Fractionation of olive leaf extracts with molecularly imprinted adsorbents working in continuous processes at the preparative scale

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Abstract

Olive leaves present high content of bioactive compounds. Despite this, they are discarded away without repurposing. These compounds are useful in a variety of industries such as food, cosmetics and pharmaceuticals. The present research proposes a continuous process of adsorption-desorption that relies on molecularly imprinting polymers (MIPs) as adsorbents, to purify the bioactive compounds. The first part of this work involves the design and synthesis of MIPs targeting quercetin, oleanolic acid, and oleuropein, which are representative compounds from the families of flavonoids, triterpenoids, and secoiridoids, respectively. Subsequently, these synthesized MIPs were assessed using solid-phase extraction to determine their retention capacities and selectivity towards bioactive compounds from the aforementioned families. This step permitted to select the materials that were suitable for continuous processes of adsorption-desorption. The criteria of this selection were based on the morphology of the materials (size, swelling etc.) and their retention capacity as well as their selectivity. For the continuous system, different-sized columns were utilized (up to 25 g of adsorbent was packed). The selected MIP was saturated with the extract and operated in recycle closed loop system, followed by desorption using various solvent fractions. Additionally, isotherm studies helped design the conditions for separating different families of bioactive compounds. The desorbed fractions were analysed using high-performance liquid chromatography (HPLC). The results show that the different desorbed fractions isolated contain specific types of bioactive compounds. It was also found that a second purification step could be considered to further increase the purity of the compounds.

Keywords: Molecular Imprinting Polymer, bioactive compounds, continuous process

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Chapter 1. Introduction

The olive tree (Olea europaea L.), belonging to the Oleaceae family, exhibits a notable capacity for drought resilience and is primarily indigenous to Mediterranean climatic conditions. It is widely cultivated in tropical and subtropical zones, particularly in nations such as Greece, Italy, Spain, Australia, Portugal, France, Cyprus, Palestine, Jordan, the USA, Morocco, Turkey, and Tunisia, where it thrives predominantly in regions sharing Mediterranean environmental characteristics. It is notable that the Mediterranean basin accounts for over 90% of the global cultivated acreage, estimated to have surpassed 11 million hectares by the year 2017 (1). In recent times, there has been a notable surge in global interest surrounding medicinal and aromatic plants, which serve as significant sources of raw materials for various industries including food, cosmetics, and pharmaceuticals. These plants are utilised to produce both traditional and innovative food products, as well as pharmaceuticals. Among these plants, the perennial olive tree has been esteemed for many centuries, with contemporary recognition stemming from its substantial phenol content, which possesses commendable antioxidant properties. Throughout the years, numerous initiatives have been proposed to assess and utilise agricultural byproducts. Many instances have revealed that discarded byproducts may contain higher concentrations of bioactive compounds compared to the primary produce.[2] [3].

Figure 1 Olive Leaf [44]

1.1. Bioactive compounds in olive leaf

Bioactive compounds are naturally occurring elements found in various food crops, which are categorised as either essential or non-essential components. In contrast to essential nutrients that are vital for human biological functions, non-essential compounds are not required for physiological processes and their absence does not lead to health disorders (3). Nevertheless, non-essential groups, particularly phytochemicals prevalent in food crops, are highly regarded for their additional health-promoting effects beyond essential nutrients. Among these effects, the antioxidative, anti-atherosclerotic, antimicrobial, and anti-inflammatory properties are particularly noteworthy. The processing methods employed in the functionalisation of foodstuffs and the production of nutraceutical supplements exert a significant influence on three key parameters: (1) bio-accessibility, which refers to the release of bio-compounds from food cell walls for absorption into the bloodstream, (2) bioavailability, indicating the availability of active compounds for physiological activity after absorption, and (3) bioactivity.

1.1.1. Polyphenols

Polyphenols are organic compounds synthesised by plants that act as protective agents against a range of environmental stressors, including UV radiation, parasites, and insect pests. The diversity of polyphenols is considerable, with over 10,000 distinct compounds identified to date. Variations in their occurrence have been observed among plant species and different plant parts. These compounds exhibit significant antioxidant and anti-inflammatory properties, conferring notable health benefits to humans, including cardiovascular protection, anticancer effects, and anti-aging properties. In addition to their preventive roles, polyphenols are being increasingly investigated for their therapeutic potential in treating various diseases. The molecular configuration of phenolics is characterised by a linkage between hydroxyl groups and an aromatic ring. The efficacy of phenolics in antioxidation is, in part, determined

by the positioning and arrangement of hydroxyl groups, as well as their presence and concentration in food matrices, whether in free or bound states. Studies have indicated that the aglycone portion exhibits superior biological activity compared to the glycoside portion (aglycones bound to glycones). Phenolics have the capacity to scavenge free radicals, which can impede the initiation phase and delay the propagation phase of lipid oxidation. This may potentially reduce the formation of volatile components resulting from hydroperoxide degradation. Olive leaves contain a plethora The phenolic compounds are primarily categorised as secoiridoids, flavonoids, and simple phenols. Among these, secoiridoids, such as oleuropein and oleuropein-aglycone, are of particular interest due to their recognised biological activity, which has attracted significant research attention. Secoiridoids, which are characteristic of the Oleaceae family to which Olea europaea L. belongs, are derived from iridoids, which are typically found in plants with a glycosidic structure originating from monoterpenes. The cleavage of cyclopentane rings leads to the formation of secoiridoids, which are commonly bound to glycosides and distinguished by the presence of an elenolic acid linkage in their molecular structures [8]. Oleuropein, the most prevalent phenolic compound found in both olive leaves and oil, displays antioxidant and free radical-scavenging properties. Its presence is not limited to the leaves of the olive plant but extends to the stems and flowers as well. As an ester of enolic acid and 3,4-dihydroxyphenyl ethanol, oleuropein serves as the primary glycoside in olives and is believed to be primarily responsible for their antioxidant and anti-melanogenesis effects is notable that olive leaves, which are rich in oleuropein, are known to confer substantially higher levels of antioxidant activity compared to olive fruit. It is notable that olive leaves, which are rich in oleuropein, are known to confer substantially higher levels of antioxidant activity compared to olive fruit.

The phenolic substances present in olive leaf play a significant role in the stability and flavour profile of the olive leaf. Among these compounds, tyrosol (4-hydroxyphenethyl

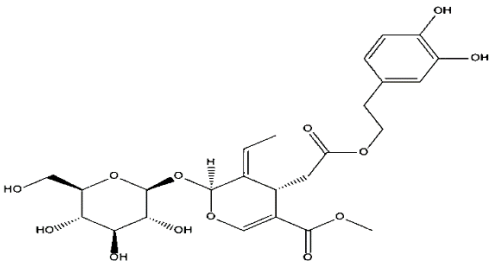
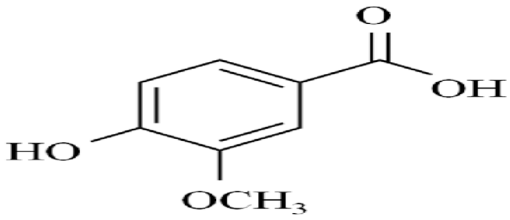
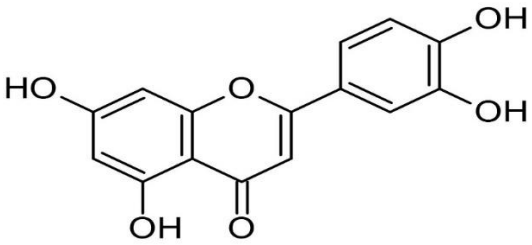
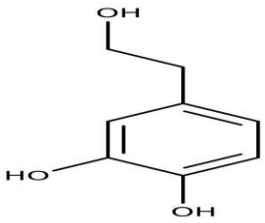
alcohol) and hydroxytyrosol (3,4-dihydroxyphenethyl alcohol) are widely recognised as primary constituents [8]. In addition, other phenolic compounds, including caffeic acid, o-coumaric acid, p-coumaric acid, ferulic acid, gallic acid, vanillic acid, p-hydroxybenzoic acid, p-hydroxyphenylacetic acid, protocatechuic acid, sinapic acid, syringic acid, tyrosol glucoside, and vanillic acid, are commonly found. Furthermore, the aglycones of oleuropein and ligstroside, along with their esters hydroxytyrosol and tyrosol with elenolic acid, diacetoxy and dialdehydic forms of these aglycones, elenolic acid, and flavonoids have also been identified in the polar fraction of olive leaf [10].

Table 1. Phenolic substance in olive leaf

Secoiridoids	(a) Oleuropein aglycone
	(b) Deacetoxy oleuropein
	(c) Oleocanthal and oleacin
	(d) Ligstroside aglycone
Phenolics	(a) Hydroxytyrosol
	(b) Tyrosol
	(c) Hydroxytyrosol glycole

Phenolic acids	(a) Gallic acid
	(b) Protocatechuic acid
	(c) p-Hydroxybenzoic acid
	(d) Vanillic acid
	(e) Caffeic acid
	(f) Syringic acid
	(g) p- and o-coumaric acid
	(h) Ferulic acid
	(i) Cinnamic acid
Flavonoids	(a) Luteolin
	(b) Apigenin
	(c) quercertin
Lignans	(a) (+) Pinoresinol
	(b) (+) Acetoxypinoresinol

Table 2. Main Phenolic substance in olive leaf [45],[46],[47],[48]

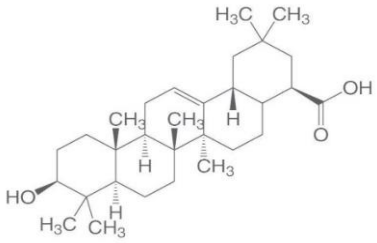
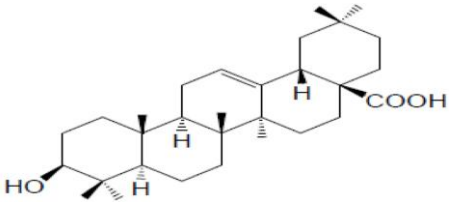
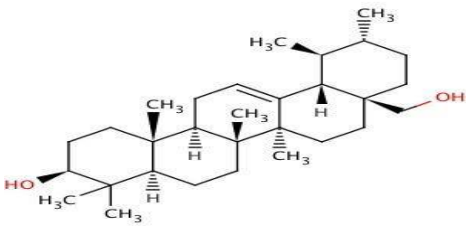
Oleuropein	
Vanillic acid	
Luteolin	
Hydroxytyrosol	

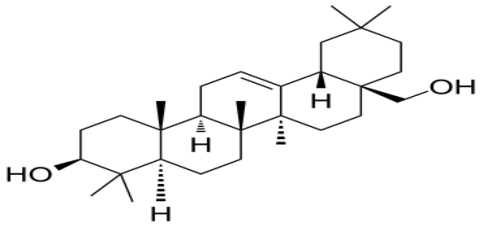
1.1.2. Triterpenoids

Triterpenoids, also known as triterpenes, are secondary metabolites that are commonly found in the outer waxes or coatings of leaves. Chemically, they are characterised by the presence of six isoprene groups, which give rise to a structure comprising thirty carbons. Triterpenoids are derived from the precursor molecule squalene, which is synthesised through the ring

formation of farnesyl pyrophosphates. These compounds can exist in either a free state or as glycosides, which are formed by the binding of carbohydrate molecules to them. Triterpenoids play a significant role in the body's defence against oxidative stress and are associated with protective effects against a number of disorders, including carcinogenic, microbial, fungal, viral, atherogenic, gastrointestinal, and hypertensive conditions. The principal triterpenes found in olives, olive tree leaves, and olive oil are oleanolic acid, maslinic acid, uvaol, and erythritol.[14]

Table 3. Triterpenoids in olive leaf [49][50][51][52]

Oleanolic acid	
maslinic acid	
uvaol	

erythrodiol	

Chapter 2. Extraction of olive leaf compounds

Classical extraction techniques commonly employed to recover active compounds from olive leaves typically involve the extensive use of organic solvents, along with agitation and/or elevated temperatures, to facilitate the diffusion and transfer of desired compounds from the matrix. These methods include percolation, solid-liquid extraction techniques such as maceration and Soxhlet extraction, distillation, and heat reflux. The selection of solvent is of paramount importance in ensuring optimal solubility and diffusivity of target ingredients. Ethanol, methanol, and hydroalcoholic solvents are the most frequently utilized solvents due to their efficacy in extracting a broad spectrum of hydrophilic and lipophilic compounds from olive leaves. However, traditional solid-liquid extraction approaches present several limitations. These include inefficient diffusivity, which leads to prolonged extraction durations; the excessive use of potentially toxic organic solvents; extended processing times; and limited adaptability. Furthermore, both thermal and non-thermal conventional extraction methods are associated with high energy consumption, production costs, and the risk of compound degradation due to prolonged exposure to solvents or elevated temperatures.

2.1. Solid-Liquid extraction

Organic solvent-based solid-liquid extraction methods are extensively utilised in industrial contexts to extract analytes from solid matrices. In recent years, there has been a notable increase in commercial initiatives aimed at extracting intracellular compounds and liquids from plant cellular tissues, which has led to a significant growth in the adoption of various solid-liquid extraction methodologies. This trend has also led to a surge in patent activity, with a particular focus on the isolation of phenols, particularly oleuropein, from olive leaves using traditional solid-liquid extraction techniques. In laboratory settings, the isolation of phenols typically employs conventional techniques such as maceration and Soxhlet extraction, utilising a range of extractants, including ethanol-water mixtures and hexane [13].

2.2. Pressurized liquid extraction

Pressurised liquid extraction (PLE) is a methodological approach to extraction that integrates heightened levels of temperature and pressure alongside liquid solvents, facilitating rapid and efficient extraction of analytes from solid matrices. In cyclically pressurised extraction, the application of pressure is directed towards the extracting liquid, wherein samples undergo periodic hydration variations within a specified time frame [14]. A number of studies have demonstrated the efficacy of PLE in a range of applications, including the extraction of pesticides from diverse food substrates, the extraction of phenolic compounds from parsley, the determination of herbicides in food samples employing non-aqueous liquid extraction, and its suitability as a technique for the fat-free extraction of polychlorinated biphenyls from fatty foods and feed matrices [15] [25].

2.3. Supercritical carbon dioxide extraction

Supercritical fluid extraction employing CO_2 represents an appealing and expedient alternative to conventional Soxhlet extraction methods, distinguished by its eco-friendly attributes. This appeal is rooted in CO_2's inherent properties, including its safety, non-toxicity, non-flammability, cost-efficiency, absence of odour and taste, high selectivity, wide availability, ease of removal, rapid action, and flexibility in adjusting extraction parameters across a broad range of temperature and pressure settings [16]. Consequently, supercritical carbon dioxide extraction (SC-CO_2) has emerged as a proficient and sustainable approach for extracting bioactive compounds (such as carotenoids, flavonoids, and phenols) from diverse fruits and vegetables. This efficacy is facilitated by the use of relatively mild extraction temperatures and the generation of residue-free extracts devoid of organic solvents. The low viscosity of SC-CO_2 facilitates thorough penetration into matrices for efficient extraction. Furthermore, its minimal latent heat of evaporation and high volatility enable residue-free removal. Furthermore, SC-CO_2 enables supercritical operations at low pressures and near-ambient temperatures. Nevertheless, it is essential to acknowledge that the primary drawback of SC-CO_2 extraction is its higher initial investment costs in comparison to traditional atmospheric pressure extraction techniques [16].

2.4. Microwave assisted extraction

The microwave-assisted extraction (MAE) method, renowned for its minimal organic solvent consumption and cost-effectiveness, has been increasingly employed as an alternative laboratory-scale extraction technique. The method significantly reduces the time required for leaching compared to conventional maceration procedures, which typically demand a minimum of five hours. MAE offers several advantages over traditional methods such as maceration, including a shortened extraction duration, improved extraction efficiency, reduced labour involvement, and heightened selectivity in extraction. These attributes position the method as a highly favourable approach for extracting phenolic compounds from olive leaves. [17].

