Supercritical Extraction of Polyphenols from Different Vegetable Matrices and their Retention and Recovery using Molecularly Imprinted Polymers

Victoria Franco Cruz

Final Dissertation submitted to

Escola Superior de Tecnologia e Gestão

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Oriented by:

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July 2019

“Temos nosso próprio tempo”

Renato Russo

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Abstract

Different kinds of vegetable residues abundant in Trás-os-Montes region, namely walnut leaf, walnut shell, almond shell, grape marc, olive leaf and onion shell (a worldwide available biomass) were considered as possible sources of polyphenols. Extraction with supercritical carbon dioxide (at T=40 °C, P=160 bar and ethanol as co-solvent), ultrasound extraction and Soxhlet extraction were alternatively used to obtain mixtures containing these bioactive compounds. Aiming at the subsequent separation and concentration of the polyphenols from the complex extracts obtained, molecularly imprinted polymers (MIPs) were used as a kind of engineered adsorbents. MIP particles synthesized by precipitation polymerization with 4-vinylpyridine (4-VP) as functional monomer and quercetin as template were applied in the retention and recovery of the polyphenols. Comparative studies with commercial polymeric adsorbents (namely with the resin DAX8) were also performed. Batch adsorption, solid phase extraction (SPE) and operation in HPLC columns packed with the adsorbents were considered in these uptake/release studies. The identification and quantification of polyphenols was performed using Liquid Chromatography with Mass Spectroscopy and Diode Array Detector (LC-MS-DAD) and also with the more straightforward HPLC-DAD technique.

With the supercritical CO_2 ($SCCO_2$) extraction conditions used, the richest phenolic profiles were obtained with onion shell and almond shell. Indeed, quercetin and many quercetin analogues (e.g. quercetin-*O*-glucoside, quercetin-*O*-diglycoside, quercetin dimer-*O*-hexoside, etc) were identified in these onion shell extracts, while quercetin, isorhamnetin-3-*O*-rutinoside or catechin (e.g.) were identified in the almond shell extracts. However, in spite of a similar composition, a higher phenolic content was measured for extracts obtained with the ultrasounds (US) or Soxhlet (SHOX) extraction, comparatively to supercritical CO_2 extraction (e.g. around 10 mg/g of total phenolic compounds with US and SHOX extraction and 1 mg/g with $SCCO_2$ for onion shell extracts, in a dry basis of plant residue).

Benefits of molecular imprinting in the designing of tailored adsorbents to be used with downstream processing of polyphenols were evidenced in this research. Indeed, a very high polyphenol retention was here shown to be possible with MIPs, even when solvents with low water content are used (e.g. ethanol/water 80/20). Thus, the hydrophobic interactions, that usually is the main driving force for adsorption with common synthetic resins, is not the unique mechanism allowing the retention of these bioactive compounds in the synthesized molecularly imprinted polymer networks. The functionalization of the materials (here with pyridyl functional groups) and the creation of imprinted tailor-made cavities (promoting analogue mechanisms to antigen/antibody or substrate/enzyme binding) were congenial for the improved performance of the MIPs. Additionally, the manipulation of the particles morphology (through precipitation polymerization), allowing a facile binding accessibility (e.g. due to surface imprinting) should also contribute for the observed superior performance of the MIPs in polyphenols retention.

The distinctive features of the MIP adsorbents were here demonstrated through the direct processing of plant extracts without water addition (e.g. using an ethanol/water 80/20 onion shell extract). A polyphenol-enriched fraction, containing quercetin and analogue molecules, was recovered at the end with the minimization of thermal treatments (only alcohol evaporation is needed if a dry residue is wanted). Thus, the simplification of the adsorption/desorption process was achieved and energetic costs can also be cut down (besides the minimization of the possible thermal degradation of the bioactive compounds). Summing up, this research demonstrate that MIP adsorbents can be helpful in the design of new and more flexible adsorption processes (e.g. working with a wider range of water content), aiming at the valorization of polyphenols present in plant extracts. Food industry, pharmaceutics and cosmetics are examples of applications fields for the principles here outlined.

Key-words: plant residues, polyphenols, extraction with supercritical CO_2, molecularly imprinted polymers (MIPs), tailored adsorbents, downstream processing

Resumo

Diferentes tipos de resíduos vegetais abundantes na região de Trás-os-montes, como a folha de nogueira, a casca de noz, a casca de amêndoa, o bagaço da uva, a folha de oliveira e a casca da cebola (uma biomassa disponível de forma mais abrangente no planeta) foram considerados como possíveis fontes de polifenóis. A extração com dióxido de carbono supercrítico (usando T = 40 °C, P = 160 bar e etanol como co-solvente), a extração por ultrassom e a extração por Soxhlet foram alternativamente utilizadas para obter misturas contendo esses compostos bioativos. Visando a subsequente separação e concentração dos polifenóis dos extratos complexos obtidos, polímeros molecularmente impressos (MIPs) foram utilizados como uma espécie de adsorventes. Partículas de MIPs sintetizadas através de polimerização por precipitação com 4-vinilpiridina (4-VP) como monômero funcional e quercetina como molécula modelo foram aplicadas na retenção e recuperação dos polifenóis. Também foram realizados estudos comparativos com adsorventes poliméricos comerciais (como a resina DAX-8). A adsorção em batch, a extração em fase sólida (SPE) e a operação em colunas de HPLC empacotadas com os adsorventes foram consideradas neste estudo para a retenção e libertação dos compostos fenólicos. A identificação e quantificação de polifenóis foi realizada utilizando cromatografia líquida com espectroscopia de massa e detector de diodo array (LC-MS-DAD) e também com a técnica mais direta de HPLC-DAD.

Com as condições de extração com CO_2 supercrítico ($SCCO_2$) utilizadas, os perfis fenólicos mais ricos foram obtidos com a casca da cebola e a casca de amêndoa. Foram identificadas quercetina e muitos análogos de quercetina (por exemplo, quercetina-*o*-glucosídeo, quercetina-*o*-diglicosídeo, quercetina dimer-*o*-hexoside, etc.) nos extratos da casca da cebola, enquanto quercetina, isorhamnetin-3-*O*-rutinoside ou catequina, por exemplo, foram identificados nos extratos da casca de amêndoa. No entanto, apesar de uma composição semelhante, um maior teor de compostos fenólicos foi medido para extratos obtidos com a extração por ultrassom (US) e Soxhlet (SHOX), comparativamente a extração com CO_2 supercrítico (por exemplo, cerca de 10 mg/g de compostos fenólicos totais com as extrações US e SHOX e 1 mg/g com $SCCO_2$ para extratos de casca de cebola, numa base seca de resíduo vegetal).

Os benefícios da impressão molecular na concepção de adsorventes adaptados a serem utilizados com o processamento a jusante de polifenóis foram evidenciados nesta pesquisa. De fato, foi possível mostrar uma elevada retenção de polifenóis com MIPs, mesmo quando solventes com baixo teor de água são utilizados (por exemplo, etanol/água 80/20). Assim, as interações hidrofóbicas, que geralmente são a principal força motriz para a adsorção com resinas sintéticas comuns, não é o único mecanismo que permite a retenção desses compostos bioativos nas redes de polímeros sintetizados com a técnica de impressão molecular. A funcionalização dos materiais (com grupos funcionais piridil) e a criação de cavidades impressas por medida (promovendo mecanismos análogos à interação antígeno/anticorpo ou substrato/enzima) foram benéficas para o melhor desempenho dos MIPs. Adicionalmente, a manipulação da morfologia das partículas (através da polimerização por precipitação), permitiu uma fácil acessibilidade aos sítios de adsorção (por exemplo, devido à impressão à superfície) que deve igualmente contribuir para o desempenho superior observado com os MIPs na retenção de polifenóis.

As características distintivas dos MIPs como adsorventes foram aqui demonstradas através do processamento direto de extratos de plantas sem adição de água (por exemplo, usando um extrato da casca da cebola em etanol/água 80/20). Uma fração enriquecida com polifenóis, contendo quercetina e moléculas análogas, foi recuperada no final com a minimização dos tratamentos térmicos (somente a evaporação do álcool é necessária se um resíduo seco for pretendido). Assim, a simplificação do processo de adsorção/dessorção foi alcançada, e os custos energéticos também poderão ser potencialmente reduzidos (para além da minimização de uma possível degradação térmica dos compostos bioativos). Resumindo, esta pesquisa demonstra que os MIPs como adsorventes podem ser úteis na concepção de novos processos de adsorção e mais flexíveis (por exemplo, permitindo trabalhar com uma quantidade de água mais ampla relativamente aos adsorventes comuns), visando a valorização dos polifenóis presentes nos extratos vegetais. A indústria alimentícia, farmacêutica e cosmética são exemplos de campos de aplicações para os princípios aqui delineados.

Palavras-chave: resíduos vegetais, polifenóis, extração com CO_2 supercrítico, polímeros molecularmente impressos, adsorventes adaptados, processamento a jusante.

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Nomenclature

4-VP — 4-Vinylpyridine

ACN - Acetonitrile

DMF - N,N- dimethylformamide

EGDMA - Ethylene glycol dimethacrylate

GA – Gallic Acid

HPLC-DAD - High Performance Liquid Chromatography with a Diode-Array Detector.

LC-DAD-ESI/MS - Liquid Chromatography coupled to Diode Array Detection and Electrospray Ionization tandem Mass Spectrometry

MIP – Molecularly Imprinted Polymers

NIP – Non-Imprinted Polymers

QUE – Quercetin

RU – Rutin

$SCCO_2$ – Supercritical CO_2

SEM - Scanning Electron Microscopy

SFE – Supercritical Fluid Extraction

SHOX – Soxhlet Extraction

SPE – Solid Phase Extraction

US – Ultrasound Extraction

UV - Ultraviolet

Chapter 1

1.1 Introduction

Source of various chemical compounds, plants, and plant residues have been used in several areas, such as medicine and diverse industries. The plants are rich in different active compounds, such as oils, phenols, flavonoids, among others, which can be found in some specific parts, such as leaves, barks, roots, etc. In order to identify which active compounds exist in a plant or in a plant residue, several steps are necessary, among them extraction of the material, characterization of the extract, separation, purification, and evaluation of the compound of interest (Gupta, Naraniwal, and Kothari 2012). In this work, walnut leaf, walnut shell, almond shell, onion shell, grape marc, and olive leaf are the source vegetables chosen for the study because they are easy to find as residues in the Trás-dos-Montes region, Portugal.

Supercritical fluid extraction (SFE) is an extraction technique considered alternative because it is environmentally friendly and efficient, since it makes use of fluid at supercritical conditions, namely, the temperature and pressure of the substance are above the critical condition. In addition, this type of extraction reduces the preparation time of the extract and also avoids the thermal degradation of phenolic compounds, being unnecessary the use of some organic solvent (Nie et al. 2010). However, it is also important to consider other extraction techniques, such as ultrasound and soxhlet, which are conventional techniques, for comparative purposes.

The objective of this work is the study of the extraction of polyphenols with supercritical CO_2 from different vegetable matrices. Also, it is aimed the study of the retention and recovery of polyphenols from these extracts, using molecularly imprinted adsorbents, namely in continuous adsorption processes (e.g. using columns packed with MIP particles). Different types of MIPs, designed and synthesized by this research group, are used for comparison with commercial adsorbents in different adsorption processes.

1.2. Organization of the Dissertation

Chapter 1 intends to introduce the topic of this thesis in the current framework and present the primary aim of the project.

In Chapter 2 are presented some general concepts about the theme.

Chapter 3 is intended for the exposure of the main equipment; reagents and the different methods are described, as well as the procedures to be used during the development of the research.

In Chapter 4 are presented and discussed the results obtained in the different experiments performed, using diverse analyses, and Chapter 5 contains a brief conclusion about all work.

The bibliographic references are presented at the end of the work.

Chapter 2

Bibliographic Revision

2.1 Phenolic Compounds

Phenolic compounds are secondary metabolites found abundantly in plants that act as natural defenders. Several phenolic compounds have already been identified, with more than 8150 flavonoids, in addition to other structural varieties. The wide diversity and distribution of polyphenols in plants led scientists to recognize that the role of phenolic compounds is not limited to the colour, taste, odour, bitterness and astringency of food, but with the result of several researches has shown that these compounds have properties as antidiabetic, anti-allergic, antimicrobial, antithrombotic, anti-inflammatory, anticancer, antihypertensive, antiarthritic, and antioxidant, which protect the human body from different diseases (Galanakis, 2018).

Thus, the scientific community and various industries, such as the pharmaceutical, biomedical, cosmetic and food industries, are now showing a great interest in polyphenols (Gomes, Dias, and Costa, 2019). In 2018 the polyphenol market was estimated at US $ 1.28 billion, with an estimated 7.2% growth up to 2025, due to the knowledge about the use of these compounds from vegetables and their health benefits, which promotes the growth of the polyphenol industry (Grand View Research, 2019).

2.2. Vegetable Residues

Different kinds of vegetable residues abundant in Trás-os-Montes region, namely walnut leaf, walnut shell, almond shell, grape marc, olive leaf and onion shell (this one worldwide distributed) are considered in this work as possible sources of polyphenols.

2.2.1. Walnut Leaf and Shell

The nuts, along with their shells and leaves have been studied and used by the pharmaceutical and cosmetic industries as well as in the medical field as a source for various types of treatments. In Portugal and in other European countries, the dry walnut leaves for infusion are still widely used (Amaral et al, 2004). The shells that are a large part of the walnut are also used in cosmetics, dental products, to polish some materials and in oil and water separation filtrations (Srinivasan and Viraraghavan, 2008). The leaves of the walnut are easily found and in large quantities and are the source of many researches, in which various phenolic compounds were identified such as naphthoquinones, flavonoids (Pereira et al, 2007), and some phenolic acids (Fukuda, Ito, and Yoshida, 2003).

FIGURE 1: (a) Walnut Leaves. Source: https://steemit.com/treetuesday/@whatisnew/english-walnut-tree and (b) Walnut Shell. Source: https://ukrainianwalnut.com/en/coarse-walnut-shell-not-crushed/

2.2.2. Almond Shell

The almond, known scientifically as Prunus dulcis or Prunus amygdalus, belongs to the family Rosaceae. In Portugal, the almond is part of a traditional culture, mainly in the region of the Algarve and Baixo Alentejo, in the south of the country, and in the region of Trás-os-Montes to the north. When the almond is processed to obtain the edible seeds, a percentage of its shells are separated and become a residual product, previously without any utility for industry, this residues were the most often discarded (Esfahlan, Jamei, and Esfahlan 2010). However, these shells, until then considered waste, have been studied and have an abundance of phenolic compounds such as phenolic acids and flavonoids, which are grouped with sugars or other polyphenols (Barreira et al, 2008).

FIGURE 2: Almond Shells. Source: http://bioplasticnews.blogspot.com/2010/12/bioplastico-baseado-em-casca-de-amendoa.html

2.2.3. Onion Shell

Onion (Allium cepa L.) is one of the most consumed vegetables in the world. Also, it is responsible for the production of a large amount of residue, such as its shell, external scales, roots and bulbs, which generates environmental problems, since it is not possible to reuse all these residues. One way to solve these problems is to reduce these leftovers using the whole plant, since the onion is rich in dietary fiber, fructooligosaccharides (Benítez et al. 2011), and flavonoids, such as quercetin, that promotes an antioxidant activities and others benefits to the human health. The onion peel has an orange color due to the presence of several phenolic compounds, such as quercetin, gallic acid, and ferulic acid (Lee et al. 2014). The quercetin average present in the onion is 347 milligrams of quercetin per kilogram of onion, a ratio of 5 to 10 times higher when compared to other vegetables (Lachman et al. 2011).

FIGURE 3: Onion Shells. Source: https://pt.depositphotos.com/43860689/stock-photo-onion-husk.html

2.2.4. Grape Marc

The grape is one of the fruits most used in industrial processes and with this end up generating an immense amount of residues, which present several environmental problems resulting from its organic composition. However, grape residues nowadays are considered a rich source of bioactive compounds that are extracted and used in the food, cosmetic and pharmaceutical industries. These compounds are mostly phenolic acids, such as gallic, vanillic and caffeic acid, as well as some flavonoids such as catechin and quercetin, and a variety of procyanidins and resveratrol (Casazza, Aliakbarian, and Perego 2011). The existence of many of these compounds makes extracts of grape residues widely used as additives for the purpose of protecting and/or enriching other products on the market. With this, some companies of cosmetics and dermocosmetics have already patented methods of processing and application of compounds derived from the extraction of this fruit (Farías-Campomanes and Meireles 2013).

FIGURE 4: Grape Marc

2.2.5. Olive Leaf

One of the most important fruit trees in the Mediterranean region, the olive tree (Olea europaea L.), also cultivated in other regions, represents 98% of the world's crop according to A. P. Pereira et al., 2007. In addition to the use of its fruit for the production of oils and olive oils, for many years olive leaf is used for the purpose of fighting diseases such as fever and malaria. Besides, the presenting antioxidant and anti-inflammatory properties from flavonoids (Benavente-García et al. 2000), they also exert hypotensive and hypoglycemic activity due to oleuropein and hydroxytyrosol present in large quantities in olive leaves. The olive leaves are also used in infusions to facilitate the absorption of bioactive compounds (Pereira et al. 2007).

FIGURE 5: Olive Leaves. Source: https://www.superpharmacy.com.au/blog/the-many-benefits-of-olive-leaf-extract

2.3. Extraction Techniques

Even though the work has as main focus the extraction with supercritical CO_2, it is important to consider other extraction techniques for comparative purposes, namely concerning the phenolic profiles achieved using different operation conditions.

