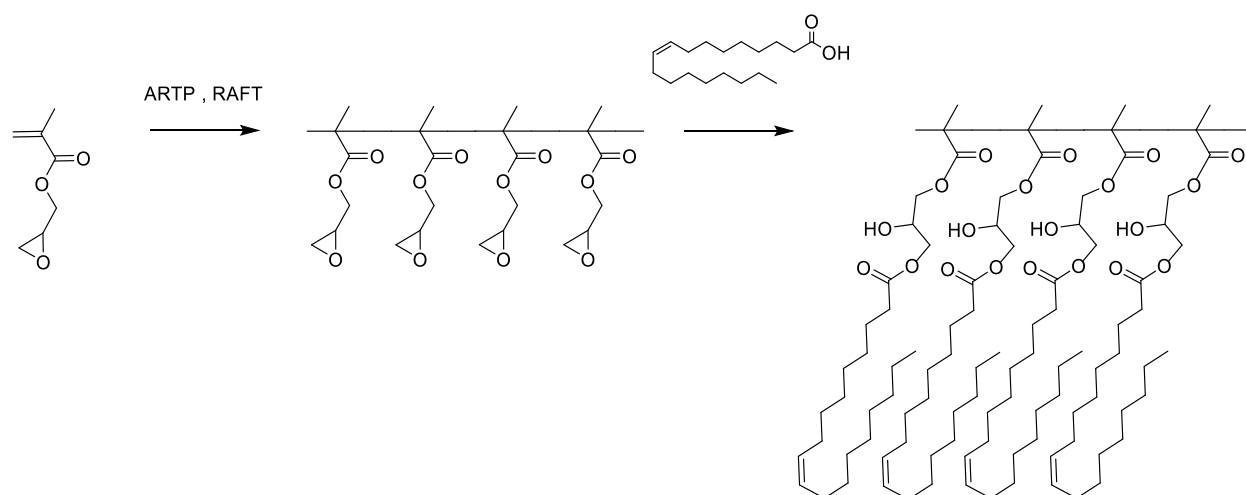


Figure 6-2: A schematic representation of the synthesis of p(GMA) homopolymers via ATRP or RAFT polymerization. It also shows the post-polymerization modification of the p(GMA) homopolymers through the reaction of OA with the epoxides in the repeating units, leading to the formation of a p(GMA-OA) homopolymer.

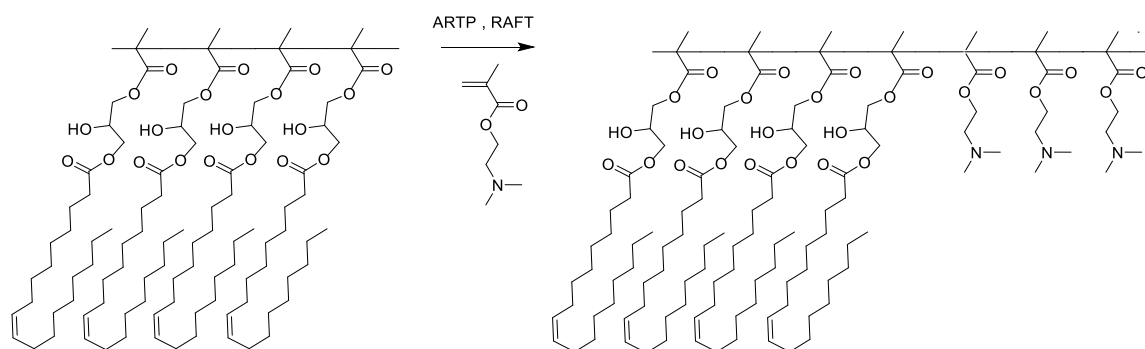


Figure 6-3: A schematic representation of the synthesis of a p(GMA-OA)-b-p(DMAEMA) copolymer via ATRP or RAFT polymerization. These polymerizations use the previously synthesized p(GMA-OA) homopolymer (**Figure 6-2**) as the macro-initiator or macro-RAFT agent, respectively.

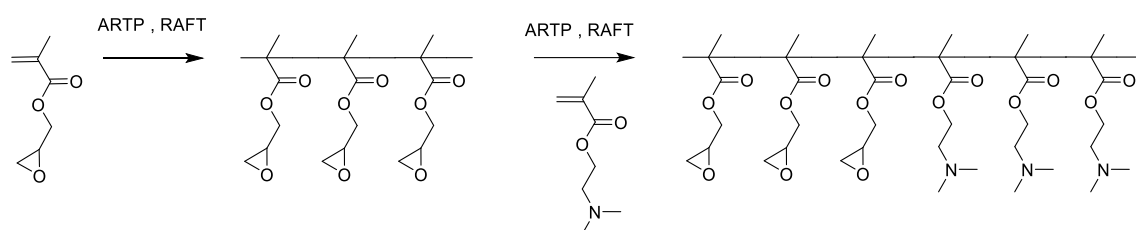


Figure 6-4: A schematic representation of the synthesis of the block copolymer p(GMA)-b-p(DMAEMA), using the corresponding previously synthesized p(GMA) homopolymers as the macro-initiator or macro-RAFT agent.

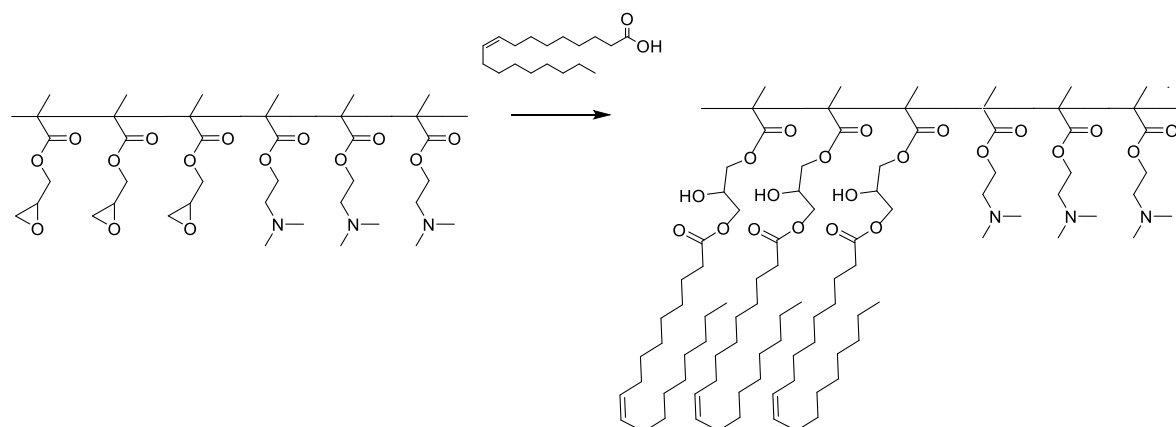


Figure 6-5: A schematic representation of the synthesis of a p(GMA-OA)-b-p(DMAEMA) copolymer through the post-polymerization modification of a p(GMA)-b-p(DMAEMA) copolymer. This route considers the reaction of OA with the epoxides in the repeating units of p(GMA)-b-p(DMAEMA) for the formation of p(GMA-OA)-b-p(DMAEMA).

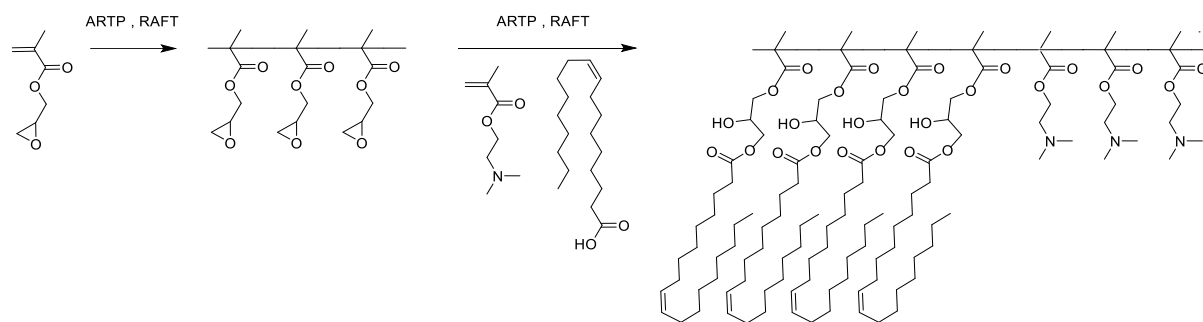


Figure 6-6: A schematic representation of the one-pot synthesis of a p(GMA-OA)-*b*-p(DMAEMA) copolymer via the simultaneous post-polymerization modification of a p(GMA) homopolymer and DMAEMA polymerization. These ATRP and RAFT processes utilize the previously synthesized p(GMA) homopolymers as the macro-initiator and macro-RAFT agent, respectively, while the epoxy groups in their moieties undergo a reaction with OA.

Table 6-2: Typical experimental conditions considered for the synthesis of poly (glycidyl methacrylate) and poly(2-(dimethylamino)ethyl methacrylate) homopolymers through the ATRP and RAFT polymerization techniques. All the reactions were performed at 60 °C and the various concentration values provided are expressed in mol/dm³.

P	Tech.	M		I		C		L		RAFT A.		S	t(h)	Yield (%)
H1	ATRP	GMA	2.6	EBrP	0.10	CuBr	0.1	BPY	0.27	-	-	Anisole	10	77
H2	RAFT	GMA	7.5	AIBN	0.02	-	-	-	-	CPDT	0.06	-	4	56
H3	ATRP	DMAEMA	2.6	EBrP	0.10	CuBr	0.1	BPY	0.27	-	-	Anisole	10	47
H4	RAFT	DMAEMA	6.4	AIBN	0.02	-	-	-	-	CPDT	0.06	-	4	38
H5	RAFT	GMA	7.5	AIBN	0.02	-	-	-	-	DBZT	0.06	-	1	10
H6	ATRP	GMA	2.6	EBrP	0.10	CuBr	0.1	PMDTA	0.1	-	-	Anisole	10	89
H7	ATRP	DMAEMA	2.6	EBrP	0.10	CuBr	0.1	PMDTA	0.1	-	-	Anisole	10	37

Table 6-3: Typical experimental conditions considered for the synthesis of poly (glycidyl methacrylate)-*b*-poly(2-(dimethylamino)ethyl methacrylate) copolymers through the ATRP and RAFT polymerization techniques. All the reactions were performed at 60 °C and the various concentration values provided are expressed in mol/dm³, except the correspondent to the used homopolymers that are presented in mg/mL.

P	Tech.	M		I		C		L		RAFT A.		S	t(h)	Mass increase (%)
C1	RAFT	DMAEMA	1.2	AIBN	8×10 ⁻⁴	-	-	-	-	H2	24 (mg/mL)	DMSO	5	170
C2	ATRP	DMAEMA	0.8	H1	40 (mg/mL)	CuBr	2.1×10 ⁻³	BPY	3.5×10 ⁻³	-	-	DMSO	5	12
C3	ATRP	DMAEMA	0.4	H1	40 (mg/mL)	CuBr	2.1×10 ⁻³	BPY	3.5×10 ⁻³	-	-	DMSO	5	24

Table 6-4: Typical experimental conditions considered for the incorporation of oleic acid in poly (glycidyl methacrylate) homopolymers. All the reactions were performed at 60 °C and the various concentration values provided are expressed in mol/dm³, except the correspondent to the used homo-/copolymers that are presented in mg/mL.

P	Polymer		OA	2-MIZ	Solvent	t(h)	Mass increase (%)
OAI1	H1	34.7 (mg/mL)	0.49	4.6×10 ⁻³	DMSO	24	40
OAI2	H2	34.7 (mg/mL)	0.49	4.6×10 ⁻³	DMSO	24	136

Table 6-5: Typical experimental conditions considered for the incorporation of oleic acid in poly (glycidyl methacrylate)-*b*-poly(2-(dimethylamino)ethyl methacrylate) copolymers. All the reactions were performed at 60 °C and the various concentration values provided are expressed in mol/dm³, except the correspondent to the used homo-/copolymers that are presented in mg/mL..

P	Polymer		OA	2-MIZ	M		I		C		L		RAFT A.		S	t(h)	Mass increase (%)
OAP1	C1	34.7 (mg/mL)	0.49	4.6×10 ⁻³	-	-	-	-	-	-	-	-	-	-	DMSO	24	280
OAP2	C2	34.7 (mg/mL)	0.49	4.6×10 ⁻³	-	-	-	-	-	-	-	-	-	-	DMSO	24	240
OAP3	C3	34.7 (mg/mL)	0.49	4.6×10 ⁻³	-	-	-	-	-	-	-	-	-	-	DMSO	24	177
OAP4	H1	34.7 (mg/mL)	0.49	4.6×10 ⁻³	DMAEMA	0.7	-	-	CuBr	2.1×10 ⁻³	BPY	3.4×10 ⁻³	-	-	DMSO	24	255



Figure 6-7: FTIR analysis of a product p(GMA-OA)-b-p(DMAEMA), a block copolymer p(GMA)-b-p(DMAEMA) and related homopolymers p(GMA) and p(DMAEMA). Specific assignment at 910 cm⁻¹ is observable in the p(GMA) homopolymer and in the p(GMA)-b-p(DMAEMA) copolymer but not in the product p(GMA-OA)-b-p(DMAEMA), indicating the reaction of the epoxide moieties of the p(GMA) block with oleic acid. On the other hand, specific assignments at 2770 cm⁻¹ (–N(CH₃)₂) groups, are also discernible in both copolymers and in the p(DMAEMA) homopolymer. The increased prominence of the band in the 2800–3000 cm⁻¹ region and the enhanced vibrational assignment at 1466 cm⁻¹ both indicate successful oleic acid incorporation into the final p(GMA-OA)-b-p(DMAEMA) product

6.3.4 GPC analysis

GPC analysis was considered in order to obtain information regarding the chain length distribution of the intermediates and final products involved in the synthesis of the target p(GMA-OA)-b-p(DMAEMA) copolymers. To this end, two alternative GPC systems running with dimethylformamide and chloroform were employed, as outlined in the experimental section. It should be noted that the GPC analysis of amphiphilic copolymers is generally problematic due to the significant solubility limitations of the materials in

conventional GPC solvents, as well as the multiple interaction effects between the copolymer's different domains (e.g., hydrophilic/hydrophobic segments) and the sorbent particles in the GPC columns. Therefore, the GPC analysis of these block copolymers often provides information about an apparent chain length distribution rather than an absolute one. Nevertheless, this information is still useful because it allows different block amphiphilic copolymers of the same kind to be compared.

Figures 6-8 to 6-11 and **Table 6-6** provide an overview of the GPC analysis results for the characterization of various homopolymers and copolymers involved in this research. The ATRP and RAFT synthesized homopolymers of p(GMA) and p(DMAEMA), products H1 to H7 in **Table 6-2**, were analyzed in DMF due to significant solubility limitations in chloroform. **Figure 6-8** presents the SEC traces observed for the p(GMA) homopolymers synthesized by ATRP and RAFT under conditions H1 and H2. The polydispersity index is estimated to be around 1.3 for both the ATRP (H1) and RAFT (H2) products, with weight-average molecular weights of 2.6×10^4 and 1.8×10^4 , respectively. Both homopolymers are suitable for further processing with the introduction of the p(DMAEMA) segment. Furthermore, these p(GMA) homopolymers exhibited a simple isolation process and a reasonable yield was achieved (77% and 56% for H1 and H2, respectively, as detailed in **Table 6-6**). **Figure 6-9**, on the other hand, presents the SEC traces observed for the p(DMAEMA) homopolymers synthesized by ATRP and RAFT under conditions H3 and H4. The polydispersity is estimated to be around 1.5 and 1.2 for these products, and the molecular weight was measured to be in a lower range, compared with the p(GMA) homopolymers (see **Table 6-2**). A more laborious isolation process was needed with the p(DMAEMA) homopolymers (see Experimental section) and lower yields were measured (47% and 38% for H3 and H4, respectively). Product H5 was investigated as an alternative to RAFT-synthesized p(GMA), using DBZT trithiocarbonate as the chain transfer agent. However, poor solubility in DMF was observed, indicating the possible formation of a high-molecular-weight polymer due to inefficient RAFT polymerization. GPC analysis of the soluble fraction (see **Figure D-13** in the Supplementary Information and **Table 6-6**) suggests that control of RAFT polymerization of GMA with DBZT is lower than with the CPDT chain transfer agent. H6 and H7 in **Table 6-2** were examined as possible alternative routes in ATRP for the synthesis of p(GMA) and p(DMAEMA) homopolymers, with PMDETA acting as the ligand instead of BPY. However, under the working conditions used, these products also

exhibited limited solubility in DMF and poor control of the polymers' molecular architecture was observed (see **Figure D-13** and **Table 6-6**).

In light of the aforementioned results concerning the p(GMA) homopolymers obtained via ATRP and RAFT polymerization, the H1 and H2 products were identified as the primary building blocks for synthesizing the desired p(GMA)-*b*-p(DMAEMA) copolymers, as outlined in **Table 6-3** and illustrated in **Figure 6-4**. The p(GMA) homopolymers were employed as either a macroinitiator (H1) in the ATRP process or a macro-chain transfer agent (H2) in the RAFT mechanism, with the objective of building the p(DMAEMA) block within the desired p(GMA)-*b*-p(DMAEMA) copolymers. The resulting copolymers C1, C2 and C3 (**Table 6-3**) were also analyzed by GPC (see also the FTIR characterization previously detailed) and the results for C2 and C3 (ATRP synthesized) are presented in **Figure 6-10** and **Table 6-6**. **Figure 6-10** shows the SEC traces for C2 and C3 in DMF (solubilization of the copolymers in chloroform was not possible) and the corresponding analysis for the H1 - p(GMA) homopolymer is also shown. Compared to the starting p(GMA) homopolymer, a shift to the high molecular weight region is observed for the copolymers, demonstrating the modification of the starting p(GMA) with the introduction of the p(DMAEMA) block (see also the average properties of C2 and C3 compared to H1 in **Table 6-6** as well as the FTIR results in **Figures D-9** and **D-10**). In contrast to C2 and C3, the C1 copolymer obtained from H2 through RAFT polymerization could not be properly solubilized for GPC characterization, indicating lower control of the RAFT process involved in the formation of the p(GMA)-*b*-p(DMAEMA) copolymer, with possible formation of free p(DMAEMA) polymer via uncontrolled radical reaction, as previously examined with the corresponding FTIR analysis (**Figure D-8**). Note also the significant increase in mass observed with C1 compared to C2 and C3, which is also consistent with the formation of free p(DMAEMA) via FRP during the RAFT process.

The incorporation of oleic acid into the p(GMA) homopolymers H1 and H2 was considered as a possible intermediate step in the formation of the final p(GMA-OA)-*b*-p(DMAEMA) copolymers. This process is detailed in **Table 6-4** and illustrated in **Figure 6-2** for the formation of the p(GMA-OA) block. FTIR analysis demonstrates successful oleic acid incorporation in H1 and H2 to form OAI1 and OAI2, respectively (see **Figure D-11**). However, limited solubility was observed in DMF, chloroform and other common solvents with products OAI1 and OAI2. This prevented GPC characterization of these

products by SEC, as well as proceeding easily to the next step of incorporating the p(DMAEMA) block into the p(GMA-OA) products.

Table 6-5 shows the synthesis conditions used in this study to produce the desired p(GMA-OA)-*b*-p(DMAEMA) copolymers. Products OAP1 to OAP3 were obtained by incorporating oleic acid into C1 to C3 p(GMA)-*b*-p(DMAEMA) copolymers, as illustrated in **Figure 6-5**. Product OAP4 is the result of a process involving the simultaneous attachment of the p(DMAEMA) block to the starting p(GMA) homopolymer (H1) via ATRP, alongside the incorporation of OA into the same p(GMA) segment. This process is depicted in **Figure 6-6**. Among the OAP1 to OAP4 p(GMA-OA)-*b*-p(DMAEMA) copolymers only OAP4 could be partially solubilized in chloroform for GPC analysis which result is presented in **Figure 6-11**. The recorded RI and light scattering signals confirm the presence of a polymer population with an apparent $\bar{M}_w = 2.5 \times 10^4$ and PDI = 1.7. Note also the FTIR results confirming the presence of a p(DMAEMA) block in OAP4 and the incorporation of OA, similarly to products OAP1 to OAP4 (see **Figure D-12**). A process similar to that considered for OAP4, which used RAFT polymerization, was found to have low polymerization efficiency.

Considering the molecular weight of the repeating unit in p(GMA) and that of OA, an ideal stoichiometric reaction between the epoxy groups and the hydroxyl groups in the fatty acid would result in a limiting value for mass increase of close to 198% (mass increase percentage defined as $100 \times (m_{p(GMA-OA)} - m_{pGMA}) / m_{pGMA}$). In order to decrease the side reactions of the epoxide groups, the reactions for the incorporation of OA were performed with a stoichiometric excess of this component close to 3. Some of the final products exceeded the 198% limit (see **Table 6-5**), which is likely due to the difficulty of removing unreacted OA from the polymer matrix. Taking more stringent cleaning steps should overcome this issue, but the presence of oleanolic acid in the final products does not limit the application of the p(GMA-OA)-*b*-p(DMAEMA) copolymers for the encapsulation and release of hydrophobic molecules, as described below. In **Table 6-7** is presented a comparison of the different synthetic approaches used here for the incorporation of OA in amphiphilic block copolymers through the reaction with GMA.

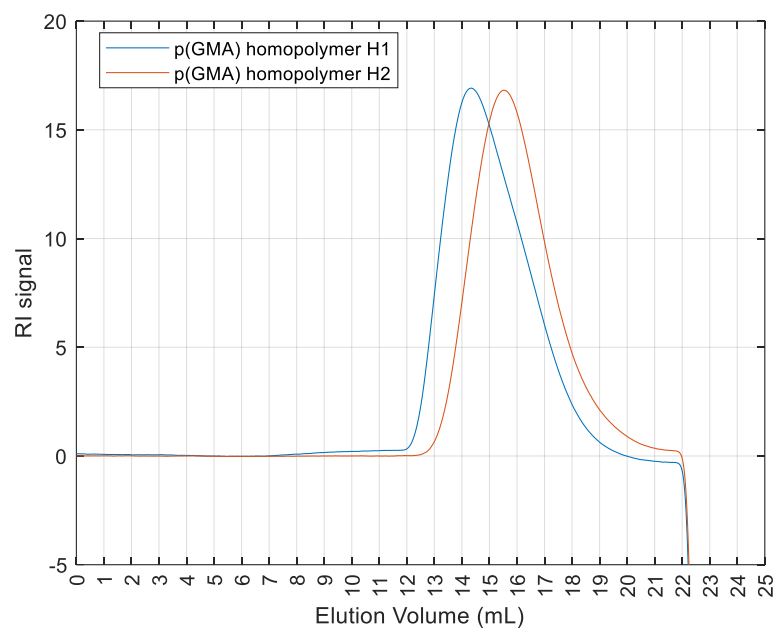


Figure 6-8: SEC/RI traces observed for different p(GMA) homopolymers synthesized by ATRP and RAFT according to the conditions **H1** and **H2** in **Table 6-1**. SEC analysis performed with DMF solvent at 50 °C and flowrate 1 mL/min.