2.5. Ultrasound assisted extraction

The investigation into the application of ultrasound in food processing has been comprehensive, with a particular focus on its physical and chemical impacts. Ultrasound induces a range of physical forces in liquids, including vibration, pressure, and mechanical agitation, even in the absence of acoustic cavitation. Moreover, acoustic cavitation results in the generation of microjets, shear forces, shock waves, radical formation, and acoustic streaming. This high-energy input not only amplifies mechanical effects in heterogeneous processes but also instigates novel chemical reactions, resulting in the formation of unexpected compounds. The application of ultrasound facilitates enhanced extraction rates by augmenting mass transfer rates and potentially disrupting cell walls via the formation of microcavities, thus yielding greater product yields within reduced processing durations. Moreover, it mitigates solvent usage, thermal degradation losses, and water and energy consumption. Ultrasound-assisted extraction (UAE) has the potential to enhance aqueous extraction and substitute organic solvents with generally recognised safe (GRAS) alternatives. Initial concerns regarding the generation of radicals by ultrasound in the context of UAE have been addressed, with the objective of enhancing the functional properties of specific food types. Despite the advent of new technologies that have made the commercial-scale extraction of bioactive compounds economically viable, the

industrial implementation of ultrasound-assisted extraction (UAE) remains a significant challenge. [18].

Chapter 3. Synthesis and Development of MIPs

Molecularly imprinted polymers (MIPs) represent a class of synthetic materials featuring artificially engineered recognition sites that possess the ability to selectively rebind a target molecule, prioritising it over closely related compounds. The synthesis of these polymers involves the polymerisation of functional monomers and the cross-linking of these monomers around a template molecule, resulting in the formation of a highly cross-linked three-dimensional polymer network. The selection of monomers is influenced by their capacity to interact with the functional groups of the template molecule. Subsequently, the template molecule is extracted, leaving behind binding sites that are characterised by shapes, sizes, and functionalities that complement the target analyte. MIPs exhibit notable stability, resilience, and resistance to a broad spectrum of pH levels, solvents, and temperature variations. Consequently, the functionality of MIPs mirrors the selective retention mechanisms observed in natural receptors, such as antibody-antigen interactions, albeit without the associated limitations in stability. Furthermore, it is notable that the synthesis of MIPs is cost-effective and straightforward, rendering them a compelling alternative to natural receptors [19].

3.1. Classification of MIPs

Three distinct methodologies for the synthesis of molecularly imprinted polymers (MIPs) have been documented: covalent, non-covalent, and semi-covalent approaches. The covalent method, initially developed by Wulff and Sarchan, entails the formation of reversible covalent bonds between the template and monomers prior to polymerisation. Upon the removal of the template, achieved through the cleavage of the corresponding covalent bonds, these bonds are reformed upon the rebinding of the analyte, resulting in a relatively uniform distribution of binding sites and the minimisation of non-specific interactions. However, this approach is constrained by the challenge of designing a template-monomer complex with readily reversible covalent bonds under mild conditions [4]. The semi-covalent approach represents an intermediate option, whereby the template is covalently bound to a

functional monomer, yet template reattachment is contingent upon non-covalent interactions.In contrast, the non-covalent method, introduced by Mosbach and colleagues, relies on weak non-covalent interactions, such as hydrogen bonding and ionic interactions, between the template molecule and selected monomers before polymerisation. This approach is favoured for MIP synthesis due to its experimental simplicity and the wide availability of monomers capable of interacting with various templates. [20].

3.2. Free Radical Polymerization

The most effective approach to synthesising Molecularly Imprinted Polymers (MIPs) is Free Radical Polymerisation (FRP). This process is renowned for its simplicity and scalability, particularly when employing acrylic acid or vinyl monomers. FRP entails the continuous generation of free radicals throughout the polymerisation process, resulting in a heightened concentration of such radicals. Nevertheless, this characteristic presents challenges as the progression and cessation of the reaction become unpredictable due to the potential interaction of highly reactive radical species, thereby yielding inactive polymer chains. [21]

3.2.1. Initiation

The initial stage of the polymerisation process is the initiation stage, during which the formation of active centres, predominantly radicals, initiates the commencement of polymerisation. Not all monomeric species possess the requisite attributes for initiation. Subsequently, one or more radicals emerge from the initiating molecules in the inaugural phase, subsequently facilitating their transfer to monomeric units in the subsequent stage [24].

3.2.2 Propagation

During radical polymerisation, the primary activity of a polymer is the elongation of its chain length, which is known as propagation. Following the initiation of a free radical, it engages with another monomeric subunit, utilising one of the pi bond electrons to form a stable bond with an adjacent carbon atom. The remaining electron instigates radicalisation in the second carbon atom, thus encompassing the entire molecule. Following the initiation of the polymer chain, propagation continues until all monomeric resources are depleted or until termination mechanisms are initiated [24].

3.2.3Termination

Termination serves as the ultimate stage in the polymerization sequence, manifesting through diverse modalities. The nuanced progression of termination encompasses distinct phases. Modulating initiator concentrations emerges as pivotal strategy for regulating polymer chain lengths; a reduction in initiator concentration fosters the generation of lengthier chains, whereas elevated concentrations precipitate the formation of numerous abbreviated chain structures [24].

3.3 Bulk Polymerization

This methodology is distinguished by its operational simplicity, involving the mixing and dissolution of constituents in a solvent in accordance with a predetermined stoichiometric ratio. Subsequently, polymerisation commences under controlled conditions of either heating or ultraviolet radiation, resulting in the formation of molecularly imprinted polymers (MIPs). Subsequently, the acquisition of small particles is achieved through a sequence of crushing, grinding, and screening procedures. However, particles produced via this method often exhibit uneven and irregular sizes. Furthermore, the imprinted binding sites of MIPs generated through this technique are often coated, which renders the removal of the template challenging and results in a diminished specific adsorption capacity [22].

3.4 Suspension Polymerization

Suspension polymerisation, defined as the dispersion of solid particles within a liquid medium, commences with liquid-liquid dispersion and concludes with solid-liquid dispersion. Among heterogeneous polymerisation techniques, it is regarded as one of the less complex methods. The process commences with the separate preparation of organic and aqueous phases, with the volume ratio of organic phase to aqueous phase typically ranging from 0.1 to 0.5 or higher. The organic phase is formulated by incorporating monomers, initiators, crosslinker molecules, and diluents, while the aqueous phase entails the dissolution of surfactants and stabilisers in distilled water [25].

3.5 Variables of the MIPs Synthesis Process

Molecularly imprinted polymers (MIPs) are typically composed of imprinted molecules (templates), functional monomers, cross-linkers, initiators, and solvents (porogens). The selection and precise dosage of these constituents during the fabrication process are of paramount importance for the production of MIPs with specific properties and functionalities [26].

3.5.1. Template

Templates represent a foundational aspect of the fabrication process of molecularly imprinted polymers (MIPs), exerting influence over the selection of functional monomers, crosslinkers, initiators, and porogens. In most cases, these templates represent the specific compounds that are subsequently analysed. It is of paramount importance that templates possess the capacity to form stable complexes with functional monomers, which necessitates the presence of inert functional groups within them. Moreover, templates must demonstrate robust thermal and chemical stability to withstand the polymerisation process without undergoing degradation [27], [28].

3.5.2 Functional Monomer

The attributes of functional monomers exert a significant influence on the efficacy of the creation of Molecularly Imprinted Polymers (MIPs). The role of functional monomers in mediating binding interactions within molecular imprinting technology is of pivotal importance. The selection of optimal monomers for imprinted material synthesis is guided by the strength and nature of template-monomer interactions, as these underpin the successful establishment of stable template-monomer complexes. In order to optimise these interactions and to tailor highly specialised binding sites for the template molecule, it is essential to align the activity of the functional monomer with that of the imprinted molecule (template). Moreover, the selection of the monomer must consider its compatibility with the solvent, which necessitates stability and solubility in the selected solvent. The optimal monomers are selected based on their propensity to exhibit cage effects or robust interactions in the specified solvent, thereby enhancing the binding cavity capacity and influencing the uniformity of the binding sites. [29,30].

3.5.3 Crosslinking agent

The crosslinker serves a uniform purpose in both surface-templated molecularly imprinted polymer (ST-MIP) and matrix-templated molecularly imprinted polymer (MT-MIP) synthesis. Primarily, it facilitates the binding of the functional monomer complex and the template to generate a rigid structural framework. This rigidity ensures that the monomer structure surrounding the template remains unchanged even after the template is removed, thereby significantly influencing the stability of the resulting MIP. Consequently, the quantity of crosslinker employed requires careful consideration. Insufficient utilisation of crosslinker may compromise the stability of the MIP and result in template leakage during synthesis. Conversely, an excess of crosslinker can impede the formation of the requisite cavities between functional monomers and templates, thereby reducing the sensitivity of the binding site. In selecting a crosslinker, it is essential to consider a number of factors,

including the nature of the functional monomer, the bond type between the functional monomer and the template, the polymerisation method, and the choice of solvent. It is of paramount importance to ensure that the functional monomer and crosslinker are compatible in terms of reactivity, as this is essential for achieving uniform distribution of monomer functional groups during random copolymerisation [31],[32].

3.5.4 Solvent

The synthesis of a molecularly imprinted polymer (MIP) is contingent upon the characteristics and quantity of the solvent employed. The selection of an appropriate porogen has a profound influence on the interaction dynamics between the target molecule and the monomer. As a dispersing agent, the porogen plays a pivotal role in the uniform formation of cavities during polymerisation. Solvents such as toluene, chloroform, dichloromethane, and acetonitrile are commonly employed in MIP synthesis procedures. The solvent plays a pivotal role in facilitating the integration of all components (monomer, template, initiator, and crosslinker) into a cohesive phase, thereby contributing to the formation of microporous polymers with discernible pores. The optimal flow-through properties of the resulting MIP necessitate the generation of sufficiently large pores, a characteristic that is directly influenced by the volume of the solvent. Consequently, the term "porogen" is often used interchangeably with the solvent in this context. [33],[34].

3.5.5 Initiator

Free radical polymerisation represents the principal methodology employed in the synthesis of surface-templated molecularly imprinted polymers (ST-MIPs) and matrix-templated molecularly imprinted polymers (MT-MIPs). This process is characterised by three sequential stages: initiation, propagation and termination. The initiation phase is contingent upon the presence of an initiator

compound, which is essential for initiating polymerisation [34]. This phase commences upon the decomposition of the initiator into original molecular fragments, termed starter pieces, which possess unpaired electrons. These electrons then engage in bonding with carbon double bonds within the functional monomer, thus initiating the polymerisation process. Consequently, the selection and utilisation of an appropriate initiator are of paramount importance, as they exert a profound influence on the success of the polymerisation process [35],[36],[37].

3.6 MIPs application

Molecularly imprinted polymers (MIPs) are employed extensively across a diverse range of domains. They serve as biomimetic agents in enzyme-linked immunosorbent assay (ELISA) tests, facilitating the examination of natural system mechanisms. Moreover, MIPs are employed in a number of other fields, including bioimaging, cell culture, drug development, cancer diagnosis, and therapeutic interventions. It is noteworthy that the development of stimuli-responsive MIPs, which are capable of reversible binding and release triggered by external factors such as pH, temperature, light, and electric fields, enhances the optimisation of drug delivery strategies. Furthermore, the reusability of MIPs renders them particularly advantageous for catalytic processes. Furthermore, MIPs can be employed as sensors. [41],[42].

Chapter4. Material and Methods

4.1. Reagents

Table 4: Reagents used in synthesis and on the process

Reagents	Molecular Form	Molecular weight	Supplier
4-vinylpyridine (4VP)	CH2CHC2H2N	105.14	Alfa Aesar
Acetonitril (ACN)	CH3CN	41.05	Fisher Chemical
Azobisisobutyronitrile (AIBN)	$[(CH_3)_2C]_2N_2$	164.21	Fluka Analytical
Methanol (MeOH)	CH3OH	32.04	Fisher Chemical
Oleuropein	C25H32O13	540.52	PanReac
Quercetin	C15H10O7	302.24	Acros Organics
Rutin	C27H30O16	610.52	Acros Organics
Vanillic acid	C8H8O4	168.14	Sigma-Aldrich
Maslinic acid	C30H48O4	472.7	Cayman Chemical
Acrylamide (AAm)	C3H5NO	71.08	Fluka Analytical
Divinylbenzene (DVB)	C10H10	130.19	Alfa Aesar
Ethylene glycol dimethacrylate (EDGMA)	C10H14O4	198.22	Sigma-Aldrich

Methacrylic acid (MAA)	C4H6O2	86.60	Sigma-Aldrich
N, N'-Methylenebisacrylamide (MBAm)	C7H10N2O2	154.17	Sigma-Aldrich
Styrene (STY)	$C_6H_5CHCH_2$	104.15	Acros organics
Oleanolic acid	C30H48O3	456.7	

4.2. Equipment

Table 5: Equipment used in this work

Equipment	**Model**	**Brand**
Analytical balance	AS/220/C/2	RADWAG
Orbital shaker	SSMI	Stuart
Vacuum pump	RE30022C	Stuart
Heating plate	VMS-C7	VWR ADVANCED
Laboratory centrifuge	Centrifuge 5810 R	Eppendorf
pH meter	inoLab	WTW
SPE system	Manifold Standard 12 – Port Model	SIGMA ALDRICH
Stirring plate	S03 series	Lbx instruments
Ultrasound	ULTRASONS-H	P SELECTA
UV-Vis Spectrophotometer	P9 Double Beam	VWR®
High-performance liquid chromatography *(HPLC-DAD)*		Knauer

4.3. Synthesis of Molecular Imprinting Polymer (MIP)

A Parr 5100 pressurised glass reactor with a maximum capacity of 1 L was used to carry out the synthesis of the quercetin MIP particles by inverse suspension polymerisation. The synthesis of a molecular imprinting polymer is a complex process involving a number of key steps. In the reaction phase (dispersed phase), quercetin (2.72 g) and 4VP (9.64 mL) were dissolved in ACN/DMF 85/15 (140 mL). The solution was then mixed in an ultrasonic bath for 15 minutes to allow intermolecular interactions between 4VP and quercetin. EGDMA (18 mL) and AIBN (1.52 g) were then added to the solution, which was then ultrasonicated for one minute. In the continuous phase, n-heptane (560 mL) and Span 80 (3.83 g) were mixed and stirred vigorously until a clear solution was obtained. The two solutions were introduced into the reactor and degassed with argon for 15 min under stirring. Polymerisation was carried out at a stirring speed of 400 rpm for a reaction time of 24 h. Once the product was obtained, the template and any unreacted material were cleaned with a solvent (methanol/acetic acid, 90/10, v.v.). The cleaning process is continued until all unreacted material and the template have been removed. Finally, the polymer was allowed to dry. The dried material was then weighed, and the conversion of the reaction determined. The table below gives an overview of the MIPs used in the experiment

Table 6: MIP used at experiment

MIP	Template	Monomer	Crosslinker	Solvent	Initiator
MIP-QUER1	Quercetin	4VP	EGDMA	ACN/DMF	AIBN
MIP-OA1	Oleanolic acid	DMAEMA/ STY	MBAm	MeOH	VA044
MIP-OA2	Oleanolic acid	DMAEMA	EGDMA	MeOH	VA044

MIP-OA3	Oleanolic acid	DMAEMA	DVB	MeOH	VA044
MIP-OA4	Oleanolic acid	MAA	DVB	MeOH	AIBN
MIP-OLA	Oleuropein	NVP	MBAm	MeOH	VA044

4.4. Solid Phase Extraction

Solid Phase Extraction (SPE) is a widely employed sample preparation technique in analytical chemistry, which facilitates the separation, purification, and concentration of analytes from complex matrices. The methodology is based on the differential affinity of compounds towards a solid adsorbent material, which is packed in a cartridge or column. In the context of this research, the adsorption and elution characteristics of the synthesized molecularly imprinted polymers (MIPs) were evaluated for both polyphenols and triterpenoids from olive leaf extracts using the solid phase extraction (SPE) technique. The selected MIP were packed (200 mg) into SPE cartridges. The SPE test consisted in four steps, which are the conditioning of the packed materials (conditioning is passing solvent of solution making homogenous condition for absorption) , the loading of the extract, the washing of the materials and finally the elution. The solvent of conditioning as well as the washing was the same as one of the extractions Ethanol/water (80/20, v.v). Real extract solution which is concentration 1.5mM and solvent Ethanol/water (80/20, v.v) used. Ethanol/water (80/20, v.v) is also used for washing step. Last step of SPE assessment is elution and (methanol/acetic acid,90/10, v.v) used. 4 ml volume passed in each step. The obtained fractions of the loadings, washings and eluted fluids were injected into the HPLC.