2.3.1. Ultrasound

The ultrasound extraction technique is used to extract phenolic compounds from plant matrices because the production of sound waves promotes cavitation near the vegetal tissue of the sample, that facilitates the rupture of the cell walls, releasing the contained chemical compounds. A suitable solvent is required to be mixed with the sample for a specific time. There are two types of systems in this type of extraction, the static system in which the extraction takes place in a closed vessel, preventing the transfer of solvent, and the dynamic system in which the solvent is supplied continuously allowing its transfer of the container and a greater adsorption of the analytes (Khoddami, Wilkes, and Roberts 2013). As one of the simplest and lowest cost extraction methods, ultrasound extraction has the capacity to be operated quickly, since it demands short processing time (Gupta et al. 2012).

2.3.2. Soxhlet

Soxhlet is an extraction technique which has the procedure of heated reflux and maceration for the extraction of lipids and phenolic compounds from solid samples, mostly using solvents that makes the desired extract insoluble in it. The process has a simple methodology, with cheap equipment, but depends on high temperatures and takes a long time. Moreover, there is other considerable disadvantages such as the need to use a large volume of solvent, the possible degradation of the compounds due to the presence of light, air, among other factors (Khoddami et al. 2013). Despite the positive results extracting phenolic compounds, Soxhlet is not selective, and there may be toxic emissions during the process (Sahena et al. 2009).

2.3.3. Supercritical CO_2

In a supercritical fluid, the pressure and the temperature are above the critical values, so the fluid can assume gas and liquid properties. In the supercritical region, a small variation in temperature and pressure can change the solvent properties. Extraction with supercritical fluids is promising due to the diffusivity, viscosity, surface tension and other properties of the fluid in question. The most used fluid in the supercritical region for the extraction process of several compounds from natural matrix is CO_2. With a temperature above 31 °C and pressure above 7.38 MPa, the CO_2 becomes supercritical, and for been an inert, cheap, readily available, odourless and tasteless solvent, it is ideal for the extraction of sensitive materials, such as vegetable matrices, and for the extraction of specific compounds into a mixture of complex compounds (Mukhopadhyay 2000; Sahena et al. 2009).

One of the main disadvantages of CO_2 as supercritical fluid is the low polarity, but it can be solved with the injection of co-solvents in order to modify the polarity of this fluid, which makes the compounds of interest eventually soluble in the extracts. The addition of the cosolvent is made in small amounts, ranging from 1% to 10% over CO_2 mass (Herrero et al. 2010). Ethanol, methanol, ethyl acetate and water are the most commonly used cosolvents. The CO_2 required for the process can be obtained as a by-product of industrial processes such as the fermentation process and fertilizers production. In this sense, the use of supercritical CO_2 as solvent in the extraction does not cause any significant increase in emissions of the gas to atmosphere, so, CO_2 supercritical is considered "environmentally friendly" (Mukhopadhyay 2000).

There are two ways in which supercritical extraction can be performed, in batch and continuous process, with an appropriate equipment using high pressures. In the batch process, the solid material is placed in the extraction reactor, and the supercritical CO_2 is introduced until the desired conditions are reached. In the continuous process, the CO_2 is continuously fed by a high-pressure pump, which causes the solute to precipitate using one or more stages (Gupta et al. 2012). Therefore, with this technique, the extracts containing phenolic compounds come out clean and stable, namely, since there is no light or air, the compounds do not present the risk of oxidizing, degrading, or rearranging (Roberto et al. 2010).

2.4. Molecularly Imprinted Polymers (MIP)

Molecularly Imprinted Polymers (MIPs) come from the creation of a tailored polymer network with specific cavities, which should fit to the desired molecule, with the use of functional monomers and appropriate synthesis conditions. To guarantee a geometric stability it utilizes a large amount of crosslinking agent, but the stiffness promoted by such agent hampers the stimulation motivated by dilation, contraction due to changes in pH and/or temperature in aqueous environments (Oliveira, Gomes, et al. 2016).

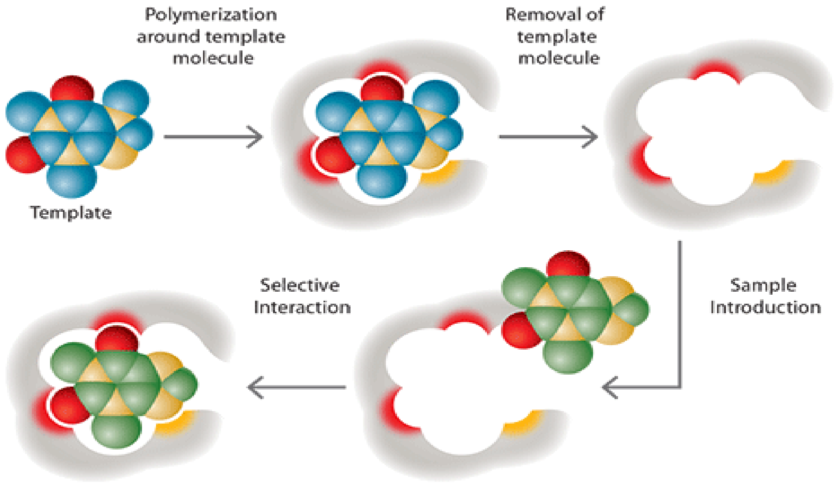


FIGURE 6: Diagram of MIP Synthesis and Selective Analyte Interaction. Source: https://www.sigmaaldrich.com/technical-documents/articles/analytical/food-beverage/analysis-aminoglycosides-pork.html

MIPs can be applied in different types of separations, such as chromatography and bio-separations, solid phase extraction systems, as well as in the development of sensors, membranes, catalysis assays and in biological processes (Oliveira, Freitas, et al. 2016). The use of MIPs as bio-adsorbents may lead to a higher retention capacity due to surface functionalization and better selectivity, because of their tailored cavities when compared to the commercial adsorbents, which justifies their application for the retention of polyphenols (Gomes et al. 2019). In this work will be studied the retention capacity of MIPs in comparison to selected commercial adsorbents, using standard polyphenols and also vegetable extracts.

Chapter 3

Experimental Section

The experimental section of this work was divided into four segments: synthesis, extraction, adsorption processes and analyses.

3.1. Materials

Polydatin (purity 95%) and gallic acid (assay 97.5-102.5%, titration) were purchased from Sigma Aldrich. Quercetin (hydrate, purity 95%) and rutin (purity 97%) were supplied by Acros Organics. These standard polyphenols were used in the synthesis and testing of the MIPs here addressed. Ethylene glycol dimethacrylate (EGDMA, 98% purity) was purchased from Sigma Aldrich. 4-vinylpyridine (4VP, 95% purity) was provided by Alfa Aesar. These compounds were used as monomers and crosslinkers in the preparation of the MIPs. AIBN of 98% purity was purchased from Sigma Aldrich and used as thermal initiator in the polymerizations performed.

Polymethylmethacrylate resin Supelite^{TM} DAX-8, an adsorbent of moderate polarity, were purchased from Sigma Aldrich and used in polyphenols retention assays, namely to perform comparative studies relatively to the performance of the MIPs prepared in this work.

Analytical reagent grades for acetonitrile (ACN), N,N-dimethylformamide (DMF), acetic acid (AcOH), methanol (MeOH), and acetone were bought from Fisher Scientific and for ethanol from PanReac. Millipore water (Milli-Q quality) was used in all the experiments unless otherwise mentioned.

3.2. Synthesis of Molecularly Imprinted Polymers

The procedure for performing the synthesis of the molecularly printed polymers was similar to that already described in the works of Oliveira, et al. (2016), Oliveira et al. (2016), Gomes et al. (2017) and Gomes et al. (2019). The method consists in the use of a polyphenol as a template, dissolved in a solvent that was a mixture of ACN with DMF. After forming a homogeneous solution, the functional monomer, 4-VP, was added to the solution and placed an ultrasound bath for 30 min to promote the interactions between the template polyphenol and the functional monomer. A homogeneous solution containing the initiator, AIBN, and the crosslinker, EGDMA, is then added. The final solution was purged with a stream of dry argon for 30 min. Finally, the polymerization reaction occurred for 24 h in a sealed vessel, using a thermostatic oil bath at 60 °C.

3.3. Extractions of polyphenols from different vegetables residues

Walnut leaf, walnut shell, almond shell, onion shell, grape marc and olive leaf were different plant sources used to obtain extracts containing polyphenols. These samples were collected in Trás-os-Montes region, Portugal. For the extraction of the phenolic compounds, all the vegetable matrices were ground without specific size.

3.3.1. Ultrasound Extraction

The hydro-alcoholic extraction ultrasounds procedure was performed using 10 g of each biomass having the EtOH/H_2O 80/20 (v/v) mixture as solvent at a temperature of 19 °C, considered as an ambient temperature at the place where the experiment was performed. The ratio of mass to solvent used was 1 g per 10 ml, the vessel was introduced into the ultrasound bath for 1 minute. The extract was then filtered and analysed by High-Performance Liquid Chromatography (HPLC-DAD) for identification of the phenolic profile.

3.3.2. Soxhlet Extraction

The Soxhlet extraction was executed using 11,5 g of the vegetable residue placed in the thimble with the help of a filter paper. 200 mL of the solvent, EtOH/H_2O 80/20 was placed in the flask and connected to the thimble and then connected to the condenser. The apparatus was placed in a thermal blanket and remained heated until two cycles were completed.

3.3.3. Supercritical CO_2 Extraction

The extraction with supercritical CO_2 was performed using a unit of supercritical reactor/extractor assembled by Paralab SA. As Gomes, Dias, and Costa (2016) described, this apparatus is composed of a Parr 970 mL steel reaction/extraction vessel with mechanical stirring, where 25 to 50 g of the biomass were placed (see Table 1). Also, it has a pumping system to supply carbon dioxide, control and purge valves, a back-pressure regulator (BPR), and a trap collector. The amount of CO_2 supplied to the vessel is controlled through a Coriolis flowmeter (mini CORI-FLOW Bronkhorst). The maximum operation temperature and pressure are T = 350 ºC and P = 200 bar. An automatic control system allows the specification of the set points for the temperature of the coil used to heat the inlet CO_2, the temperature of the reactor and the extractor, the temperature of the BPR, and the temperature of the trap. In this work the desired temperature and pressure profiles were 40 °C and 160 bar, respectively, also 5 to 10% of ethanol was added as co-solvent. The operational conditions were maintained for 2 hours (see e.g. Figure 7 and Appendix I). The obtained extract was also analysed with HPLC-DAD.

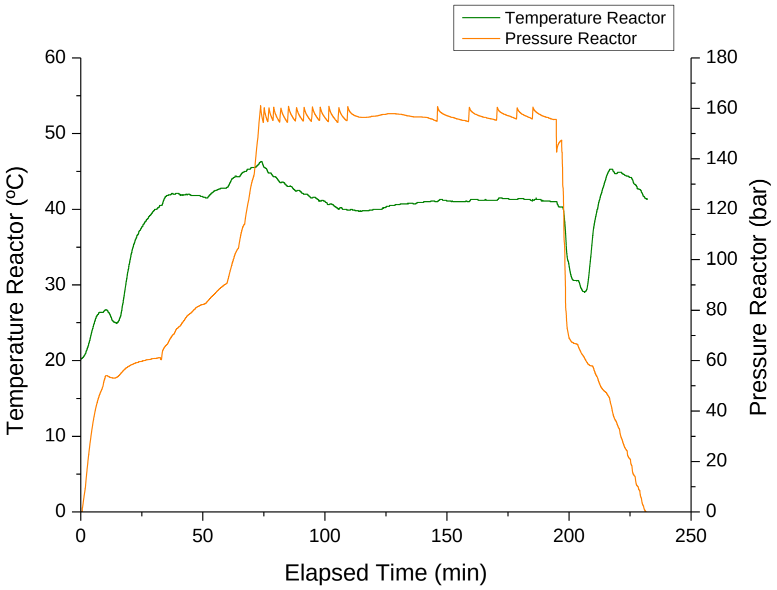


FIGURE 7: Profiles of temperature and pressure observed for the onion peel extraction with supercritical CO_2 at set-point conditions T = 40 ºC and P = 160 bar and including 5% of ethanol as co-solvent

TABLE 1: Conditions of the experiments performed with the supercritical CO_2 extraction

Vegetable Matrix	Mass (g)	Temperature Reactor (ºC)	Pressure Reactor (bar)	Co-Solvent (%)	Time (hr)
Almond Shell	50,00	40	160	5	2
Olive Leaf	50,00	40	160	5	2
Onion Shell	25,01	40	160	5	2
Onion Shell	30,16	40	160	10	2
Walnut Shell	50,01	40	160	5	2

3.4. Solid Phase Extraction

Based on the methodology performed in the works of Oliveira, et al. (2016), Gomes et al. (2017) and Gomes et al. (2019), the solid phase extraction method (SPE) was performed with 50 mg of the selected dry adsorbents placed in extraction cartridges. First the materials were washed and conditioned with $EtOH/H_2O$, monitoring each wash using UV spectrometry, until the response was zero. With the cleaned and conditioned material, 4 fractions were withdrawn, passing a volume of 5 mL of each solvent, for approximately 15 min each. For each fraction a different solvent was used, and thus performing two steps, retention and recovery of the polyphenols. A blank fraction was also collected with the same solvent where the material was conditioned. The first loading fraction was performed using as solvent the solution containing a standard phenolic compound, or the extracts obtained above, that were added to the cartridges. Solvent in a hydrophilicity gradient was used for the release steps, namely, the first step of the release was performed with a solvent which liberated the hydrophilic compounds, and, in the second stage a different solvent was used to recover the more hydrophobic compounds. Thereafter, the washing step was performed using $EtOH/H_2O$. Finally, the elution fraction was performed using a solvent with higher elution strength, such as MeOH/AcOH. The collected fractions, corresponding to the loading, washing and elution steps, were analysed using an HPLC-DAD solvent gradient process.

3.5. Batch Adsorption

The methodology used in the present work was based on the method described by Oliveira et al. (2016), where fixed amounts of the selected adsorbent material were replicated, with seven samples, including a blank solution. Specifically, 50 mg material were mixed with 5 mL of $MeOH/H_2O$ solution containing the standard polyphenol at different concentrations. The mixture was equilibrated at room temperature for about 24 h under 60 rpm agitation using an orbital shaker. After equilibration, a small sample was collected and centrifuged, and the concentration measured by UV monitoring at a wavelength of 280 nm. From these absorbance data, the adsorbed amount can be calculated.

3.6. Continuous Adsorption Process

The continuous adsorption and desorption process were performed to evaluate the performance of commercial adsorbents and the MIPs synthesized for the retention and release of polyphenols present in vegetable extracts. Thus, the materials were packed in HPLC columns. For each material were used different columns sizes ranging from 4.6 to 8 mm of internal diameter and 18 to 50 mm length. First, the packed column was conditioned with the same solvent present at the extract, after that, the saturation step was operated at a flow rate of 1 ml/min of the extract and at the room temperature (22 °C). Samples were taken at different times and measured in UV until detection of the saturation point, and thus allowed to proceed with the release of the compounds. The release step was made in fractions, with solvent in a hydrophilicity gradient. The release steps were performed at a flow rate of 1 ml/min and at 45 ºC. The Table 2 shows the composition of the solvent used with each fraction and the time instant corresponding to the duration of the sampling.

TABLE 2: Polyphenol release fractions and composition of the solvent used

Fractions	**Time (min)**	**Solvent A[1] (mL)**	**Solvent B[2] (mL)**	**A/B (v/v)**	**Total Volume (mL)**
1	20	18	7	72/28	25
2	7	6,9	8,1	46/54	15
3	3	5,25	9,75	35/65	15
4	6	2,55	12,45	24/76	15
5	9	1,275	13,725	8,5/91,5	15
6	5	-	15	0/100	15

[1] H_2O/MeOH (90/10), with pH = 3 adjusted with acetic acid. [2] MeOH/AcOH (90/10)

3.6.1. Modelling of the column's dynamics in Continuous Adsorption Process

This method was based on the approach used by Gomes et al. (2019), which consists of the description of the adsorption/desorption processes involved in the continuous running of columns packed with the synthetized MIPs, and commercial adsorbents, as DAX-8. It was performed in MATLAB® considering the mass balance equations for the mobile and stationary phases, as represented by the partial differential equations (PDE) (1) and (2), respectively, also the interfacial liquid/solid equilibrium of the solute is described by equation (3). When a mass transfer coefficient in the liquid phase is also used, the condition of the following kind is represented by equation (4). The equation (5) is the initial condition for equations (1) and (2) and the classical Danckwerts boundary conditions at column inlet and outlet are described by equations (6) and (7), respectively.

$$\varepsilon \frac{\partial C}{\partial t} - \varepsilon D_{ax} \frac{\partial^2 C}{\partial x^2} + \varepsilon \mathrm{u} \frac{\partial C}{\partial x} + (1-\varepsilon) \frac{\partial q}{\partial t} = 0 \quad (1)$$

$$\frac{\partial q}{\partial t} = k_{fS}(q^* - q) \quad (2)$$

$$q^* = f_{eq}(C^*) \quad (3)$$

$$k_{fS}(q^* - q) = k_{fL}(C - C^*) \quad (4)$$

$$t = 0, 0 \leq x \leq L, C = C_0 \quad (5)$$

$$x = 0, t > 0, \mathrm{u}C - D_{ax} \frac{\partial C}{\partial x} = \mathrm{u}C_{in} \quad (6)$$

$$x = L, t > 0, \frac{\partial C}{\partial x} = 0 \quad (7)$$

The parameters involved are the porosity of the bed (ε), the interstitial velocity of the mobile phase in the column (u), the axial dispersion coefficient (D_{ax}) and the mass transfer coefficients in liquid and solid phases (k_{fL}, k_{fS}, respectively), the axial position in the column (x), time (t), the concentration of the solute in the mobile phase (C) and the volume-average concentration of solute adsorbed in the particles (q). C^* is the concentration of solute in the liquid phase at the equilibrium interface, L represents the length of the column, C_0 the initial concentration of solute in the column and C_{in} the concentration of the solute at column inlet. This model was used to fit the experimental measurements to the theoretical predictions, namely with the breakthrough curves.