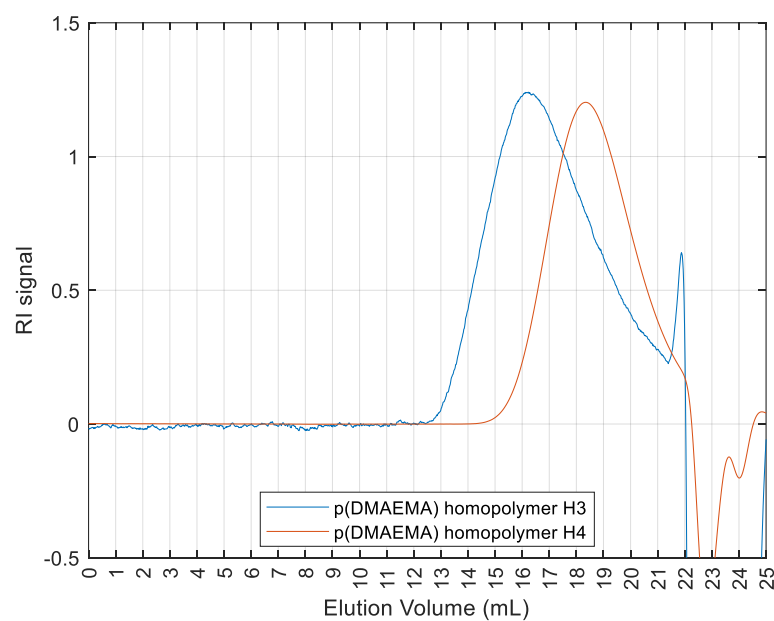


Figure 6-9: SEC/RI traces observed for different p(DMAEMA) homopolymers synthesized by ATRP and RAFT according to the conditions **H3** and **H4** in **Table 6-1**. SEC analysis performed with DMF solvent at 50 °C and flowrate 1 mL/min.

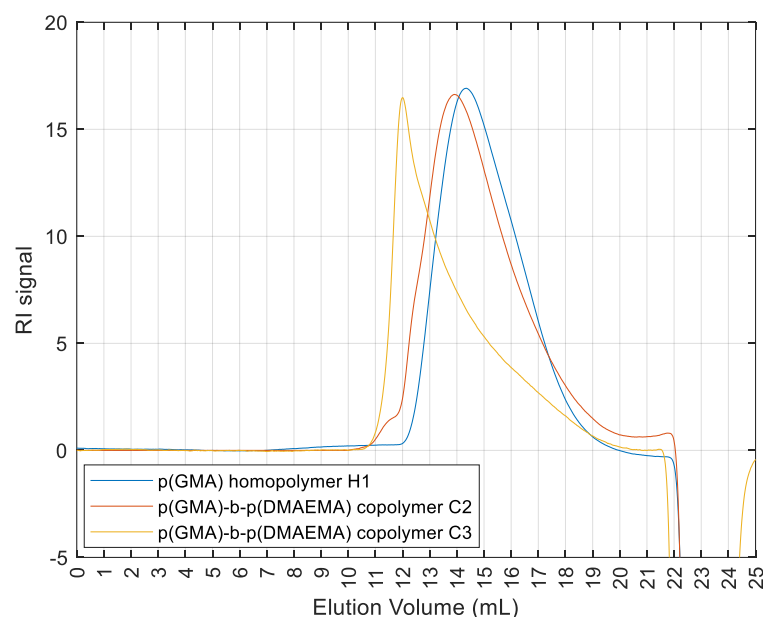


Figure 6-10: SEC/RI trace observed for the **H1** p(GMA) homopolymer synthesized by ATRP and the related p(GMA)-*b*-p(DMAEMA) homopolymers synthesized by ATRP according to the conditions **C2** and **C3** in Table 6-2. SEC analysis performed with DMF solvent at 50 °C and flowrate 1 mL/min.

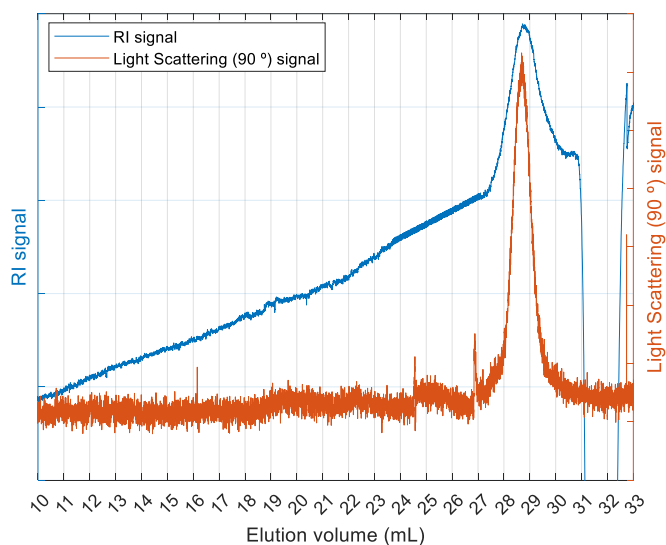


Figure 6-11: SEC/RI trace observed for the **OAP4** p(GMA-OA)-*b*-p(DMAEMA) copolymer synthesized according to the conditions in Table 6-5. SEC analysis performed with chloroform solvent at 30 °C and flowrate 1 mL/min. The refractive index (RI) signal and light scattering (LS) signal at the 90 °detector are here presented.

Table 6-6: Overview of the results obtained with the GPC analysis of various homopolymers and copolymers involved in this research.

Polymer	Product	Polymerization Technique	$\bar{M}_n$	$\bar{M}_w$	PDI
p(GMA)	H1	ATRP	2.0×10^4	2.6×10^4	1.3
p(GMA)	H2	RAFT	1.4×10^4	1.8×10^4	1.3
p(DMAEMA)	H3	ATRP	9.1×10^3	1.4×10^4	1.5
p(DMAEMA)	H4	RAFT	5.7×10^3	7.1×10^3	1.2
p(GMA)	H5*	RAFT	4.2×10^4	6.1×10^4	1.5
p(GMA)	H6*	ATRP	8.4×10^3	1.4×10^4	1.6
p(DMAEMA)	H7*	ATRP	9.0×10^3	3.1×10^4	3.5
p(GMA)- <i>b</i> -p(DMAEMA)	C2	ATRP	2.7×10^4	4.3×10^4	1.6
p(GMA)- <i>b</i> -p(DMAEMA)	C3	ATRP	1.9×10^4	2.9×10^4	1.6
p(GMA-OA)- <i>b</i> -p(DMAEMA)	OAP4*	ATRP	1.5×10^4	2.5×10^4	1.7

* A limited solubility was observed for H5, H6 and H7 in DMF while the analysis for OAP4 was performed in chloroform.

Table 6-7: Brief comparison of the different synthetic approaches considered in this study for the incorporation of oleic acid in amphiphilic block copolymers through the reaction with glycidyl methacrylate.

Reaction route	Observations
Synthesis of methacrylated oleic acid from glycidyl methacrylate and oleic acid, followed by ATRP/RAFT polymerization	Difficult due to branching/crosslinking, low ATRP/RAFT polymerization yield, low solubility in successive steps leading to p(GMA-OA)- <i>b</i> -p(DMAEMA)
Incorporation of oleic acid in ATRP/RAFT p(GMA) homopolymers followed by polymerization with DMAEMA	Limited solubility of the p(GMA-OA) products hindering the incorporating the p(DMAEMA) block
Incorporation of oleic acid in ATRP/RAFT p(GMA)- <i>b</i> -p(DMAEMA) copolymers	p(GMA-OA)- <i>b</i> -p(DMAEMA) was achieved and self-assembly in well-defined spherical aggregates was observed
Simultaneous ATRP attachment of the p(DMAEMA) block and incorporation of oleic acid in p(GMA) homopolymers	p(GMA-OA)- <i>b</i> -p(DMAEMA) was achieved and self-assembly in well-defined spherical aggregates was observed. Hard removal of OA in excess

6.3.5 SEM and TEM analysis

The developed p(GMA-OA)-*b*-p(DMAEMA) copolymers were applied in this work for the encapsulation of oleanolic acid due to their amphiphilicity. Both the formation of solid dispersions and microemulsions/nanodispersions with these materials were scrutinized, as detailed in the section below. SEM and TEM analyses were performed to gain information on the morphological features of these solid-state and liquid dispersions, as presented in **Figures 6-12 and 6-13**.

The solid particles shown in **Figure 6-12** are the result of dispersing the p(GMA-OA)-*b*-p(DMAEMA) copolymers in THF in the presence of oleanolic acid. This is followed by evaporating the solvent in order to create a polymeric carrier. These images reveal amorphous materials that are dispersed in size and form agglomerates with dimensions of up to around 1 μm . At microscale, nucleus of agglomeration can be observed, with sizes ranging from approximately 1 μm (see **Figure 6-12(6)**). Note that these dry particles undergo a significant morphological change when in contact with a solvent, specifically an aqueous mixture. The hydrophilic p(DMAEMA) domain can be solvated by water, resulting in significant swelling of these particles, particularly in acidic environments, due to the ionization of the tertiary amine groups in the DMAEMA polymer moieties. The details of these features are provided below in the context of delivering oleanolic acid using the polymeric carrier p(GMA-OA)-*b*-p(DMAEMA).

The TEM images presented in **Figure 6-13** correspond to the liquid dispersions that were prepared using p(GMA-OA)-*b*-p(DMAEMA) for the encapsulation of oleanolic acid. The copolymers were first dispersed with oleanolic acid in THF, and then transferred to an aqueous solvent via dialysis in a cellulose bag. Notably, **Figures 6-13(1) and 6-13(2)** show well defined spherical particles with size 20 to ~ 100 nm with an external shell with thickness around 5 to 8 nm that were achieved using the p(GMA-OA)-*b*-p(DMAEMA) amphiphilic copolymer OAP1. These results demonstrate the self-assembly ability of the developed amphiphilic materials in the transition from THF to water, forming a hydrophilic corona based on the p(DMAEMA) block and a hydrophobic p(GMA-OA) core, which can encapsulate oleanolic acid.

Figure 6-13(3) also shows the nanodispersion achieved using the p(GMA-OA)-*b*-p(DMAEMA) amphiphilic copolymer, containing spherical particle aggregates dispersed over a range of sizes up to ~ 200 nm.

Interestingly, core/shell nanoparticles with a size of up to 500 nm were observed when the self-assembled aqueous dispersions were generated using a p(GMA-OA)-*b*-p(DMAEMA) material synthesized by ATRP with the simultaneous incorporation of OA (OAP4), as shown in **Figure 6-13**. These results demonstrate that the synthesis conditions influence the molecular architecture of amphiphilic copolymers, which in turn affects the morphology of self-assembled nanostructures. See [26] for more information on the relationship between the size of hydrophobic and hydrophilic blocks in copolymers and the size distribution of aggregates.

Overall, the TEM results presented in **Figure 6-13** demonstrate that the proposed synthesis routes lead to the formation of amphiphilic copolymers of the type p(GMA-OA)-*b*-p(DMAEMA), which are capable of self-assembling in an aqueous environment to form nano-carriers for the encapsulation of hydrophobic molecules, such as oleanolic acid. In general, however, the size and morphology of polymer self-assemblies of this kind (e.g., spherical and cylindrical micelles, vesicles, and single- and multi-chain polymer nanoparticles) depend on their specific molecular architecture [26]. For example, the ratio between the hydrophobic and hydrophilic blocks is crucial in this context [26,46]. Indeed, simulations demonstrate that the molecular structure of amphiphilic block copolymers plays a critical role in the formation of aggregates, particularly regarding the size of the hydrophobic and hydrophilic segments, namely the amphiphile's surface area, the volume and maximum effective length of the hydrophobic chain [26]. The self-assembly behavior of these systems is also affected by the polymer concentration, as well as the concentration and type of other species (e.g., metal ions [46]). Consequently, various approaches are being considered to fine-tune the properties of these advanced materials, including their mechanical properties [47]. Therefore, further refinement of the ATRP and RAFT reaction conditions proposed in this work may be considered to enable even more precise tailoring of the molecular architecture of the p(GMA-OA)-*b*-p(DMAEMA) copolymers, with the aim of designing nano-carriers that exhibit improved features compared to those shown in **Figure 6-13**. Various techniques (e.g., solvent-switch approach, polymer rehydration, etc., [26]) and working conditions (e.g., polymer concentrations) may also be employed to customize these nanostructures.

6.3.6 Application with encapsulation and delivery of oleanolic acid

Oleanolic acid is a pentacyclic triterpenoid found in many plants, including the olive tree, where it occurs alongside oleic acid. Notably, triterpenoids such as oleanolic and maslinic acids have been investigated for use in medicinal chemistry due to their antiproliferative and apoptosis-inducing effects on cancer cells, as well as their potential in HIV chemotherapy and other biological applications [48-58]. However, oleanolic acid is poorly soluble in water (estimated solubility ~ 4.6 mg/L at 20 °C) and is poorly absorbed from the gastrointestinal tract (permeability $\sim 10^{-6}$ cm/s), resulting in an absolute oral bioavailability of around 0.7% for doses of 25 and 50 mg/kg [48,52]. Therefore, many researchers are focusing on developing delivery systems for oleanolic acid in order to enhance its bioavailability and improve the efficacy of therapies. In this context, it is especially important to consider controlled drug delivery systems, which aim to transport drugs directly to target sites while minimizing side effects and reducing the required dosage. Attempts to increase the solubility of oleanolic acid in water have included using solid dispersions, compounding with β -cyclodextrin, and changing the crystal form using spray drying, among other methods. Conversely, the main drug delivery systems that have been explored for the controlled delivery of oleanolic acid are solid lipid nanoparticles, liposomes, microemulsions, nanodispersions, phospholipid complexes, nanostructured lipid carriers, nanoparticles and micelles [52]. Tailored drug delivery systems offer significant advantages over traditional plant-active formulations. These include the potential for intracellular uptake, sustained release and protection against physical and chemical degradation [52]. Polymers play a key role in the development of such kinds of drug delivery systems for oleanolic acid, specifically as carriers in solid dispersions and as components for microemulsions, nanodispersions, nanoparticles or micelles.

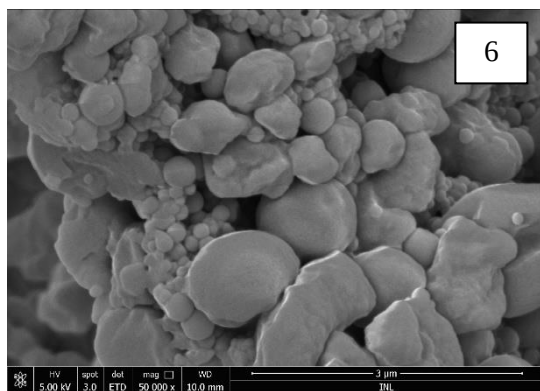
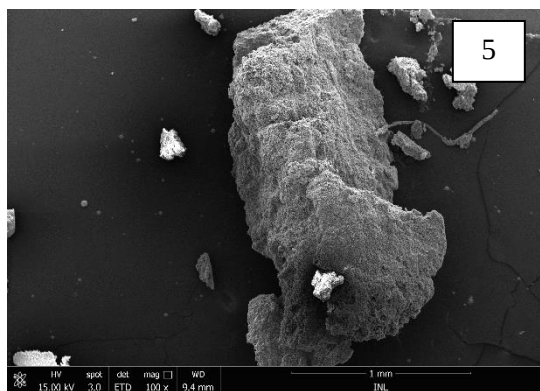
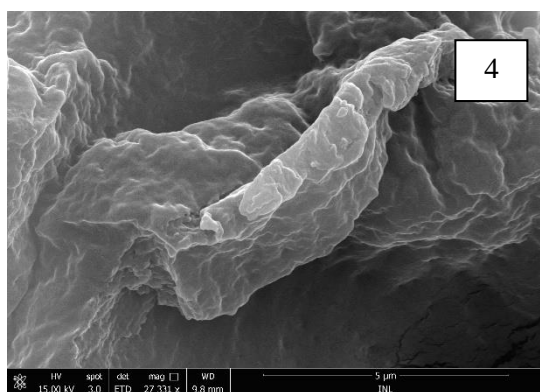
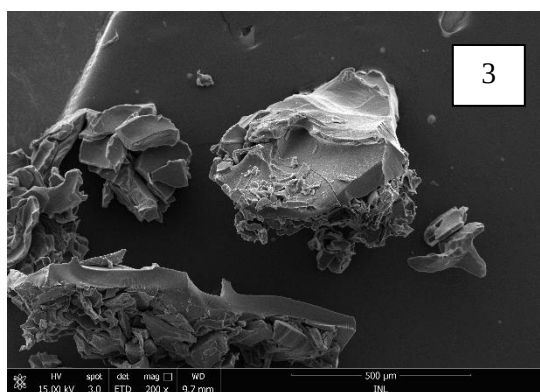
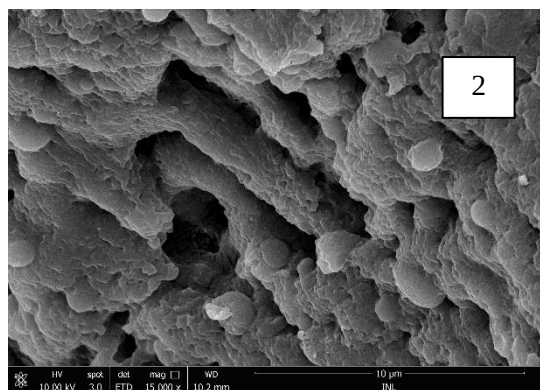
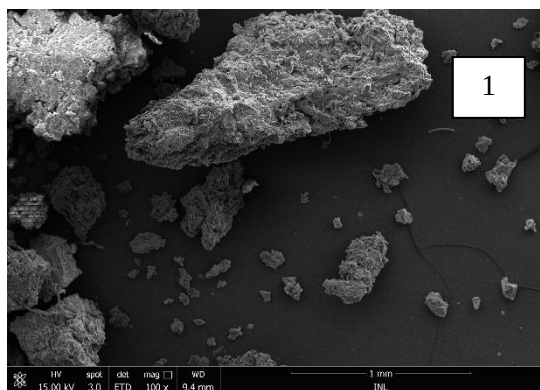


Figure 6-12: SEM images of solid particles of the p(GMA-OA)-b-p(DMAEMA) copolymers synthesized in this research. 1 and 2 – OAP1, 3 and 4 – OAP2, 5 and 6 – OAP4.

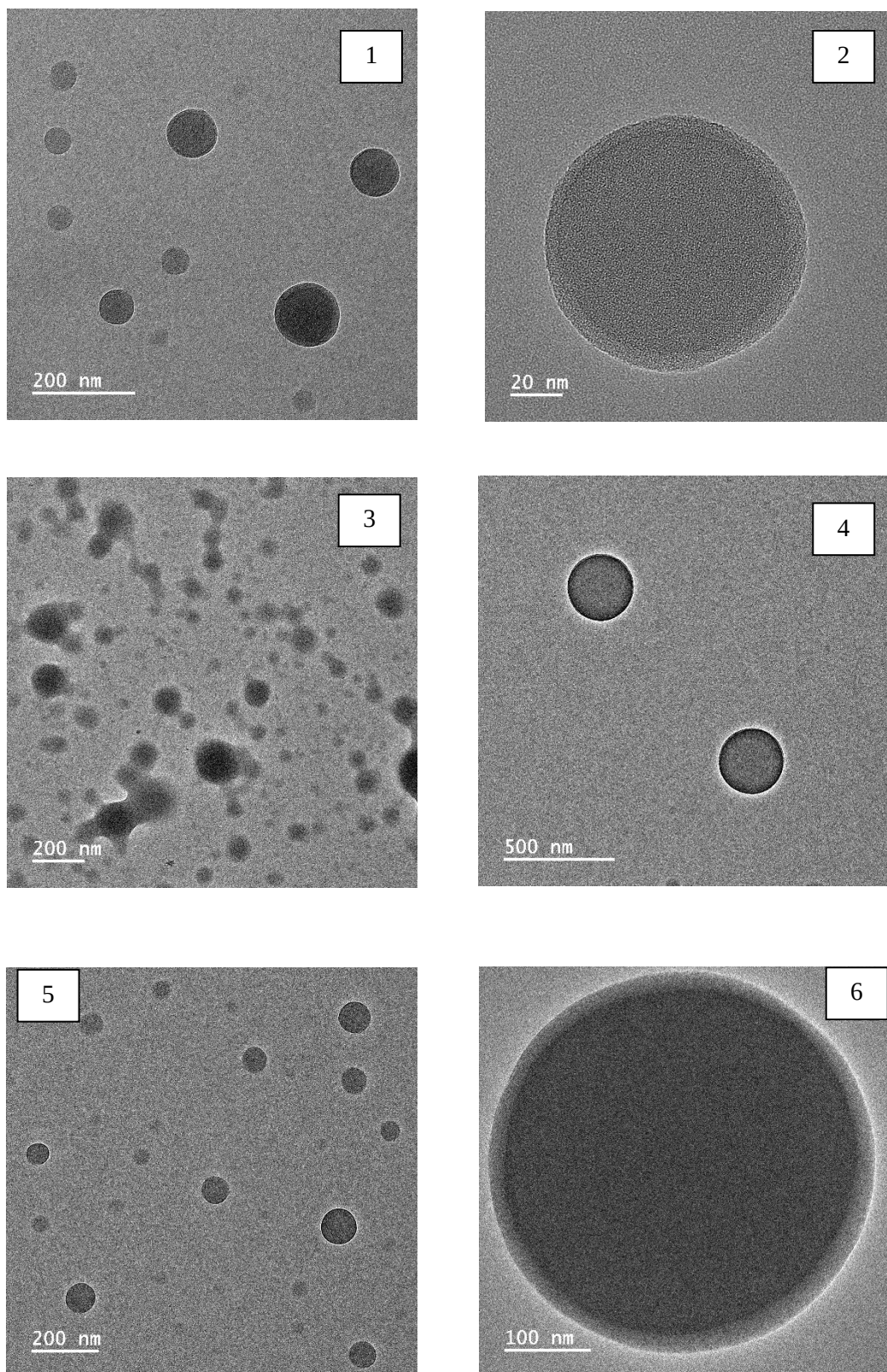


Figure 6-13: TEM images of aqueous dispersions produced with different p(GMA-OA)-b-p(DMAEMA) copolymers synthesized in this research. 1 and 2 – OAP1, 3 – OAP2, 4 – OAP3, 5 and 6 – OAP4 (see **Table 6-5**).

Following this line of thought, the encapsulation and delivery of oleanolic acid using the developed p(GMA-OA)-*b*-p(DMAEMA) copolymers is here analyzed. The low aqueous solubility and limited permeability of oleanolic acid result from the presence of nonpolar functional groups in its structure, specifically the hydrophobic regions of the bulky ring structures characteristic of pentacyclic triterpenoids. The current approach explores the possibility of a strong hydrophobic interaction between the oleic acid moieties incorporated into the p(GMA-OA)-*b*-p(DMAEMA) copolymers and oleanolic acid (see **Figure D-2** depicted in SI). These hydrophobic interactions are believed to facilitate the encapsulation of oleanolic acid in an aqueous environment. The hydrophilic functional groups in the DMAEMA moieties act as the hydrophobic/hydrophilic interface. Simple visual confirmation of the ability of p(GMA-OA)-*b*-p(DMAEMA) copolymers to encapsulate hydrophobic compounds was obtained using Nile Red as a model molecule. Indeed, a color change was observed as the surrounding solvent was varied, starting with a mixture of Nile Red and p(GMA-OA)-*b*-p(DMAEMA) in THF and approaching an aqueous environment through a dialysis process in a cellulose bag (see **Figure D-14** in SI). This color change indicates the encapsulation of Nile Red within the hydrophobic portion of the p(GMA-OA)-*b*-p(DMAEMA) aggregates, specifically the OA-containing moieties.