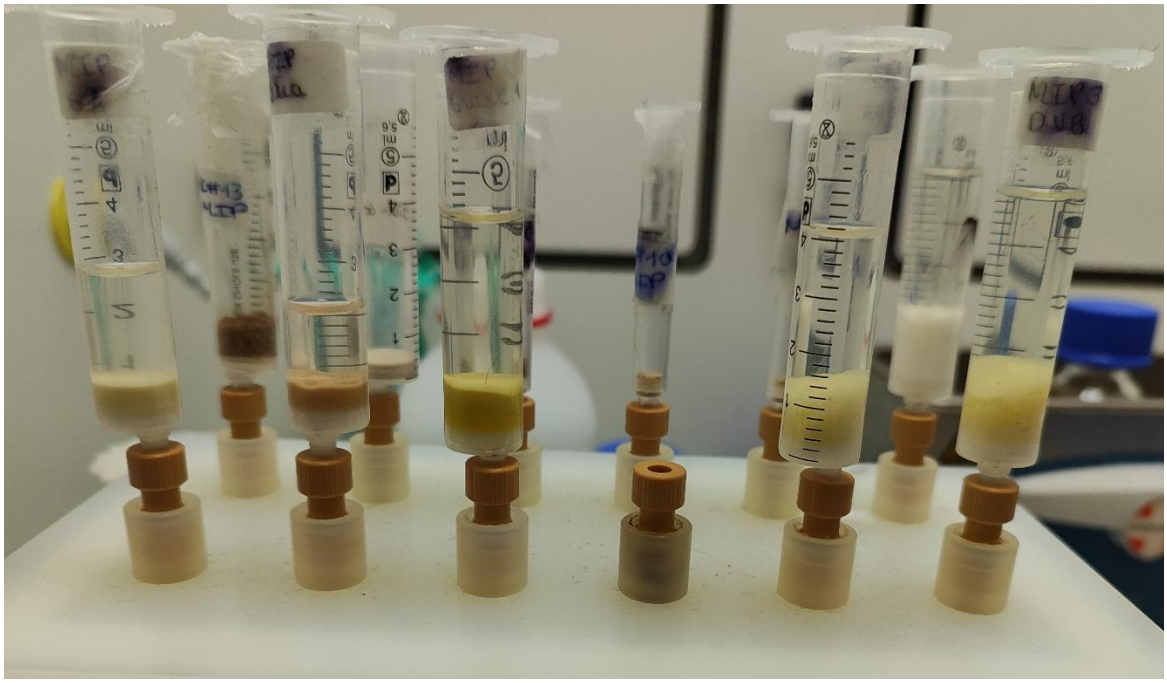


Figure 2 : SPE assessment

4.5. Process for isotherm

The adsorption isotherm is formed when an adsorbate and adsorbent interact for an adequate duration, resulting in a dynamic equilibrium between the interface concentration and the adsorbate concentration in the bulk solution. This equilibrium is of great importance for the design and optimisation of engineered adsorption processes. The efficacy of such processes is contingent upon a number of experimental variables, including pH, temperature, initial adsorbate concentration, the characteristics of the adsorbent, and the presence of coexisting ions. The experiment was conducted using a column with a length of 50 mm, a diameter of 4.6 mm, and a packing 295 mg of MIP_quer1. Subsequently, an equimolar solution prepared with pure compounds was passed through the column in a continuous system, initial volume of the solution is 30 ml and it recycle 2h and approximately four times the total volume being processed. At this juncture, the adsorbent reached its maximum adsorption capacity, signifying the completion of the adsorption process. The desorption process employs the same continuous system previously used for the adsorption process, with the solvent (methanol/acetic acid, 90/10, v/v) being mixed. Subsequently, sample injected to HPLC and the results obtained.

4.6. Purification of real extract in continuous system

For preparative-scale purification, a 250 mm-long, 20 mm-diameter column was employed, with 25.10 grams of MIP_quer1 packed into it. The continuous system employs a three-step purification process to extract bioactive compounds from a real extract. This process involves conditioning, adsorption and desorption by fractionation. In the experiment, a continuous recycle process was employed, whereby the solvent or solution was recycled within the system. Initially, the column was conditioned using a solvent mixture comprising ethanol and water in a 65/35 ratio by volume. Secondly, the adsorption process was conducted at room temperature and for a period of 24 hours. The real extract solution, designated as VR-2 from NATAC, was employed with a concentration of 3.4 mg/ml, a total volume of 720 ml, a solvent comprising ethanol/water (65/35, v.v.), and a flow rate of 2 ml/min. Once the saturation point has been reached, water with a pH of 3 is passed through the system in order to remove any impurities, such as sugars. The desorption process is continued using the fractionation method which is used different solvent mixture and table 7 represent solvent gradient. The desorption process is conducted at a temperature of 45 degrees Celsius in a closed loop, with a total volume of 500 ml of solvent and a flow rate of 2 ml per minute over a period of 12 hours. All samples were subjected to analysis using high-performance liquid chromatography.

Figure 3: Different size of column used in continuous process

Table 7: Solvent gradient used in desorption

Flow rate	Elution steps	Time (h)	V total (ml)	Solvent
2 ml/min	Elution 1	24	500	H2O ph.=3
	Elution 2	12	500	H2O
	Elution 3	12	500	EtOH/H2O 10/90
	Elution 4	12	500	EtOH/H2O 20/80
	Elution 5	12	500	EtOH/H2O 30/70
	Elution 6	12	500	EtOH/H2O 40/60
	Elution 7	12	500	EtOH/H2O 50/50
	Elution 8	12	250	EtOH/H2O 60/40
	Elution 9	12	250	EtOH/H2O 70/30
	Elution 10	12	250	EtOH/H2O 80/20
	Elution 11	12	250	EtOH/H2O 90/10
	Elution 12	12	250	EtOH
	Elution 13	12	250	MeOH
	Elution 14	12	250	MeOH/Acetic Ac 90/10

4.7. HPLC

HPLC is an device that was employed in this research. A Jasco MD-4010 photo diode array (PDA) detector was employed for the HPLC-DAD analyses. A Nucleosil® C18 analytical column, with dimensions of 15 cm in length and 4.6 mm in internal diameter, and a particle size of 5 µm, was selected for use at a temperature of 45 °C. The flow rate was set at 1 mL/min, and the absorbance changes were monitored at both 280 and 360 nm. The mobile phases employed for chromatographic analysis were as follows: (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 employed, commencing with 100% solvent A and concluding with 100% solvent B. This method was utilised for the analysis of polyphenols. A different method was employed for the analysis of triterpenoids, which involved the use of a solvent comprising acetonitrile and water (85/15) in conjunction with 0.5ml of phosphoric acid. The flow rate was set at 1 ml/min and the experiment was conducted at room temperature.

4.8.UV-vis

UV-vis is basic analytical device to examine analyte this is a type of absorption spectroscopy in which UV-visible light is absorbed by the molecule. The one used for this work is the P9 Double Beam UV-Visible Spectrophotometer from VWR®

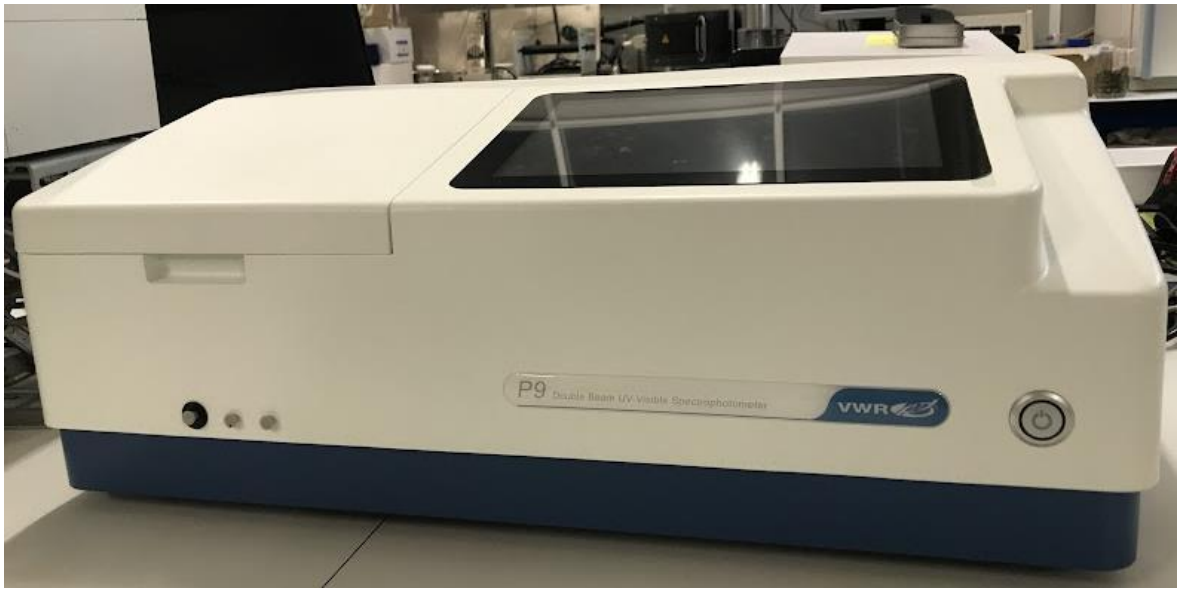


Figure 4: Uv-vis used in this work

4.9. FTIR

Fourier transform infrared spectroscopy (FTIR) is a highly effective analytical technique employed to obtain the infrared spectrum of absorption, emission, photoconductivity, or Raman scattering of a solid, liquid, or gas. In the context of molecularly imprinted polymers (MIPs), Fourier transform infrared spectroscopy (FTIR) is employed to identify functional groups and confirm the successful imprinting of the template molecule within the polymer matrix. The apparatus employed was a PerkinElmer Spectrum Two, and the characterisation was conducted in attenuated total reflectance (ATR) mode between 4000 cm-1 and 450 cm-1.

Chapter 5. Result and Discussion

This chapter presents the findings from the experiments conducted on the synthesis and application of Molecularly Imprinted Polymers (MIPs) for the purification of bioactive compounds from olive leaf extracts. The discussion includes an analysis of the data obtained from the various stages of the Solid Phase Extraction (SPE) process, and the implications of these results for the development of efficient and selective MIPs.

5.1. SPE for triterpenoids with pure compound

To assess the adsorption capacity of the synthesised MIPs, solid-phase extraction (SPE) experiments were conducted utilising oleanolic acid and maslinic acid in an equimolar manner.

Figure 5 presents the results of the loading phase of the equimolar mixture of the aforementioned triterpenoids. The highest retention was observed for mip_OA3, followed by mip_OA2. Oleanolic acid was the most retained in both cases, due to its role as the imprinting compound. This rule, however, is not applicable to mip_OA1, as it retains maslinic acid to a greater extent than oleanolic acid. The adsorption capacity of triterpenoids on oleanolic acid-imprinted materials is dependent on the quantity of crosslinker and functional monomer utilised in the polymerisation recipe. As the quantity of crosslinker increases, the retention of the corresponding material diminishes, as anticipated, given that the crosslinker lacks functional groups that can form hydrogen bonds with the triterpenoids. The materials imprinted with oleuropein and quercetin, designated as mip_OLEA and mip_QUERC1, respectively, exhibited low retention for the triterpenoids. This outcome serves to emphasise the pivotal role of the imprinting template in determining the selectivity of the final MIP towards target compounds.

Figure 6 illustrates the outcome of the washing phase. The washing step also exhibited a similar trend to the loading step, with high retention observed for mip_OA3, mip_OA4, and other compounds. This is an expected result.

Figure 7 depicts the outcome of the elution phase. Furthermore, the elution step exhibited a similar trend with a slight variation. MIPs mip_OA3 and mip_OA4 demonstrated a higher outcome, whereas the outcomes for the remaining MIPs aligned with the expected trend.

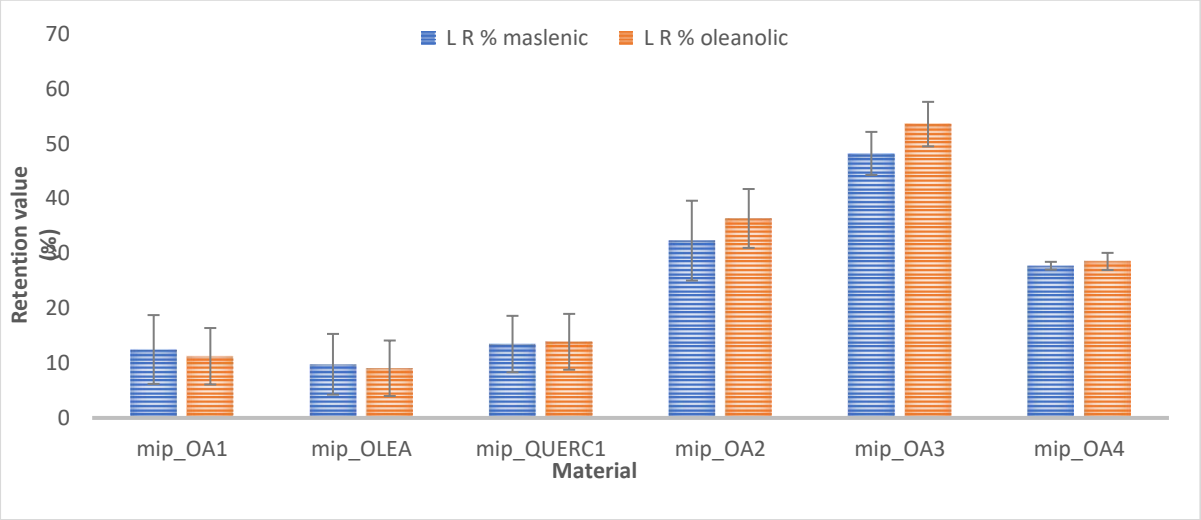


Figure 5: Loading step of the SPE assessment using an equimolar mixture of oleanolic acid and maslenic acid (2.25 mg ml^{-1} each) dissolved in ethanol-water (9:1; v.v), (4ml).

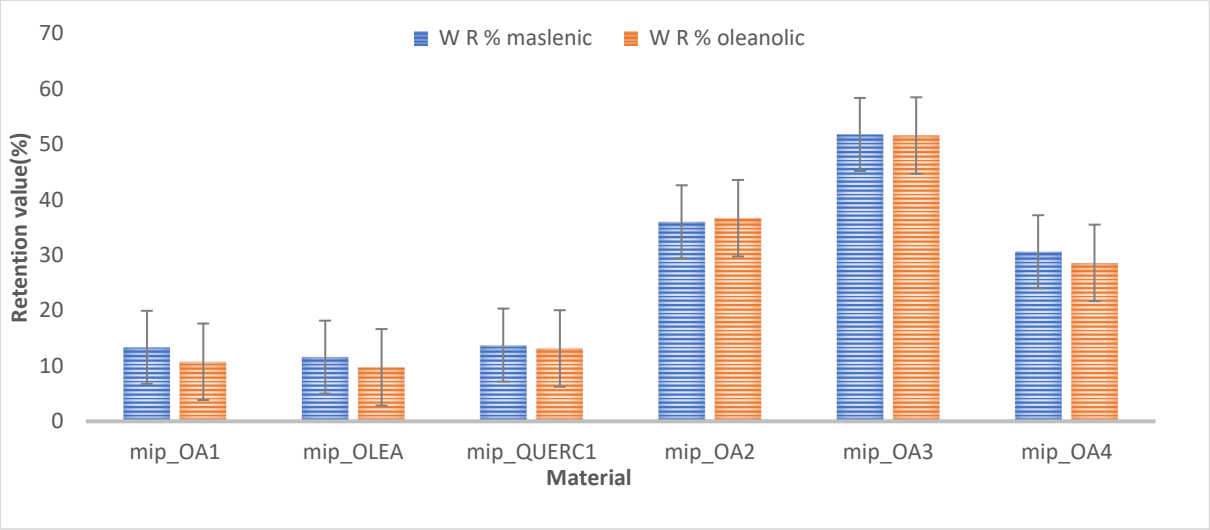


Figure 6: Washing step of the SPE assessment passing mixture of the solvent ethanol-water (9:1; v.v), (4ml).