3.7. SEM Analysis

The morphology of the synthesized MIP and NIP particle was analysed by SEM of the dried products, at the Materials Center of the University of Porto (CEMUP). A SEM FEI Quanta 400FEG instrument with the EDS (Energy Dispersive Spectroscopy) system Edax Genesis X4M was used in these analyses.

3.8. Polyphenols Identification through gradient HPLC-DAD

The HPLC equipment used was a Jasco MD-4010 photo diode array detector. The stationary phase was a C18 Nucleosil 100 analytical column with 150 mm in length and an internal diameter of 4.6 mm, with a particle size of 5 µm (MACHEREY-NAGEL), at room temperature (22 ºC). The flow rate was 1 ml/min and the absorbance changes were monitored at 280 nm and 320 nm. The mobile phases for chromatographic analysis were: (A) acetonitrile/water (10/90), with pH = 3 adjusted with acetic acid and (B) acetonitrile/water (90/10), with pH = 3 adjusted with acetic acid. The linear gradient method was used starting with 100% of solvent A and ending with 100% of solvent B.

3.9. Polyphenols Identification through Liquid Chromatography with Mass Spectroscopy and Diode Array Detector (LC-DAD-ESI/MS^n)

These analyses were performed at the Centro de Investigação da Montanha (CIMO). The chromatographic analysis was performed in the same way as in the Bessada et al. (2016) work, which used 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/MS^n), a quaternary pump, an auto-sampler (kept at 5 ºC), a degasser and an automated thermostatted column compartment. Chromatographic separation was achieved with a Waters Spherisorb S3 ODS-2C18 (3 µm, 4.6 mm × 150 mm, Waters, Milford, MA, USA) column thermostatted at 35 ºC. 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. Double online detection was carried out in the DAD using 280 and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet. MS detection was performed in negative mode, using a Linear Ion Trap LTQ XL mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an ESI source. Nitrogen served as the sheath gas (50 psi); the system was operated with a spray voltage of 5 kV, a source temperature of 325 ºC, a capillary voltage of - 20 V. The tube lens offset was kept at a voltage of - 66 V. The full scan covered the mass range from m/z 100–1500. The collision energy used was 35 (arbitrary units). Data acquisition was carried out with Xcalibur® data system (ThermoFinnigan, San Jose, CA, USA).

Chapter 4

Experimental Results and Discussion

4.1. Polymerization Conditions Used in the Synthesis of MIPs

In the present work, the MIPs were synthesized considering two polymerization technique, MIP 1 was obtained by suspension polymerization and others MIPs by precipitation polymerization, with the objective of being used as adsorbents and also to be compared to commercial synthetic materials often used in adsorption processes for polyphenols. NIPs were prepared in parallel, following the same experimental procedure as for MIPs, without template molecule. Table 3 describes the polymerization conditions used in the synthesis of the MIPs considering the following parameters:

- Y_M – The mass fraction of functional monomer plus crosslinker in the solution (%)
- Y_I – The mole fraction of initiator comparatively to functional monomer plus crosslinker (%)
- Y_{CL} - The mole fraction of crosslinker in the mixture of functional monomer plus crosslinker (%)
- $Y_{FM/T}$ – The mole ratio between the functional monomer and the template molecule

TABLE 3: Polymerization conditions used in the preparation of different MIPs

MIP	Template	Functional Monomer	Solvent	Y_M	Y_I	Y_{CL}	$Y_{FM/T}$
MIP 1	Polydatin	4VP	MeOH/H_2O 80/20	62,4	1,8	55,7	6,0
MIP 2	Quercetin	4VP	ACN/DMF 91/9	6,4	5,2	49,1	5,0
MIP 3	Quercetin	4VP	ACN/DMF 91/9	5,6	5,2	49,1	5,0
MIP 4	Quercetin	4VP	ACN/DMF 91/9	20,0	5,0	49,9	20,3

Note that the MIP 1 (MIP 7 in Gomes et al. (2017), and MIP 13 in Gomes et al. (2019)) and the MIP 2 (MIP 9 in Gomes et al. (2019)) were already tested in previous works. The MIP 3 was synthesized to replace MIP 2, since there was not enough mass of MIP 2 to perform all experiments that were planned along this research. The MIP 4 was synthesized to try the change of the particle size, and thus, to operate the packed column with more pressure stability.

The molecular imprinted technique is based on the specific interactions, such as hydrogen bonding, between the functional monomer and template. Figure 8 presents the expected interaction between the functional monomer, 4-VP, used in this work and three different templates.

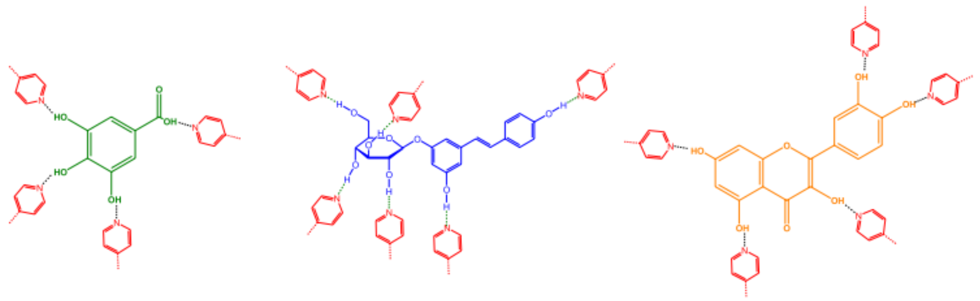
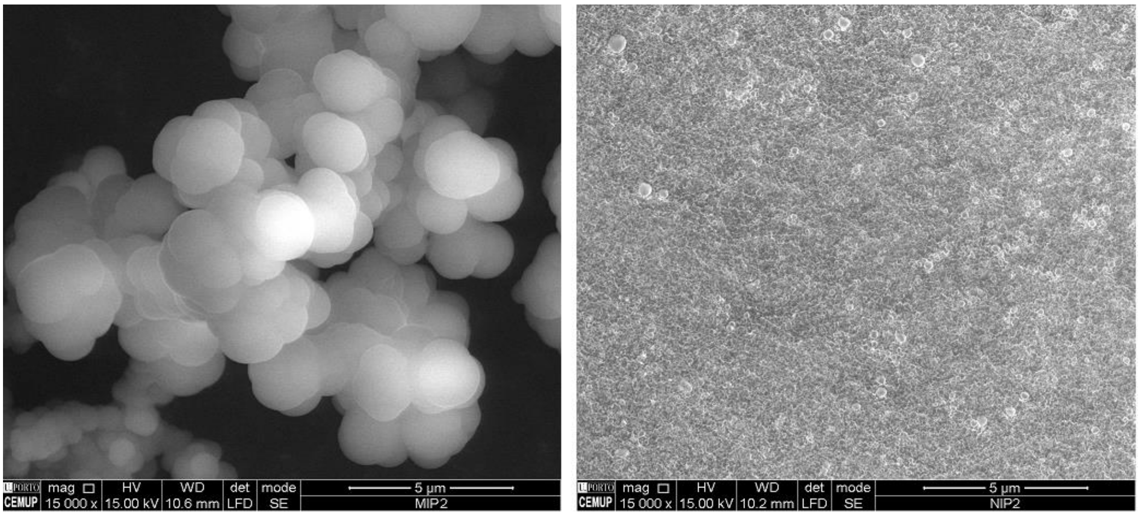

(a) (b) (c)

FIGURE 8: Depiction of some expected interactions between three possible polyphenol template molecules (a gallic acid, b polydatin and c quercetin) and functional monomers used for molecular imprinting. The interaction of pyridyl functional groups with the templates was here selected for illustration purposes. Source: (Gomes et al. 2019).

The morphology of the MIPs and NIPs synthesized particles was obtained by SEM of dried materials. SEM Images (Figure 9) showing the morphology of MIP 2 and NIP 2 and the size of particles is around 1µm. Through the SEM images, is possible to see that the presence/absence of template molecule affects the product morphology. The impact of the synthesis conditions on morphology of MIPs, using SEM analysis, can be found in Gomes et al. (2019), namely for MIP1 MIP2 and NIP2.



(a) (b)

FIGURE 9: SEM images showing the morphology of polyphenol adsorbents synthesized in this work. (a) MIP2 and (b) NIP2

4.2. Comparison between MIPs and Commercial Adsorbents Using Standard Polyphenols

The performance of the synthesized MIPs for the retention and release of polyphenols was evaluated by three different techniques experiments: batch adsorption, continuous adsorption and solid phase extraction. The comparison between synthesized MIPs and a commercial synthetic resin, DAX-8, it was also made in the present work.

4.2.2. Batch Adsorption

In the first phase of the project were carried out adsorption experiments using a standard of quercetin as analyte, at different concentrations, with MeOH/H_2O as solvent in different alcohol/water proportions and using also DAX-8 as a possible commercial adsorbent. The objective was to assess the adsorption performance of the different materials with a relatively simple chemical system (the use of a standard polyphenol leads to an easier interpretation of the results).

After adsorption, the UV absorbance was measured at a wavelength of 280 nm. The initial and final concentration of the template before and after equilibration C_0 and C, respectively, were used in Equation (8) to obtain the adsorbed amount of quercetin in the different materials.

$$q = \frac{V\,(C_0 - C)}{m_s} \tag{8}$$

Where q is the amount adsorbed (µmol/g), C_0 is the initial concentration and C is the equilibrium concentration of the analyte (mM), V is the volume of solution used (mL) and m_s the mass of the adsorbent (mg).

To describe the adsorption equilibrium, if a light solute layer was formed, in this case of quercetin, on the surface of the solid, the Langmuir isotherm can be used, which is represented by the following Equation (9).

$$q_e = q_{max} \frac{k_L C_e}{1 + k_L C_e} \tag{9}$$

Where q_{max} and k_L are two parameters calculated using the experimental data, q_{max} (mM) representing the maximum adsorbed amount of solute on the surface and k_L

(mM^{-1}) the ratio between the adsorption and desorption kinetic constants.

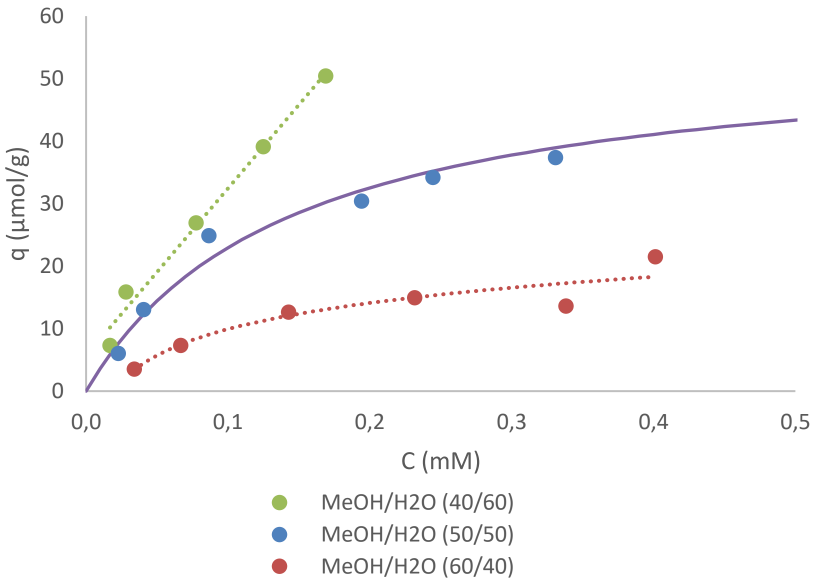


FIGURE 10: Experimental data and Langmuir isotherms for the batch adsorption of quercetin in DAX-8 considering $MeOH/H_2O$ as solvent with different compositions alcohol/water (the effect of the hydrophobic interactions is assessed with DAX-8).

Results presented in Figure 10 show that DAX-8 had a better performance when using $MeOH/H_2O$ 40/60 as the solvent, adsorbing a higher amount of quercetin as a consequence of the increase of the hydrophobic interactions. These results show that these effects (hydrophobic interactions) are a major driving force for adsorption when this class of materials is used. In Figure 11 is presented the comparison for the batch adsorption of quercetin in DAX-8 and MIP 1 using $MeOH/H_2O$ 50/50 as solvent. With these conditions, a very similar retention capacity is observed with the two materials (note that MIP 1 is a polydatin MIP, as described in Table 1, that was here considered just for comparative purposes).

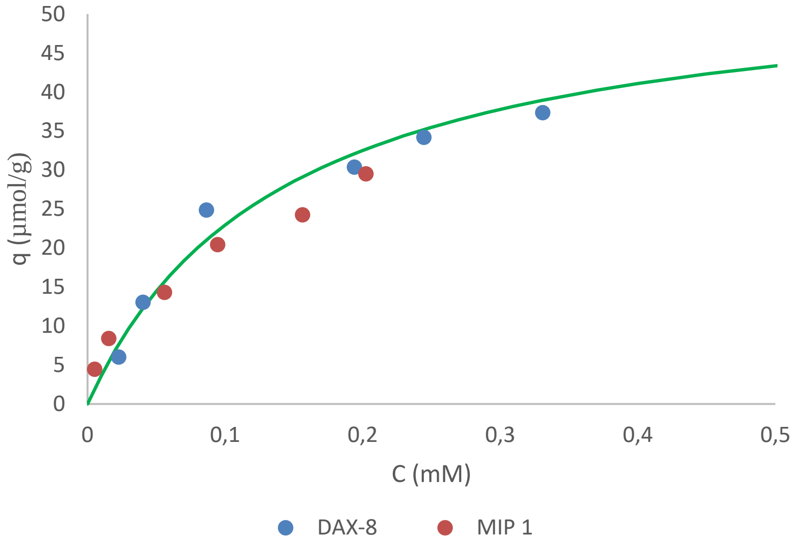


Figure 11: Experimental data and Langmuir isotherm for the batch adsorption of quercetin in DAX-8 and MIP 1 considering MeOH/H_2O 50/50 as solvent.

With these conditions, the obtained results show that the retention of quercetin was similar in the DAX-8 and MIP 1. This can be explained by the fact that the cavities of the MIP 1 are not favourable for a good quercetin adsorption, since the template used to synthesize this MIP was polydatin. Additionally, the specificity of MIPs is hampered in aqueous media due to the strong effect of hydrophobic interactions. Indeed, the main purpose of the results above presented was to highlight the important effect of hydrophobic effects when adsorption with polymeric materials is performed.

4.2.3. Continuous Adsorption Process

The second adsorption test was performed with a continuous operation process with MeOH/H_2O 50/50 as solvent. In these studies, the three adsorbents were used, MIP 1, MIP 2 and DAX-8, each packed in a different HPLC column.

To calculate the adsorbed amount (q) of quercetin in this process, it was necessary to estimate the area below the saturation curve (UV measurements were performed at 280 nm). This calculation was done by the trapezoid rule and from it is possible calculate q in µmol/g using Equation (10).

$$q = \frac{solute\ absorbed}{mass\ of\ material} \qquad (10)$$

The final values for each concentration are presented in Figure 12 (see also Table 4). As can be seen, the highest adsorbed amount of quercetin is given with the use of MIP 2 as an adsorbent, which, unlike MIP 1, was synthesized using quercetin as a template, so, it has a higher affinity for this polyphenol.

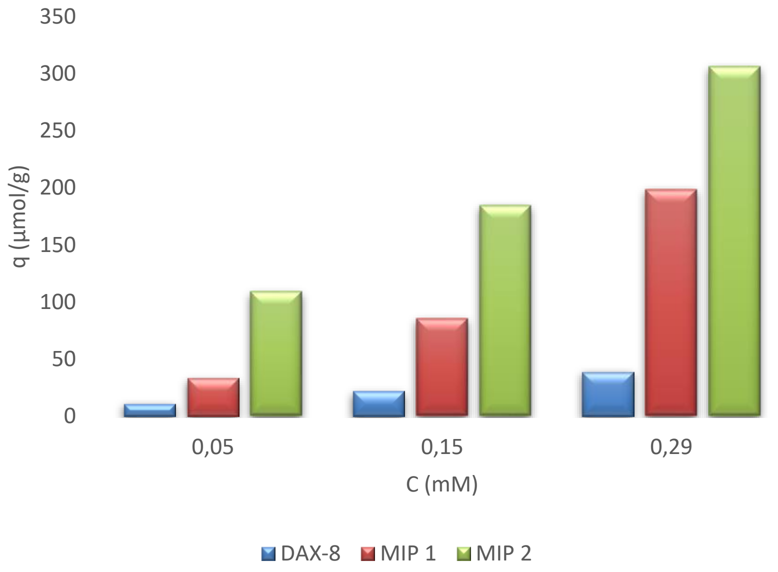


FIGURE 12: Amount of quercetin absorbed by DAX-8, MIP 1 and MIP 2 in a continuous absorption process with MeOH/H_2O 50/50 as solvent.