Encapsulation of oleanolic acid in solid dispersions was performed using p(GMA-OA)-*b*-p(DMAEMA) materials as a polymeric carrier and a solvent evaporation method with THF. On the other hand, microemulsions/nanodispersions with encapsulated oleanolic acid were also prepared using p(GMA-OA)-*b*-p(DMAEMA) through dispersion in THF followed by dialysis in cellulose bag (see experimental section). Precipitation of material during transition to an aqueous environment was observed to a small extent, namely with OAP1, indicating that the formation of polymer chains that are not suitable for self-assembly is plausible. As discussed above, the presence of free p(DMAEMA) is possible in the case of OAP1. In both cases, the amount of oleanolic acid encapsulated was estimated by dispersing the solid particles in a large excess of methanol [53], followed by HPLC-DAD analysis of the resulting liquid phase (see the quantification of oleanolic acid by HPLC-DAD in the SI, **Figure D-17**). Concentrations of oleanolic acid in solid dispersions were observed to be up to 0.21 mg/mg, while concentrations in particle dispersions were estimated to be up to 0.17 mg/mL.

Dried solid particles and dispersions of p(GMA-OA)-*b*-p(DMAEMA) with oleanolic acid encapsulated were thereafter scrutinized as drug delivery systems, specifically

concerning the dynamics of oleanolic acid release. Following recent related studies, EtOH/PBS 30/70 was considered as the reference medium for oleanolic acid release [53]. Solid particles were directly dispersed in EtOH/PBS 30/70 while aqueous dispersions were dialyzed against EtOH/PBS 30/70. In both cases, the liquid phase was sampled along the time (exterior of dialysis bag with dispersions) for HPLC-DAD quantification of the contained oleanolic acid. **Figure 6-14** shows the dynamics of oleanolic acid release that were measured under these conditions. A logarithmic scale is used to represent the fraction of released oleanolic acid, which highlights the differences in magnitude observed for the various surrounding conditions (constant temperature of 25 °C). Indeed, a sustained release of oleanolic acid is observed in EtOH/PBS 30/70 for at least 24 hours (region **I**). For solid particles, a steady value for OA delivery of around 1% (concentration in the liquid ~6 mg/L) is observed, while for aqueous dispersions in the same conditions, a negligible value of less than 0.1% is estimated (note the impact of diffusion through the cellulose bag on this system). To demonstrate the high amount of oleanolic acid encapsulated in the particles, the release was triggered by altering the composition of the surrounding liquid phase. This was achieved by adding methanol to the system to create a mixture of (EtOH/PBS 30/70)/MeOH 50/50 (region **II**). A substantial increase in oleanolic acid release was observed, with measured values ranging along the time from 20 to 70% for solid particles, and from 7 to 30% for dispersions. An additional delivery stage was induced by the further addition of methanol, which changed the composition of the liquid phase to (EtOH/PBS 30/70)/MeOH 20/80 (region **III**). The amount released was found to approach 100% for the solid particles, while a steady value of around 70% was achieved with the dispersion.

To assess the potential impact of pH on oleanolic acid release due to the presence of DMAEMA moieties in the p(GMA-OA)-*b*-p(DMAEMA) copolymers used for encapsulation, experiments involving fluids at pH 3 and 7.6 were conducted. The temperature was also increased to 40 °C because p(DMAEMA) is thermoresponsive with lower critical solution temperature (LCST) at around 50 °C (aggregation observed because of hydrogen bond disruption). An acetate buffer was used to achieve a pH of 3, while a PBS buffer was used to achieve a pH of 7.6. **Figure 6-15** presents the results obtained under these conditions, showing a significant difference in oleanolic acid release for both solid particles and dispersions when the pH of the surrounding fluid is varied. Indeed, a faster release dynamic is observed with both solid particles and dispersions when the surrounding pH is 7.6, compared with the corresponding delivery profiles at pH

3. This is highlighted in **Figure 6-15** during regime **I**, where EtOH/buffer 30/70 was used. Note the negligible release of oleanolic acid at pH 3 over a period of up to around 100 hours, for both solid particles and dispersions. Notably, the morphology of the polymer particles and dispersion stability is also affected by the pH, with observation of swelled material at pH 3 and collapsing of the material at pH 7.6, as showed in **Figures D-15 and D-16** of the SI. These observations confirm the pH responsiveness of the copolymers, which is consistent with the expected behavior induced by p(DMAEMA): swelling in acidic conditions and collapsing at alkaline pH due to ionization and deionization, respectively. The lower amount of oleanolic acid released in acidic conditions (pH 3) is likely due to the low ionization of this compound compared to pH 7.6, resulting in reduced solubility in the surrounding fluid. The confirmation for a substantial additional amount of oleanolic acid encapsulated in the solid particles and dispersions was again worked out by changing the composition of the release fluid through the addition of methanol. The correspondent results are also presented in **Figure 6-15** with the inclusion of region **II** (fluid composition (EtOH/buffer 30/70)/MeOH 50/50) and **III** (fluid composition (EtOH/buffer 30/70)/MeOH 20/80). Note the relevant boost in oleanolic acid release observed in these regions even for the systems with the initial use of EtOH/buffer 30/70 at pH 3. This is a consequence of the increase of solubility of oleanolic acid in the surround fluid when methanol is added, thus confirming the potential of the encapsulation vehicles to deliver high amounts of this molecule. In region **III** (highest composition in methanol) similar release fractions are observed for the initial systems at pH 3 and pH 7.6, approaching 100% for both solid particles and 70% for both dispersions.

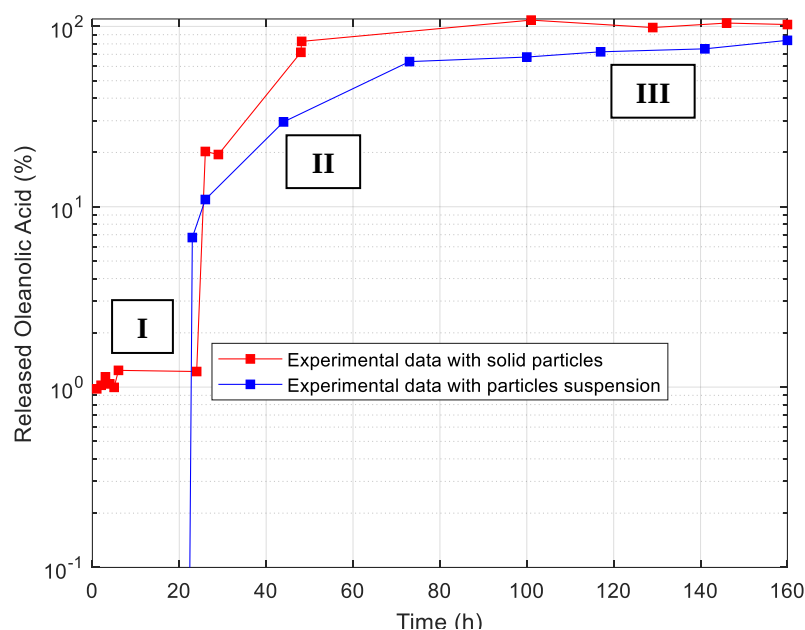


Figure 6-14: Measured dynamics of oleanolic acid release from encapsulation solid particles and particle dispersions of copolymers of p(GMA-OA)-b-p(DMAEMA). The composition of the release fluid was changed along the time according to the following: **I** – EtOH/PBS 30/70, **II** – (EtOH/PBS 30/70)/MeOH 50/50, **III** – (EtOH/PBS 30/70)/MeOH 20/80. Experiments with PBS solution at pH=7.6 and T=25 °C. The concentration of oleanolic acid in the release fluid was measured by HPLC-DAD. Log scale is used to represent the released oleanolic acid, highlighting the observed differences in magnitude.

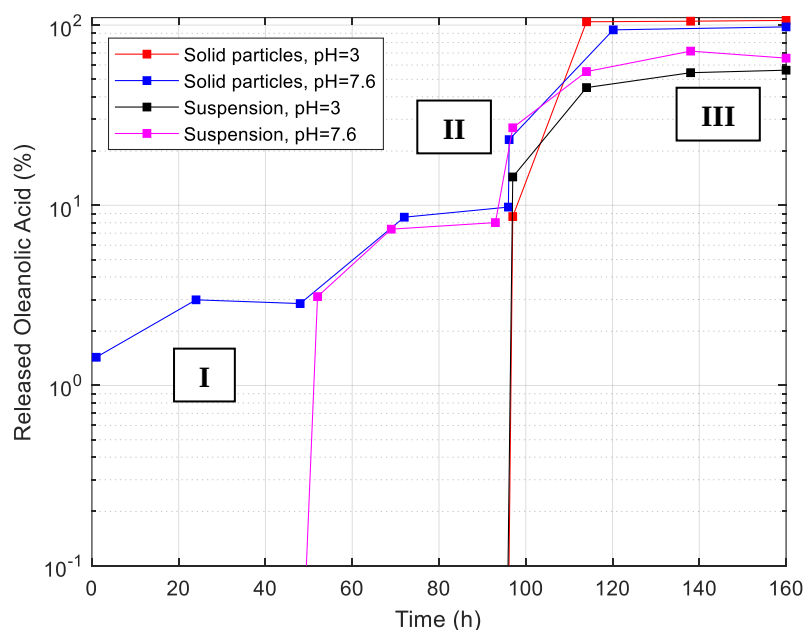


Figure 6-15: Measured dynamics of oleanolic acid release from encapsulation solid particles and particle dispersions of copolymers of p(GMA-OA)-b-p(DMAEMA). The composition of the release fluid was changed along the time according to the following: **I** – EtOH/buffer 30/70, **II** – (EtOH/buffer 30/70)/MeOH 50/50, **III** – (EtOH/buffer 30/70)/MeOH 20/80. Experiments with PBS solution at pH=7.6 and acetate buffer at pH=3. All the experiments at T=40 °C. The concentration of oleanolic acid in the release fluid was measured by HPLC-DAD. Log scale is used to represent the released oleanolic acid, highlighting the observed differences in magnitude.

6.4. Conclusion

This research focused on assessing different reaction routes using glycidyl methacrylate to synthesize amphiphilic block copolymers that incorporate oleic acid in the hydrophobic part. The hydrophilic segment was built up with the 2-(dimethylamino)ethyl methacrylate monomer. The main goals of this work were to find reaction routes that lead to low-impact branching and/or crosslinking mechanisms involving epoxides and oleanolic acid, and to synthesize p(GMA-OA)-*b*-p(DMAEMA) copolymers in a straightforward and scalable way. The synthesis of block copolymers was considered using both ATRP and RAFT polymerization.

The reaction route involving the synthesis of methacrylated oleic acid from glycidyl methacrylate and oleic acid proved problematic due to branching/crosslinking, a low ATRP/RAFT polymerization yield, and the difficulty of solubilizing the reactants in successive steps, resulting in p(GMA-OA)-*b*-p(DMAEMA) copolymers. However, higher synthesis efficiency was observed when glycidyl methacrylate moieties were modified by post-polymerization with oleic acid. This research identified the following routes for efficiently synthesizing the desired p(GMA-OA)-*b*-p(DMAEMA) materials:

- a) RAFT or ATRP synthesis of the p(GMA) block, followed by attachment of the p(DMAEMA) block and final incorporation of oleic acid into the p(GMA)-*b*-p(DMAEMA) block copolymers to produce the desired p(GMA-OA)-*b*-p(DMAEMA) material.
- b) ATRP synthesis of the p(GMA) block, followed by simultaneous attachment of the p(DMAEMA) block and incorporation of oleic acid to get the p(GMA-OA)-*b*-p(DMAEMA) material.

Working with DMSO solvent and a stoichiometric excess of oleic acid compared to epoxide groups was found to be particularly important for reducing branching and crosslinking during the step involving the incorporation of oleic acid into the copolymers. FTIR and GPC analyses were used to assess the progress of the chemical composition and molecular architecture of the intermediate homo- and co-polymers, and to demonstrate the successful production of the intended p(GMA-OA)-*b*-p(DMAEMA) materials. FTIR analysis demonstrated the total conversion of epoxides through reaction with oleic acid. The synthesized amphiphilic block copolymers were considered as a potential

vehicle for encapsulating and delivering oleanolic acid, a pentacyclic triterpenoid that is found in many plants, including the olive tree, where it occurs alongside oleic acid. SEM and TEM analyses were performed to elucidate the morphology of these vehicles when considering the use of solid polymer dispersions and aqueous dispersions for encapsulating and delivering oleanolic acid. The ability of the p(GMA-OA)-*b*-p(DMAEMA) copolymers to self-assemble in an aqueous environment was demonstrated by observing nano-dispersions, including well-defined spherical particles up to ~100 nm in size with an external shell around 5–8 nm thick. Spherical aggregates with a size of up to ~500 nm were observed in aqueous dispersions when using a p(GMA-OA)-*b*-p(DMAEMA) copolymer synthesized through the simultaneous attachment of the DMAEMA block and the incorporation of oleic acid. The loading capacity of oleanolic acid was estimated to be 0.21 mg/mg in solid polymer dispersions, and 0.17 mg/mL in aqueous particle dispersions. These are relevant values when considering the use of the materials to release oleanolic acid in aqueous environments. The dynamics of oleanolic acid release was measured under different conditions, including changes in solvent composition, pH and temperature. The results demonstrate sustained release of the encapsulated oleanolic acid and morphological transitions induced by pH/temperature changes due to the ionization/deionization of the tertiary amines contained within the hydrophilic p(DMAEMA) domain in acidic/alkaline conditions.

Overall, the proposed synthesis routes lead to the efficient formation of amphiphilic copolymers of the type p(GMA-OA)-*b*-p(DMAEMA), thus creating hybrid natural-synthetic tailored materials. These copolymers are capable of self-assembling in an aqueous environment to form nano-carriers that can encapsulate hydrophobic molecules such as oleanolic acid. Further refinement of the ATRP and RAFT reaction conditions presented in this work could improve the tailoring of the molecular architecture of the p(GMA-co-OA)-*b*-p(DMAEMA) copolymers and customization of nano-carriers, including their mechanical properties and drug release capabilities. Various alternative techniques (e.g., solvent-switch approach, polymer rehydration, etc.,) and working conditions, such as the polymer concentration and formulation of the dispersion, may also be employed to customize these nanostructures. The application of the class of amphiphilic copolymers here developed to the sustained release of other kinds of natural hydrophobic molecules, namely related pentacyclic triterpenoids with proved therapeutic effects, such as maslinic acid or betulinic acid, is also another future promising line of research in this area.

6.5 References

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CHAPTER 7. Concluding Remarks and Future directions

7.1 Concluding remarks

The growing demand for sustainable strategies to valorize agricultural sub-products has stimulated the development of advanced materials capable of selectively recovering valuable bioactive compounds. In this context, controlled radical polymerization (CRP) techniques, particularly atom transfer radical polymerization (ATRP) and reversible addition–fragmentation chain transfer (RAFT), provide powerful tools for designing functional polymeric and hybrid systems with tailored properties. Cellulose and other natural substrates are ideal for such developments as they combine renewable resources with advanced synthetic approaches. This thesis explored these strategies to create innovative adsorbents and delivery systems that bridge the gap between material design and practical applications in biomass valorization and circular bioeconomy.

Chapter 2 demonstrates the functionalization of microcrystalline cellulose with poly(4-vinylpyridine) (P(4VP)) brushes via ATRP to yield hybrid adsorbents for the recovery of polyphenols from grape pomace, almond residues, almond wastewater and olive oil effluents. The material's morphology was ideal for continuous sorption/desorption, outperforming commercial resins, native cellulose, and cellulose-polyacrylonitrile hybrids. A polyphenol retention rate of ~70–77% was achieved due to the strong binding of pyridyl groups. Simple sorption and desorption made it possible to separate compounds such as malvidin-3-O-glucoside, isorhamnetin-3-O-rutinoside and secoiridoid/flavonoid-rich fractions. This demonstrates the potential of MCC-P(4VP) in downstream processing and biorefining. The results also highlighted ATRP as a versatile tool for designing natural–synthetic adsorbents. However, future research is needed into molecular imprinting, the ratio of cellulose to synthetic materials, cost–benefit analysis and controlling the population of polymers in systems involving hydrogen-bonding monomers such as 4VP. Based on this results, **Chapter 3** explores surface-molecularly imprinted cellulose–synthetic hybrid particles synthesized via ATRP using quercetin as a template. These particles outperformed both native cellulose and non-imprinted hybrids due to the presence of quercetin-shaped cavities and strong 4VP–flavonoid interactions. The particles enabled the selective enrichment of luteolin-7-O-glucoside, apigenin-7-O-glucoside and quercetin in olive leaf extracts, with enrichment factors of up to four. The study confirmed the suitability of hydroalcoholic solvents for processing these materials and demonstrated their potential for scaling up for industrial biomass valorization.

Expanding on the molecular imprinting approach of Chapter 3, **Chapter 4** integrates pyridyl functionalities to enhance selectivity and performance in winemaking residues. The chapter describes the synthesis of hybrid cellulose–synthetic particles containing pyridyl groups and quercetin-imprinted binding sites via ATRP. The functionalization process was confirmed, and SEM analysis revealed clear surface modification compared with native cellulose. The materials exhibited strong polyphenol binding (IF ~8) and maintained high retention, even in hydroalcoholic solvents. The materials were successfully applied to winemaking residues, enabling the efficient fractionation of flavonols and anthocyanins with enrichment factors of up to 5 for quercetin, 12 for quercetin-3-O-glucuronide and 4 for flavonols such as myricetin and quercetin glucosides. This work reinforced a sustainable approach by combining cellulose-based adsorbents with intensified sorption/desorption processes and the valorization of agro-industrial residues, in line with the UN 2030 Agenda. After addressing the development of hybrid cellulose–synthetic adsorbents for polyphenol fractionation in Chapters 3 and 4, **Chapter 5** focuses on the use of gram-scale synthetic molecularly imprinted adsorbents for the valorization of winemaking by-products, specifically spent diatomaceous earth from wine filtration, and their application in a pilot sorption/desorption prototype. An optimized inverse suspension polymerization process was used to synthesize a gram-scale molecularly imprinted sorbent that preserves pyridyl–polyphenol interactions and is suitable for large-scale continuous processes. This material was then used in a pilot-scale sorption/desorption prototype with hydroalcoholic solvents to produce phenolic-enriched fractions. These fractions exhibited significantly higher total phenolic content and antioxidant activity than the initial extract. Notably, the most enriched fraction exhibited ~3.8-fold higher TPC and up to 6.4-fold higher antioxidant activity. Some of the purified fractions even outperformed raw grape pomace extracts. This study integrated material development, process design and real-extract applications to present a proof of concept with the potential to be extended to other biomass sources within the framework of sustainability and circular bioeconomy. Having concluded the development of hybrid sorbent materials in Chapter 5, **Chapter 6** turns to the design of release vehicles, focusing on amphiphilic block copolymers as nanocarriers for hydrophobic bioactive compounds. This chapter demonstrated the feasibility of synthesizing amphiphilic block copolymers of the type P(GMA-OA)-b-P(DMAEMA) by combining post-polymerization modification of glycidyl methacrylate with oleic acid and controlled radical polymerization techniques (ATRP and RAFT). The most effective methods involved the

sequential or simultaneous incorporation of DMAEMA and oleic acid. Using dimethyl sulfoxide (DMSO) as the solvent and ensuring there was an excess of oleic acid was crucial to minimizing branching and crosslinking. FTIR and GPC analyses confirmed the successful preparation of the intended copolymers. These materials self-assembled into well-defined nanoaggregates, enabling the encapsulation and controlled release of oleanolic acid with a high loading capacity. Release studies revealed responsiveness to pH and temperature, which is linked to the ionization/deionization of the DMAEMA segment. These results validate amphiphilic block copolymers as a versatile vehicle for hydrophobic bioactive compounds and highlight the importance of fine-tuning polymerization and formulation conditions to optimize performance.

This thesis demonstrates how natural substrates combined with controlled radical polymerization enable the design of advanced hybrid adsorbents and delivery vehicles for biomass valorization. The developed materials proved effective for selectively recovering, purifying, and delivering bioactive compounds from agriculture sub-products, while showing compatibility with scalable processes. These findings highlight their potential as enabling technologies for sustainable biorefining, contributing to the circular bioeconomy and global sustainability goals.

7.2 Future Directions

Based on the results presented in this thesis, there are several potential areas of research that could further advance the development and application of hybrid and polymer-based materials for biomass valorization.

First, the design of next-generation hybrid adsorbents should focus on integrating molecular recognition features with tailored cellulose–synthetic architectures. Strategies such as combining ATRP grafting with surface imprinting, or tuning cellulose/synthetic incorporation ratio, may enhance selectivity while maintaining sustainability. A comprehensive cost–benefit and life-cycle analysis is to be also necessary to assess the industrial feasibility of these adsorbents for large-scale applications.

Secondly, scaling up sorption processes with hybrid materials remains a critical step. While the successful operation of a pilot sorption/desorption prototype demonstrates technical feasibility, future work should address long-term performance, regeneration cycles and integration into continuous biorefining operations. Applying this approach to

other agro-industrial residues could increase its impact and enhance resource recovery in line with circular bioeconomy strategies.

Thirdly, the development of amphiphilic block copolymers as release vehicles creates new opportunities for the design of multifunctional systems. Future studies should optimize polymerization conditions to refine the molecular architecture and explore alternative self-assembly techniques (e.g. solvent-switch or rehydration methods) to create a greater variety of particle morphologies. These systems could be extended beyond oleanolic acid to encapsulate a wide range of hydrophobic bioactive, and further investigation could be conducted into their behavior in response to stimuli under physiological conditions.

Fourthly, integrating artificial intelligence (AI) and machine learning (ML) could accelerate material design and process optimization. Predictive models trained on structure–activity relationships, adsorption kinetics or process parameters could support the virtual screening of monomer–template systems, the optimization of polymerization conditions and even real-time process control. Using AI/ML tools would reduce the experimental workload, improve reproducibility and pave the way for adaptive, smart separation systems

Finally, exploring hybrid material platforms that combine sorption, fractionation and controlled release functionalities within a single system shows strong potential. Such multifunctional materials could significantly improve the efficiency of biorefining processes by facilitating the extraction and delivery of bioactive compounds.

Overall, these future directions aim to expand the scope and applicability of the materials developed in this work, consolidating their role as enabling technologies for sustainable biomass valorization and contributing to the advancement of the circular bioeconomy.

Overall, the aim of these future directions is to expand the scope and applicability of the materials developed in this work, thereby consolidating their role as enabling technologies for the sustainable utilization of biomass and contributing to the development of the circular bioeconomy.

Appendices

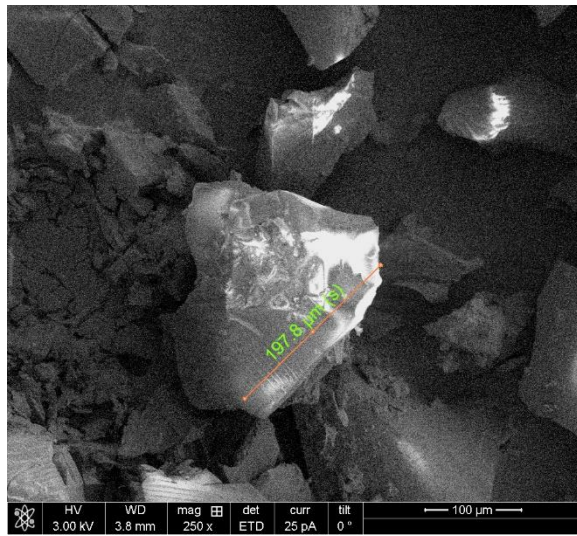
Appendix A - Supporting Information for Chapter 2

Table A-1: Different sets of synthesis conditions used with the ATRP grafting of P(4VP) and PAN on MCC. The initial concentration of macro-initiator MCC-Br was changed along these runs while the remaining conditions were the same (see also Table 1 in the main document). The estimated global monomer conversion (free + grafted polymer), as well as the estimated mass grafting ratio in the hybrid particles (mass synthetic polymer per mass of cellulose), are also presented for each run.