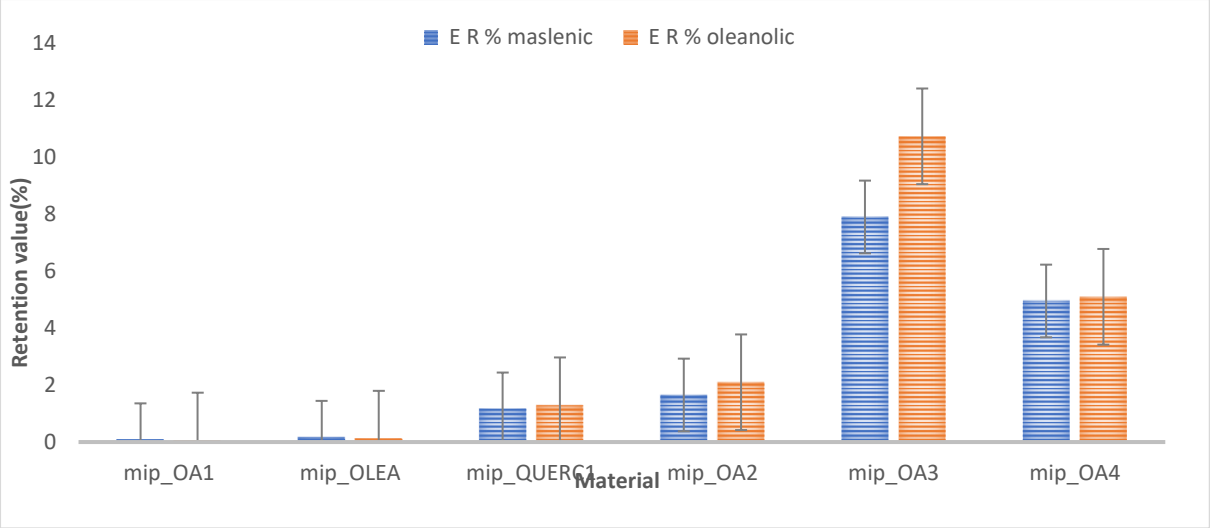


Figure 7: Elution step of the SPE assessment passing mixture of solvent Methanol-Acetic Acid (9:1; v.v), (4ml).

5.2.. SPE for polyphenols standard

The objective of this study is to evaluate the adsorption capacity of MIP for polyphenols. The SPE experiment employed an equimolar amount of vanillic acid, rutin, oleuropein and quercetin.

Figure 8 illustrates the retention of four different polyphenols in distinct MIPs. The results demonstrate that oleuropein is the least retained compound in all MIPs, likely due to its weak binding with the polymer, which results in low retention. For MIP_QUER1, it is evident that quercetin is retained in a high amount, which is consistent with the template of MIP being quercetin. This result is expected. Moreover, MIP_OA4 exhibits a markedly low retention for all compounds in comparison to the other MIPs. This is attributed to the hydrogen bonding and the low crosslinker amount, which collectively result in a weaker binding between the compounds and the polymer. Vanillic acid exhibits a high retention in almost all materials, with the exception of mip_QUER1. This is due to the fact that the vanillic acid configuration is small and adsorbs a high amount.

Figure 9 illustrates the results obtained from the washing phase. Furthermore, the washing phase was found to be correlated with the loading phase. It can therefore be concluded that the compound with the highest retention in the loading step will also have the highest outcome in the washing phase.

Figure 10 illustrates the outcome of the elution phase. The results demonstrate the strength of the binding ability of quercetin with MIP, despite the use of a strong solvent, which resulted in the retention of quercetin in the material.

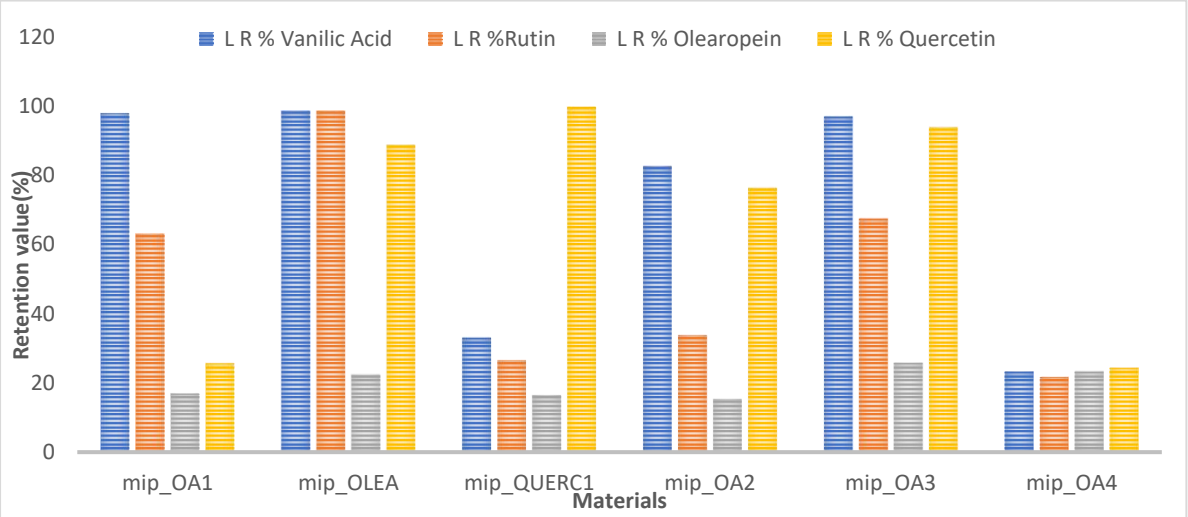


Figure 8: Loading step of the SPE assessment using an equimolar mixture of vanillic acid, rutin, oleuropein and quercetin (1.5 mM each) dissolved in ethanol-water (9:1; v.v), (4ml).

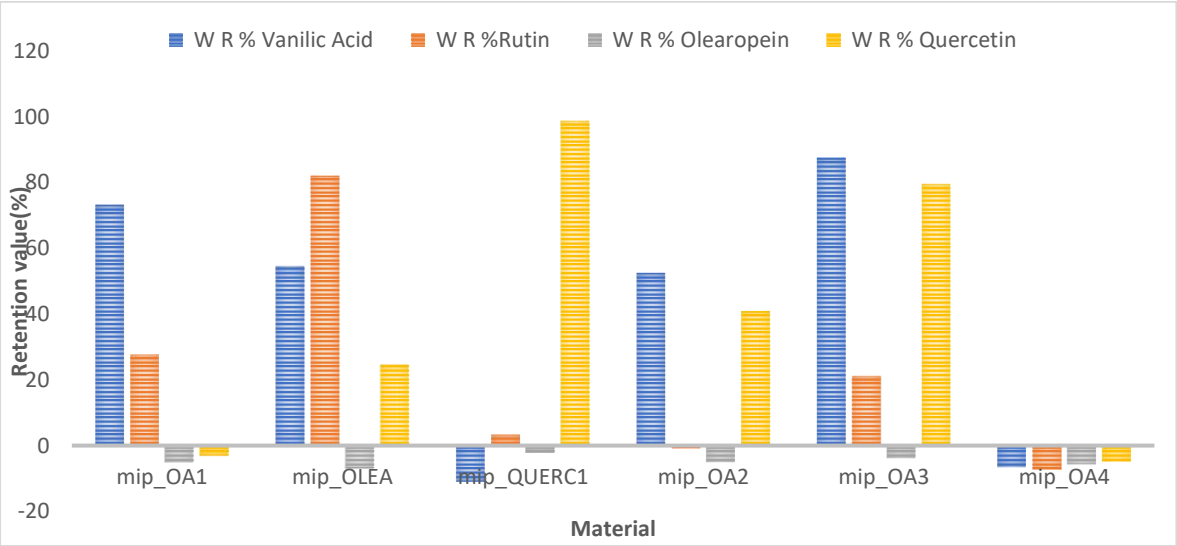


Figure 9: Washing step of the SPE assessment passing mixture of the solvent ethanol-water (9:1; v.v), (4ml).

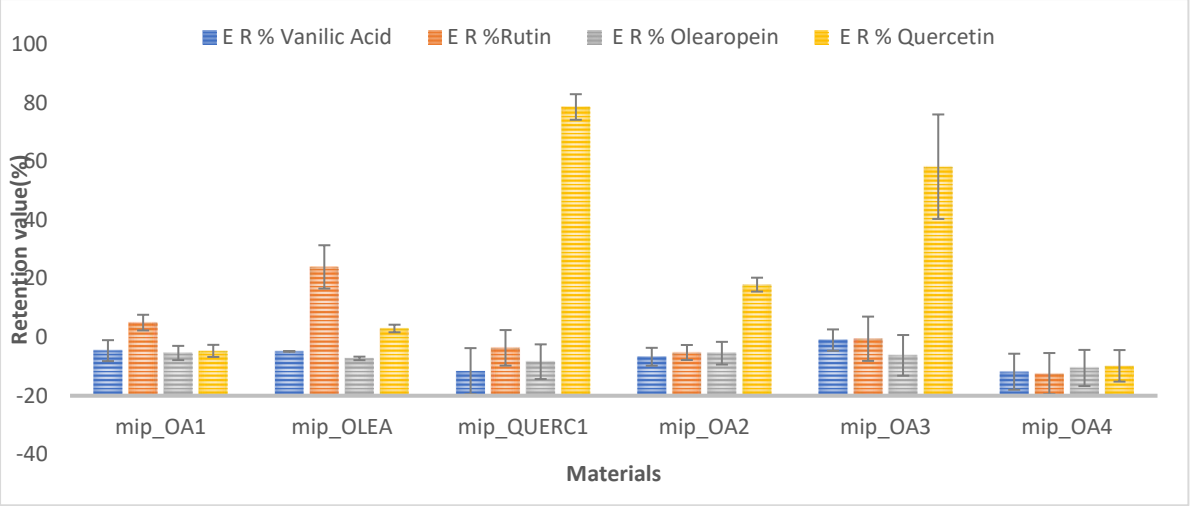


Figure 10: Elution step of the SPE assessment passing mixture of the solvent Methanol-Acetic acid (9:1; v.v), (4ml).

5.3.. SPE for Triterpenoids real extract

Subsequently, the adsorption capability of MIP for triterpenoids was investigated in the presence of pure compounds. The experiment was conducted using a real extract that is known to contain a high concentration of triterpenoids. The extract was subjected to solid-phase extraction (SPE) prior to the commencement of the experiment.

Figure 11 illustrates the results obtained during the loading step of the real extract. The loading phase of the real extract also exhibited a similar pattern to that observed in the previous experiment, in which equimolar amounts of maslinic acid and oleanolic acid were used. The results demonstrated that MIP_OA2 and MIP_OA3 exhibited high retention, in accordance with the previous findings. It can be observed that the retention values for mip_OLEA, Mip_quer1, and Mip_oa1 are lower than those of the other compounds, as would be expected.

Figure 12 provides an illustration of the results obtained during the washing phase. A significant difference was observed between maslinic acid and oleanolic acid. All MIPs retained oleanolic acid to a greater extent than maslinic acid, likely due to the fact that four of the six MIPs were synthesised

using oleanolic acid as a template. The reason for this is that oleanolic acid has a higher binding ability with the polymer than maslinic acid.

Figure 13 illustrates the outcome of the elution phase. The elution phase exhibited a similar trend to the washing step, with oleanolic acid demonstrating a higher retention than maslinic acid. The reason for this result is the same as that previously stated, namely the binding ability and template.

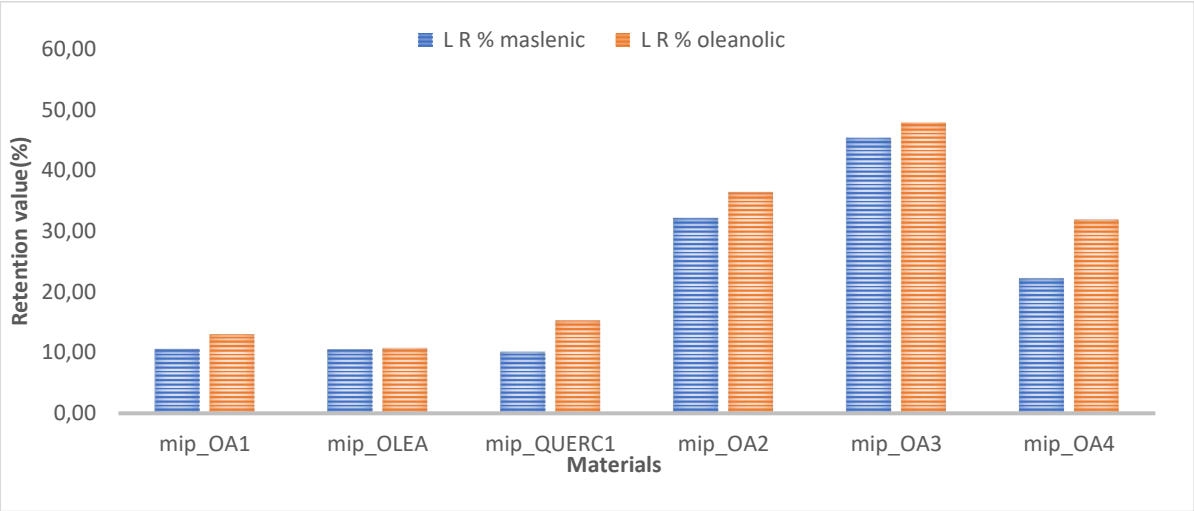


Figure 11: Loading step of the SPE assessment using an real extract trp30 (1.5 mM) dissolved in ethanol-water (9:1; v.v), (4ml).

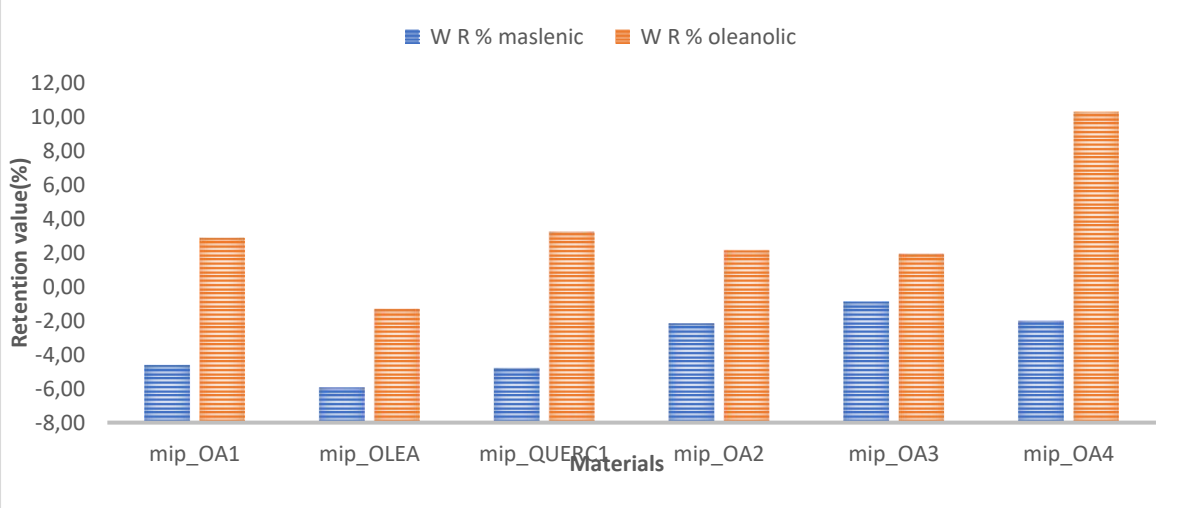


Figure 12: Washing step of the SPE assessment passing mixture of the solvent ethanol-water (9:1; v.v), (4ml).

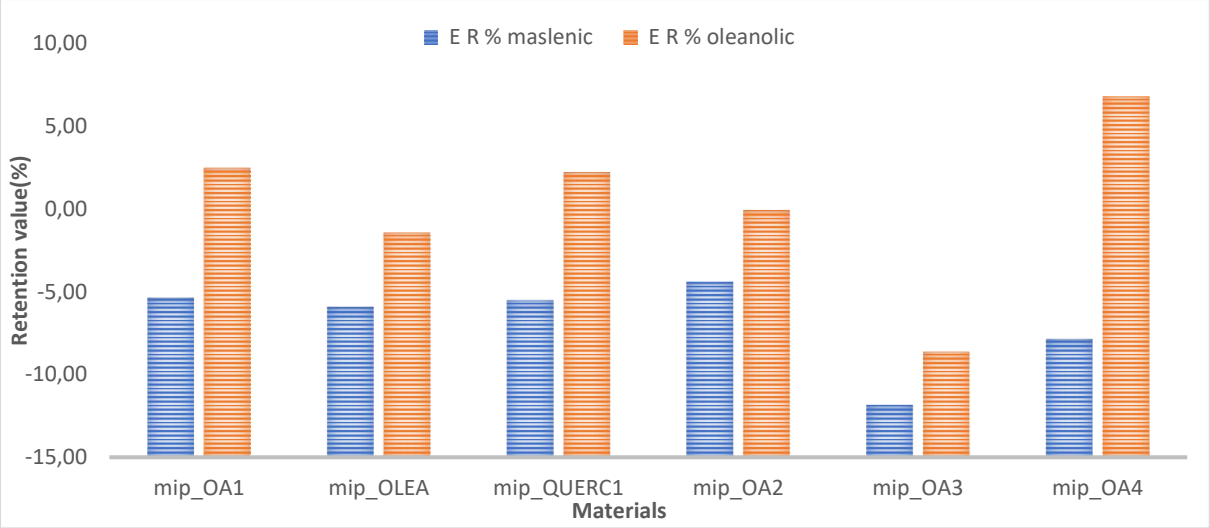


Figure 13: Elution step of the SPE assessment passing mixture of the solvent methanol-acetic acid (9:1; v.v), (4ml).