Results presented in Figure 12 highlight that the adsorption in MIPs (specially in MIP 2) is not exclusively due to hydrophobic interactions and the functionalization of the materials surface (4VP was used as functional monomer in MIP 1 and in MIP 2) enhance their retention capability for quercetin. Moreover, the morphology introduced by the molecular imprinting mechanism also benefits the performance of the materials in continuous operation (e.g. boosting the mass transfer between liquid and solid phases). These aspects become apparent when the batch and continuous process performances of DAX-8 and MIP 1 are compared (see Figure 11 and Figure 12).

4.2.3.1. Experimental and Simulated Breakthrough Curve

The information provided by the breakthrough curves, namely the adsorbent retention capacity in relation to the solute under the operating conditions, it is fundamental to design an efficient continuous process in order to valorise the polyphenols present in the plant extracts. Thus, the results obtained with the selected MIPs (MIP1 and MIP2), and with DAX-8 were theoretically analysed in the framework of the modelling of the continuous adsorption process that was before presented (see section 3.6.1). The involved partial differential equations were solved using the function *pdepe* of Matlab and parameter estimation was performed using the function *lsqnonlin* (also of Matlab).

The experimental data and simulated curves presented in Figures 13 to 15 illustrate these issues, showing also the outcome of the modelling tools used in the analysis of the continuous adsorption process (see also parameter estimation presented in Table 5 considering the Langmuir isotherm). All runs were carried out at T= 22 ºC. Standard quercetin was used as the target polyphenol, with three different concentrations and the considered eluent was MeOH/H_2O 50/50. Note that, for each adsorbent, individual estimations for k_L and q_{max} were produced (see Table 5) instead of the global optimization of the data. Indeed, additional work concerning the global estimation of these parameters can be performed but the goal of this analysis was just the observation of the different performance of the materials with the quercetin polyphenol template.

In fact, when the performances of different adsorbents are compared considering above described operation conditions, the usefulness of materials tailored through molecular imprinting becomes clear. Using quercetin as reference molecule, much higher retention capabilities are observed with MIPs comparatively to DAX8 when MeOH/H_2O 50/50 is used as eluent.

TABLE 4: The measured adsorbed amounts of quercetin (µmol/g) in continuous processes using MeOH/H_2O 50/50 as solvent. These values were estimated using the breakthrough curves and the trapezoidal rule for integration.

Concentration (mM)	MIP 1	MIP 2	DAX-8
0,05	32,987	109,210	10,734
0,15	85,569	184,761	21,949
0,29	198,734	306,444	38,831

TABLE 5: Langmuir isotherm parameter (k_L) and the estimated maximum retention capacity in Langmuir isotherm (q_{max}) for different materials and concentrations using MeOH/H_2O 50/50 as solvent. These estimations were obtained through fitting of the different experimental breakthrough curves to the theoretical model used.

Material	Concentration (mM)	k_L (mM^{-1})	q_{max} (mM)
DAX -8	0,05	0,186	1632,376
	0,15	0,134	1491,239
	0,29	0,131	1398,420
MIP 1	0,05	0,242	2123,354
	0,15	0,220	1984,765
	0,29	0,209	2968,659
MIP 2	0,05	0,304	4158,481
	0,15	0,282	2367,566
	0,29	0,293	2035,628

These observations are a preliminary demonstration that with the adsorption mechanism of polyphenols in the MIPs, higher retention/selectivity capabilities are in principle possible, namely in comparison with the non-specific commercial adsorbents. These issues will be extended with the analysis of much more complex mixtures of polyphenol molecules, in particular the practical case with industrial relevance correspondent to the processing of real plant extracts.

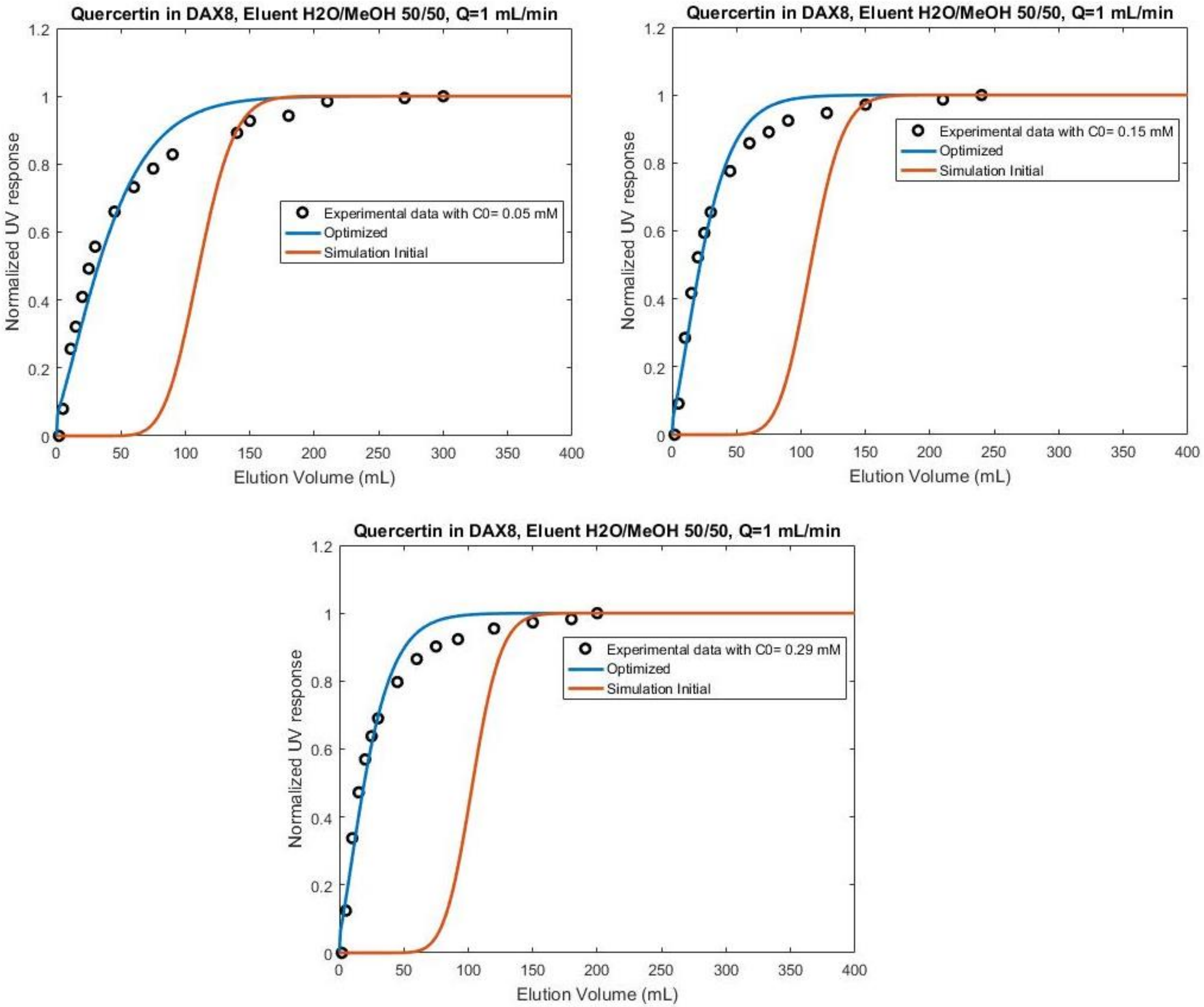


FIGURE 13: Experimental results (symbols) and simulations (lines) for the continuous adsorption of quercetin in a column with dimensions L×D = 23×4.6 mm×mm packed with 230,85 mg of DAX-8.

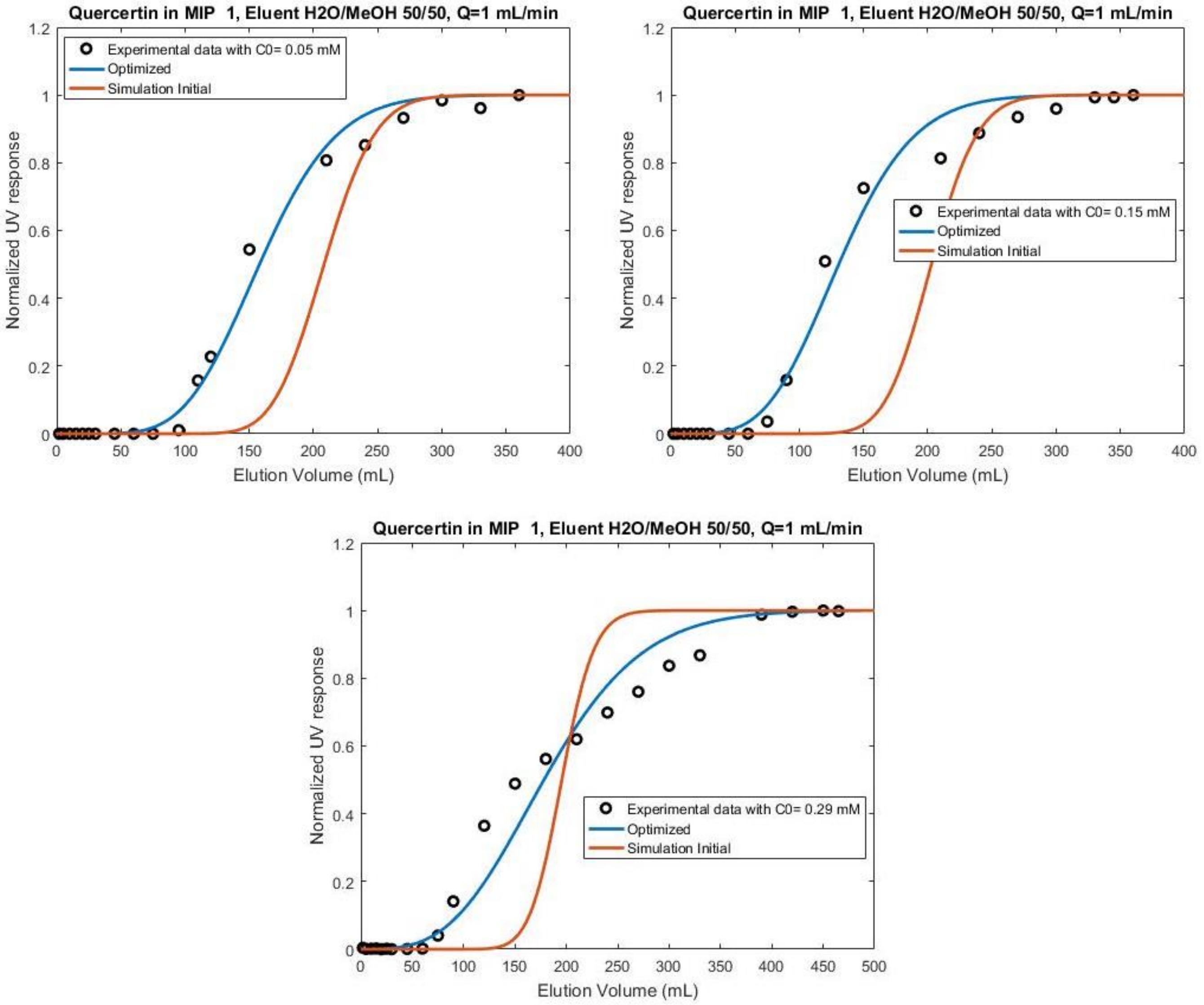


FIGURE 14: Experimental results (symbols) and simulations (lines) for the continuous adsorption of quercetin in a column with dimensions L×D = 33×8 mm×mm packed with 251 mg of MIP 1.

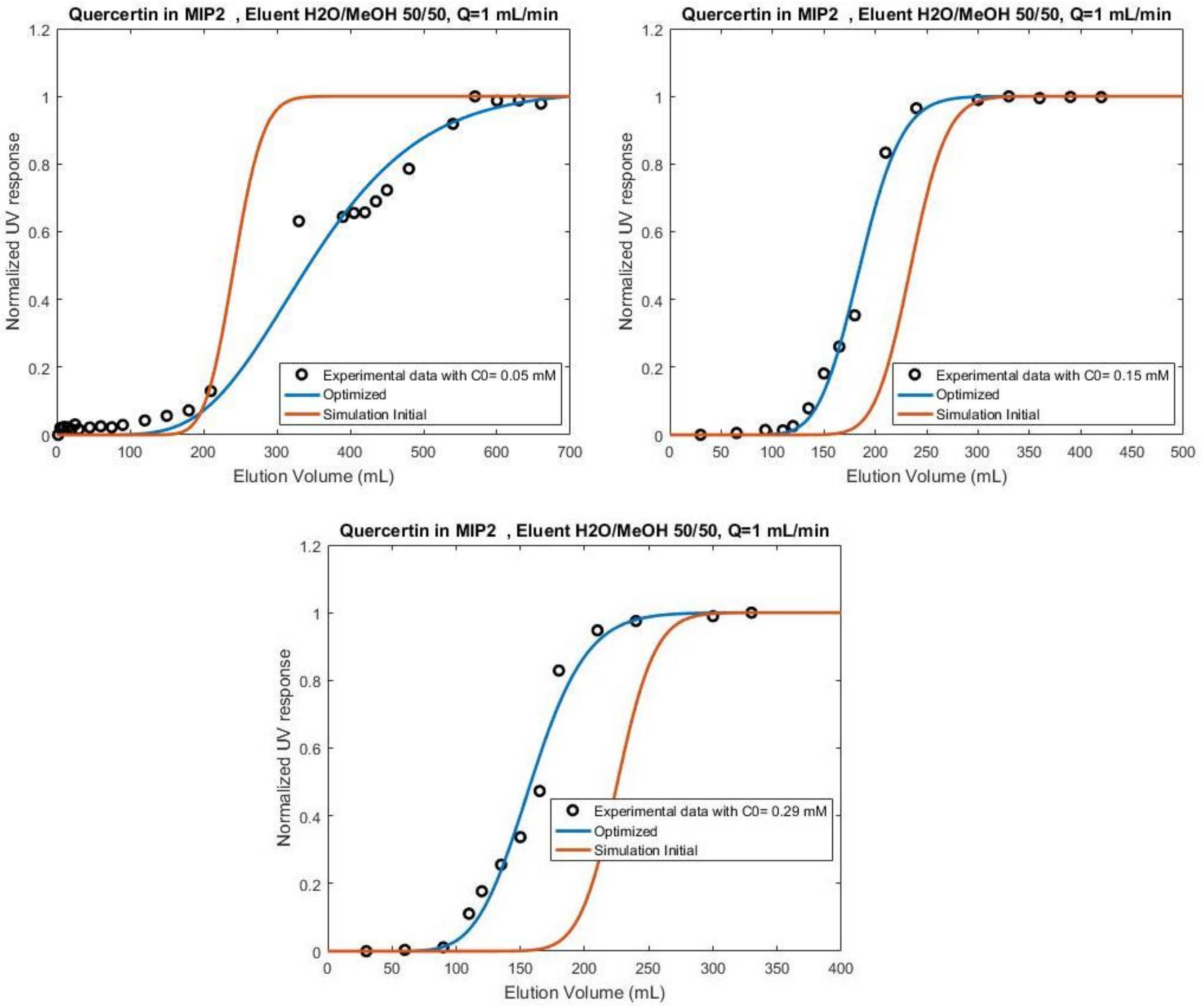


FIGURE 15: Experimental results (symbols) and simulations (lines) for the continuous adsorption of quercetin in a column with dimensions L×D = 50×4.6 mm×mm packed with 150 mg of MIP 2.

4.2.4. Solid Phase Extraction

In Figure 16 are presented results for the SPE retention of the standard polyphenol molecules gallic acid, rutin and quercetin, mixed in EtOH/H_2O 80/20, using MIP 3 (a replica of MIP 2) and considering also the comparison with the commercial adsorbent DAX-8.

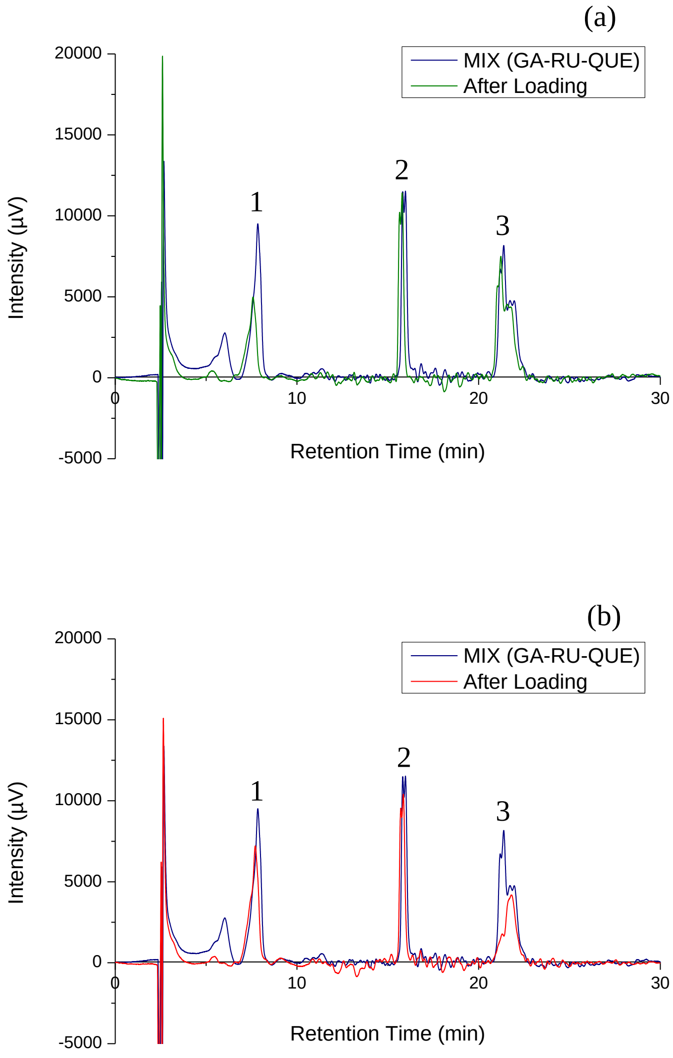


FIGURE 16: SPE assessment of (a) DAX-8 and (b) MIP 3 concerning the retention of: (1) Gallic Acid, (2) Rutin and (3) Quercetin. SPE loading was performed using a mixture of the polyphenols with individual concentration 0.04 mM in EtOH/H_2O 80/20. Analyses performed by HPLC-DAD

The retention capabilities were calculated by subtraction and normalization of the area of the peaks (initial peak area and peak area after loading), and it is possible to see a higher retention capability after loading of quercetin for the MIP with these conditions, when compared to the DAX-8. 47,6% of quercetin was retained in MIP 3 while only 10.4% of quercetin was retained in the commercial resin, which proves the efficiency of the MIP when used as an adsorbent for the retention of polyphenols, mainly due to its functionality (introduced by the 4-VP monomer) and some specificity (geometric matching generated by molecular imprinting).