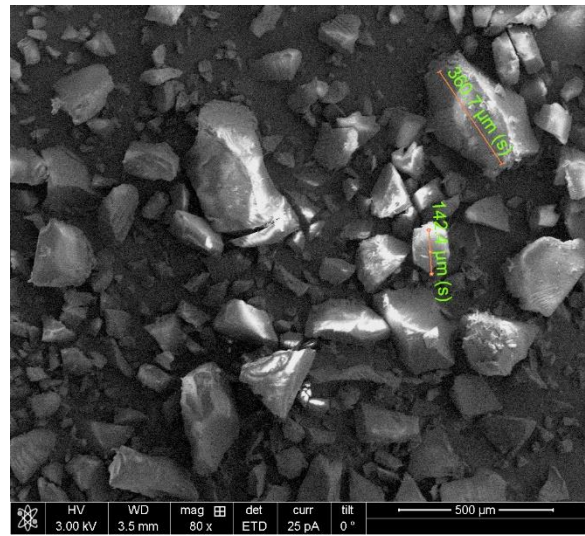
Product	Initial MCC-Br concentration (g/L)	Estimated global monomer conversion	Estimated mass grafting ratio in the hybrid particles (mass synthetic polymer/cellulose)
MCC-P(4VP)-A	9.511	0.50	9
MCC-P(4VP)-B	4.750	0.34	38
MCC-P(4VP)-C	0.979	0.18	100
MCC-PAN-A	12.365	0.26	1.8
MCC-PAN-B	6.200	0.17	1.7
MCC-PAN-C	1.251	0.21	6.2

Table A-2: Results for the N₂ adsorption/desorption measurements with representative materials considered in this research.

Product	BET specific surface area m ² /g	Micropores volume mm ³ /g
Native MCC	1	-
MCC-P(4VP)	1	-
MCC-PAN	3	-
P(4VP-EGDMA)	-	-
P(4VP-AN-DVB)	4	-



(a)



(b)

Figure A-1: SEM images showing the morphology of synthetic adsorbent particles synthesized in this research. (a) P(4VP-EGDMA) (b) P(4VP-AN-DVB).

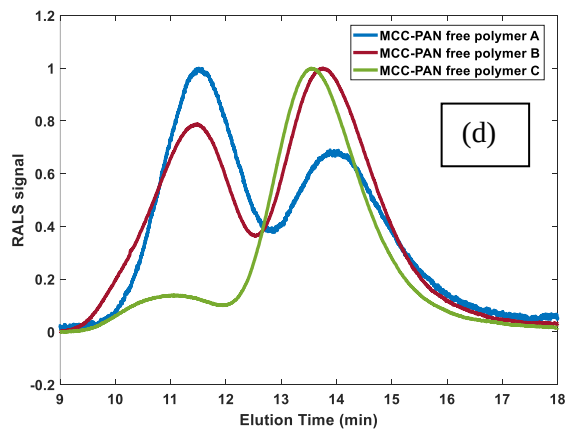
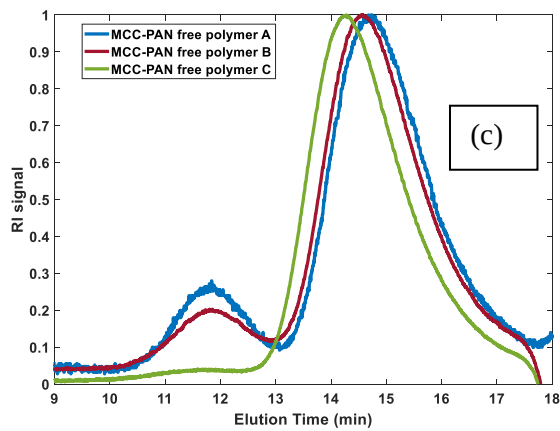
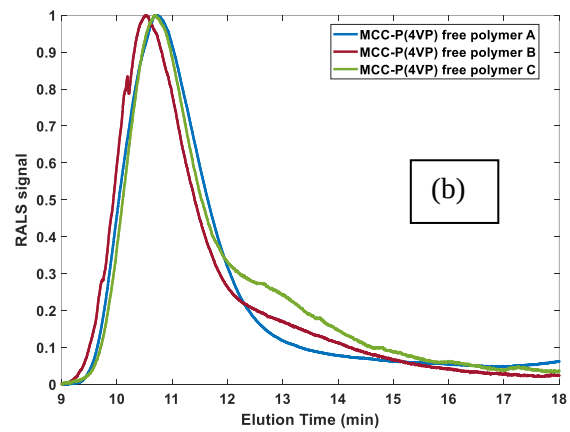
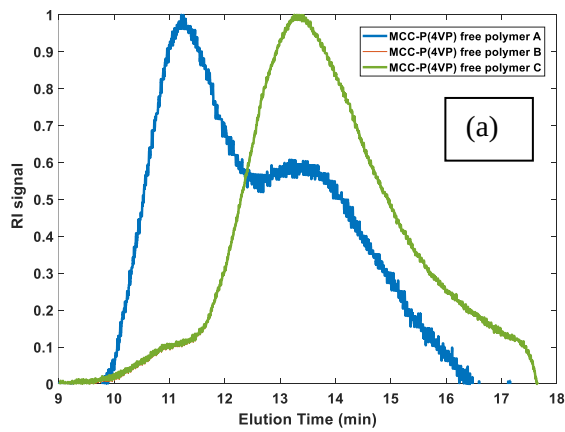


Figure A-2: SEC analysis of the free polymer formed during the cellulose grafting with P(4VP) and PAN considering different synthesis conditions (see Table 2-1 in the main document and Table A-1). (a) and (b) are correspondent to the MCC- P(4VP) grafting process, while (c) and (d) are relative the MCC- PAN system.

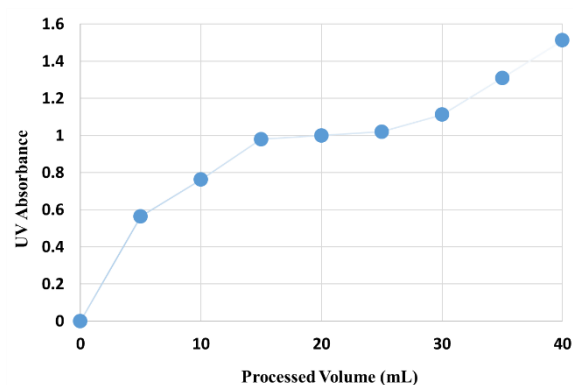


Figure A-3: Dynamics of adsorption observed for the processing of a grape pomace extract in a SPE column with the MCC-(4VP) hybrid particles (200 mg).

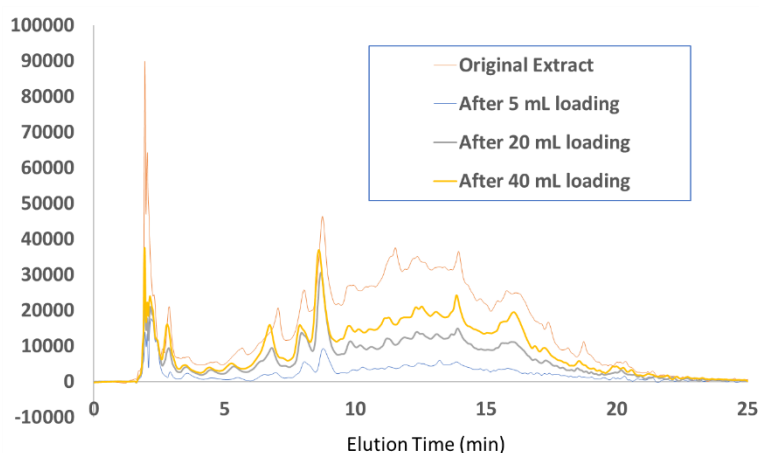


Figure A-4: HPLC-DAD analysis of samples collected at the SPE column outlet correspondent to different processed volumes on feeding a grape pomace extract into a SPE column containing the MCC-P(4VP) hybrid particles. UV absorption measured at 280 nm.

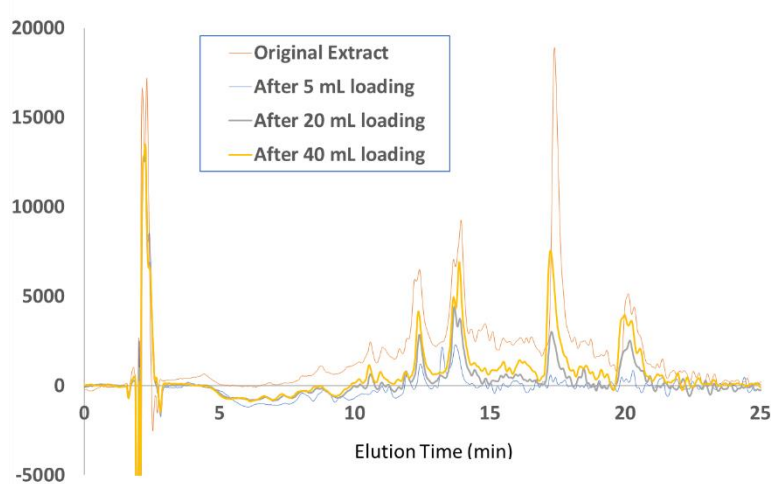


Figure A-5: HPLC-DAD analysis of samples collected at the SPE column outlet correspondent to different processed volumes on feeding a grape pomace extract into a SPE column containing the MCC-P(4VP) hybrid particles. UV absorption measured at 360 nm.

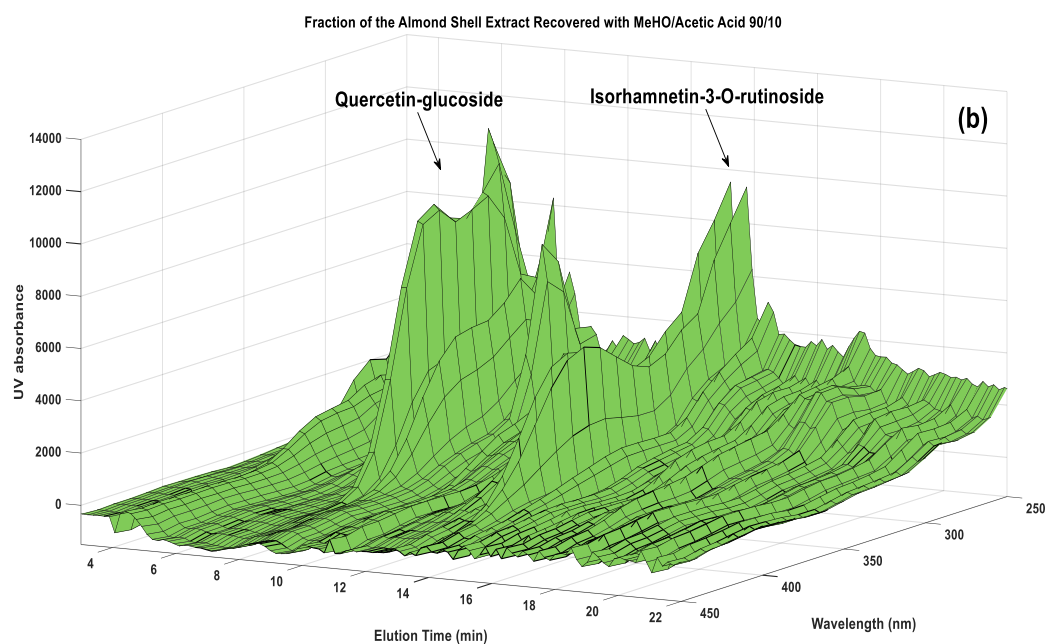
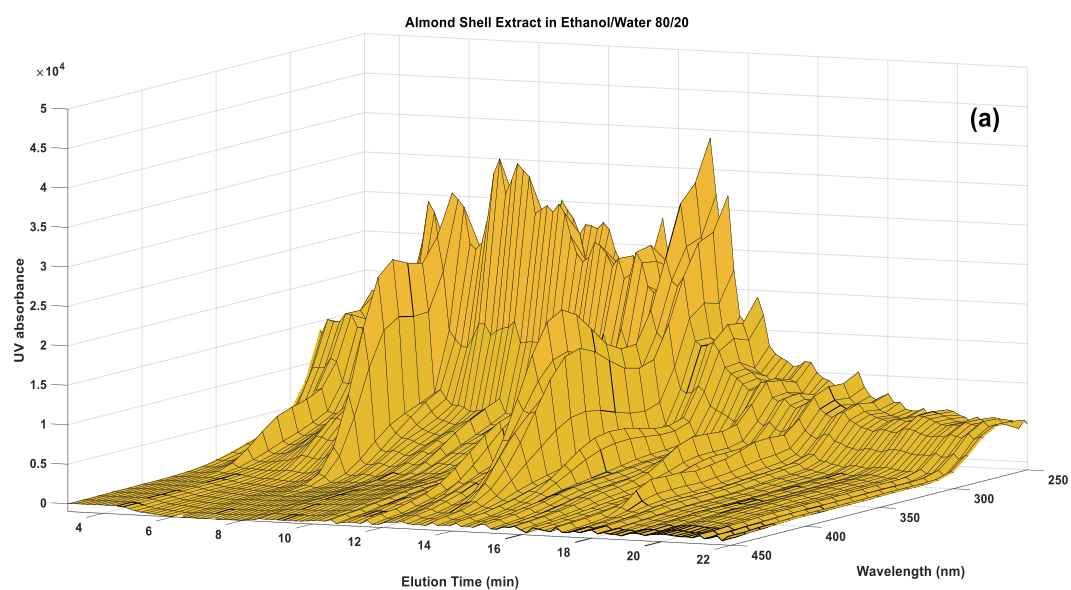


Figure A-6: HPLC-DAD analysis of: (a) an almond shell extract obtained in EtOH/water 80/20 and (b) a fraction of this extract recovered in MeOH/acetic acid 90/10 after loading the almond shell extract with the MCC-P(4VP) adsorbent.

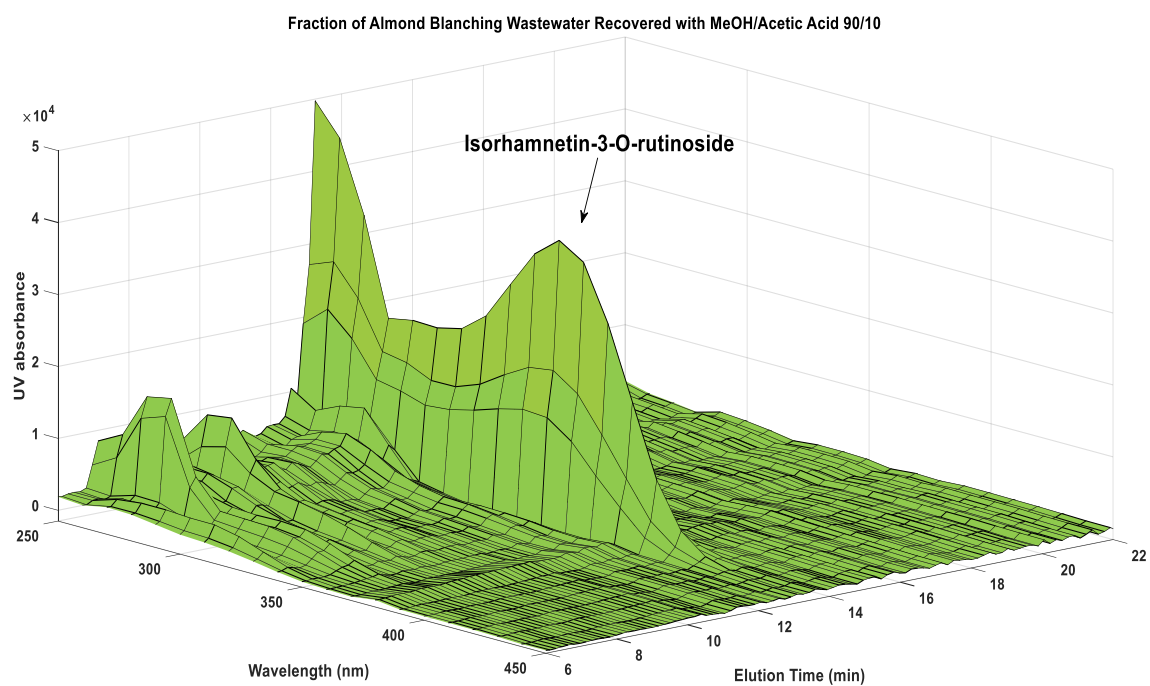
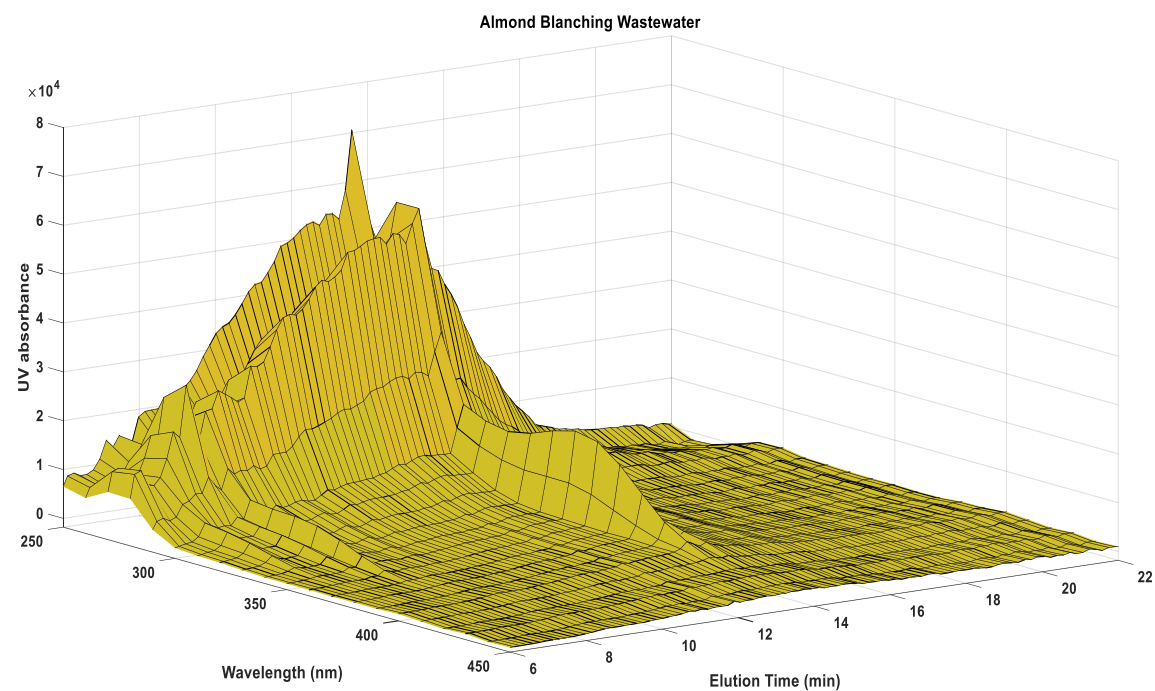


Figure A-7: HPLC-DAD analysis of: (a) almond blanching wastewater (b) a fraction of this extract recovered in MeOH/acetic acid 90/10 after loading the almond blanching wastewater with the MCC-P(4VP) adsorbent.

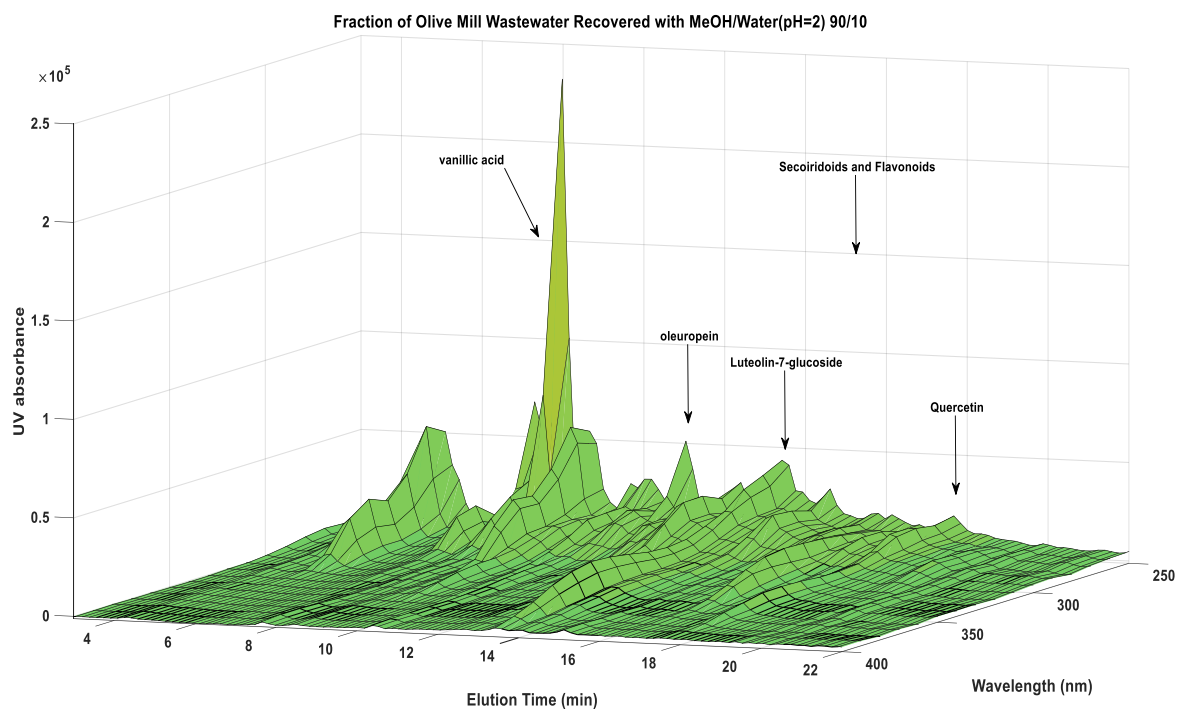
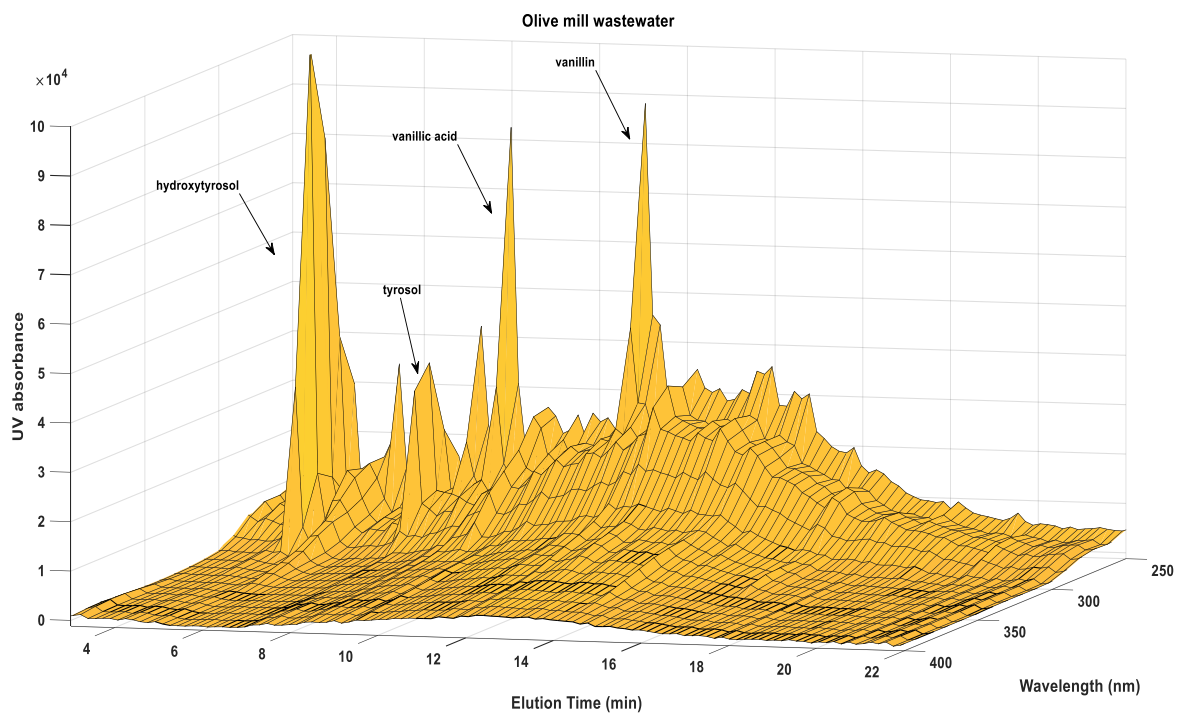


Figure A-8: HPLC-DAD analysis of: (a) olive mill wastewater (b) a fraction of this extract recovered in MeOH/Water(pH=2) 90/10 after loading the olive mill wastewater with the MCC-P(4VP) adsorbent.