5.4. SPE for polyphenols real extract

Following the understanding of the adsorption capacity of MIPs for polyphenols using pure compounds, an experiment was conducted with a real extract, which has a high polyphenol content, using solid-phase extraction (SPE).

Figure 14 illustrates the outcome of the loading phase, which was conducted using a sample with a high polyphenol content. MIP_olea exhibits a high degree of retention relative to other MIPs, likely due to the template's polyphenol nature. Furthermore, the bar chart demonstrates that oleuropein is retained to a lesser extent than other polyphenols due to its weaker binding with the polymer. Although verbascose and luteolin have strong binding with the polymer, this is the reason for their high retention.

Figure 15 illustrates the outcome of the washing phase. The outcome of the washing steps follows the same pattern as that of the loading phase, which is to be expected.

Figure 16 illustrates the outcome of the elution phase. The weak binding capability of oleuropein was almost completely removed from the polymer, while a residual amount remained in the polymer material for verbascose and luteolin.

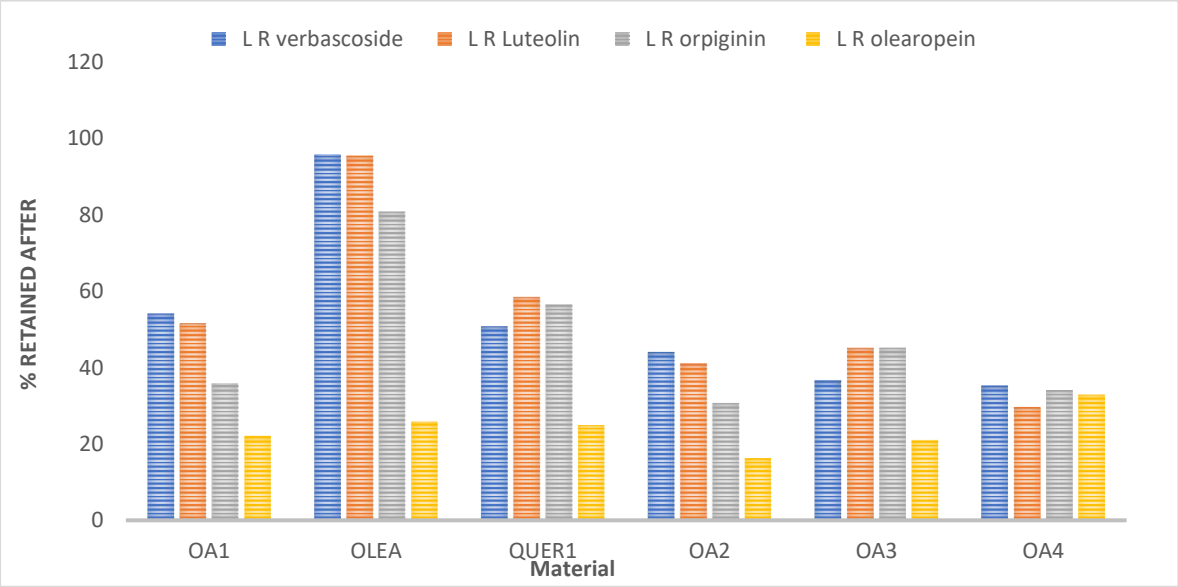


Figure 14: Loading step of the SPE assessment using an real extract N20 (1.5 mM) dissolved in ethanol-water (9:1; v.v) (4ml).

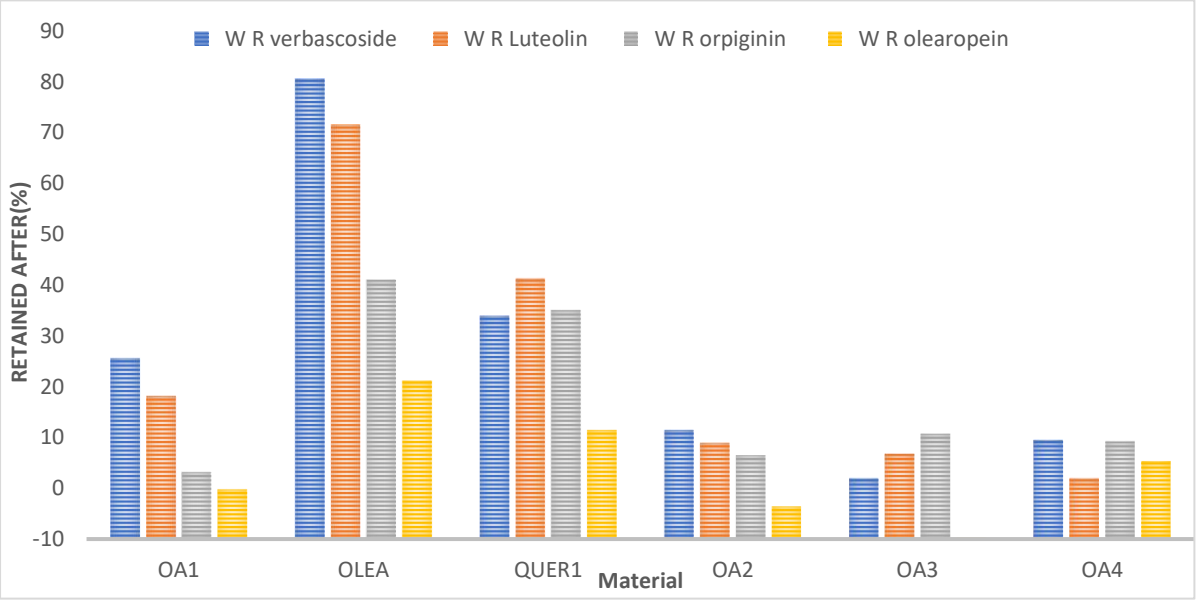


Figure 15: Washing step of the SPE assessment passing mixture of the solvent ethanol-water (9:1; v.v), (4ml).

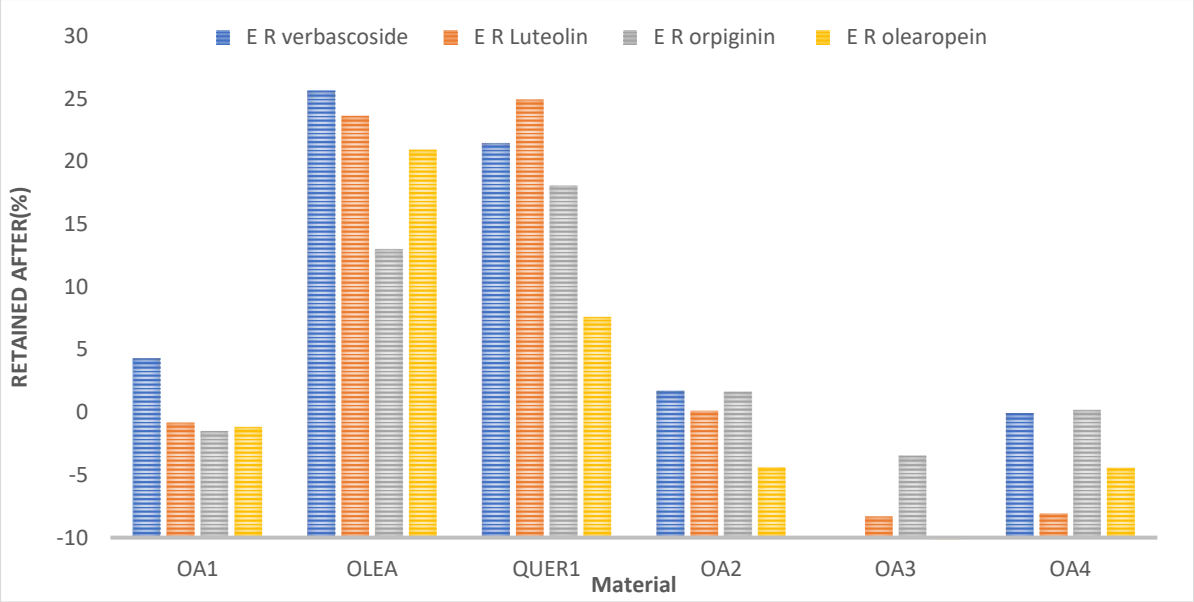


Figure 16: Elution step of the SPE assessment passing mixture of the solvent methanol-acetic acid (9:1; v.v), (4ml).

5.5. Saturation and fractionation of industrial extract on SPE

Following the acquisition of data pertaining to the retention ability of MIP for triterpene and polyphenol compounds, the subsequent stage of the process is the evaluation of saturation capacity and fractionation by solvent gradient. Therefore, the actual extract N20 was loaded, which is known to contain a high concentration of polyphenols.

Figure 17 presents the results obtained from the saturation step of the real extract. The graph illustrates the correlation between the initial value and the saturation line. From the graphs, it is evident that at 20 ml of the volume, the MIP has already reached saturation. However, upon continuous loading, there is a shift between molecules.

Figures 17, 18 and 19 illustrate the fractionation desorption process using pure water and an ethanol-water mixture, respectively. Figure 15 demonstrates that the use of water as a solvent enables the separation of phenolic acids from other compounds. Figure 16 illustrates the separation of secoridoids using a small amount of ethanol. Figure 17 illustrates that an increase in ethanol concentration in the solvent results in the formation of strong bonded compounds, such as luteolin, quercetin and so forth.

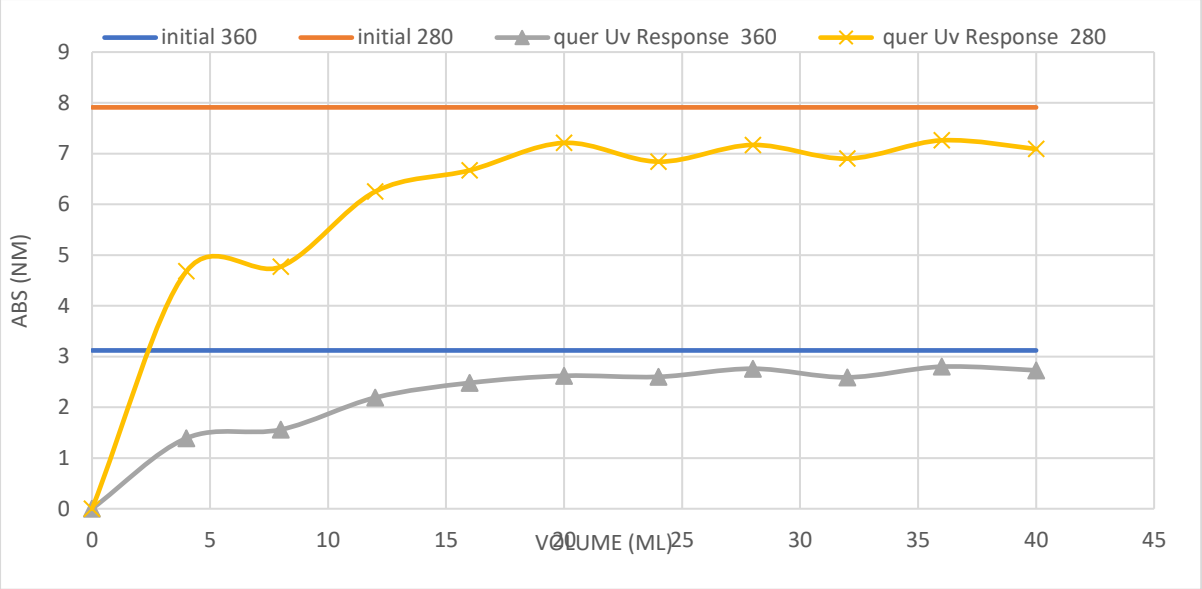


Figure 17: Saturation of mip_quer1 wavelengths of Uv-vis (280 nm,360 nm)

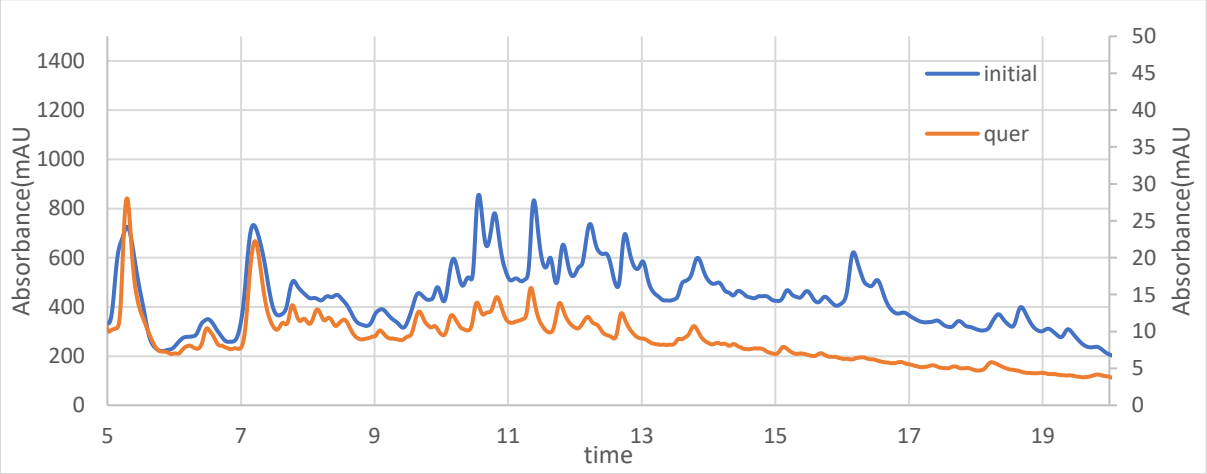


Figure 18: Desorption by pure water

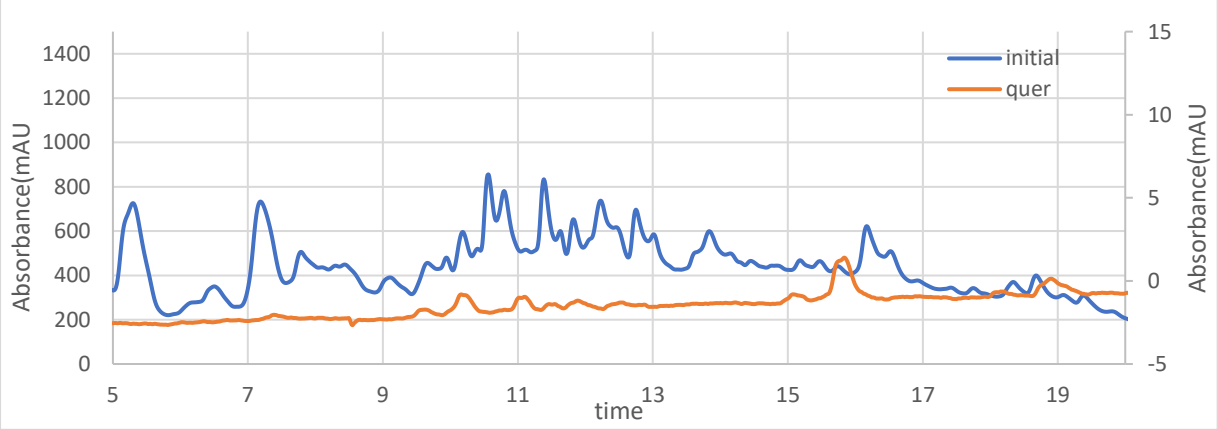


Figure 19: Desorption water-ethanol (20/80 v.v)

5.6. Isotherm for polyphenols

The assessment of the adsorption and desorption capacity of the synthesised MIPs necessitates the construction of an isotherm curve, which represents a pivotal step in the process of designing the preparative scale separation of the compounds from one another.