Knowing the good efficiency of the MIP as an adsorbent material with the previous adsorption conditions, the same mixture of standard polyphenols, gallic acid, rutin and quercetin was used but considering EtOH/H_2O 50/50 as solvent in the loading step. These results are presented in Figure 17 (note that a deviation in the peaks location is observed in the HPLC-DAD analyses due to the instrument operation in different days).

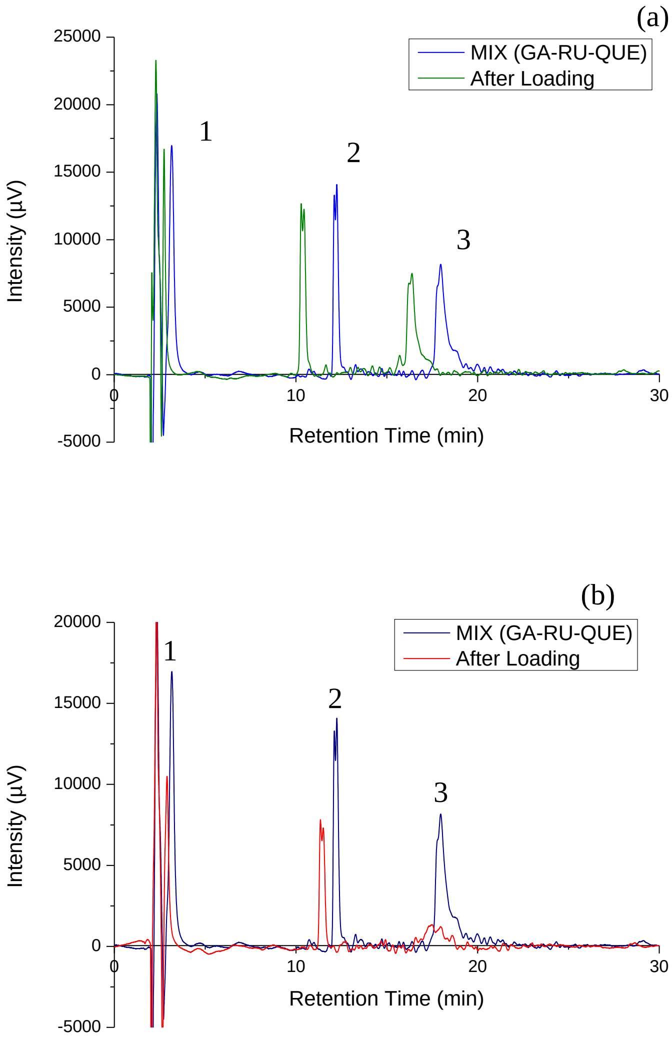


FIGURE 17: SPE assessment of (a) DAX-8 and (b) MIP 3 concerning the retention of: (1) Gallic Acid, (2) Rutin and (3) Quercetin. SPE loading was performed using a mixture of the polyphenols with individual concentration 0.04 mM in EtOH/H_2O 50/50. Analyses performed by HPLC-DAD

As the quercetin has a low polarity, which is to say that it is a relatively hydrophobic molecule, it was expected that with the increase of the proportion of water in the solvent the retention of quercetin in the material would be greater, as was evidenced for some polyphenols in the study of Silva, Pompeu, Larondelle, and Rogez, (2007). Indeed, the results show that with EtOH/H_2O 50/50, comparatively to EtOH/H_2O 80/20, the quercetin retention increased from 10.4 to 12,01% in DAX-8 and with MIP 3 there was an increase from 47,6 to 85,11%.

These results prove that in adsorption processes of polyphenols, the MIPs (namely the quercetin MIPs here studied), as adsorbents, present a higher retention and selectivity capabilities than commercial alternatives. These latter materials need higher water content to retain more polyphenol and the correspondent specificity of the materials is lower. Below, in this work, these important issues are extended in the framework of real plant extracts.

4.3. Extracts obtained

To obtain the extracts of different vegetable matrices, three types of extractions were considered.

With the ultrasound extraction technique, five different extracts were obtained and analysed in HPLC-DAD as described in chapter 3. In Figure 18 is presented the phenolic profile obtained with each vegetable matrix.

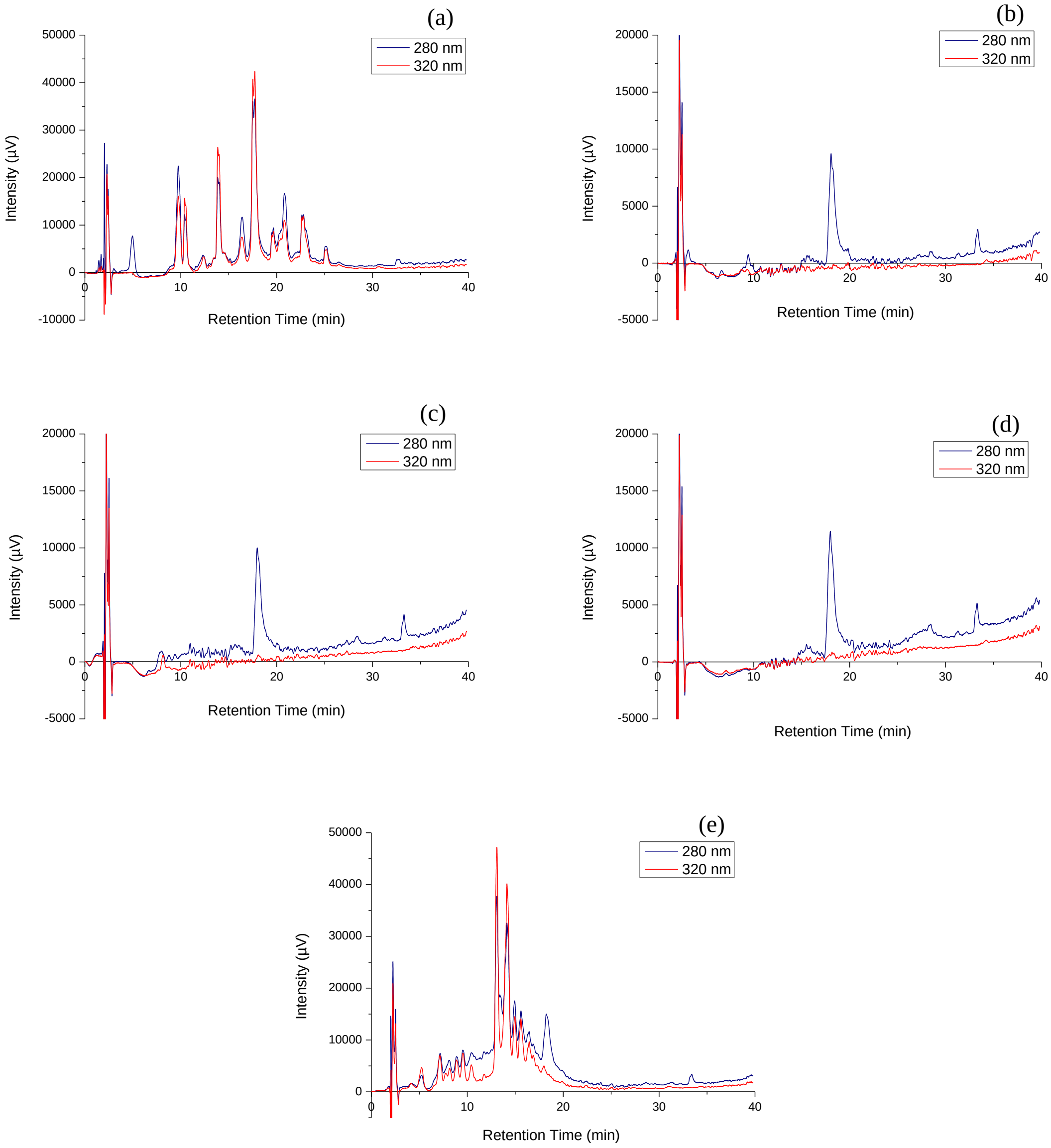


FIGURE 18: Phenolic profiles measured with HPLC-DAD gradient analysis for different plant extracts obtained using the ultrasound extraction technique. (a) Onion shell extract. (b) Grape marc extract. (c) Almond shell extract. (d) Walnut shell extract. (e) Walnut leaf extract.

With the extraction using supercritical CO_2 were made five extractions of different matrices and also analysed in HPLC-DAD, as can be seen in Figure 19. The extraction profiles observed with each matrix, concerning the temperature, pressure, CO_2 flow rate and total CO_2 mass supplied to the $SCCO_2$ apparatus are presented in the appendix section I.1.

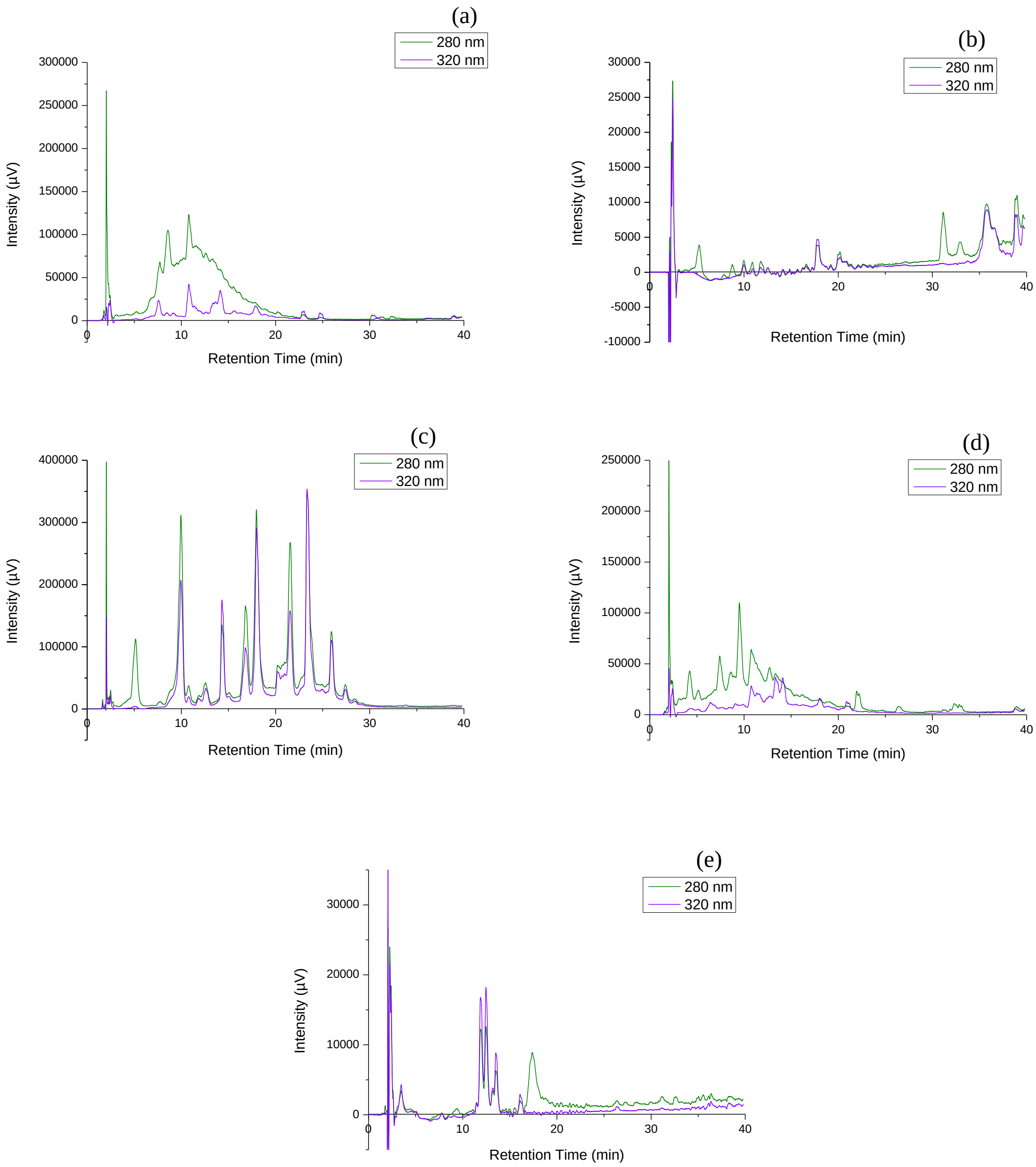


FIGURE 19: Phenolic profiles measured with HPLC-DAD gradient analysis for different plant extracts obtained considering supercritical CO_2 extraction. (a) Almond shell extract. (b) Onion shell extract with 5% of ethanol as co-solvent. (c) Onion shell extract with 10% of ethanol as co-solvent. (d) Walnut shell extract. (e) Olive leaf extract.

The unique vegetal matrix that was used to obtain a soxhlet extract was the onion shell (see Figure 20), in order to make a comparison between the phenolic profiles obtained with the other two extractions reported above. In fact, richer phenolic profiles were consistently obtained with the onion shell (namely concerning the presence of quercetin) and, based on this observation, this research was thereafter mostly focused on this plant residue.

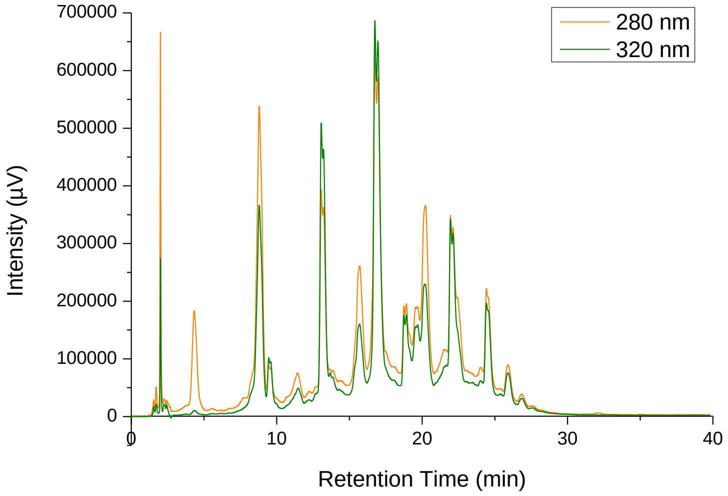


FIGURE 20: Onion shell phenolic profile measured with HPLC-DAD gradient analysis for a plant extract obtained considering Soxhlet extraction

In Figure 21 is compared the HPLC-DAD analysis of an onion shell extract with the chromatogram correspondent to a mixture of gallic acid, rutin and quercetin standard polyphenols. Some similitude in the location of the quercetin peak is observed comparatively to a dominant peak in the onion shell extract, indicating a possible strong presence of this polyphenol in the plant residue, as below further explored.

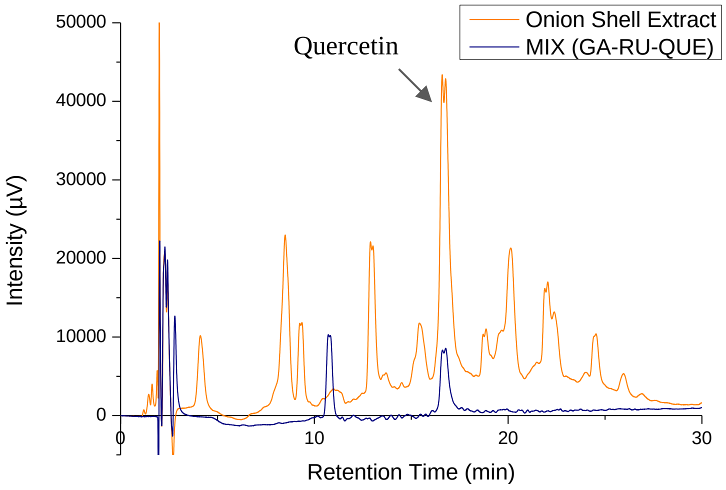


FIGURE 21: Identification of quercetin in the onion shell extract obtained by ultrasound extraction. A mixture of gallic acid, rutin and quercetin at 0.04 mM in EtOH/H_2O 80/20 was considered to perform this preliminary identification. UV analysis at wavelength of 280 nm

As can be seen in Figure 22, the phenolic profile of the onion shell obtained with different extraction technics present the same peaks with a slight difference of intensity between them. This seems to show that almost the same polyphenol molecules are present in the different onion shell extracts but at different concentrations. The results for the estimation of the total phenolic compounds (TPC) of the extracts are shown in Table 6. The extracts obtained by Soxhlet and ultrasound extraction present a higher concentration of TPC than the extract obtained by supercritical CO_2.

TABLE 6: Results for the total phenolic compounds (TPC) expressed in (mg/g) estimated to be present in onion shell extracts obtained considering three different extraction techniques.