Appendix B - Supporting Information for Chapter 3

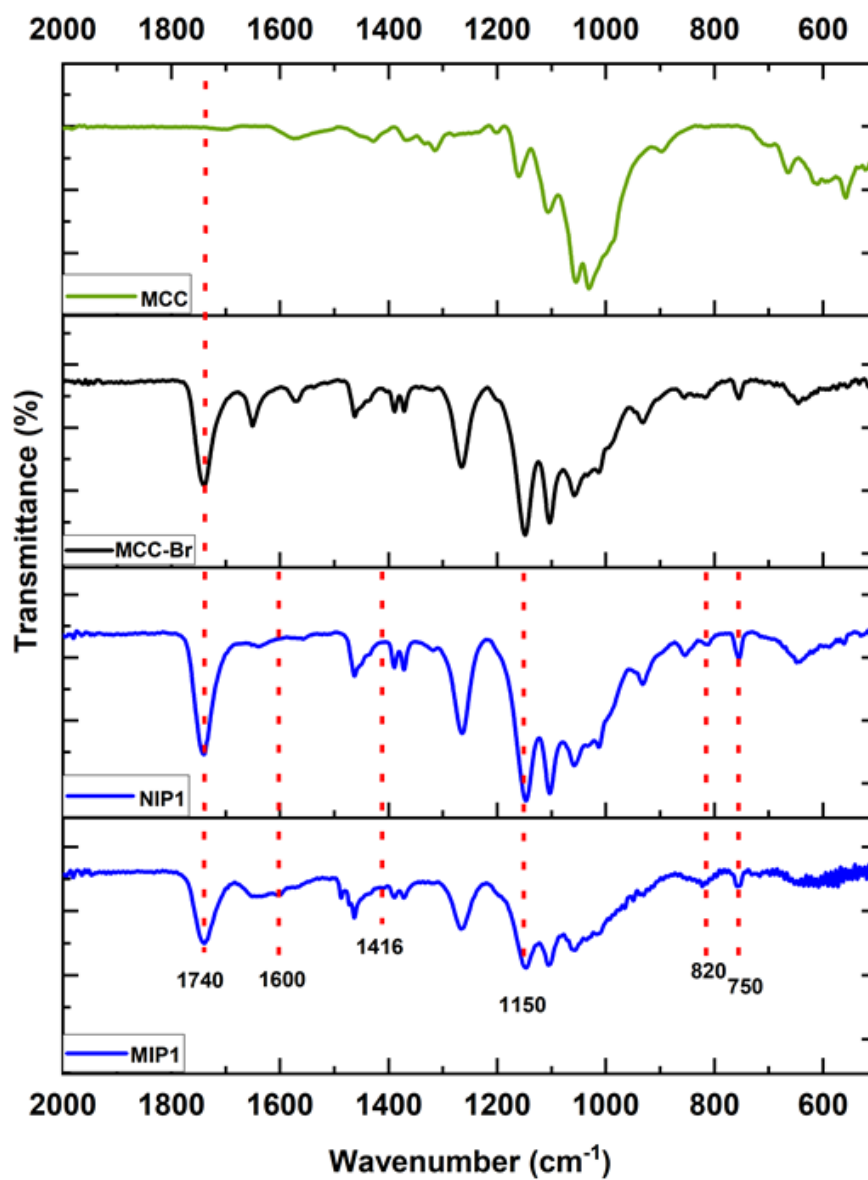


Figure B-1: Comparative FTIR spectra for pristine microcrystalline cellulose (MCC), the product resulting from the immobilization of α -bromoisobutryl bromide in MCC (MCC-Br), NIP1 and MIP1.

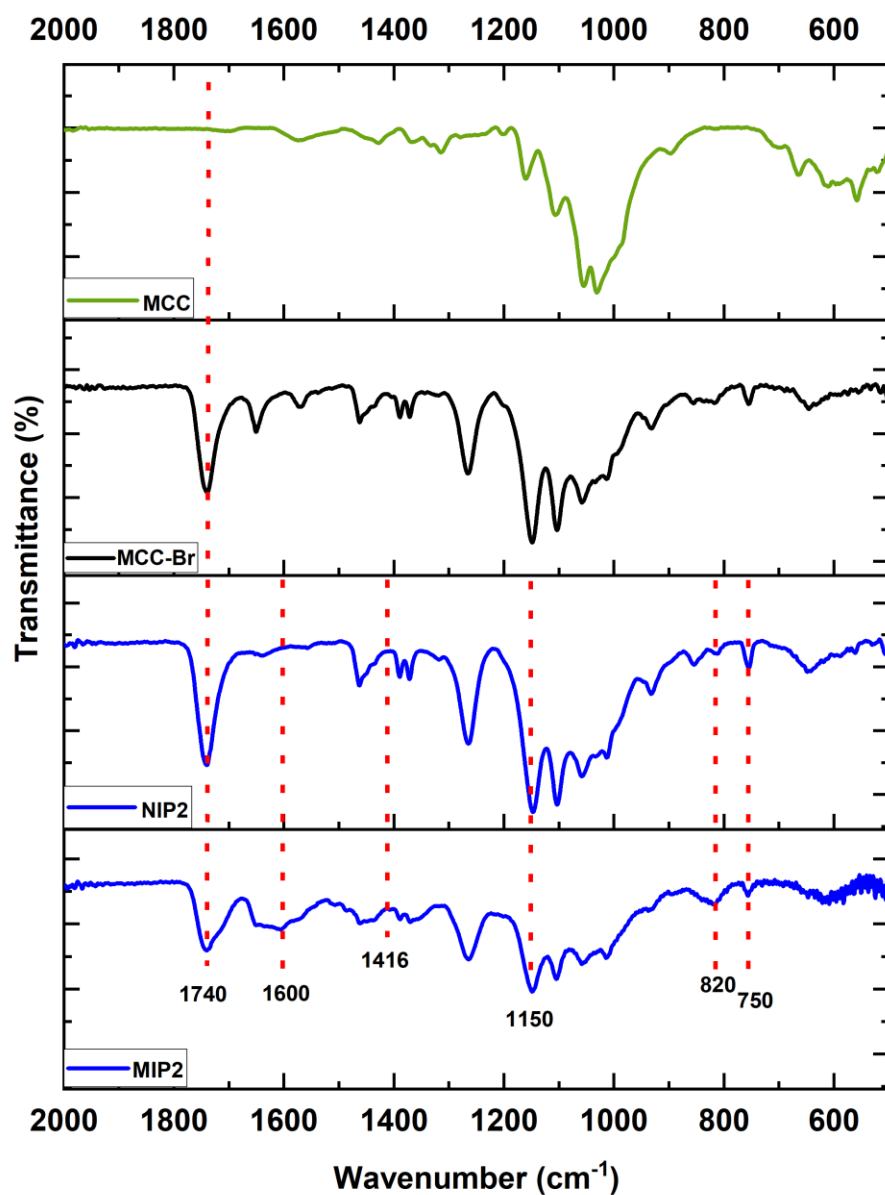


Figure B-2: Comparative FTIR spectra for pristine microcrystalline cellulose (MCC), the product resulting from the immobilization of α -bromoisobutyryl bromide in MCC (MCC-Br), NIP1 and MIP1.

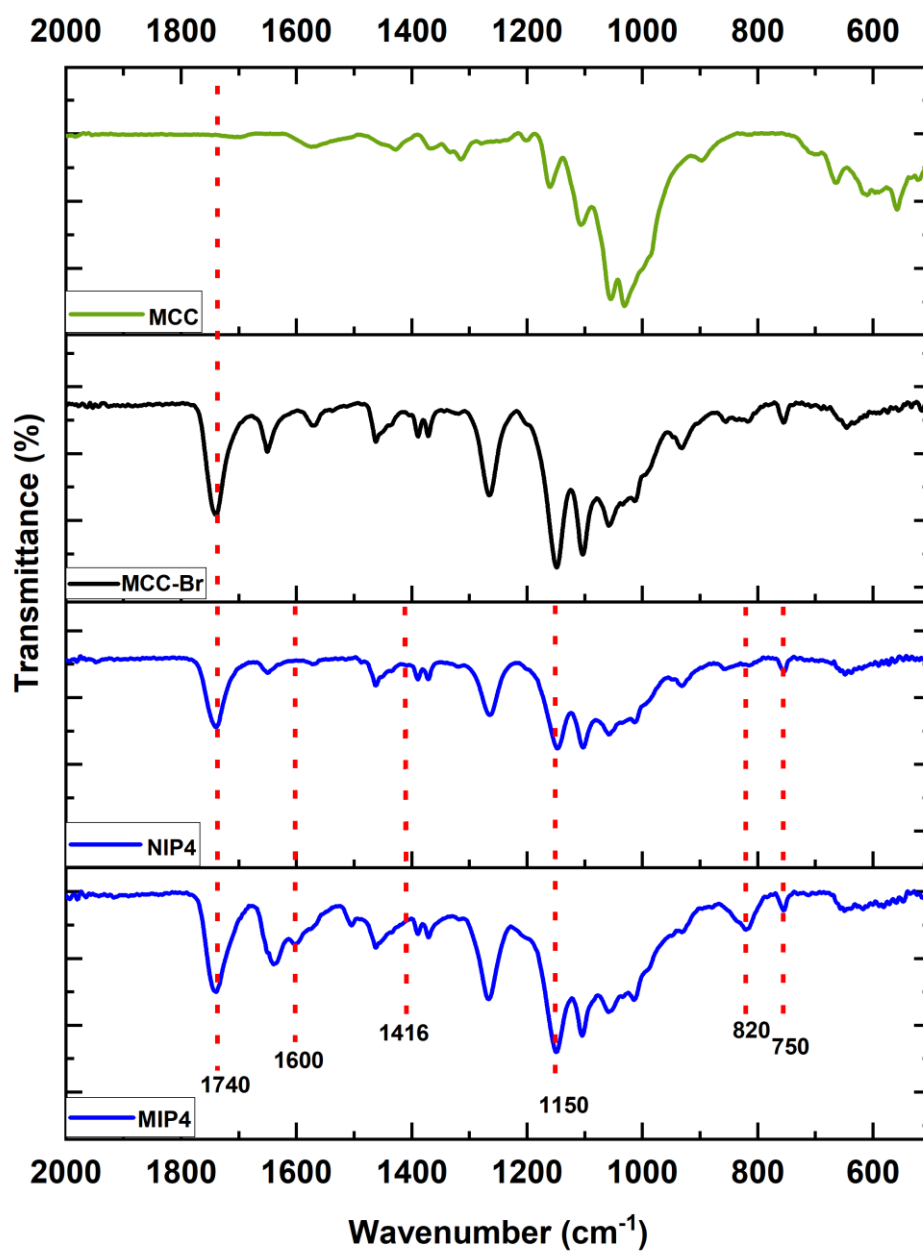


Figure B-3: Comparative FTIR spectra for pristine microcrystalline cellulose (MCC), the product resulting from the immobilization of α -bromoisobutyryl bromide in MCC (MCC-Br), NIP4 and MIP4.

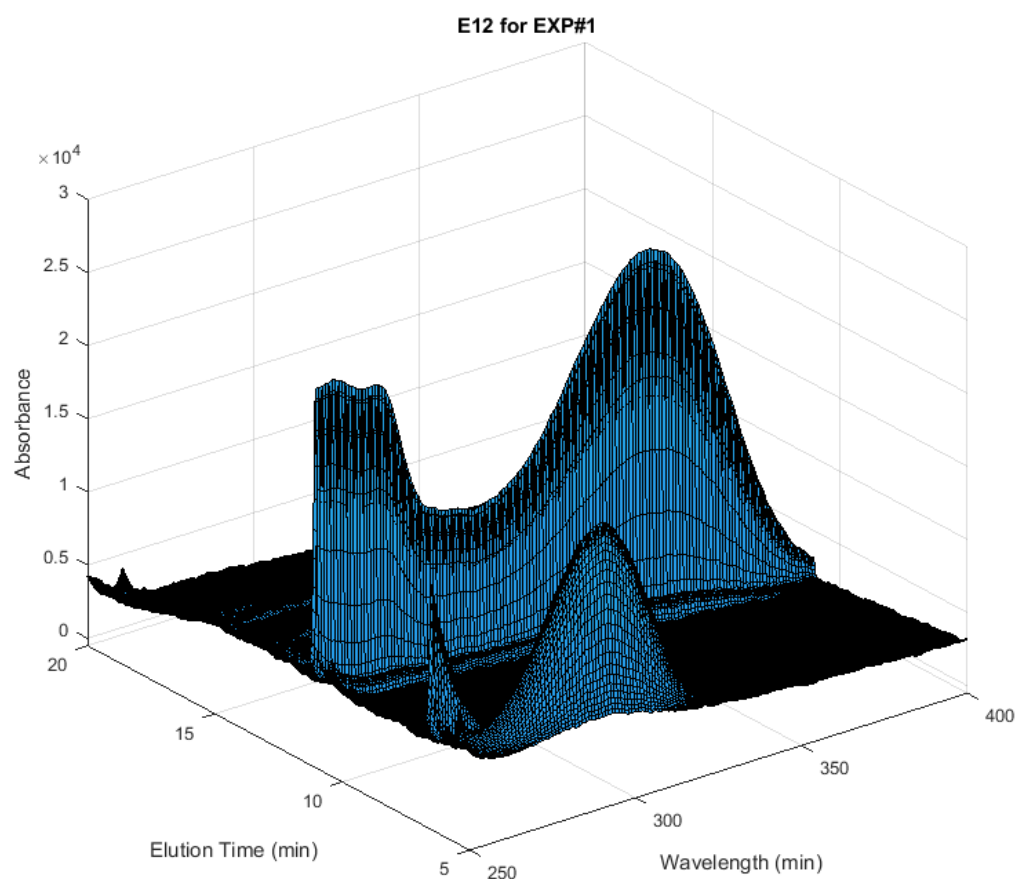


Figure B-4: 3D HPLC-DAD chromatogram for a fraction collected during the desorption of MIP3 particles pre-loaded with the olive leaf extract in ethanol/water 80/20 at 0.5 mg/mL. Fraction recovered in methanol/acetic acid 90/10 showing a high enrichment in the flavonoid luteolin-7-O-glucoside.

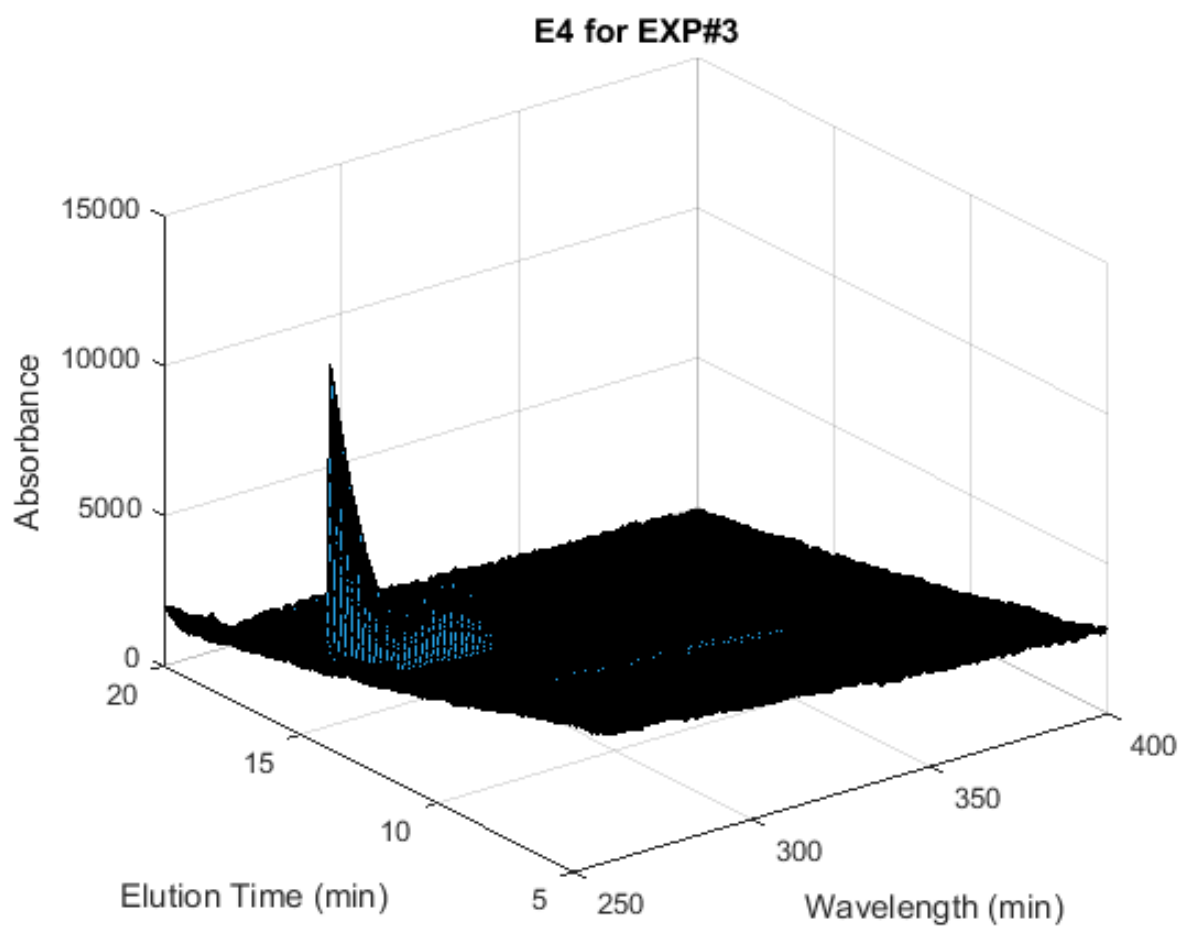


Figure B-5: 3D HPLC-DAD chromatogram for a fraction collected during the desorption of MIP3 particles pre-loaded with the olive leaf extract in ethanol/water 80/20 at 5 mg/mL. Fraction recovered in water showing an enrichment in oleuropein.

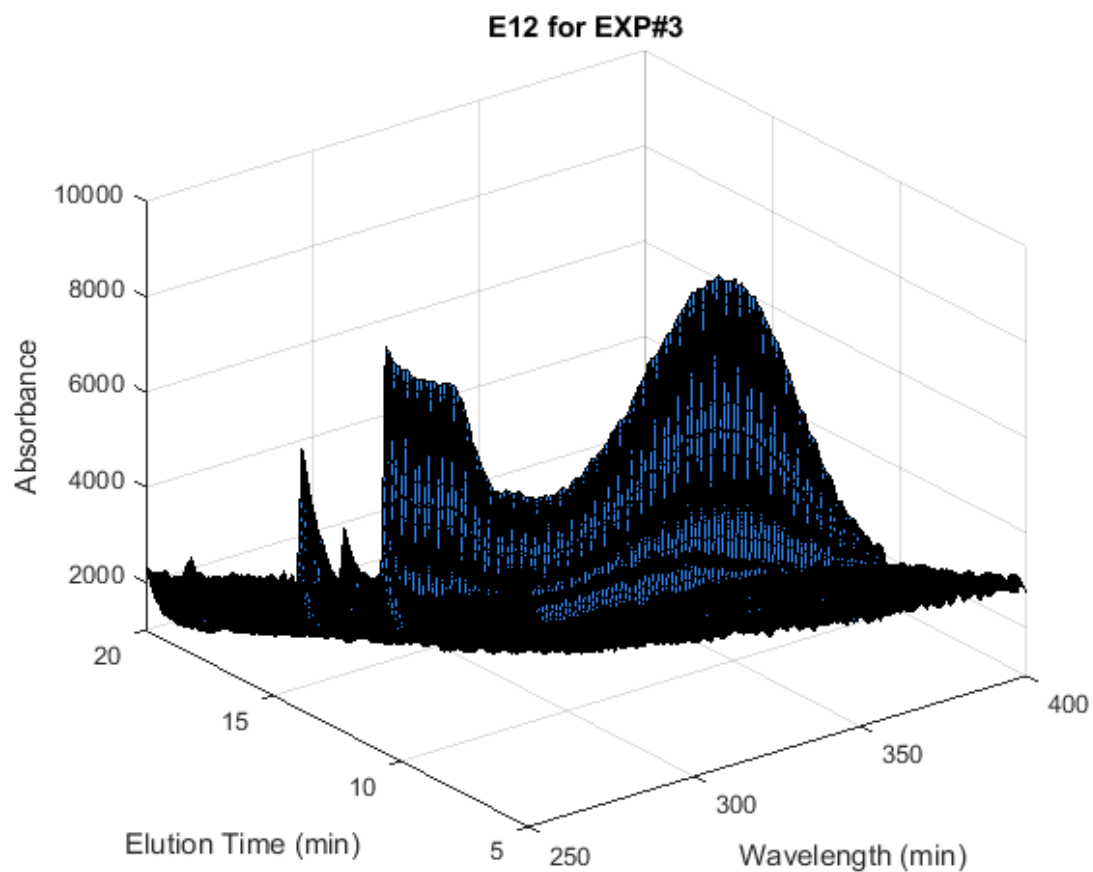


Figure B-6: 3D HPLC-DAD chromatogram for a fraction collected during the desorption of MIP3 particles pre-loaded with the olive leaf extract in ethanol/water 80/20 at 5 mg/mL. Fraction recovered in ethanol/water 30/70 showing an enrichment in the flavonoid luteolin-7-O-glucoside.