Figure 20 illustrates the adsorption process of polyphenols. From the graph, it is evident that at 10 ml of loading solution, vanillic acid and oleuropein were already saturated. Although quercetin reached saturation at 40 ml and more volume passed due to the adsorbent, it was synthesised using quercetin as a template. This explains the high capacity for quercetin to reach saturation.

Figure 21 illustrates the desorption of compounds. Due to the relatively weak binding between the MIP and oleuropein and vanillic acid, these compounds desorbed in the first fraction, which passed approximately 5 ml of solvent. While there was a significant increase in the amount of quercetin desorbed, this then decreased gradually. Following the replacement of the solvent, there was a marked increase in the quantity of desorbed compounds due to the influence of the polar solvent. While vanillic acid and oleuropein had already been desorbed from the MIP,

Figure 22, 23, 24, 25 illustrate four distinct isotherms for polyphenols. It can be observed that all isotherms exhibit a tendency towards selectivity for the separation of quercetin. However, when ethanol-water (50:50 v/v) is used as a solvent, vanillic acid and oleuropein display a greater selectivity.

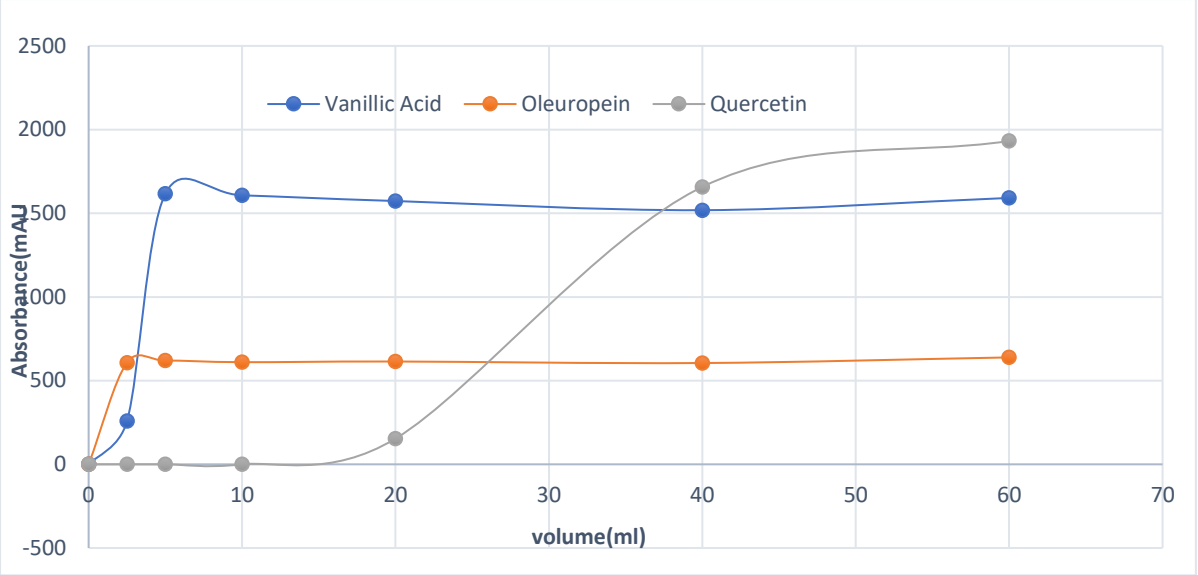


Figure 20: Adsorption step of vanillic acid, oleuropein, quercetin (each 0.1 mM), solvent ethanol-water (80:20, v.v), T=25 °C.

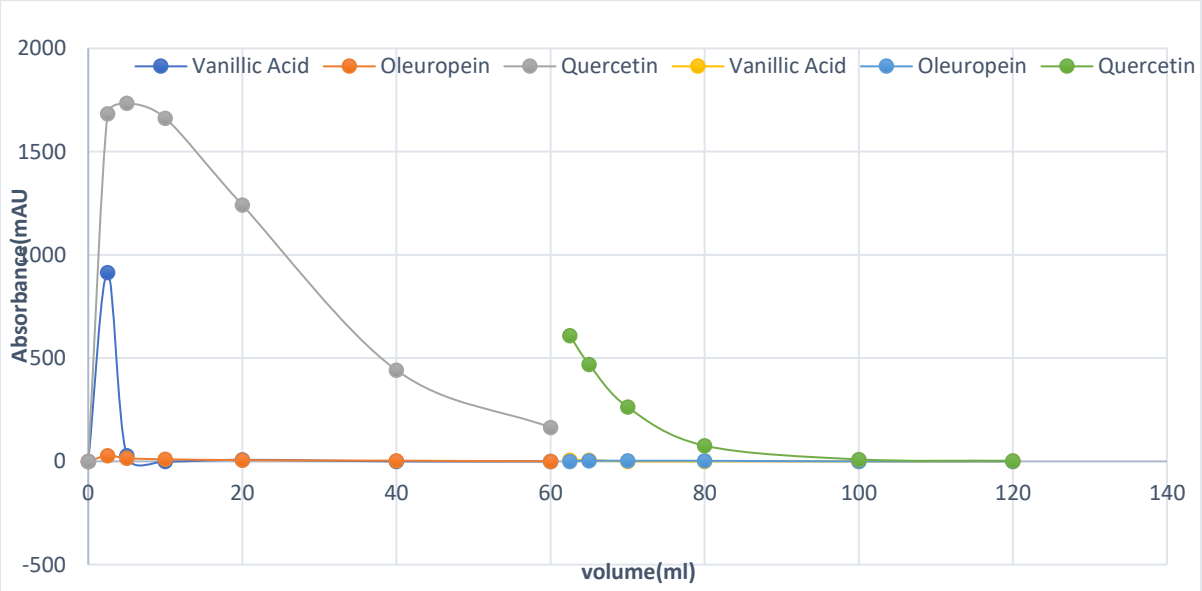


Figure 21: Desorption step of vanillic acid, oleuropein, quercetin, solvent ethanol-water (80:20, v.v), methanol-acetic acid (90:10, v.v) respectively (0-60 ml,60-120 ml), T=25 °C.

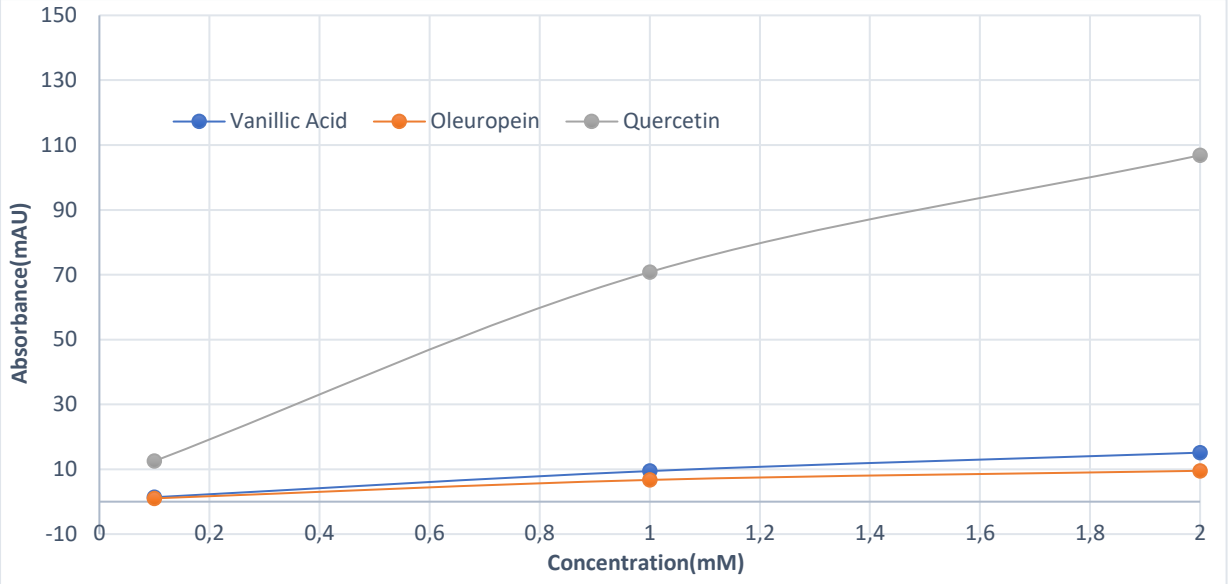


Figure 22: Isotherm curve of polyphenols, solvent ethanol-water (80:20, v.v), T=25 °C

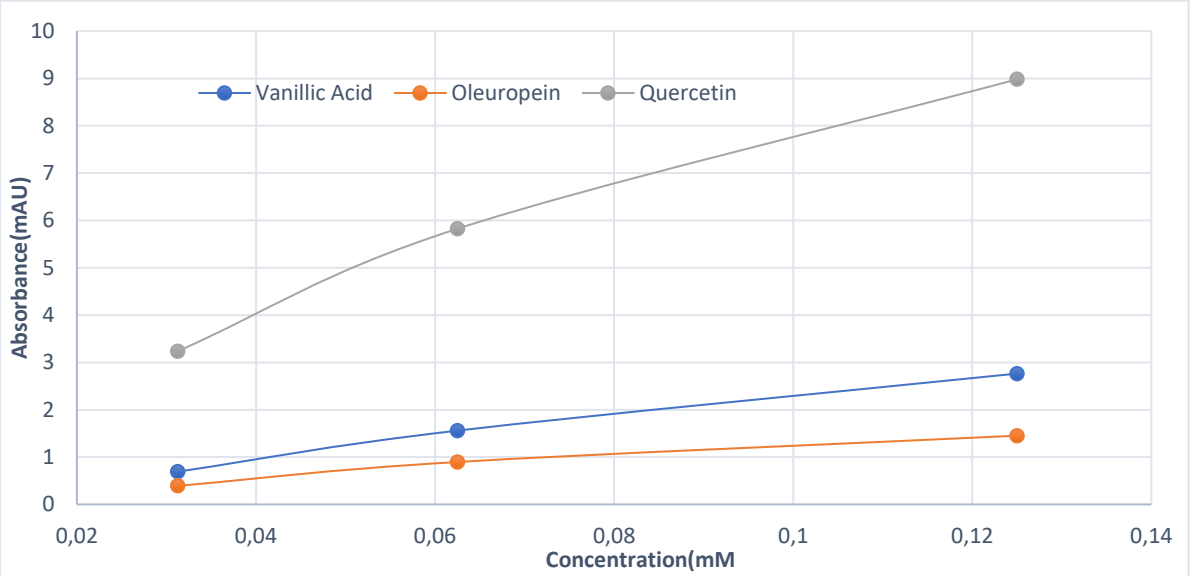


Figure 23: Isotherm curve of polyphenols, solvent ethanol-water (50:50, v.v), T=25 °C.

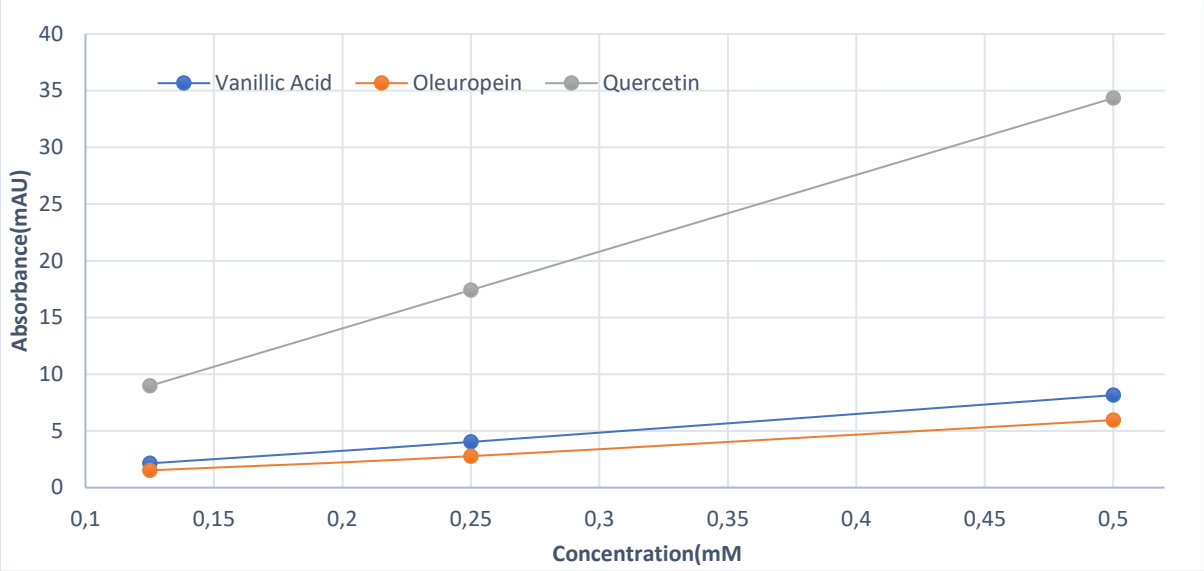


Figure 24: Isotherm curve of polyphenols, solvent ethanol-water (65:35, v.v), T=25 °C.

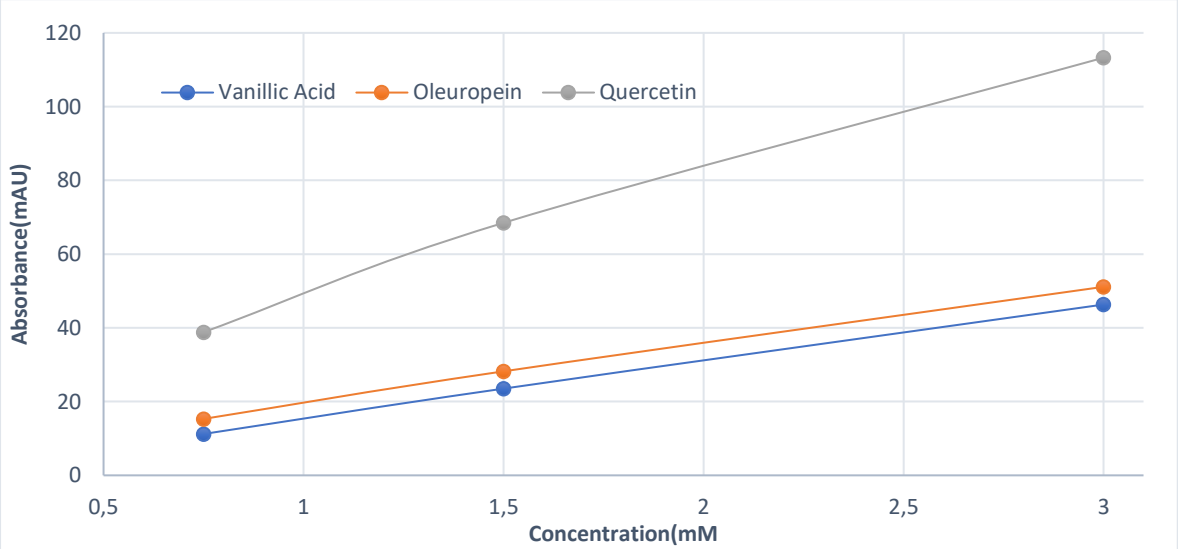


Figure 25: Isotherm curve of polyphenols, solvent ethanol pure, T=25 °C.

5.7. Isotherm for Triterpenoids

In order to evaluate the adsorption and desorption capabilities of the synthesised MIPs, it is of the utmost importance to analyse the isotherm curve in order to design a preparative-scale process for the separation of compounds from one another.

Figure 26 presents data obtained from the adsorption phase of maslinic acid and oleanolic acid. The most striking trend is the significant increase in adsorption amount observed in 10 ml of volume. Subsequently, the material reached saturation and remained stable.

Figure 27 illustrates the desorption process by fractionation. The graph demonstrates that 20 ml of solvent is sufficient to desorb the majority of the compound. Upon changing the solvent, there was a significant increase in the amount of compound, which is to be expected. Figures 28, 29, 30 and 31 illustrate the results of the isotherm for triterpenoids. It is evident that an increase in the water content of the solvent mixture results in a greater ease of separation between maslinic acid and oleanolic acid. Figure 29 illustrates a more effective separation of triterpenoids when methanol/water (50/50, v/v) is employed as the solvent.