Extractions	Mass dried onion shell (g)	TPC (mg/g)
Soxhlet Extraction	11.50	11.28
Supercritical Extraction	30.16	1.21
Ultrasound Extraction	10.00	10.22

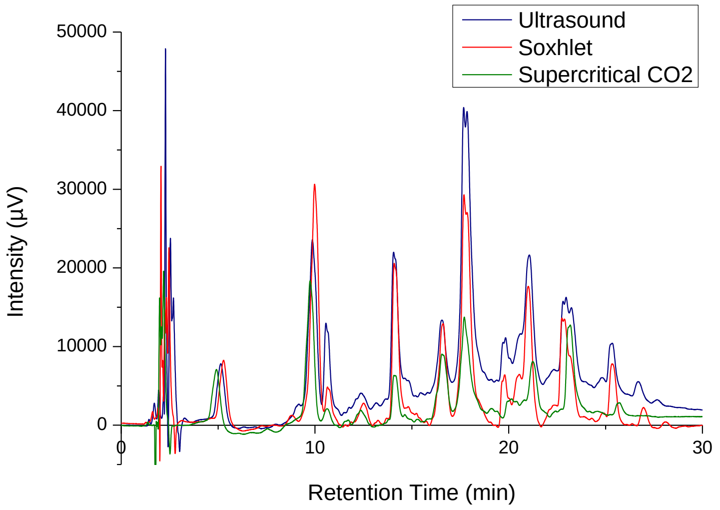


FIGURE 22: Comparison of phenolic profiles, measured with HPLC-DAD gradient analysis, for onion shell extracts obtained by ultrasound, soxhlet and supercritical CO_2 techniques. UV monitoring at wavelength 280 nm.

4.4. Identification of Phenolic Compounds Present in Onion Shell and Almond Shell Extracts.

The phenolic compounds present in onion shell extracts and almond shell extracts were identified using the LC-DAD-ESI/MS^n method, which analysis conditions were described in chapter 3 (topic 3.9). The extracts considered for this analysis were the onion shell US and almond shell SFE. The tentative identification of phenolic compounds was based on retention time (Rt), UV-vis (λmax), spectral mass (MS) and specific fragmentation (MS^2) patterns in alignment with literature. In the case of quercetin, the identification was also confirmed by comparison with pure standard. Data of retention time (Rt), molecular formula, molecular weight (Mw), molecular ion ($[M-H]^-$), main fragment ions (MS^2) was used and a tentative identification of the phenolic compounds is presented in Table 7. Figure 23 shows the HPLC phenolic profile of an onion shell obtained by ultrasound extraction (a) and an almond shell extract obtained by supercritical extraction (b). Thus, it was possible to identify at least eleven phenolic compounds present in the onion shell extract and nine compounds present in the almond shell extract (Bessada et al. 2016; Campone et al. 2018; Rockenbach et al. 2012; Smeriglio et al. 2016).

TABLE 7: A Retention time (Rt), molecular formula, molecular weight, wavelengths of maximum absorption (λmax), mass spectral data [M-H]$^-$, relative abundances of fragment ions (MS^2) and tentative identification of the phenolic compounds present in onion shell and almond shell extracts.

Peak	Rt (min)	Molecular formula	λ_{max} (nm)	[M-H]$^-$ (m/z)	MS^2 (m/z)	Mw (g/mol)	Tentative Identification	References
1	5.37	$C_7H_6O_4$	260 294	152.99	109 (100)	154.12	protocatechuic acid	(Bessada et al. 2016)
2	6.98	$C_{14}H_{14}O_6$	280	289.00	245 205 179	290.26	*(+)* catechin	(Smeriglio et al. 2016)
3	10.51	$C_{45}H_{38}O_{18}$	285	865.40	739 695 577 287	866.77	Procyanidin trimer	(Rockenbach et al. 2012)
4	10.88	$C_{15}H_{10}O_8$	294	317.13	299 191 273 207	318.24	2-(3,4-Dihydroxybenzoyl)-2,4,6-trihydroxy-3(2H)-benzofuranone	(Campone et al. 2018)
5	13.99	$C_{45}H_{38}O_{18}$	280	865.00	739 695 577 289 287	866.77	Procyanidin trimer	(Rockenbach et al. 2012)
6	14.15	$C_{27}H_{30}O_{17}$	265 342	625.32	463 301	626.52	Quercetin-*O*-diglycoside	(Campone et al. 2018)
7	17.69	$C_{27}H_{30}O_{16}$	280	609	300-255	610.52	Rutin	(Bessada et al. 2016)
8	18.76	$C_{21}H_{20}O_{12}$	280	463.28	301 151	464.38	Quercetin-O-hexoside	(Bessada et al. 2016)
9	21.92	$C_{28}H_{32}O_{16}$	280 340	623.41	315 300 271	624.56	Isorhamnetin-3-*O*-rutinoside	(Smeriglio et al. 2016)
10	23.15	$C_{21}H_{20}O_{12}$	253 365	463.28	301 151	464.38	Quercetin-*O*-glucoside	(Campone et al. 2018)
11	23.26	$C_{22}H_{22}O_{12}$	278 350	477.29	314 285 243 151	478.40	Isorhamnetin-3-*O*-glucoside	(Smeriglio et al. 2016)
12	29.13	$C_{22}H_{14}O_{11}$	294	453.18	299	454.23	Protocatecoyl quercetin	(Campone et al. 2018)
13	31.44	$C_{15}H_{10}O_7$	255 370	301.22	273 257 178 151	302.24	Quercetin	(Campone et al. 2018)
14	34.45	$C_{36}H_{28}O_{19}$	255 303 368	763.30	611 299	764.60	Quercetin dimer-*O*-hexoside	(Bessada et al. 2016)
15	37.94	$C_{36}H_{28}O_{19}$	255 303 368	763.30	611 299	764.60	Quercetin dimer-*O*-hexoside	(Bessada et al. 2016)
16	39.41	$C_{36}H_{28}O_{19}$	255 303 368	763.30	611 299	764.60	Quercetin dimer-*O*-hexoside	(Bessada et al. 2016)
17	42.14	$C_{30}H_{18}O_{14}$	271 302 364	601.21	499 299	602.46	Quercetin dimer	(Campone et al. 2018)
18	47.53	$C_{45}H_{26}O_{21}$	303	901.2	499 299 601	902.68	Quercetin Trimer	(Campone et al. 2018)

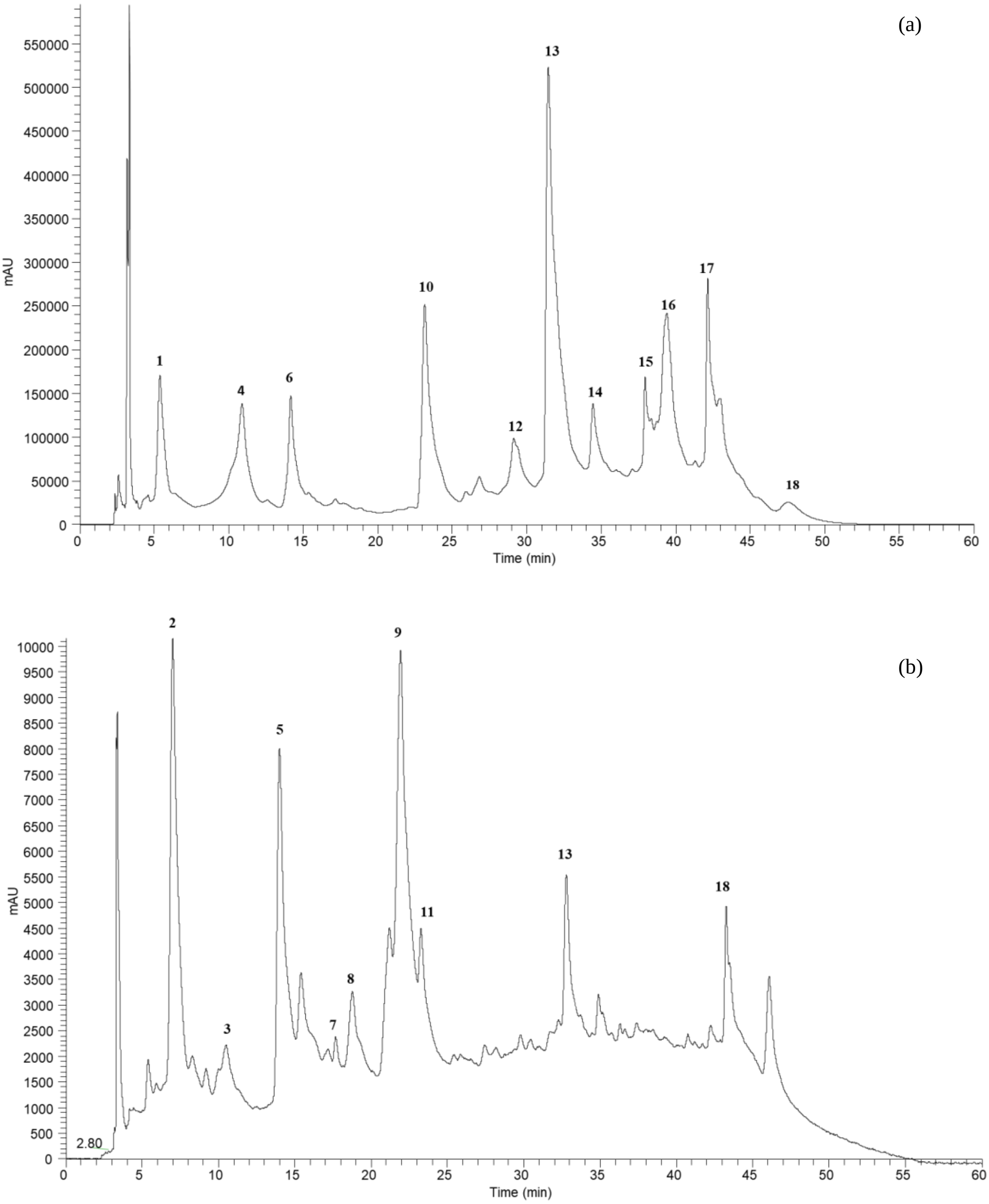


FIGURE 23: HPLC chromatograms of the phenolic compounds present in (a) an onion shell extract (recorded at 280 nm) and (b) an almond shell extract (recorded at 320 nm). LC-DAD-ESI/MS^n was used in this work to identify/quantify phenolic compounds in the plant extracts.

As an example, Figure 24, show the methodology used to identify quercetin (peak 13 in Figures 23(a) and (b)) with the aid of the Xcalibur^{TM} software. First, considering the total ion current (TIC) chromatogram (Figure 24 (a)) of the onion shell US extract, was selected the retention time of peak 13 (Rt=32 min), then through the PDA chromatogram (Figure 24 (b)), were identified the wavelengths of maximum absorption corresponding (λmax = 255 and 370 nm). For the selected retention time and considering the value of molecular ion [M-H]$^-$ at 301.22 m/z (Figure 24 (c)), the fragmentation peaks (MS^2), at 273, 257, 178 and 151 m/z (Figure 24 (d)) were considered. By comparison with literature it was possible conclude that the compound is correspondent to quercetin. The other phenolic compounds were identified considering the same criteria.

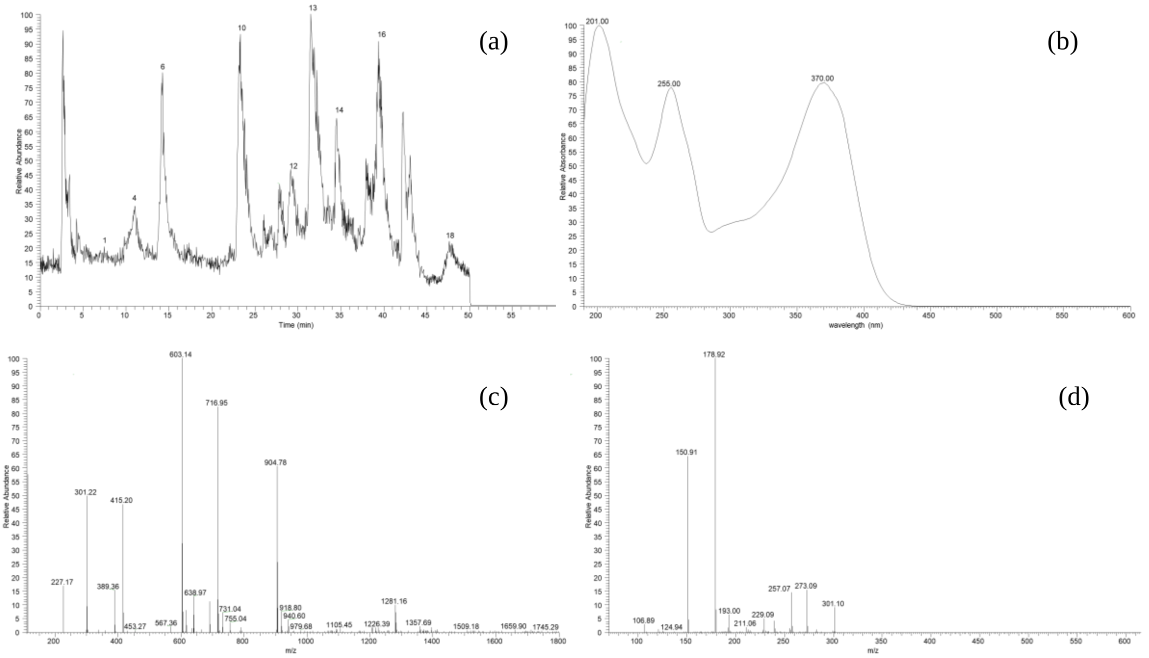


FIGURE 24: Results for the LC-DAD-ESI/MS^n analysis performed to identify quercetin in onion shell extract obtained by the ultrasounds technique. The total ion chromatogram (LC-TIC) (a), the photodiode array (PDA) signals at Rt= 32 min (b), molecular ion values [M-H]$^-$ at Rt= 32 min (c) and the fragmentation ions (MS^2) relative to m/z=301.22 (d) are here presented.

4.5. Efficiency of MIPs in Phenolic Compounds Retention and Recovery with Vegetables Extracts

After obtaining all the extracts, the almond shell sample resulting from supercritical CO_2 operation and all the extracts of the onion shell were selected to be used as target in the continuous adsorption process, and in the SPE analysis to evaluate the efficiency of the MIPs synthetized in the retention and recovery of the contained phenolic compounds.

4.5.1. Retention and Recovery of Polyphenols with Different Adsorbents

After proving in the previous sections that MIPs have a better polyphenol retention capacity than commercial adsorbents in a wither range of operation conditions (see previous results concerning the quercetin standard retention), further SPE experiments were performed in order to compare MIPs synthesized with the commercial DAX-8 resin. Now, the main goal was to evaluate the performance of the different materials with real plant extracts, which is of practical interest for application in many industries (e.g. food industry, pharmaceutics and cosmetics).

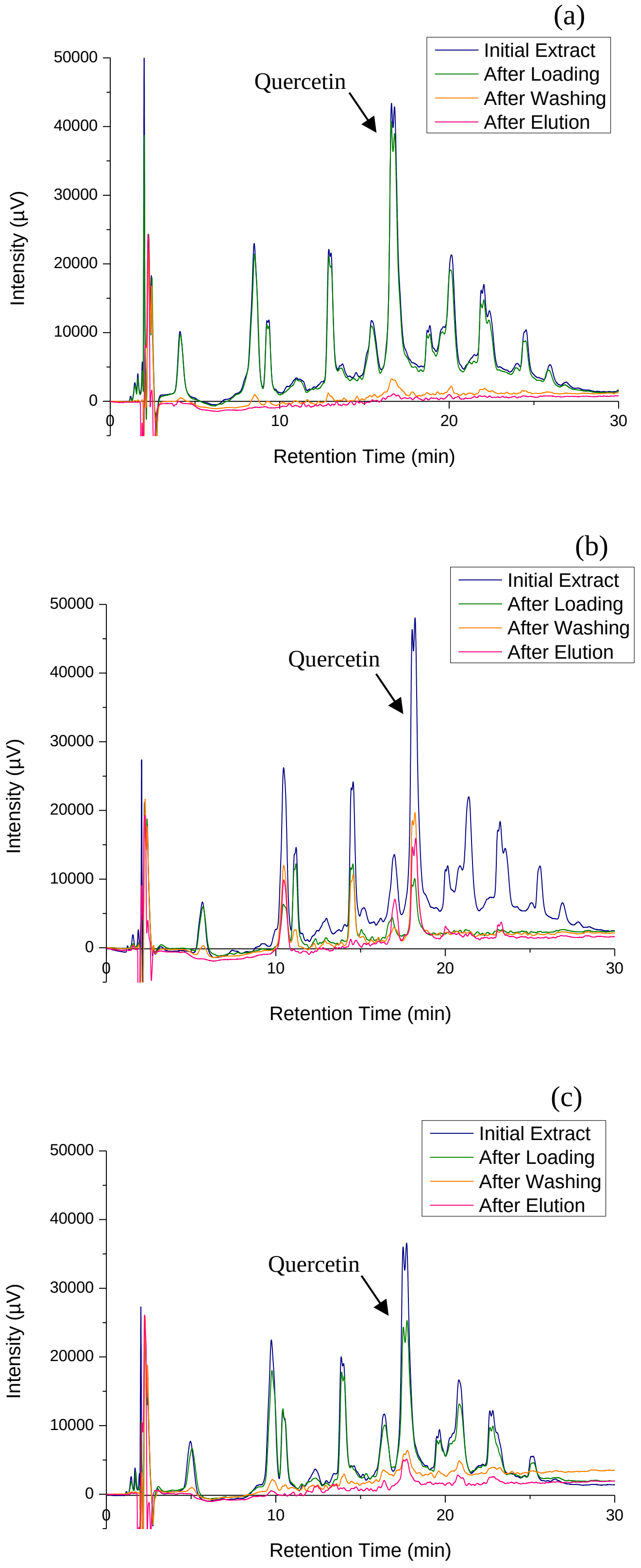


FIGURE 25: SPE assessment of (a) DAX-8 (b) MIP 3 and (c) MIP 4 concerning the phenolic compounds retention and recovery when plant extracts are used. Loading was performed using the onion shell extract obtained by ultrasound extraction in EtOH/H_2O 80/20, washing with EtOH/H_2O 80/20 and elution with MeOH/Acetic Acid 90/10 (UV analysis at 280 nm).