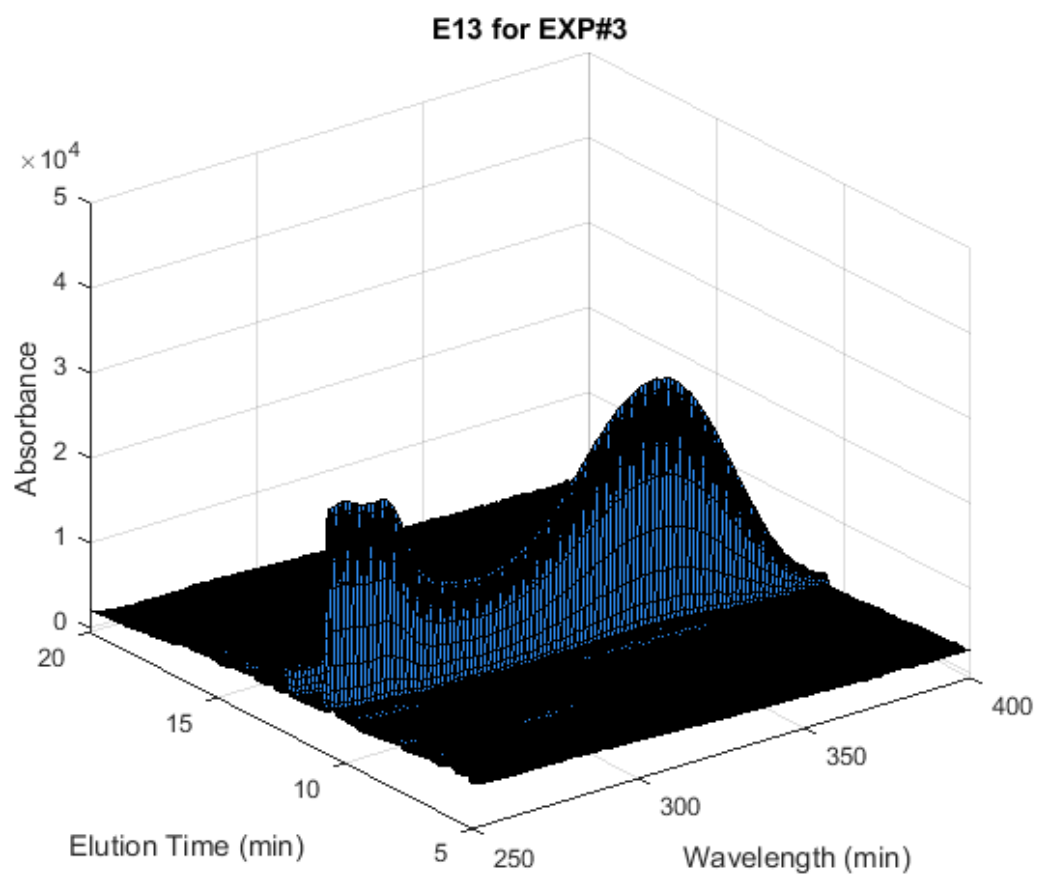


Figure B-7: 3D HPLC-DAD chromatogram for a fraction collected during the desorption of MIP3 particles pre-loaded with the olive leaf extract in ethanol/water 80/20 at 5 mg/mL. Fraction recovered in ethanol/water 50/50 showing a high enrichment in the flavonoid luteolin-7-O-glucoside.

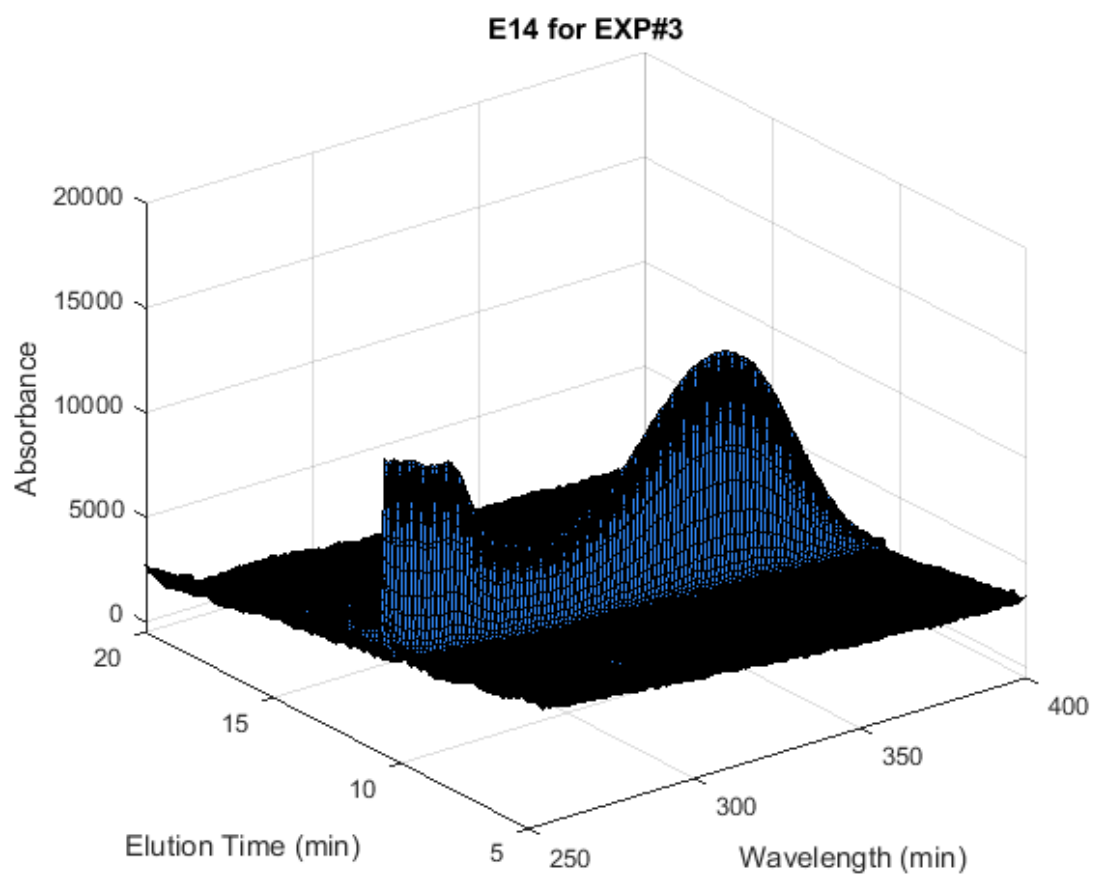


Figure B-8: 3D HPLC-DAD chromatogram for a fraction collected during the desorption of MIP3 particles pre-loaded with the olive leaf extract in ethanol/water 80/20 at 5 mg/mL. Fraction recovered in ethanol/water 80/20 showing a high enrichment in the flavonoid luteolin-7-O-glucoside.

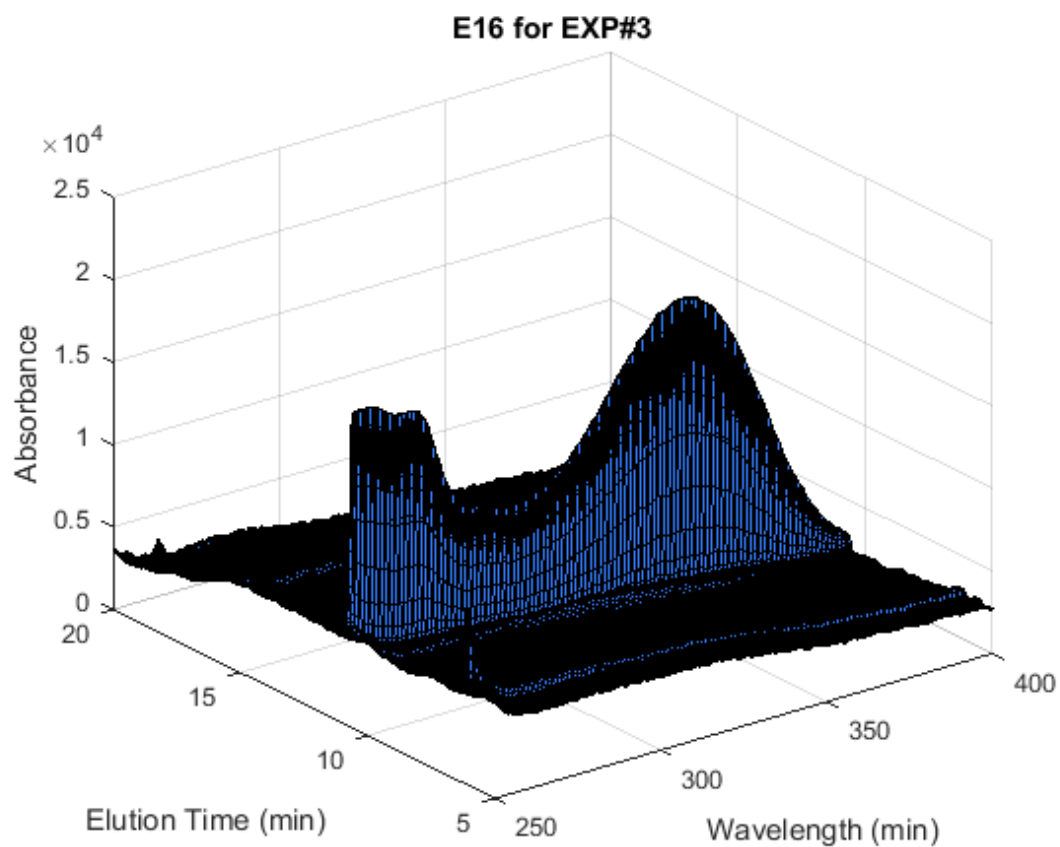


Figure B-9: 3D HPLC-DAD chromatogram for a fraction collected during the desorption of MIP3 particles pre-loaded with the olive leaf extract in ethanol/water 80/20 at 5 mg/mL. Fraction recovered in methanol/acetic acid 90/10 showing a high enrichment in the flavonoid luteolin-7-O-glucoside.

Appendix C-Supporting Information for Chapter 4

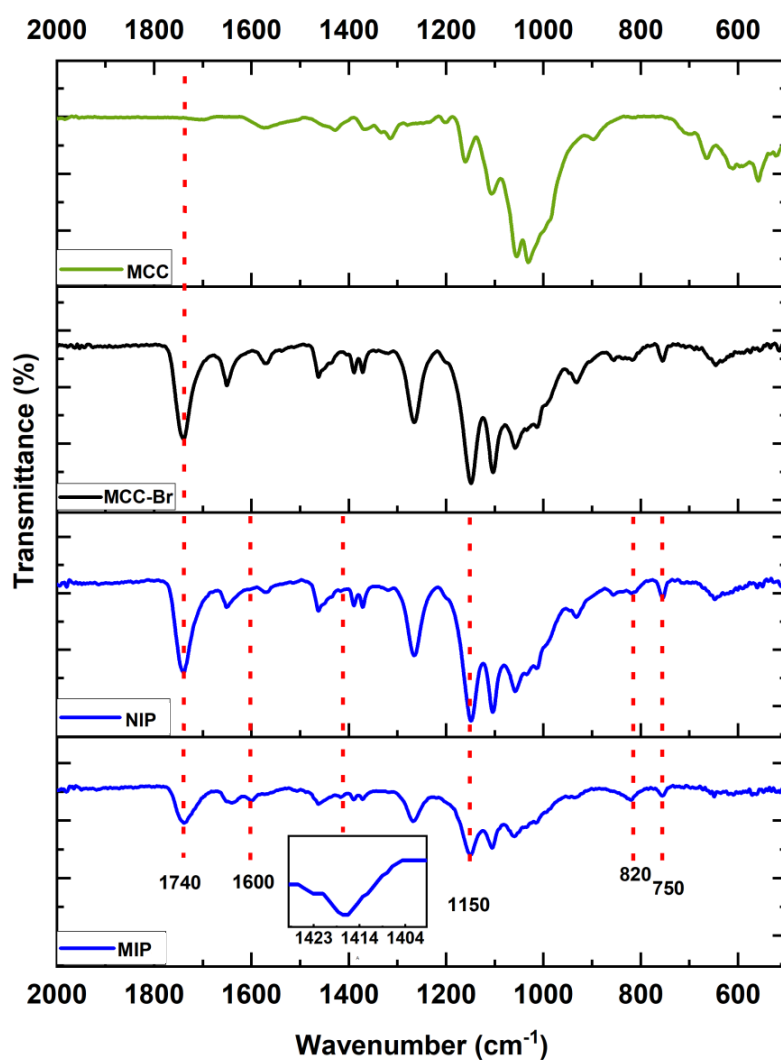


Figure C-1: Comparative FTIR spectra for pristine microcrystalline cellulose (MCC), the product resulting from the immobilization of α -bromoisobutyryl bromide in MCC (MCC-Br), NIP and MIP hybrid cellulose-synthetic particles.

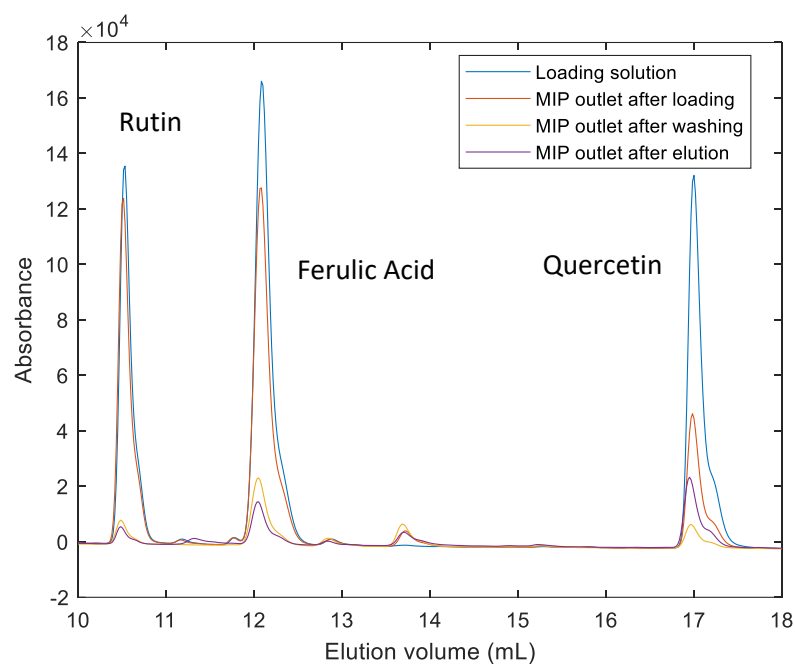


Figure C-2: HPLC-DAD analyses for the solutions collected at MIP SPE column outlet after the loading, washing and elution steps. Competitive sorption/desorption assessment using a ferulic acid/rutin/quercetin solution in ethanol/water 80/20 with concentration 0.1×10^{-3} M for each compound, 2.5 mL of volume of liquid phase and 25 mg of adsorbent in the SPE cartridges. UV measurements at 280 nm are here presented.

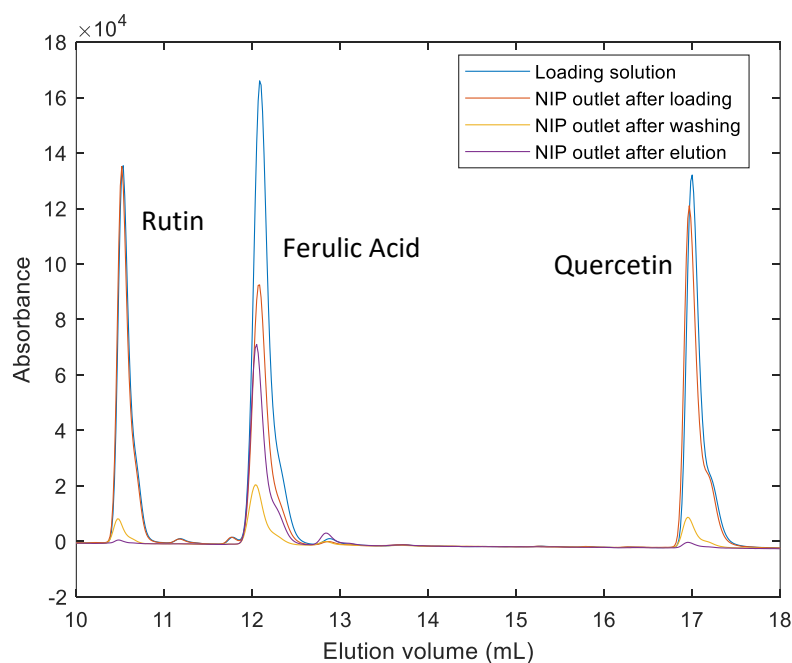


Figure C-3: HPLC-DAD analyses for the solutions collected at NIP SPE column outlet after the loading, washing and elution steps. Competitive sorption/desorption assessment using a ferulic acid/rutin/quercetin solution in ethanol/water 80/20 with concentration 0.1×10^{-3} M for each compound, 2.5 mL of volume of liquid phase and 25 mg of adsorbent in the SPE cartridges. UV measurements are 280 nm is here presented.

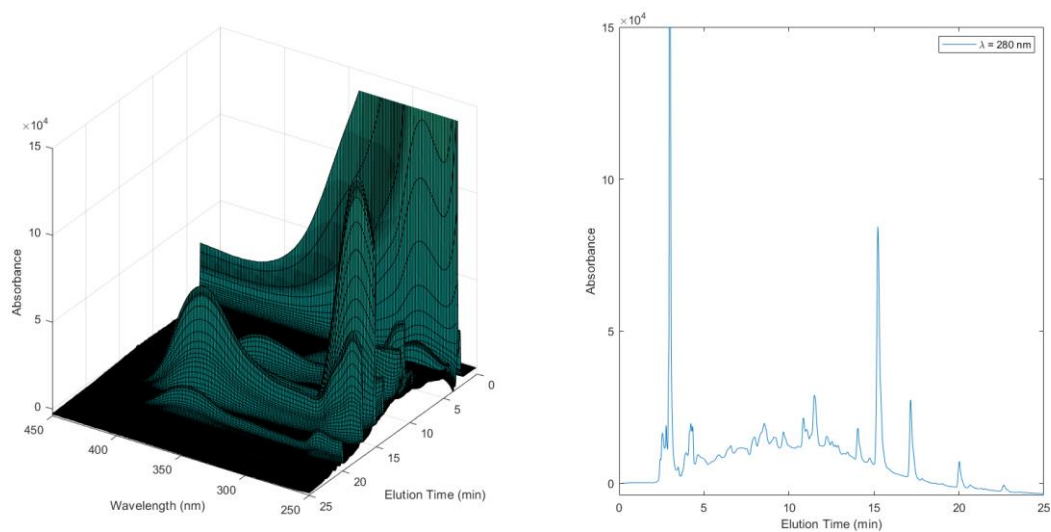


Figure C-4: 3D HPLC-DAD chromatogram (a) and correspondent 2D view at 280 nm (b) for a diatomaceous earth extract used in this work. A different view of the 3D plot is here presented and the system peak is also included (see correspondent Figure in the main text of the paper).

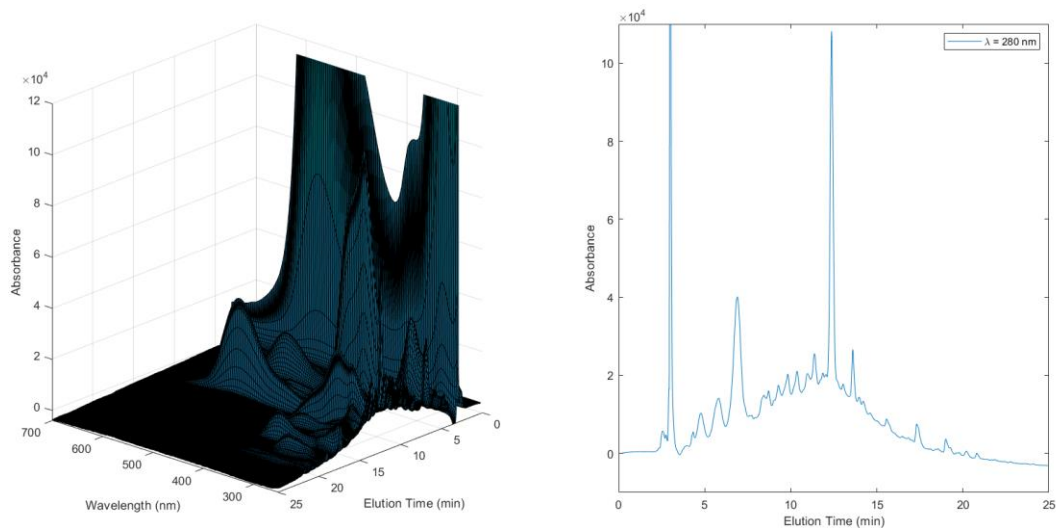


Figure C-5: 3D HPLC-DAD chromatogram (a) and correspondent 2D view at 280 nm (b) for a grape pomace extract used in this work. A different view of the 3D plot is here presented and the system peak is also included (see correspondent Figure in the main text of the paper).

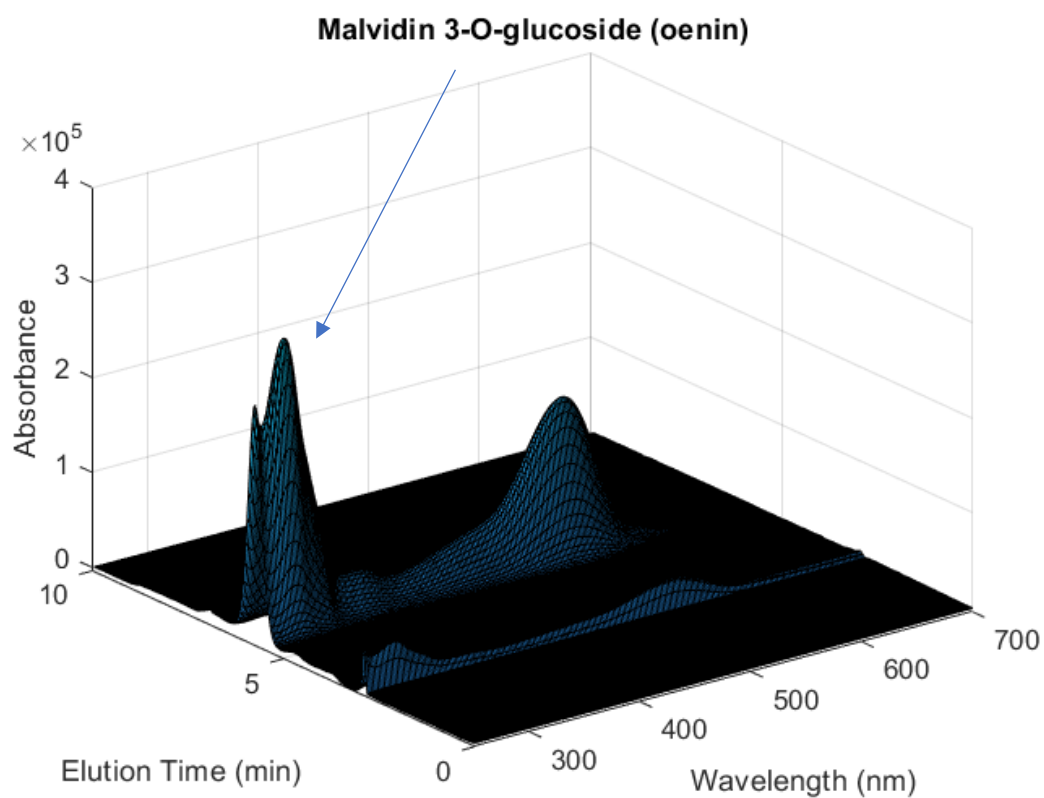


Figure C-6: 3D HPLC-DAD chromatogram for the standard anthocyanin malvidin 3-O-glucoside (oenin).