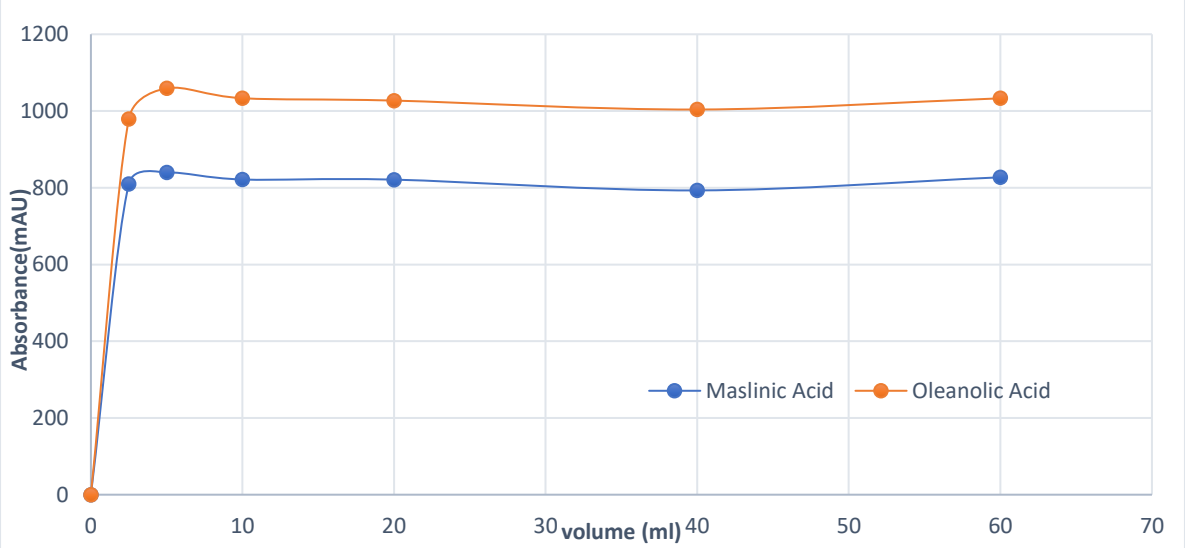


Figure 26: Adsorption step of maslinic acid, oleanolic acid (each 0.1 mM), solvent ethanol-water (80:20, v.v), T=25 °C.

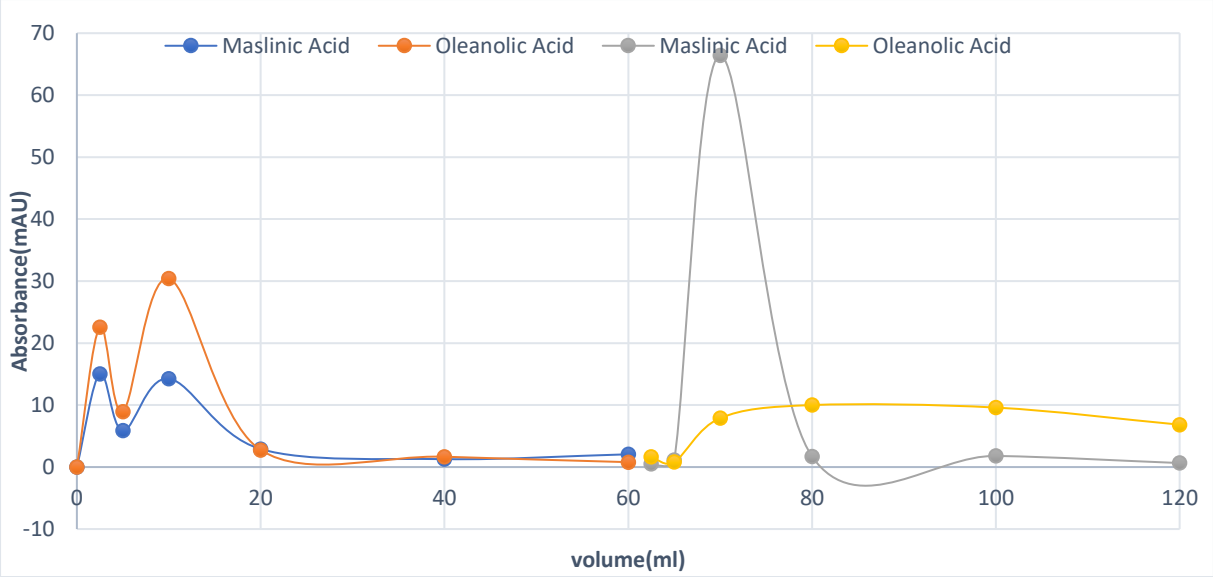


Figure 27: Desorption step of maslinic acid and oleanolic acid, solvent ethanol-water (80:20, v.v), methanol-acetic acid (90:10, v.v) respectively (0-60 ml,60-120 ml), T=25 °C.

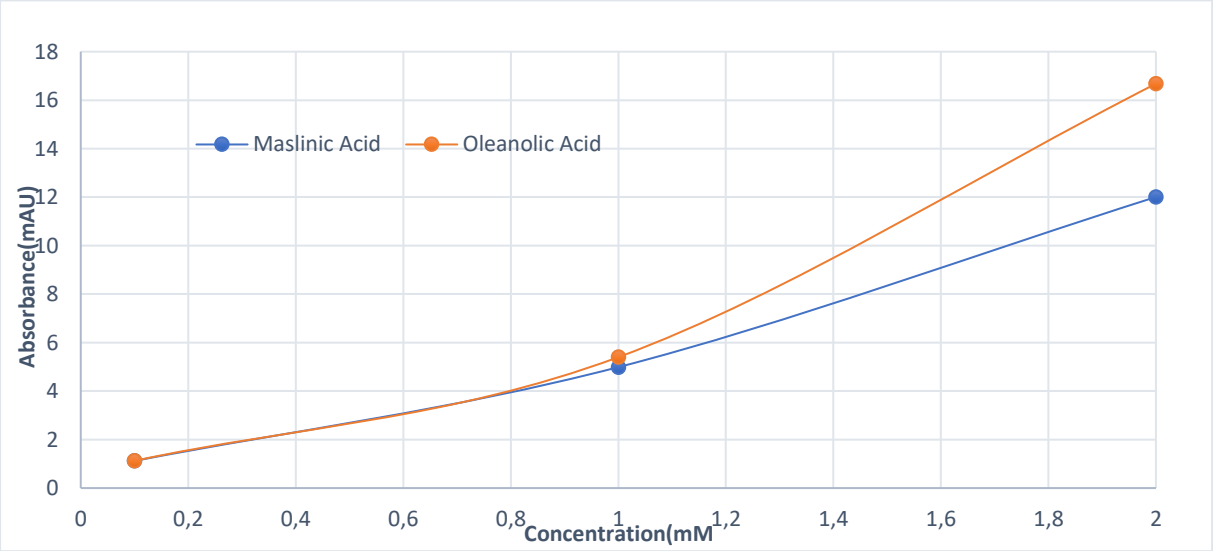


Figure 28: Isotherm curve of triterpenoids, solvent ethanol-water (80:20, v.v), T=25 °C.

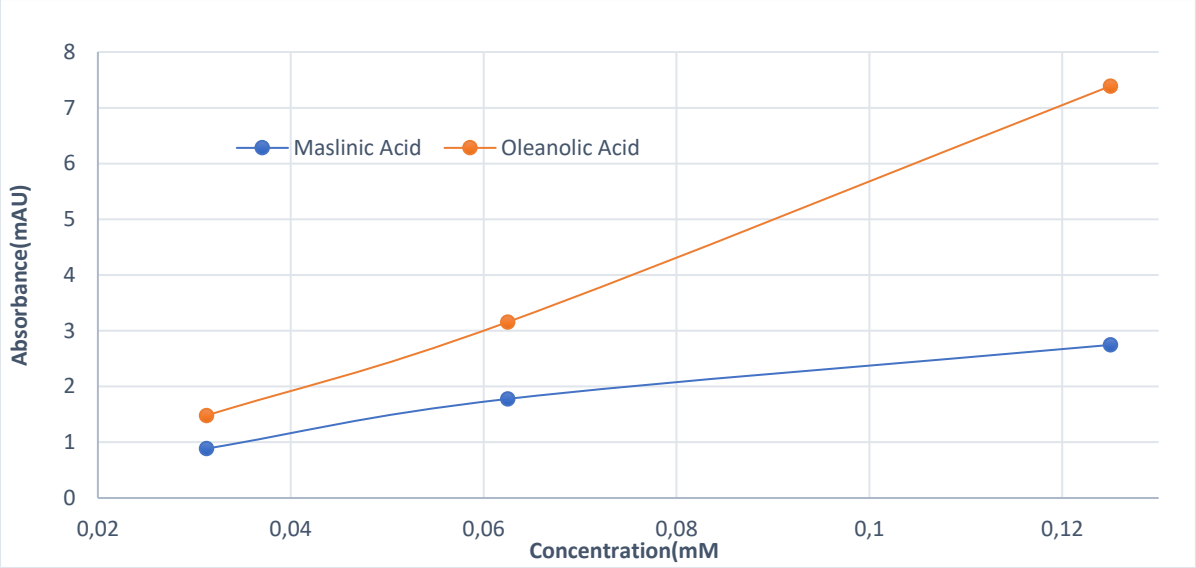


Figure 29: Isotherm curve of triterpenoids, solvent ethanol-water (50:50, v.v), T=25 °C.

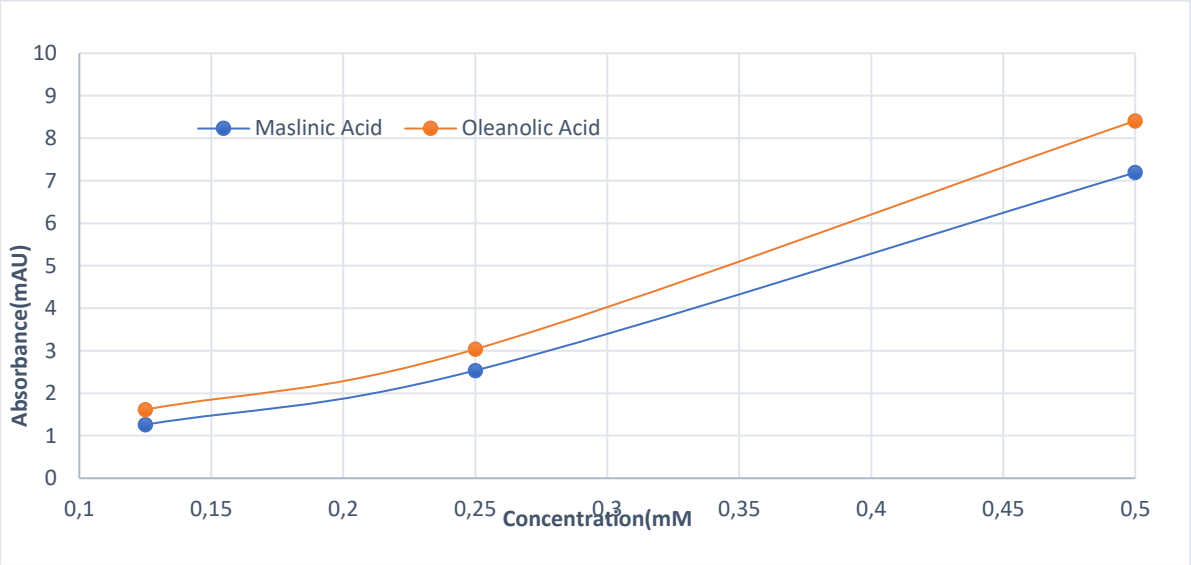


Figure 30: Isotherm curve of triterpenoids, solvent ethanol-water (65:35, v.v), T=25 °C.

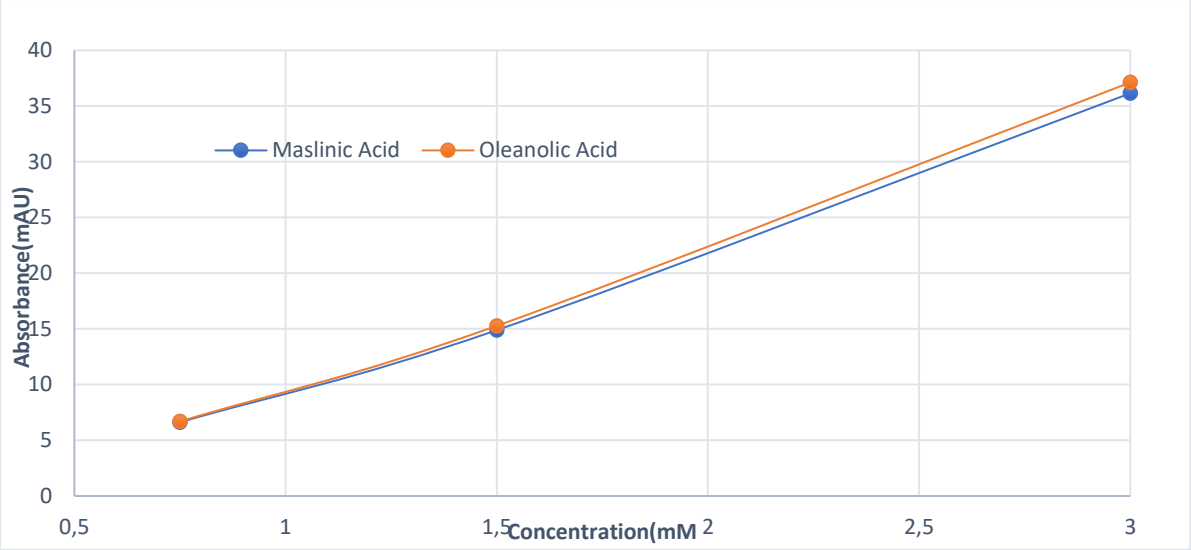


Figure 31: Isotherm curve of triterpenoids, solvent ethanol pure, T=25 °C.

5.8. Real extract adsorption and saturation

The objective of this study is to assess the purification and fractinoantion desorption. Prior to commencing the adsorption process, it is essential that the adsorbent is saturated.

Figure 32 illustrates the saturation of the column which was packed with mip_quer1 and represent chromatogram for polyphenols family. The difference between the initial line and the saturation line provides an approximation of the amount retained in the column by calculating area difference between the peak. Due to the nature of MIP polyphenols, a considerable amount was retained, with a particular preference for flavonodis.

Figure 33 illustrates the saturation of triterpenoids. It is evident that the discrepancy between the saturation and initial lines is minimal, which is indicative of a low retention rate. This is attributed to the nature of MIP, which results in a low retention of triterpenoids.

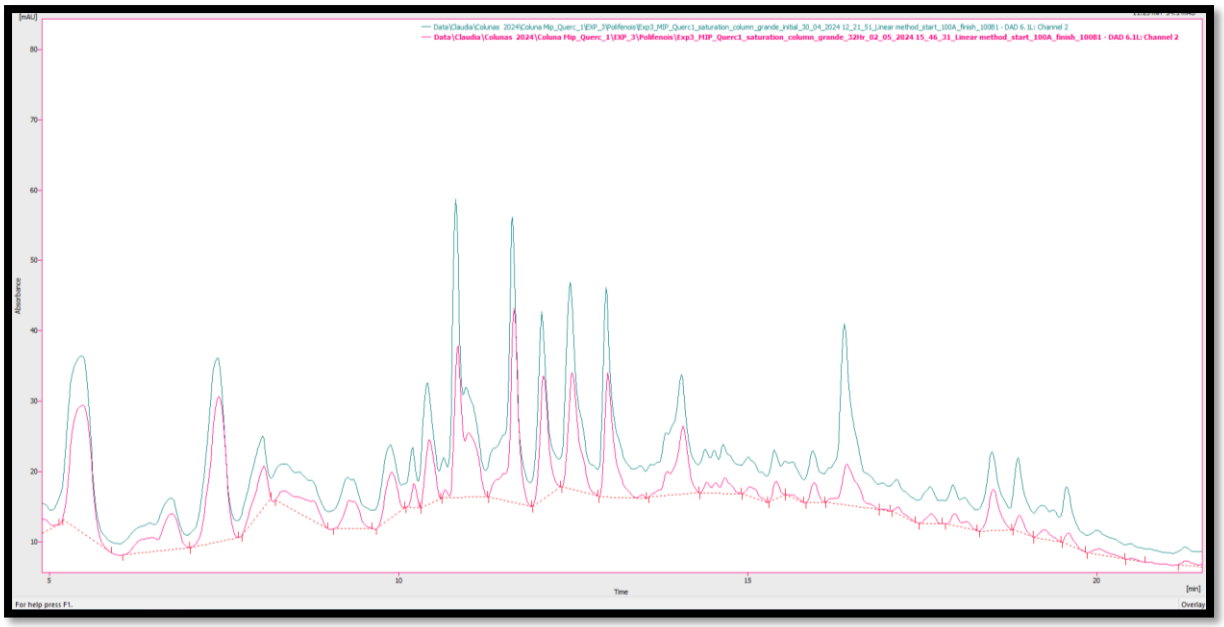


Figure 32: Comparison between initial sample and saturation after 24 hours in MIP_quer1 (for polyphenols), green line represents initial solution and red line represent fraction.