In Figure 25 it is possible to see that after the loading there was a high decrease in the phenolic peaks when using MIPs 3 and 4 as adsorbent materials, which means that there was a good retention of polyphenol in these imprinted polymer networks (MIP 3 presents a better performance than MIP 4, indicating the effect of morphology in the adsorption process). These plots also show that, after the washing and elution steps, there was recovery of some of the retained polyphenols. In opposite, the decrease in the quercetin peak with the use of DAX-8 as adsorbent material it is very small, indicating the low retention achieved with this material with the selected operation conditions (loading with EtOH/H_2O 80/20). As a consequence, after loading, was not possible a good recovery of the polyphenols. Therefore, these results once again prove that the MIPs allow to perform more flexible adsorption processes, namely with less impact of the hydrophobic interactions.

Indeed, results presented in Figure 25 are a main outcome of this research because prove that the produced MIP adsorbents are able to work with a diverse range of operation conditions. Previously, the good retention achieved with MIPs when working with solutions containing high water content was demonstrated (e.g. EtOH/H_2O 50/50 or even considering the operation with a higher amount of water). Now, the possibility to work also with low hydrophilic solutions (EtOH/H_2O 80/20) is also demonstrated for MIPs (even with real plant extracts) but not for DAX-8 (hydrophobic interactions are a main driving force for adsorption in this case).

Table 8 shows the percentage of quercetin retention in the cases described above, which was calculated by subtracting and normalizing the area of the involved peaks (note that, after identification of the peaks by LC-DAD_ESI/MS, individual or global phenolic compounds retention/recovery can be performed).

TABLE 8: Quercetin retention in DAX-8, MIP 3 and MIP 4 when a onion shell extract is processed with EtOH/H_2O 80/20 and using these materials as adsorbents.

Adsorbent Material	Extract	Solvent	Retention (%)
DAX-8	Onion Shell by Ultrasound	EtOH/H_2O 80/20	7,32
MIP 3	Onion Shell by Ultrasound	EtOH/H_2O 80/20	88,49
MIP 4	Onion Shell by Ultrasound	EtOH/H_2O 80/20	25,86

Note, however, as commented above, the MIP 4 presents an inferior performance comparatively to MIP 3 in polyphenol retention, highlighting the effect of the synthesis conditions on the materials adsorption efficiency.

4.5.2. Continuous Process Adsorption with MIP 4

The parameter Y_M considered in the molecular imprinting impacts on the morphology of the synthesized MIPs, which means that this parameter modifies at least the particle size of the materials. Efficiency of the molecular imprinting process (e.g. the formation of specific binding cavities) can also be affected by the change of Y_M. MIP 4 is the material produced with the highest value in this parameter, so it is the one that has larger particles. This synthesis was performed in this research to evaluate the impact on the use of the different materials in continuous process adsorption experiments. Indeed, some testing was performed with this material in order to try to decrease the backpressure problem that is observed with too small particles (e.g. when using MIPs 2 and 3).

The HPLC column was first packed with 250,6 mg of MIP 4 and, after column stabilization, in a typical continuous adsorption experiment, a flow rate of 1 mL/min of plant extract (e.g. the extract of the onion shell obtained with the ultrasound technique in EtOH/H_2O 80/20) was feed to the system up to the saturation point of the material in the column. A typical saturation profile is presented in Figure 26, showing the operation up to 190 mL.

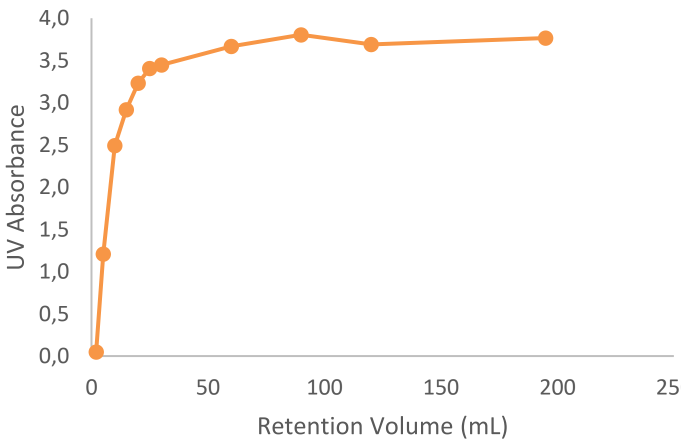


FIGURE 26: Saturation curve obtained in a HPLC column packed with MIP 4 when processing directly an onion shell extract (obtained by ultrasound extraction in EtOH/H_2O 80/20). The UV absorbance presented in the plot is correspondent to the analysis of the fluid collected at column outlet.

After saturation, the release of the phenolic compounds was performed using a kind of solvent gradient process. The composition of the solvents used with this goal was previously presented in Table 2. In Figure 27 is presented the HPLC-DAD analysis of initial onion shell extract, along with the different fractions collected. These results show the ability to obtain quercetin–rich fractions (namely fractions 4 to 6) with the implementation of this continuous adsorption process.

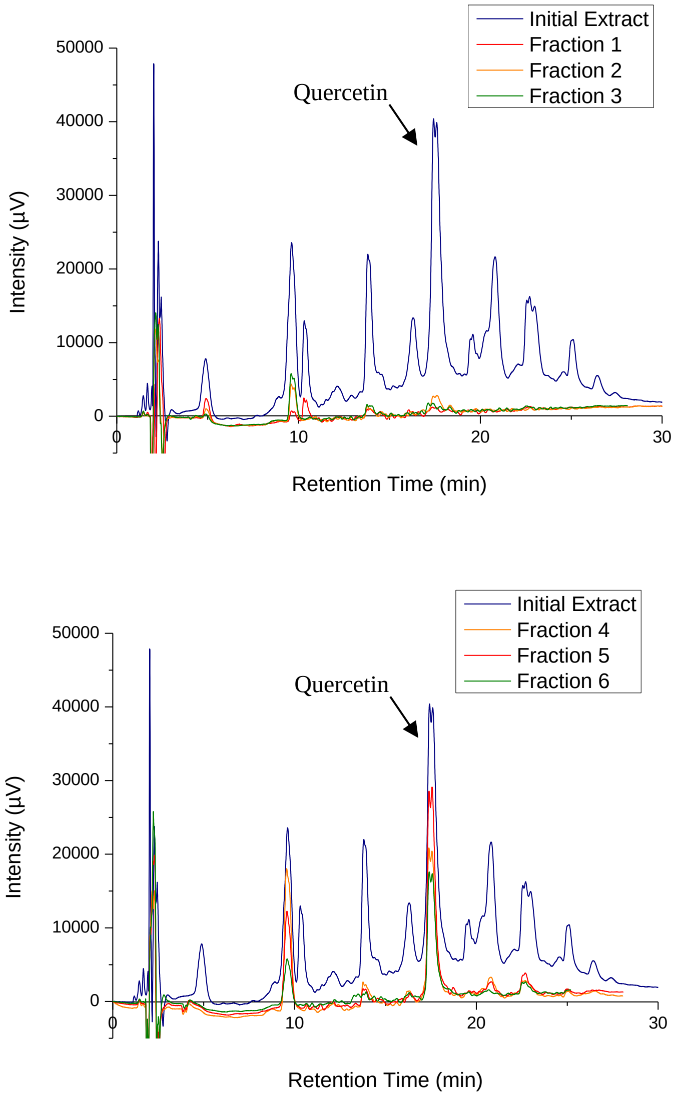


FIGURE 27: HPLC-DAD chromatograms of the onion shell extract (initial extract) obtained by US and the fractions (Table 2) recovered after the saturation of a HPLC column containing MIP 4 (UV analysis at 280 nm).

Similar results are presented in Figures 28 and 29 but considering an onion shell extract obtained by the soxhlet technique in EtOH/H_2O 80/20 (in this case the saturation of the column was reached in 150 mL).

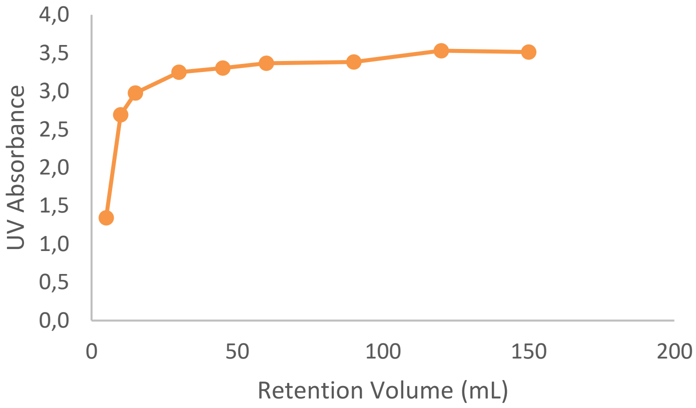


FIGURE 28: Saturation curve obtained in a HPLC column packed with MIP 4 when processing directly an onion shell extract (obtained by soxhlet extraction in EtOH/H_2O 80/20). The UV absorbance presented in the plot is correspondent to the analysis of the fluid collected at column outlet.

Results presented in Figure 29 confirm again the usefulness of the MIP material and of the adsorption process considered to deal with the onion shell extract and produce fractions enriched with polyphenols, namely quercetin-enriched fractions. The desorption conditions used in this case are presented in the legend of Figure 29.

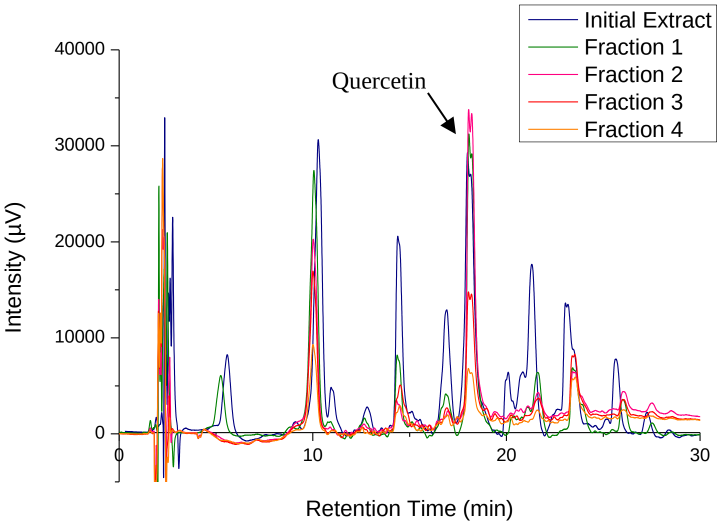


FIGURE 29: HPLC-DAD chromatograms of the onion shell extract (initial extract) obtained by soxhlet and of the recovered fractions after the saturation in MIP 4. With the fraction 1 was used 5 mL EtOH/H_2O 80/20 as solvent and the fractions 2, 3, 4 were obtained using 10, 5 and 5 mL, respectively of MeOH/Acetic Acid (90/10) as eluent solvent (UV analysis at 280 nm).

Therefore, with the modification of the size of the particles of the MIP, it was possible to operate the continuous process in stable conditions. Since this procedure is similar to an industrial scale adsorption process, the use of MIPs as adsorbents has an industrial interest in promoting selective and efficient adsorption without the need for large amounts of water in the solvent.

However, due to the different retention capacities above reported for MIP 4 and MIP 3, new experiments were performed with the solid phase extraction technique. The re-evaluation of MIP 3 with real plant extracts was thus performed. The next section presents the results obtained in this context.

4.5.3. Solid Phase Extraction Experiments with MIP 3 and Plant Extracts

The following results were obtained with experiments performed in SPE aiming at the evaluation of MIP 3 with real plant extracts. An almond shell extract obtained with $SCCO_2$ operation (see Figure 30 bellow) was considered in this context. Moreover, onion shell extracts obtained with the SCCO2 technique, soxhlet and ultrasounds extraction were also processed in a similar way (see results thus obtained in Figures 31 to 34). Results present in these Figures highlight again the ability of the material to retain phenolic compounds contained in the different plant extracts used. Note again that a low hydrophilic solvent was here used, and a similar outcome is not possible with conventional adsorbents like DAX-8.

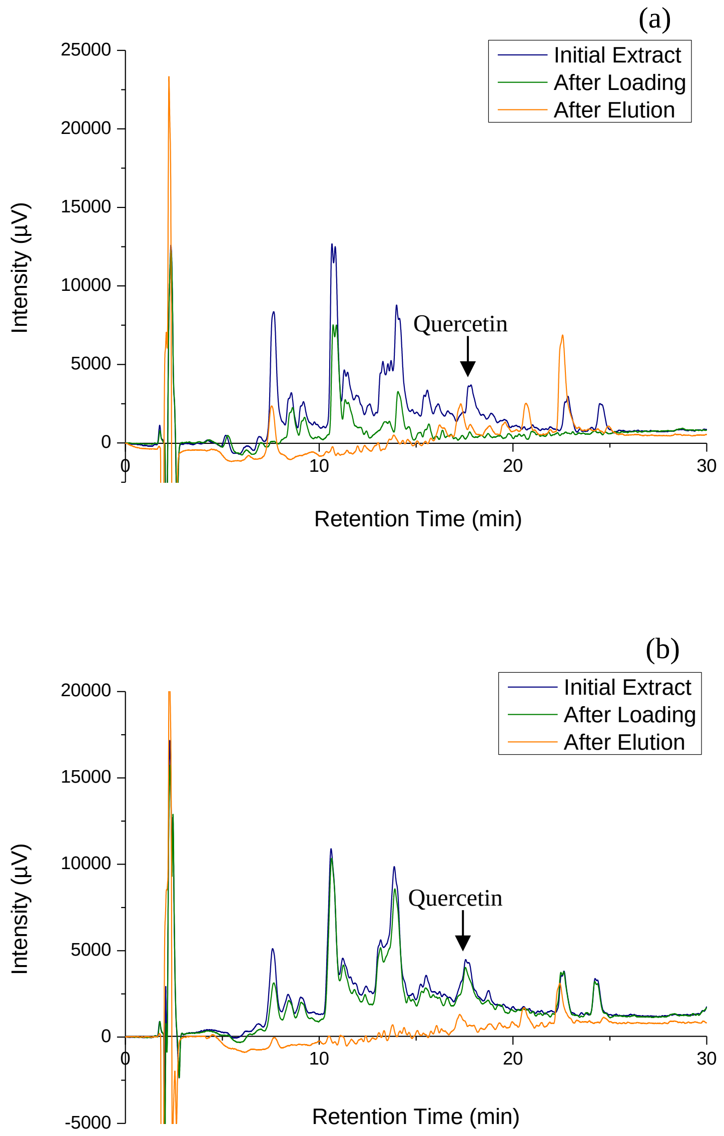


FIGURE 30: HPLC-DAD chromatograms of the almond shell extract (initial extract) obtained with SCCO2, fraction recovered after their retention in MIP 3 (loading) and fraction eluted. As loading solvent was used EtOH/H_2O with two different proportions (a) 70/30 and (b) 80/20. In elution step was used MeOH/Acetic Acid (90/10).

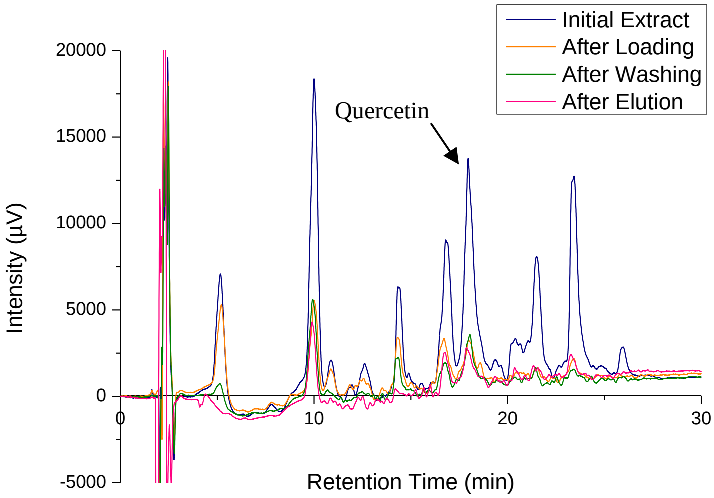


FIGURE 31: HPLC-DAD chromatograms of the onion shell extract (initial extract) obtained by SCCO2, fraction recovered after their retention in MIP 3 (loading) and fraction eluted. As loading solvent was used EtOH/H_2O 80/20. In elution step was used MeOH/Acetic Acid (90/10).