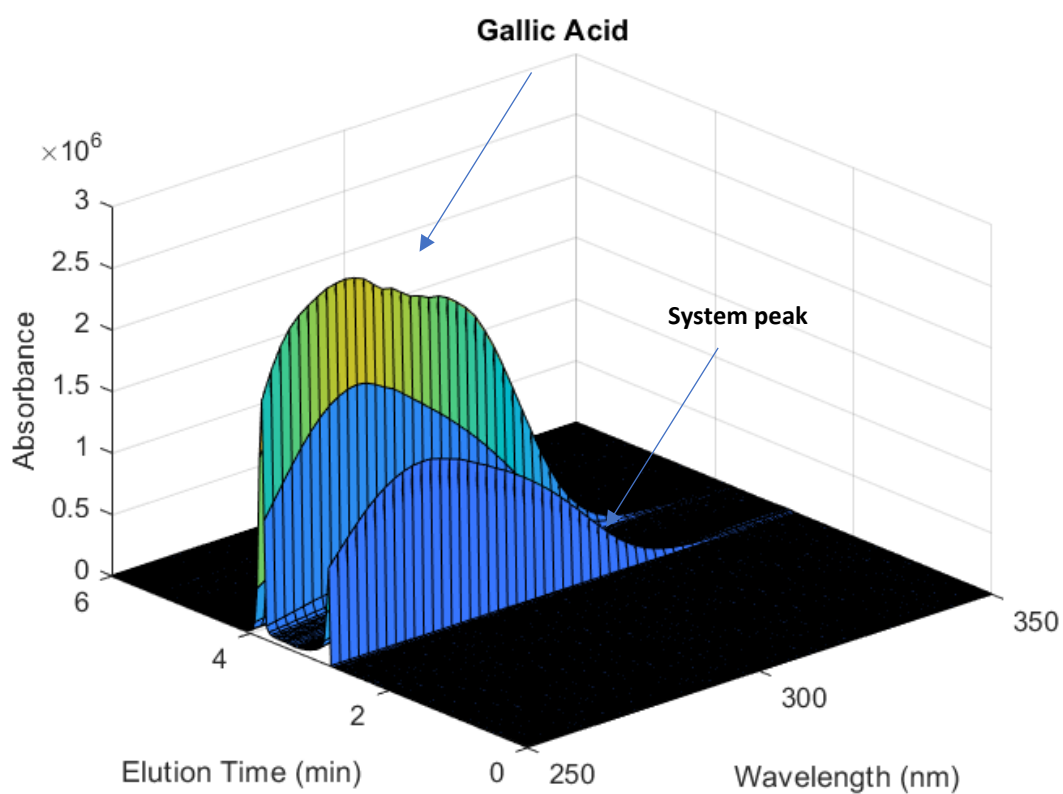


Figure C-7: 3D HPLC-DAD chromatogram for the standard gallic acid.

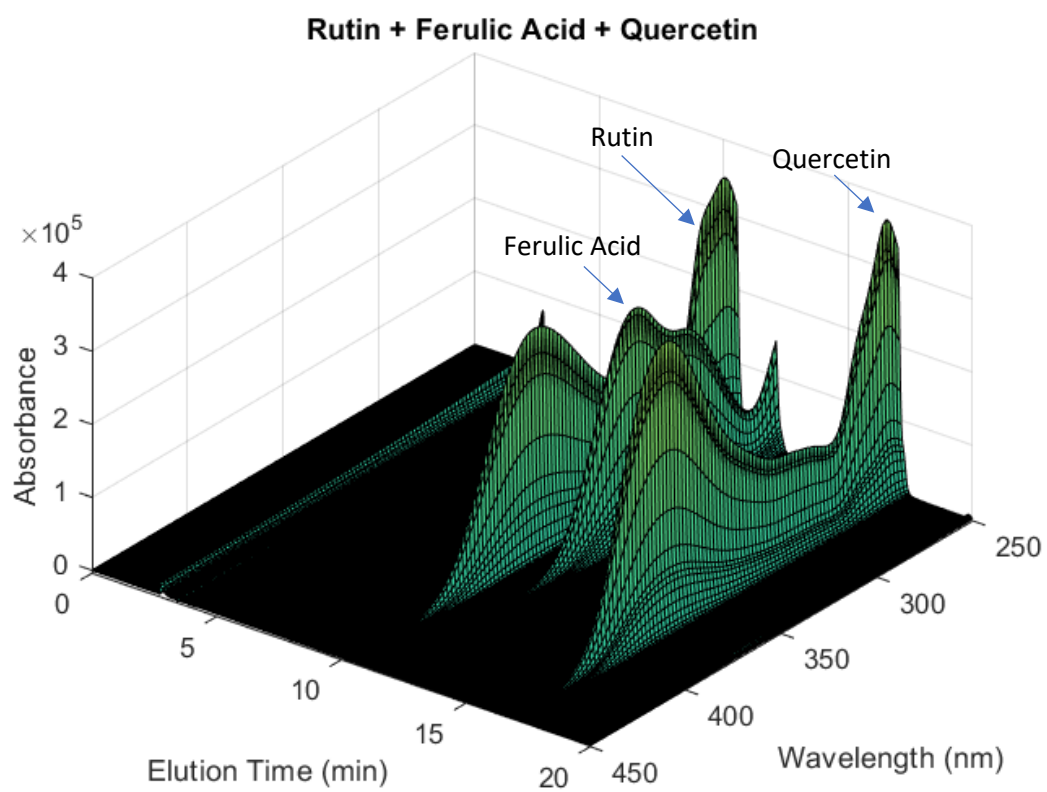


Figure C-8: 3D HPLC-DAD chromatogram for the mixture of standard compounds rutin + ferulic acid + quercetin.

Appendix D-Supporting Information for Chapter 6

Additional schemes depicting the synthesis of vinyl monomers from oleic acid, the incorporation of oleic acid in pre-formed polymers and the potential of p(GMA-OA)-b-p(DMAEMA) copolymers for the encapsulation of oleanolic acid.

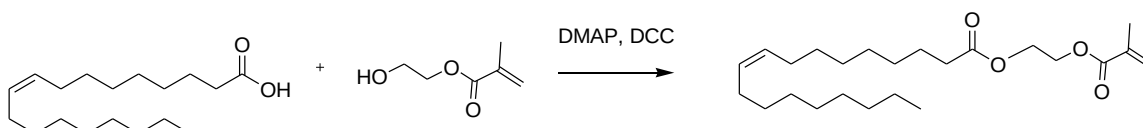


Figure D-1: Depiction for the synthesis of methacrylated oleic acid via Steglich esterification with HEMA.

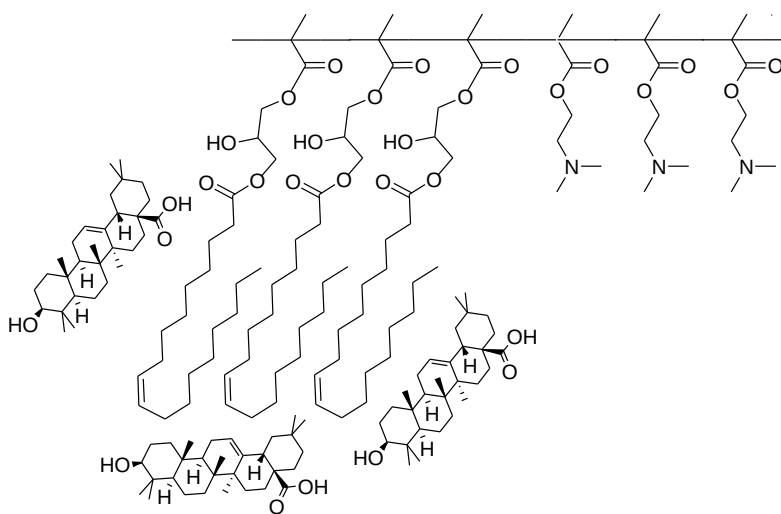


Figure D-2: Depiction of the expected interactions of oleanolic acid with the hydrophobic block of p(GMA-OA)-b-p(DMAEMA) copolymers. These hydrophobic interactions are explored to encapsulate oleanolic acid and the subsequent use of the aggregates formed in aqueous media by the p(GMA-OA)-b-p(DMAEMA) copolymers to deliver the contained oleanolic acid

Additional FTIR analysis for different products obtained in the framework of the synthesis of vinyl monomers from oleic acid, the incorporation of oleic acid in pre-formed polymers and final p(GMA-OA)-*b*-p(DMAEMA) copolymers.

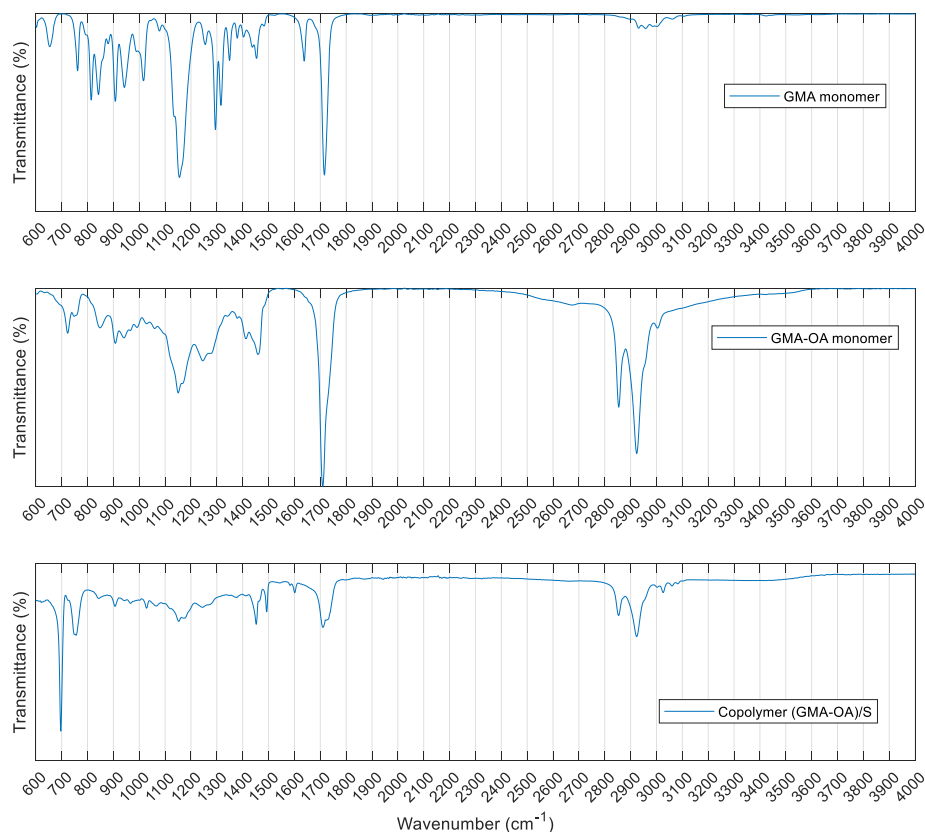


Figure D-3: FTIR analysis for the GMA monomer, the methacrylated oleic acid monomer obtained according to the synthesis conditions of Run 4 in Table 6-1 and the copolymer formed through the free radical polymerization of this monomer with styrene. The substantial decrease of the specific assignment at 910 cm⁻¹, correspondent to epoxide groups, is observable in the MOA monomer in comparison with GMA, indicating the reaction of the epoxides with oleic acid. In the MOA monomer spectrum, the increased prominence of the band in the 2800–3000 cm⁻¹ region and the enhanced vibrational assignment at 1466 cm⁻¹ both indicate also the successful synthesis of the methacrylated oleic acid monomer. For the copolymer synthesized from the MOA monomer and styrene, strong assignments at 700 and 750 cm⁻¹ demonstrate the incorporation of the latter in the final product, while the 1700 cm⁻¹ and 2800–3000 cm⁻¹ vibrations demonstrate the presence of the MOA moieties.

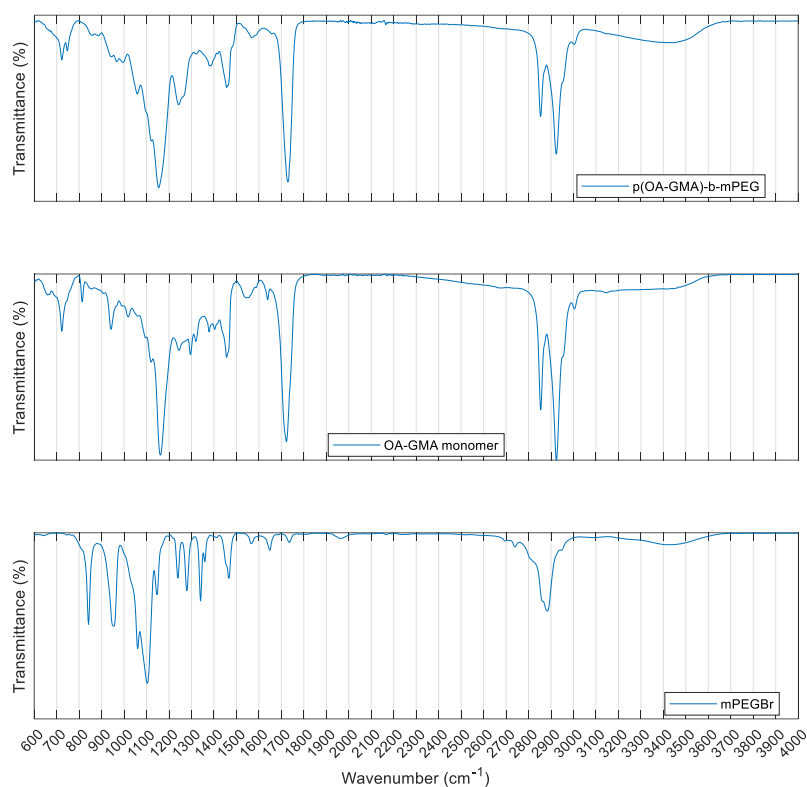


Figure D-4: FTIR analysis for the mPEGBr macroinitiator, MOA monomer obtained through reaction of OA and GMA and for the block copolymer p(GMA-OA)-b-mPEG synthesized via ATRP of mPEGBr with the MOA monomer. The depletion of the peak at around 1630 cm^{-1} demonstrates the consumption of vinyl functionality in the MOA monomer.

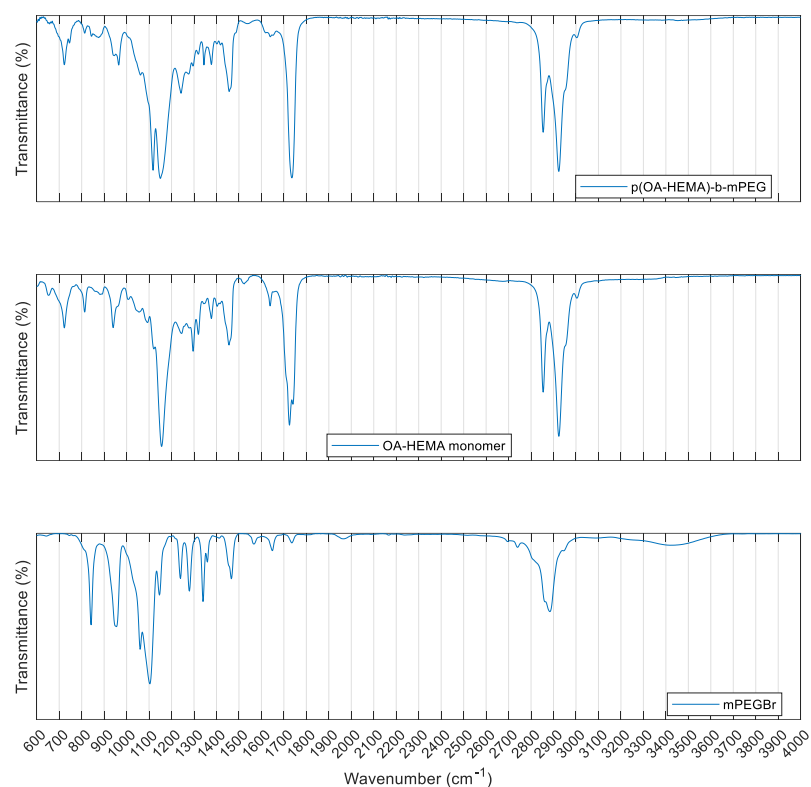


Figure D-5:FTIR analysis for the mPEGBr macroinitiator, MOA monomer obtained through esterification of OA and HEMA and for the block copolymer p(HEMA-OA)-b-mPEG synthesized via ATRP of mPEGBr with the MOA monomer. The depletion of the peak at around 1630 cm⁻¹ demonstrates the consumption of vinyl functionality in the MOA monomer.

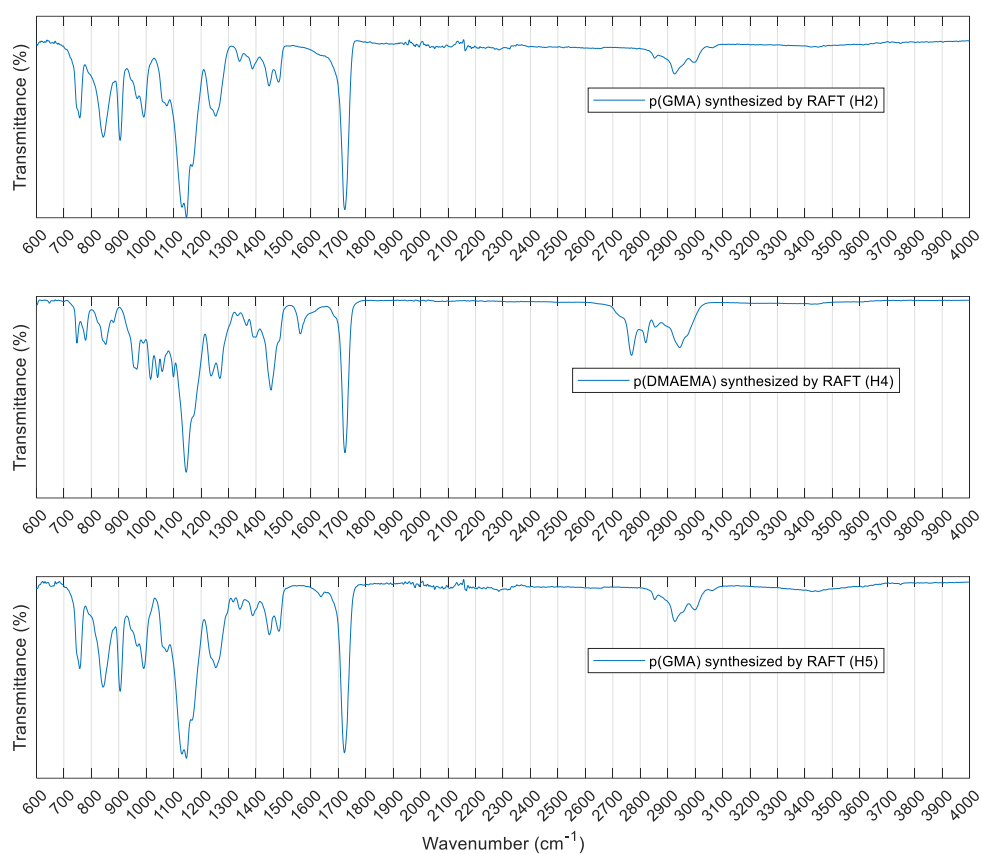


Figure D-6: FTIR analysis for RAFT synthesized p(GMA) and p(DMAEMA) homopolymers. The products H2, H4 and H5 described in Table 6-2 are here compared. Specific assignments at 910 cm⁻¹ for p(GMA) and 2770 cm⁻¹ for p(DMAEMA) are evidenced in these spectra.

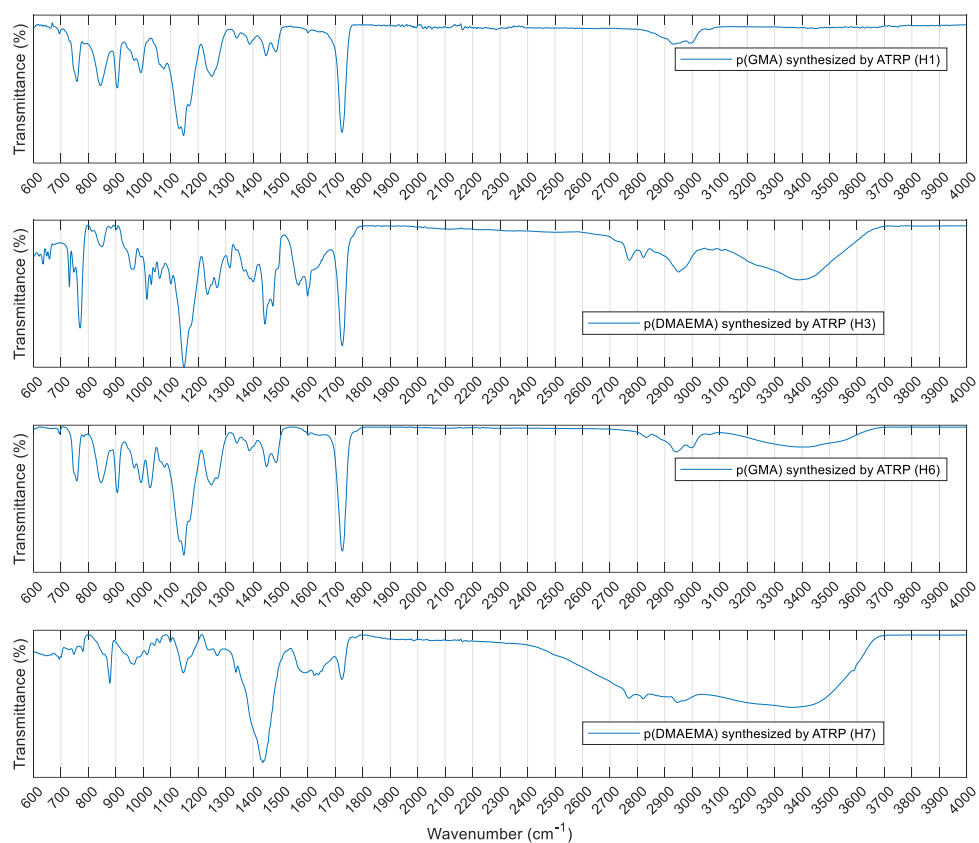


Figure D-7: FTIR analysis for ATRP synthesized p(GMA) and p(DMAEMA) homopolymers. The products H1, H3, H6 and H7 described in Table 6-2 are here compared. Specific assignments at 910 cm⁻¹ for p(GMA) and 2770 cm⁻¹ for p(DMAEMA) are evidenced in these spectra.

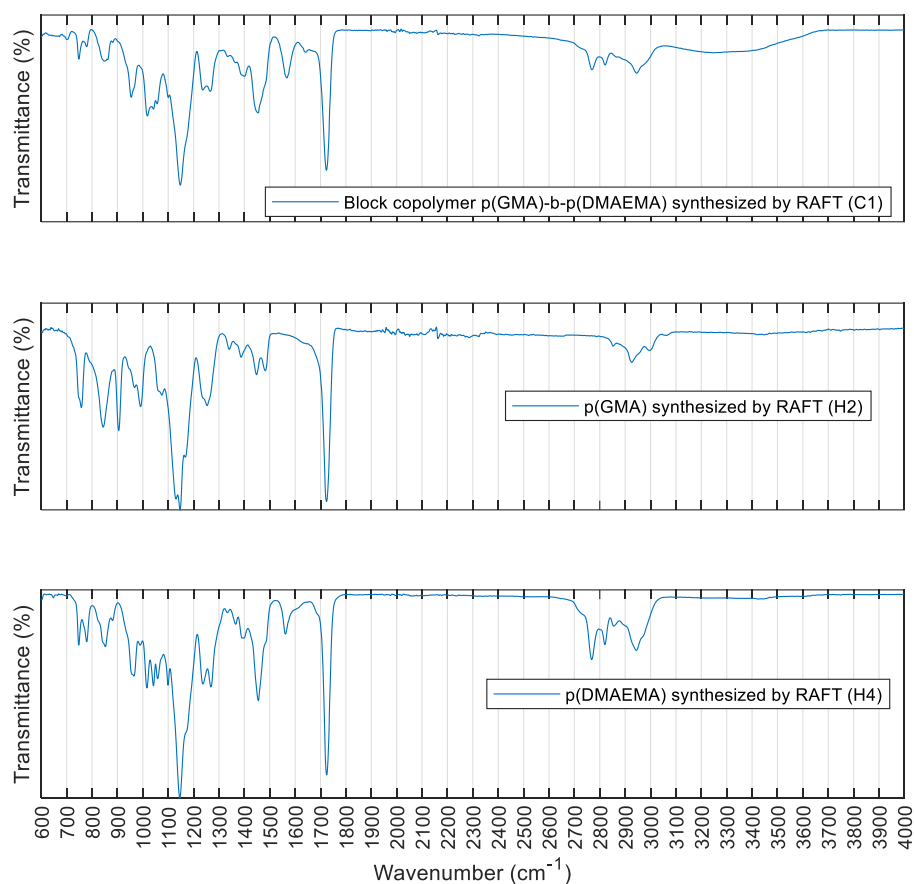


Figure D-8: FTIR analysis of the block copolymer p(GMA)-*b*-p(DMAEMA), synthesized by RAFT and labelled as product **C1** in Table 6-3. Also presented for comparison purposes are the FTIR analyses of RAFT-synthesized homopolymers H2 and H4 of p(GMA) and p(DMAEMA), respectively. Specific assignments around 2770 cm⁻¹ are discernible in the copolymer and in the p(DMAEMA) homopolymer. These FTIR observations indicate the presence of p(DMAEMA) in the final product. Specific assignment at 910 cm⁻¹ is not fully observable in the copolymer **C1** as compared with the p(GMA) homopolymer. Overall, these results are compatible with formation of a fraction of free p(DMAEMA) homopolymer in the RAFT process considered for homopolymer synthesis (**C1** Table 6-3). More clear evidence for p(GMA)-*b*-p(DMAEMA) copolymer formation is presented below with analysis of products **C2** and **C3**.