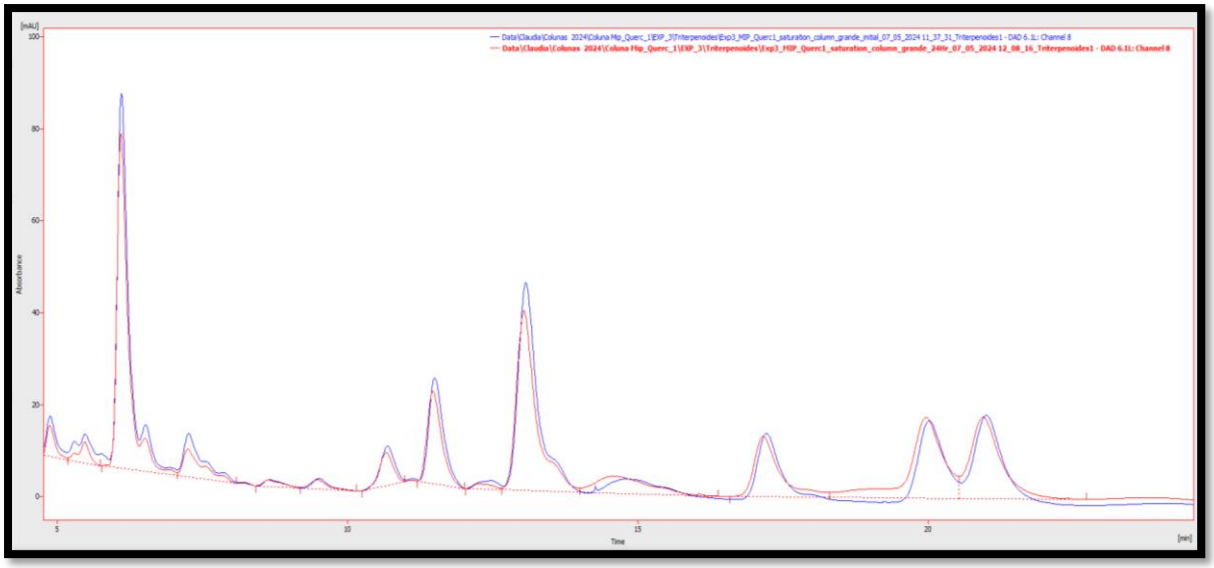


Figure 33: Comparison between initial sample and saturation after 24 hours in MIP_quer1 (for triterpenoids), blue line represents initial solution and red line represent fraction.

5.9. Real Extract Desorption by Fractionation

Figure 34 illustrates the outcome of the fractionation desorption of polyphenols. A comparison can be made between the initial solution and the fraction obtained by purifying the extract with pure ethanol, which allows flavonoids to be separated and the initial extract to be purified. A further purification of the extract could result in a greater degree of purity for a specific compound.

Figure 35 illustrates the fractionation desorption process for triterpenoids. A comparison between the initial solution and the purified fractions reveals that maslinic acid and oleanolic acid can be purified, although further purification is required.

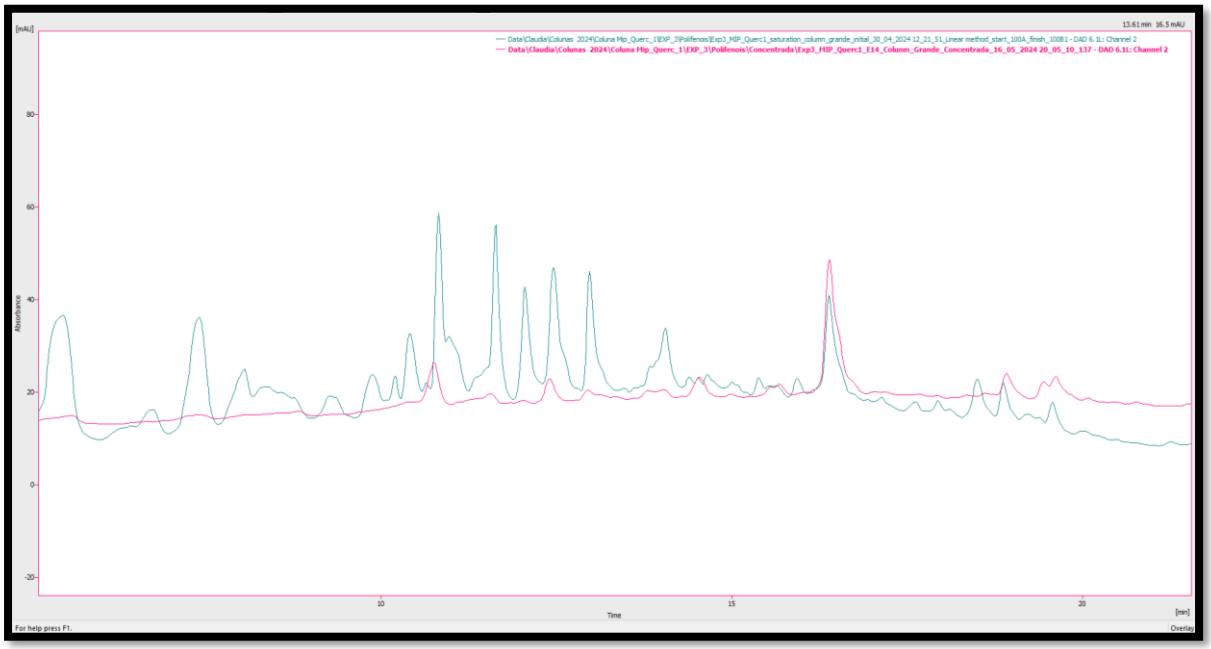


Figure 34: Comparison between initial solution and fraction of solvent (pure methanol) in MIP_quer1, (polyphenols), green line represents initial solution and red line represent fraction.

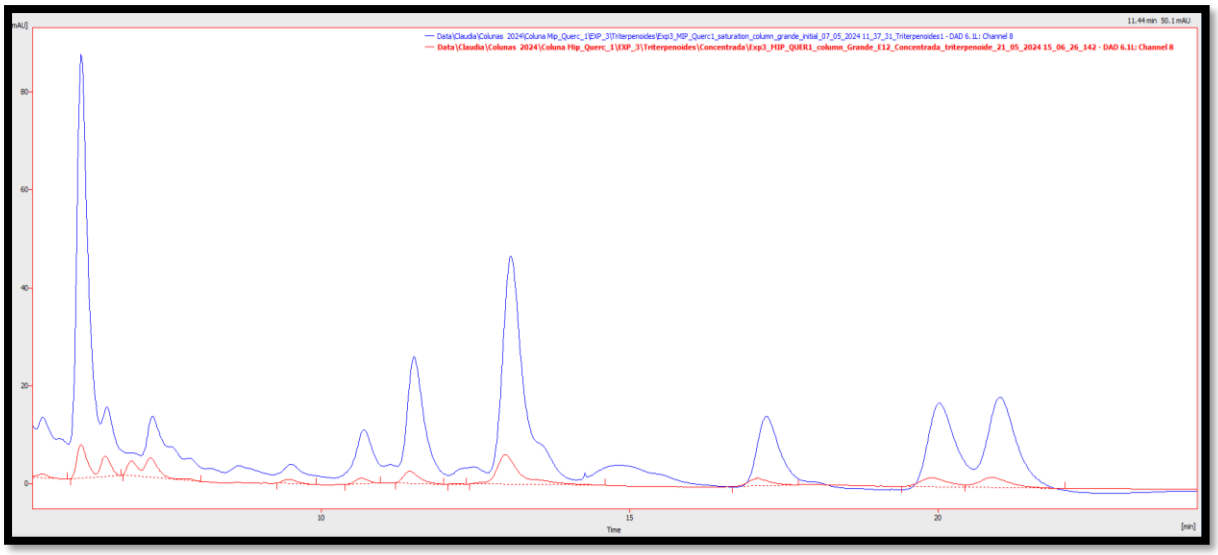


Figure 35: Comparison between initial solution and fraction of solvent (pure ethanol) in MIP_quer1, (triterpenoids), blue line represents initial solution and red line represent fraction.

5.10. Second Step Purification

The second purification step is crucial for the purpose of increasing the purity of a specific compound.

Figure 36 represents the outcome of the second purification stage of the initial compound, which was purified using pure methanol. The initial extract was purified to obtain a more purified form of rutin, which can be further purified to increase its purity.

Figure 37 illustrates the results of the second step in the purification of the titerpenoids family. Furthermore, the second purification step resulted in a notable increase in the purity of maslinic acid.

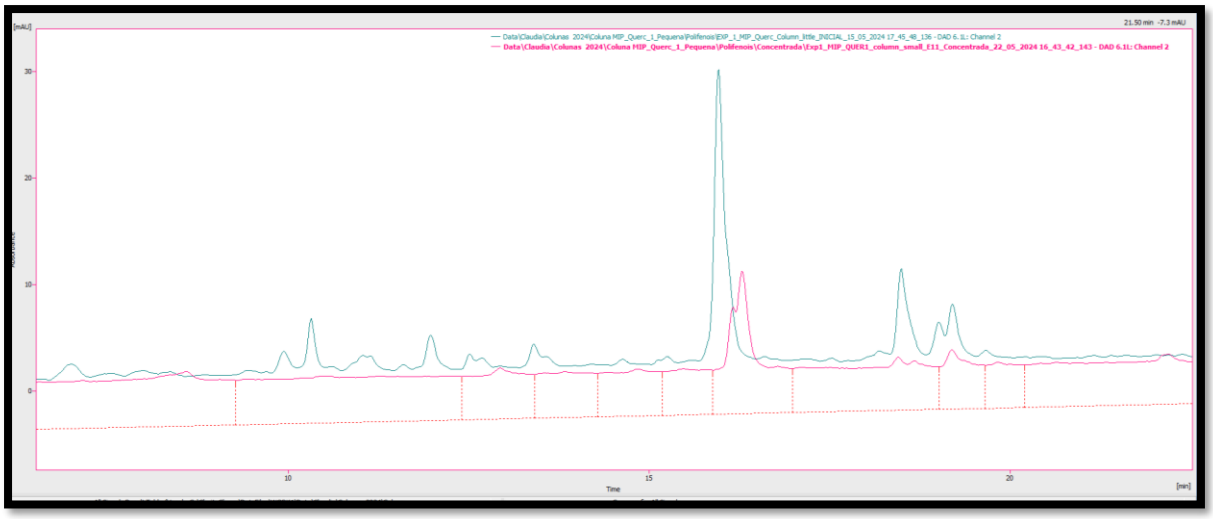


Figure 36: Comparison between initial solution and fraction of solvent (pure methanol) in MIP_quer1, (polyphenols), green line represents initial solution and red line represent fraction.

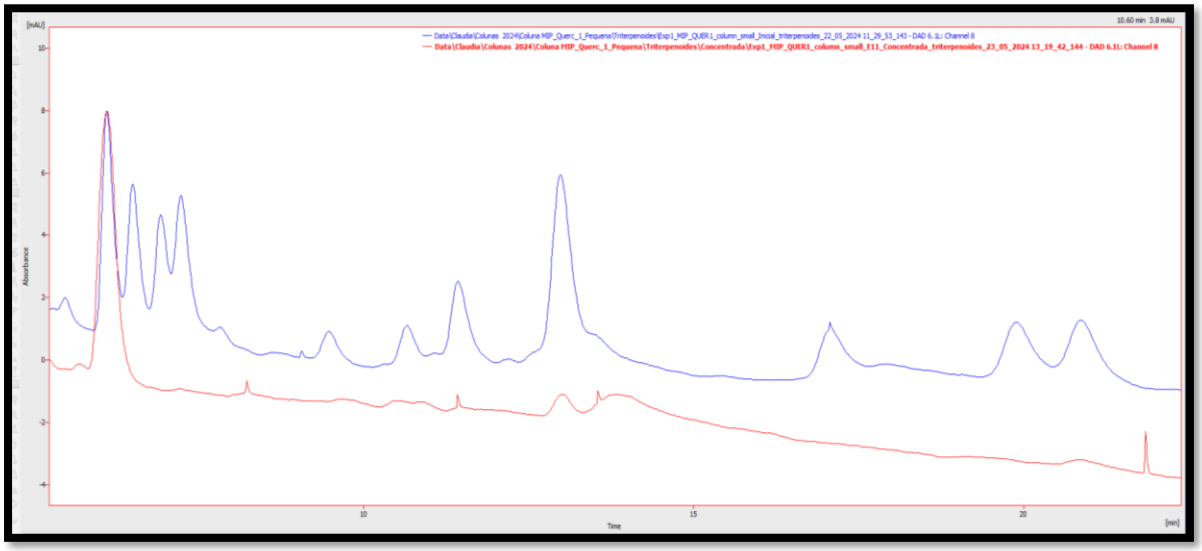


Figure 37: Comparison between initial solution and fraction of solvent (pure ethanol) in MIP_quer1, (triterpenoids), blue line represents initial solution and red line represent fraction.

5.11. Conclusions

This research study is dedicated to the valorisation of bioactive compounds from olive leaves extracts. For this purpose, molecularly imprinted polymers (MIPs) were produced using inverse suspension polymerization, intended to be utilized as an adsorbent for the fractionation of bioactive compounds. The imprinting compounds for the MIPs included quercetin, oleanolic acid, and oleuropein, which are representative flavonoids, triterpenoids, and secoiridoids, respectively. During the synthesis step, different ratios of crosslinker and monomer were used to evaluate their influence on the MIPs performance. The synthesised MIPs were evaluated using solid-phase extraction (SPE) to assess their selectivity and retention capacities. Standard compounds from different families and the real extract were employed in the SPE assessment. The final results of the SPE assessment demonstrated that MIP_quer1 exhibits a high retention capacity for polyphenol families and was utilized as an adsorbent in a continuous system. The competitive adsorption of triterpenoids, secoiridoids, flavonoids, and phenolic acids in MIP_quer1 was studied in a continuous system using standard molecules of maslinic acid, oleanolic acid, vanillic acid, oleuropein, and quercetin. The measured adsorption isotherms demonstrated that MIP_quer1 particles have a significantly stronger binding capacity for quercetin compared to vanillic acid and oleuropein. Conversely, oleanolic acid exhibited a stronger binding with the adsorbent compared to maslinic acid. The obtained data were utilized to design and scale up the sorption/desorption processes, facilitating the fractionation of bioactive compounds present in olive leaf extracts. To accomplish this, a preparative scale column was packed with 25 grams of absorbent. Because of the MIP's strong flavonoid binding capacity, it was demonstrated that even when dealing with an industrial olive leaf extract at high concentrations (e.g., 5–10 mg/mL) and aqueous mixtures with high alcohol content (e.g., ethanol volumetric fraction over 50%), a simple adsorption process remains feasible. Various solvent gradients were employed during the desorption process, resulting in fractions with compositions different from the initial extract. Fractions with low alcoholic content were enriched with non-flavonoids such as oleuropein, whereas fractions with alcoholic content ranging from 40% to 90% were strongly enriched with glycosylated flavonoids and triterpenoids. Additionally, flavonoid aglycones such as quercetin and luteolin were enriched in fractions with an alcohol content exceeding 90%. Furthermore, some fractions underwent a second purification step,

resulting in increased purity of specific compounds such as luteolin and maslinic acid. Overall, the results of this study demonstrate the effectiveness of the developed materials and sorption/desorption conditions for process upscaling within the circular bioeconomy, particularly for extracting bioactive compounds from olive leaf extract.

5.12. Further Improvement

This study has established a foundation for the effective utilisation of MIPs in continuous processes. Nevertheless, several avenues for further enhancement have been identified. The primary objective is to enhance the specific MIPs for the triterpenoid family. On the other hand, scaling up the process to an industrial level is a goal worth pursuing in future work.

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