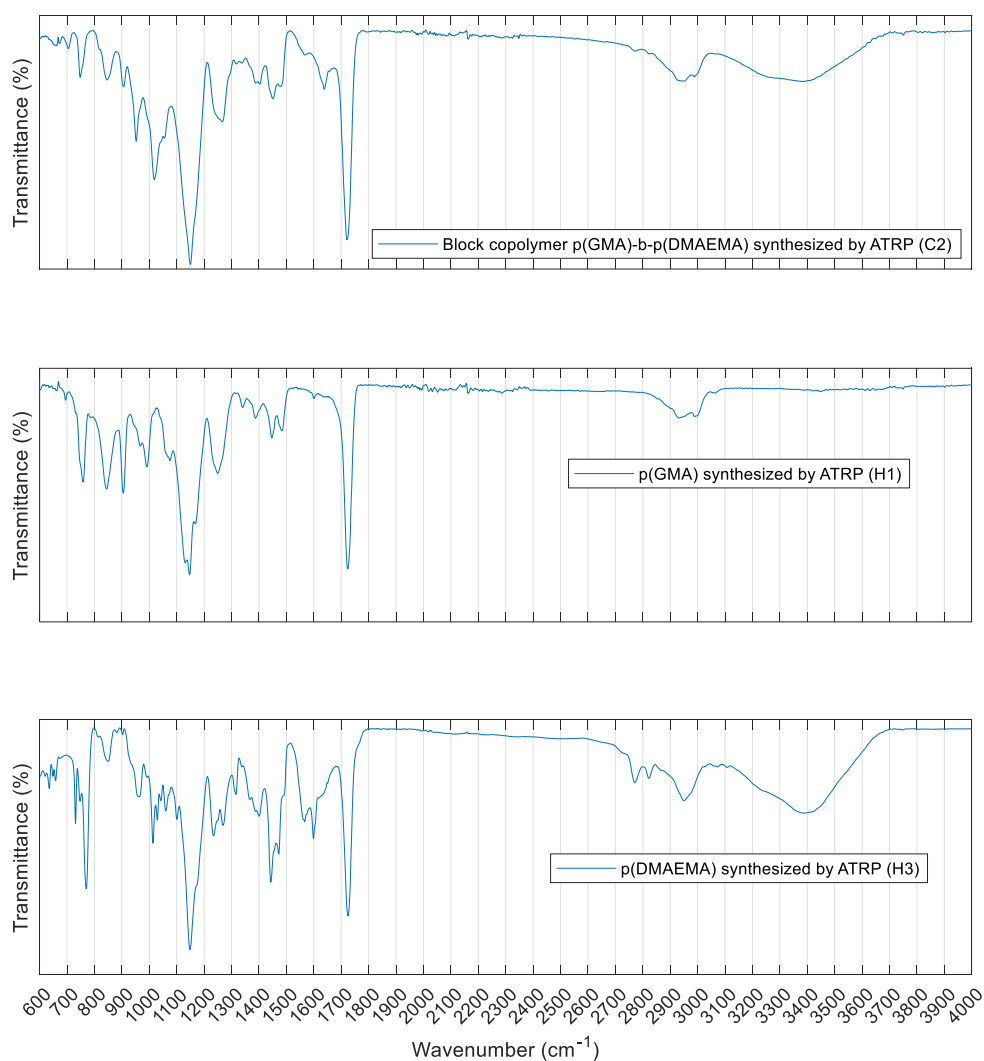


Figure D-9: FTIR analysis of the block copolymer p(GMA)-b-p(DMAEMA), synthesized by ATRP and labelled as product **C2** in Table 6-3. Also presented for comparison purposes are the FTIR analyses of the ATRP-synthesized homopolymers H1 and H3 of p(GMA) and p(DMAEMA), respectively. Specific assignment at 910 cm⁻¹ is observable in the copolymer **C2** as well as in the p(GMA) homopolymer. On the other hand, specific assignments around 2770 cm⁻¹ are also discernible in the copolymer and in the p(DMAEMA) homopolymer. These FTIR observations indicate the successful formation of the p(GMA)-b-p(DMAEMA) block copolymer.

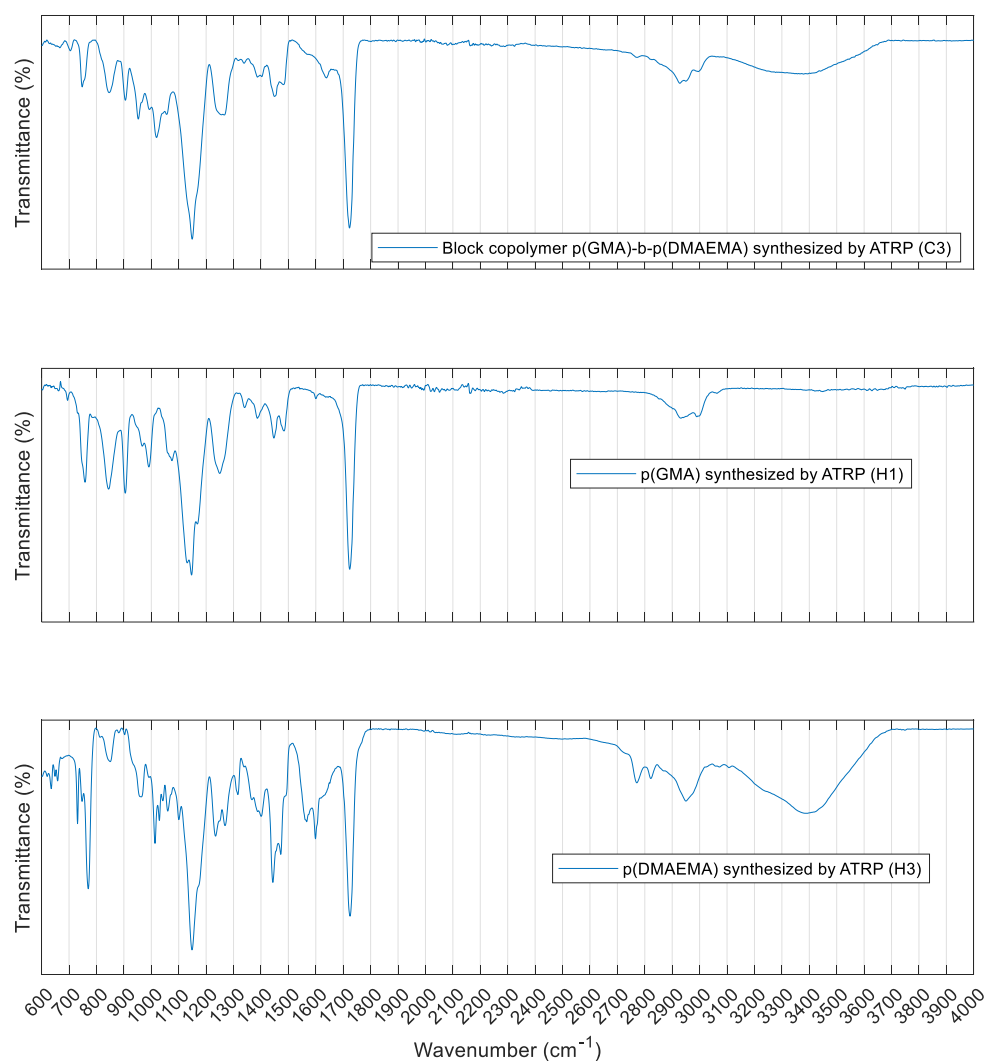


Figure D-10: FTIR analysis of the block copolymer p(GMA)-b-p(DMAEMA), synthesized by ATRP and labelled as product **C3** in Table 6-3. Also presented for comparison purposes are the FTIR analyses of the ATRP-synthesized homopolymers H1 and H3 of p(GMA) and p(DMAEMA), respectively. Specific assignment at 910 cm⁻¹ is observable in the copolymer **C3** as well as in the p(GMA) homopolymer. On the other hand, specific assignments around 2770 cm⁻¹ are also discernible in the copolymer and in the p(DMAEMA) homopolymer. These FTIR observations indicate the successful formation of the p(GMA)-b-p(DMAEMA) block copolymer.

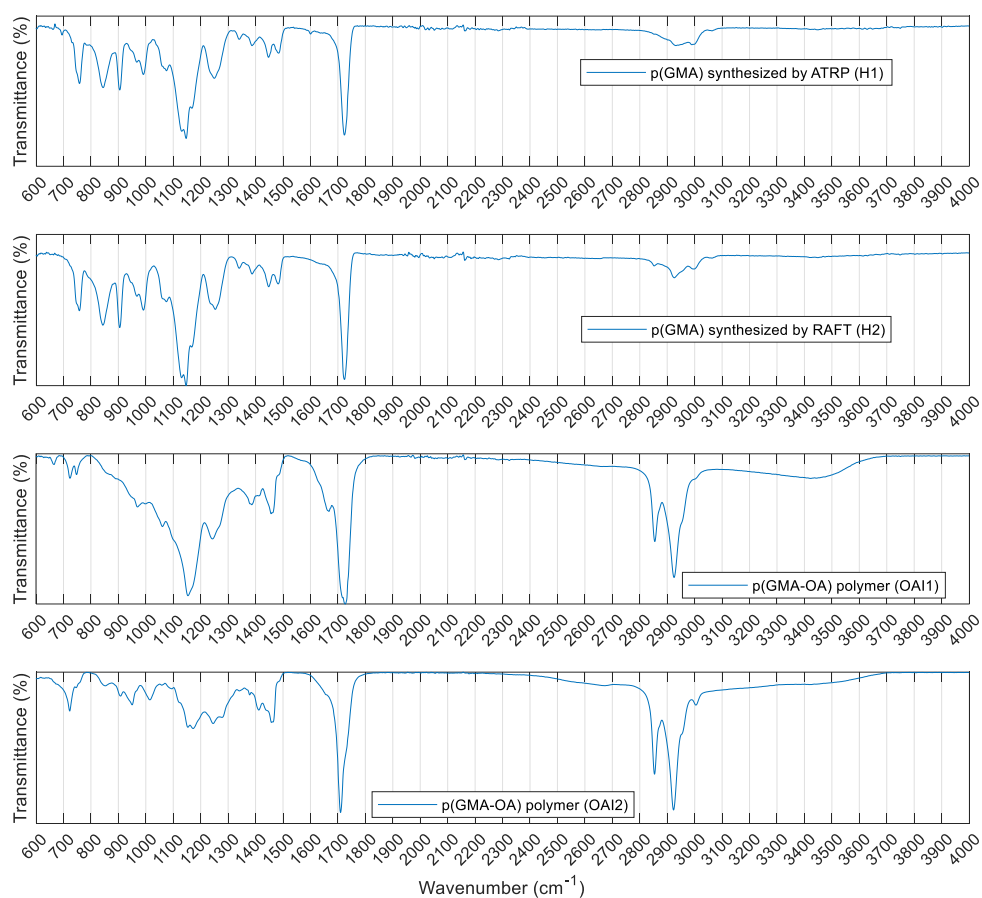


Figure D-11: FTIR analysis of polymers p(GMA-OA), labelled as **OAI1** and **OAI2** in Table 6-4. Also presented for comparison purposes are the FTIR analyses of the p(GMA) homopolymers H1 and H2 synthesized by ATRP and RAFT polymerization, respectively. Specific assignment at 910 cm⁻¹ is not observable in the p(GMA-OA) **OAI1** product indicating the full reaction of the epoxide moieties of the p(GMA) blocks with oleic acid. A very high depletion of epoxide moieties is also observed for **OAI2**. The increased prominence of the band in the 2800–3000 cm⁻¹ region and the enhanced vibrational assignment at 1466 cm⁻¹ both indicate successful oleic acid incorporation into the final p(GMA-OA) polymers.



Figure D-12: FTIR analysis of different block copolymers p(GMA-OA)-b-p(DMAEMA), labelled as **OAP1**, **OAP2**, **OAP3** and **OAP4** in Table 6-5. Specific assignment at 910 cm^{-1} is not observable in these products indicating the reaction of the epoxide moieties of the p(GMA) blocks with oleic acid. The increased prominence of the band in the 2800–3000 cm^{-1} region and the enhanced vibrational assignment at 1466 cm^{-1} both indicate successful oleic acid incorporation into the final p(GMA-OA)-b-p(DMAEMA) products. On the other hand, specific assignments around 2770 cm^{-1} are also discernible, indicating the presence of the DMAEMA moieties in the final products.

Additional SEC/RI traces for different products obtained in the framework of the synthesis homopolymer, copolymers, incorporation of oleic acid in pre-formed polymers and final p(GMA-OA)-*b*-p(DMAEMA) copolymers.

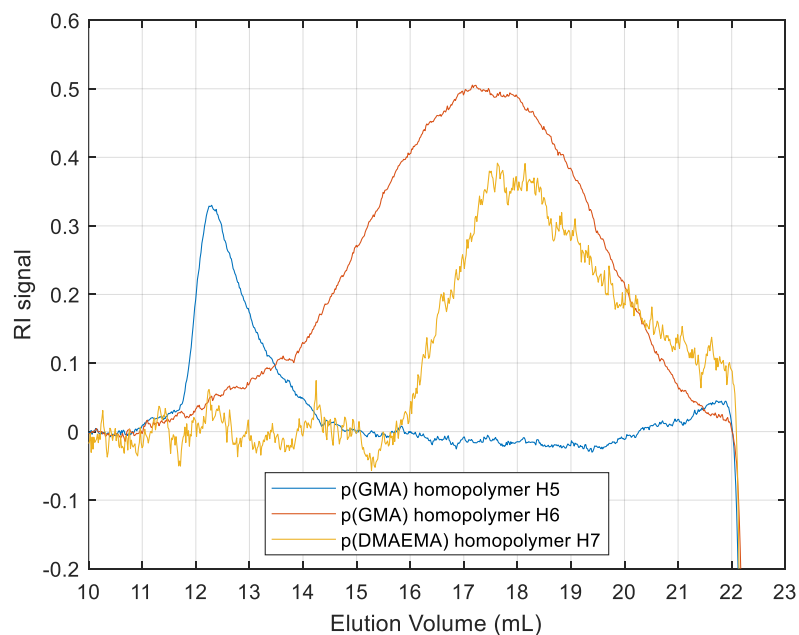


Figure D-13: SEC/RI traces observed for the DMF soluble part of the homopolymers **H5**, **H6** and **H7**. A low solubility for these polymers was observed in DMF indicating a low control of the ATRP and RAFT polymerization processes when using the synthesis conditions in Table 6-2. SEC analysis performed with DMF solvent at 50 °C and flowrate 1 mL/min.

Additional photos and analytical results illustrating the use of p(GMA-OA)-*b*-p(DMAEMA) copolymers for the encapsulation and delivery of hydrophobic molecules, namely Nile Red and Oleanolic acid.

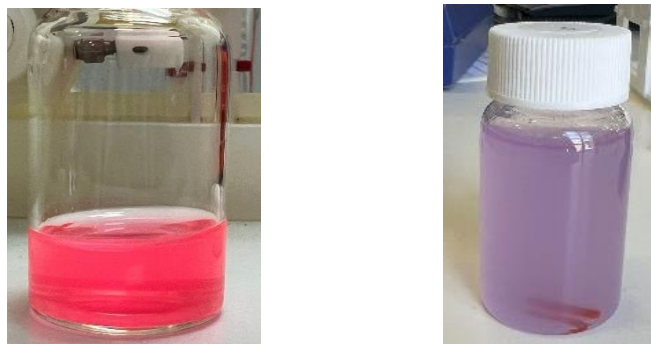


Figure D-14: Visual confirmation of the self-assembly of synthesized amphiphilic p(GMA-OA)-*b*-p(DMAEMA) copolymers, demonstrating the formation of aggregates that encapsulate Nile Red. The image on the left shows a THF solution containing the copolymer and Nile Red. The picture on the right shows a 50:50 THF/water solution formed by the dropwise addition of water to the initial solution. The color change indicates the encapsulation of Nile Red within the hydrophobic portion of the p(GMA-OA)-*b*-p(DMAEMA) aggregates.

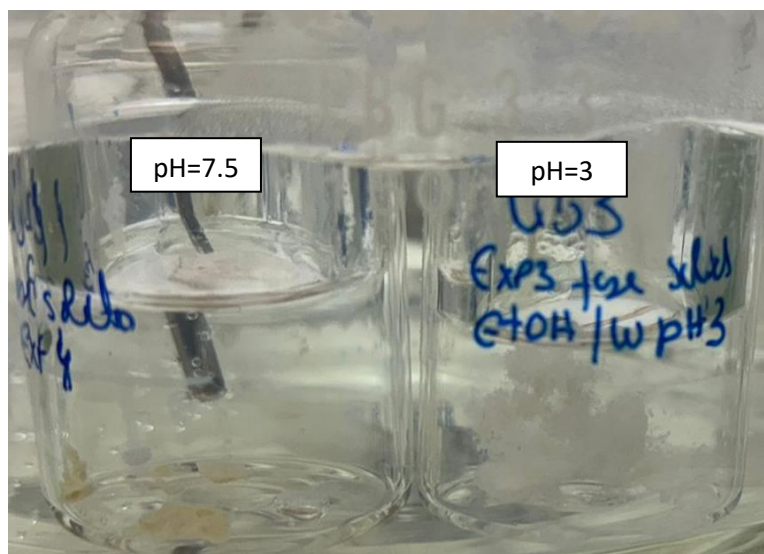


Figure D-15: Images of block copolymer p(GMA-OA)-b-p(DMAEMA) particles, pre-loaded with oleanolic acid, and dispersed in 30/70 ethanol/water solutions at pH 7.5 (left) and pH 3 (right), both at 40 °C. Swollen particles are observed at pH 3, while material collapse occurs at pH 7.5. Under acidic conditions, the ionization of the tertiary amine groups of DMAEMA creates a repulsive polycationic effect in the p(DMAEMA) block.

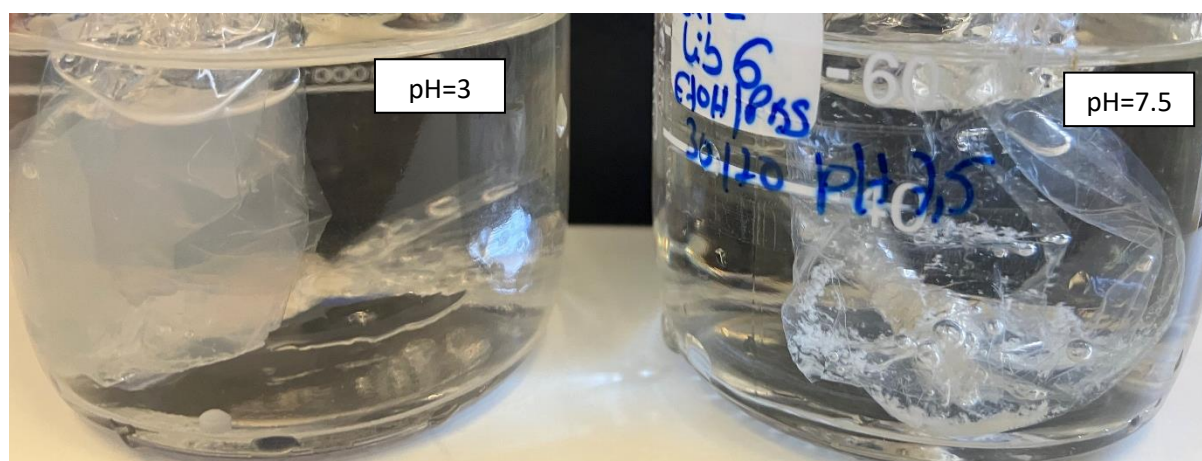


Figure D-16: Images of block copolymer p(GMA-OA)-b-p(DMAEMA) suspensions pre-loaded with oleanolic acid and dispersed in 30/70 ethanol/water solutions at pH 3 (left) and pH 7.5 (right) at 40 °C. Both systems were placed under dialysis in cellulose bags. A stable suspension is observed at pH 3, while particle precipitation occurs at pH 7.5. Under acidic conditions, the ionization of the tertiary amine groups of DMAEMA creates a repulsive polycationic effect in the p(DMAEMA) block that stabilizes the suspension.

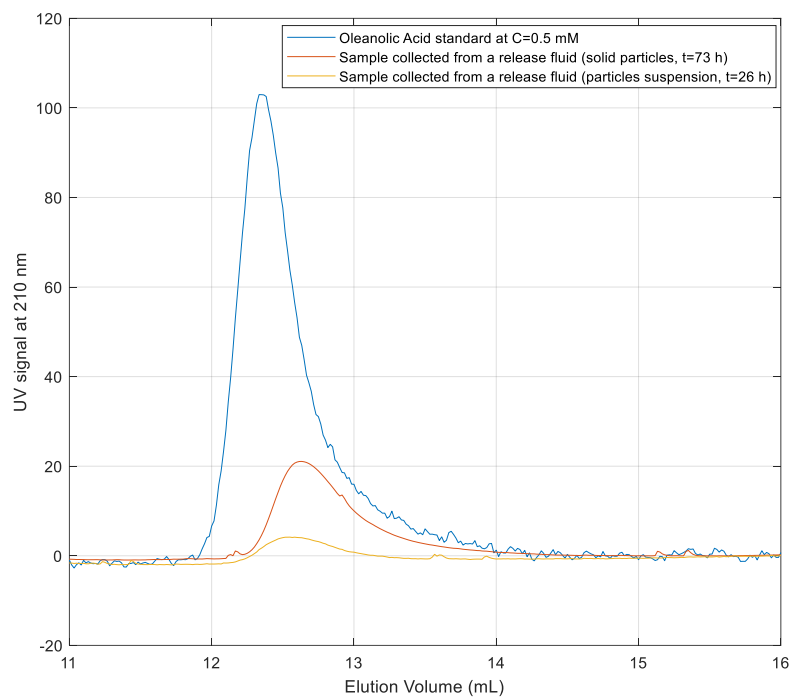


Figure D-17: Illustration of HPLC-DAD analysis used for the quantification of oleanolic acid released from p(GMA-OA)-*b*-p(DMAEMA) encapsulation materials. The HPLC-DAD chromatogram of the oleanolic acid standard at a concentration of 0.5 mM is presented alongside the analysis of the release fluid samples from experiments involving solid particle and particle suspension encapsulation systems.