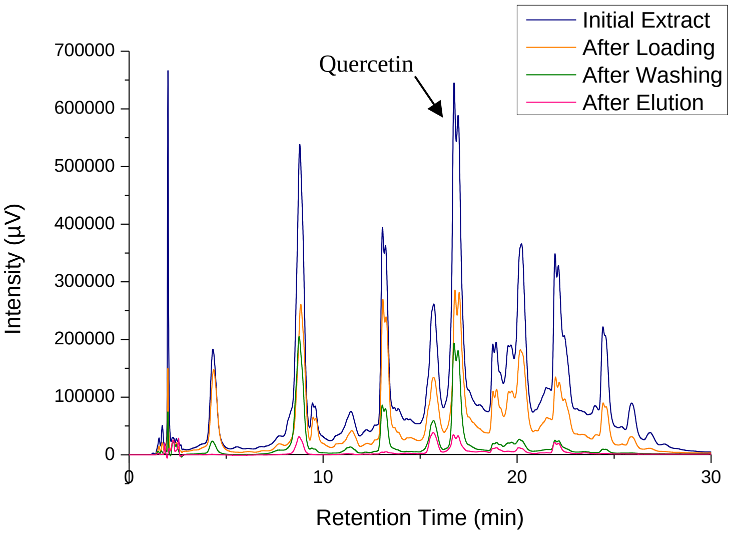


FIGURE 32: HPLC-DAD chromatograms of the onion shell extract (initial extract) obtained by soxhlet, fraction recovered after their retention in MIP 3 (loading) and fraction eluted. As loading solvent was used EtOH/H_2O 80/20. In elution step was used MeOH/Acetic Acid (90/10).

Note also that the experiment correspondent to Figure 33 was carried out by increasing the mass of the adsorbent material from 50 to 500 mg in order to try a very high retention of the phenolic compounds. Thus, the material was saturated using 200 mL of the onion shell extract obtained by ultrasound extraction (a bigger extract volume was thus processed in this conditions).

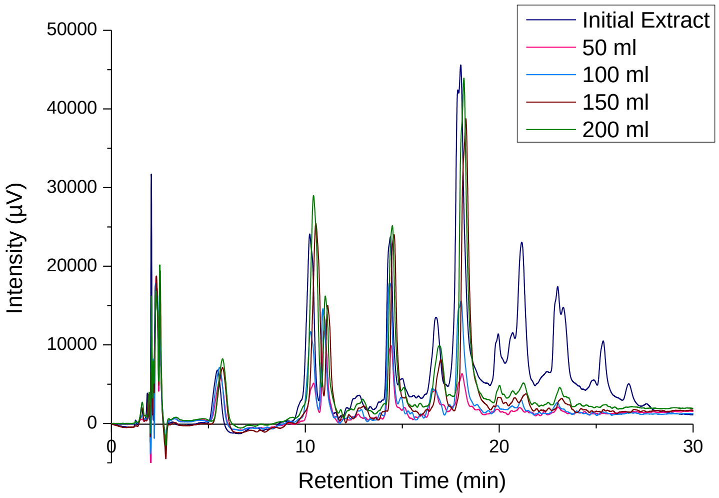


FIGURE 33: HPLC-DAD chromatograms of the onion shell extract (initial extract) obtained by ultrasounds and the fractions correspondent to the saturation of MIP 3 with different extract volumes (from 50 to 200 mL).

After saturation of the MIP material, as presented in Figure 33, different recovery steps were performed by collecting successive fractions. In these release fractions, three solvents were used, starting from a hydrophilic solvent to a more hydrophobic solvent. In the first stage of the release, ultrapure water was used as solvent, then the solvent used was pure ethanol, and finally, the last step, was carried out using pure methanol as solvent (potentially with higher elution strength).

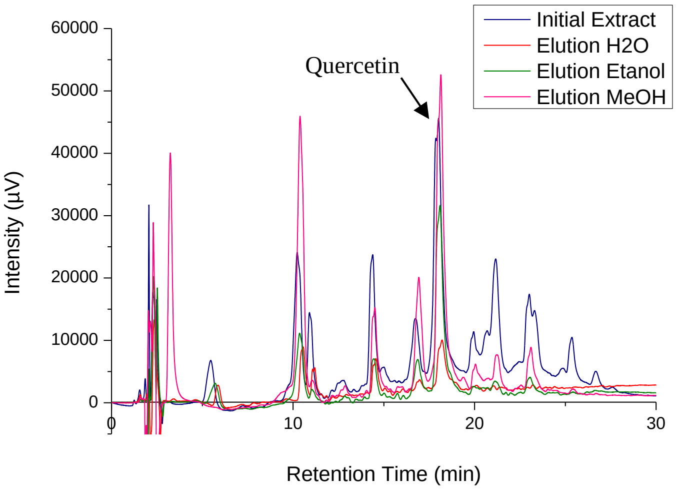


FIGURE 34: HPLC-DAD chromatograms of the onion shell extract (initial extract) obtained by ultrasounds and fractions recovered after the saturation of MIP 3 with this plant extract. Different recovery steps were performed using ultrapure water, ethanol and methanol.

With the last simplified fraction obtained after release (in pure methanol), the solvent contained in the fraction was evaporated to give a dry residue, which was again dissolved in EtOH/H_2O 80/20. This final solution was analysed in LC-DAD-ESI/MS. Figure 35 shows the chromatogram of the phenolic compounds present as a result of this analysis.

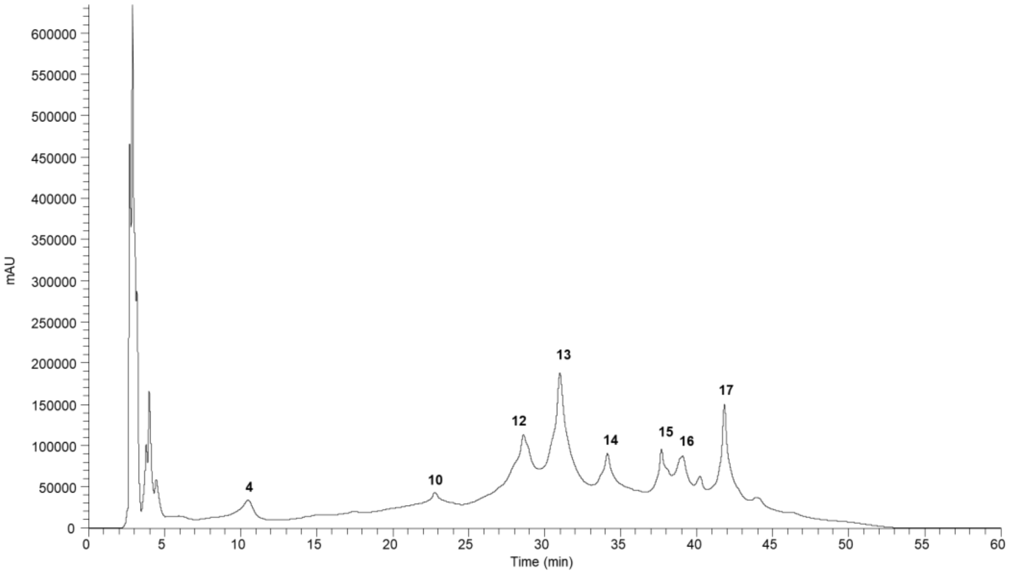


FIGURE 35: A HPLC chromatograms of the phenolic compounds present in the final fraction obtained after SPE saturation of MIP 3 with a onion shell extract. A concentration of C=1 mg of dry residue/mL was used in the re-dissolved sample (UV analysis recorded at 280 nm).

Results presented in Figure 35 (see also Table 7) allows to conclude that the residue resulting from the adsorption/desorption process has a rich phenolic profile, mainly with the presence of quercetin and quercetin derivatives.

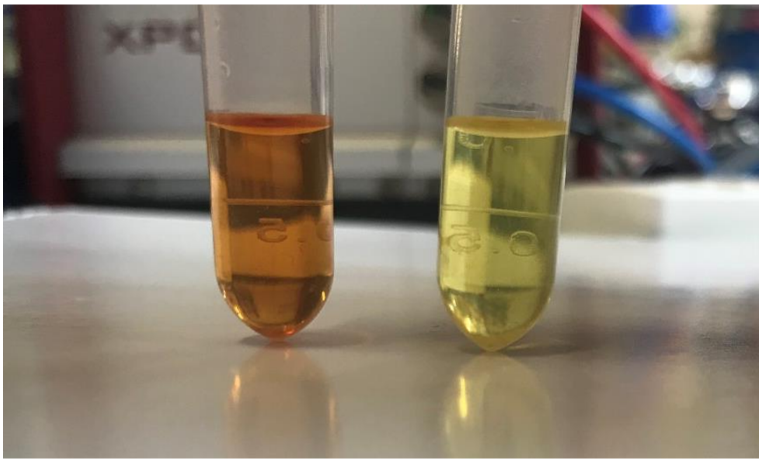


FIGURE 36: On the left, onion peel extract by US, and on the right, final fraction in EtOH/H_2O 80/20.

In Figure 36 is presented the visual comparison between the initial onion shell extract (left side of the figure) and the solution obtained after re-dissolution of the solid residue resulting from the above described adsorption/desorption process. A clear colour changing, and the yellow colour of the solution obtained after the extract processing is characteristic of the presence of quercetin (and quercetin derivatives). Thus, these final results prove again the usefulness of the developed MIP adsorbents and the associated adsorption/desorption processes in the valorisation if plant residues, namely to obtain phenolic-rich products with application in food industries, pharmaceutics or cosmetics.

Chapter 5

5.1. Conclusions

In this study, different kinds of vegetable residues abundant in Trás-os-Montes region, namely walnut leaf, walnut shell, almond shell, grape marc, olive leaf and onion shell were used as sources of polyphenols with potential economic valorization. Extraction with supercritical carbon dioxide (namely using as reference conditions T=40 °C, P=160 bar and ethanol as co-solvent), ultrasound extraction and soxhlet extraction were used to obtain mixtures containing these bioactive compounds that are potentially useful in many application fields (e.g. food industries, pharmaceutics, cosmetics). With the supercritical CO_2 ($SCCO_2$) extraction conditions used, the richest phenolic profiles were obtained when onion shell and almond shell residues were considered. The onion shell extract obtained with supercritical CO_2 was compared with the ultrasound and soxhlet extracts. However, despite a similar composition, a higher phenolic content was measured for extracts obtained with the ultrasounds (US) or the soxhlet (SHOX) techniques, comparatively to supercritical CO_2 extraction (e.g. around 10 mg/g of total phenolic compounds with US and SHOX extraction and 1 mg/g with $SCCO_2$ for onion shell extracts, in a dry basis of plant residue).

The identification and quantification of polyphenols was performed using Liquid Chromatography with Mass Spectroscopy and Diode Array Detector (LC-MS-DAD) and also with the more straightforward HPLC-DAD technique. As result of these analysis, the quercetin and many quercetin analogues (e.g. quercetin-*O*-glucoside, quercetin-*O*-diglycoside, quercetin dimer-*O*-hexoside, etc) were identified in the onion shell extracts, while quercetin, isorhamnetin-3-*O*-rutinoside or catechin were identified in the almond shell extracts.

Aiming at the subsequent separation and concentration of the polyphenols from the complex extracts obtained, molecularly imprinted polymers (MIPs) were used as a kind of engineered adsorbents materials. Comparative studies with commercial polymeric adsorbents, namely with the resin DAX-8 were also performed in this work. With the adsorption techniques considered (batch adsorption, solid phase extraction (SPE) and continuous adsorption), that were here used in the uptake/release studies, was proved that the MIP particles, which were synthesized by precipitation polymerization with 4-VP as functional monomer and quercetin as template, had a very high polyphenol retention, even when solvents with low water content are used (e.g. ethanol/water 80/20), namely when compared with the commercial polymeric adsorbent, the DAX-8, which polyphenol adsorption mechanism is mostly based on the hydrophobic interactions.

The distinctive features of the MIP adsorbents were demonstrated in this work through the direct processing of plant extracts without water addition. It was proved that the hydrophobic interactions are not the unique mechanism that allows the retention of these bioactive compounds in the synthesized molecularly imprinted polymer networks. The functionalization of the materials, with pyridyl functional groups, and the creation of imprinted tailor-made cavities were congenial for the improvement performance of the MIPs. Additionally, the manipulation of the particle's morphology allowed a facile binding accessibility and contributed for the observed superior performance of the MIPs in polyphenols retention.

A polyphenol-enriched fraction, containing quercetin and analogue molecules, was recovered at the end with the minimization of thermal treatments, and only alcohol evaporation was needed in order to obtain a dry residue. Thus, the simplification of the adsorption/desorption process was achieved, and as consequence the energetic costs can also be cut down. Likewise, the minimization of the possible thermal degradation of the bioactive compounds is another potential benefit of the adsorption/desorption process explored in this work. Considering the results obtained, this research demonstrate that MIP adsorbents can be helpful in the design of new and more flexible adsorption processes, aiming at the valorization of polyphenols present in plant extracts.

5.2. Limitations and Future works

The low efficiency of the supercritical CO_2 polyphenol extraction observed in this research, namely when compared with ultrasounds and soxhlet extraction, is one of the shortcomings of this work. Thus, new operation conditions with $SCCO_2$ should be considered in order to try the tackle of this issue. Further studies with other supercritical P-T combinations and other amounts/types of co-solvents should be performed with the different plant residues here addressed. Eventually, very different operation conditions need to be selected with the diverse plant residues used (walnut leaf, walnut shell, almond shell, grape marc, olive leaf and onion shell). Indeed, the solubility and migration rate of different polyphenols present in the diverse residues in the $SCCO_2$/co-solvent should be addressed with dissimilar operation conditions.

The recovery process of the polyphenols after their adsorption in MIPs should also be enhanced in future work. The precise quantification of the fraction recovered and the development of conditions to maximize the recovery efficiency should be worked out. The design of solvent gradient processes and/or the operation with trains of different MIPs are some possibilities to be considered in future researches.

Another important issue to be considered with the use of MIPs in adsorption processes is concerned with their reusability (number of cycles of adsorption/desorption without significant loss of efficiency). This problem is connected with the aggressive operation conditions eventually used (e.g. high temperatures and/or inadequate pH limits). It is known that the ester linkage of the crosslinker considered in the synthesis of the MIPs used in this work (EGDMA) is prone to hydrolysis in strong acidic or alkaline conditions (acid or base catalysed ester hydrolysis). Thus, the synthesis and evaluation of similar MIPs with a different crosslinker (e.g. divinylbenzene) should also be considered in future works

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Appendix

Appendix I

I.1. Extraction Profiles Observed During the Operation with Supercritical CO_2.

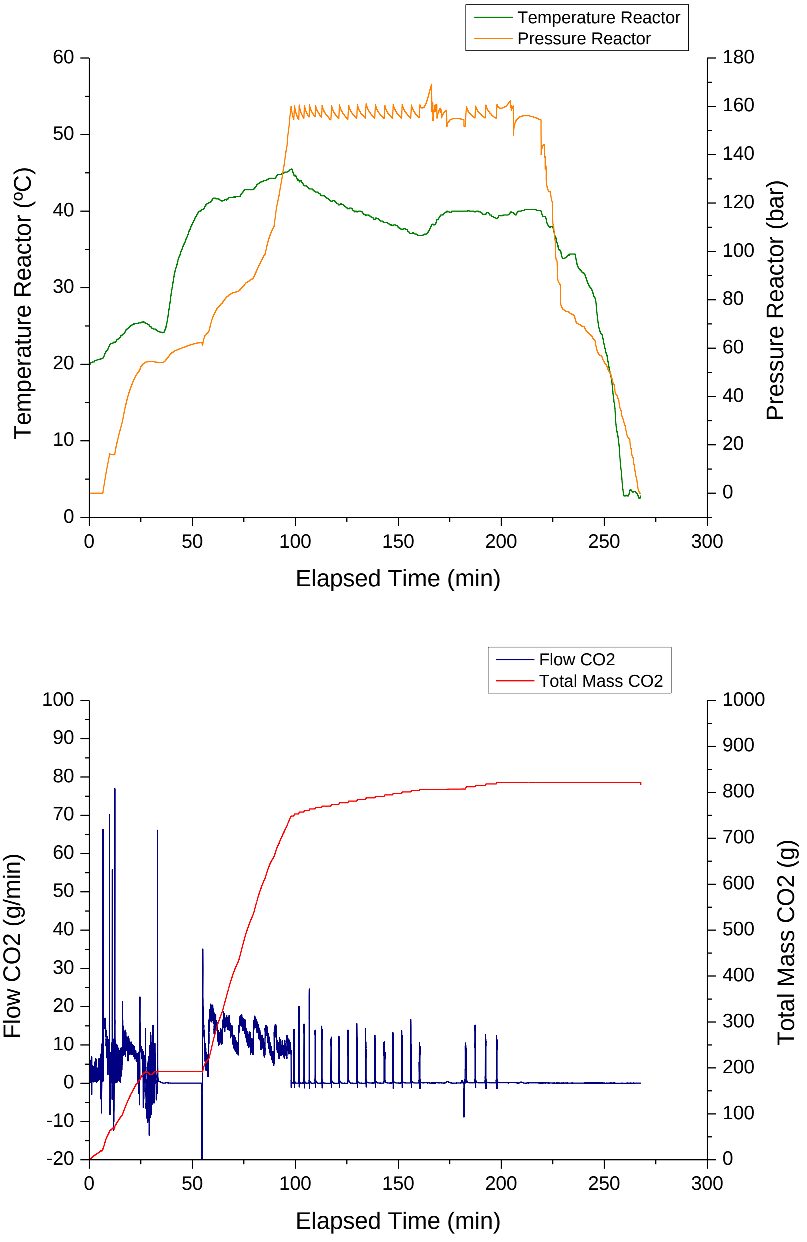

FIGURE 37: Profile of (a) temperature and pressure and (b) flow and total mass of CO_2 correspondent to the almond shell extraction with supercritical CO_2 at conditions close to T = 40 ºC and P = 160 bar with 5% ethanol as co-solvent.

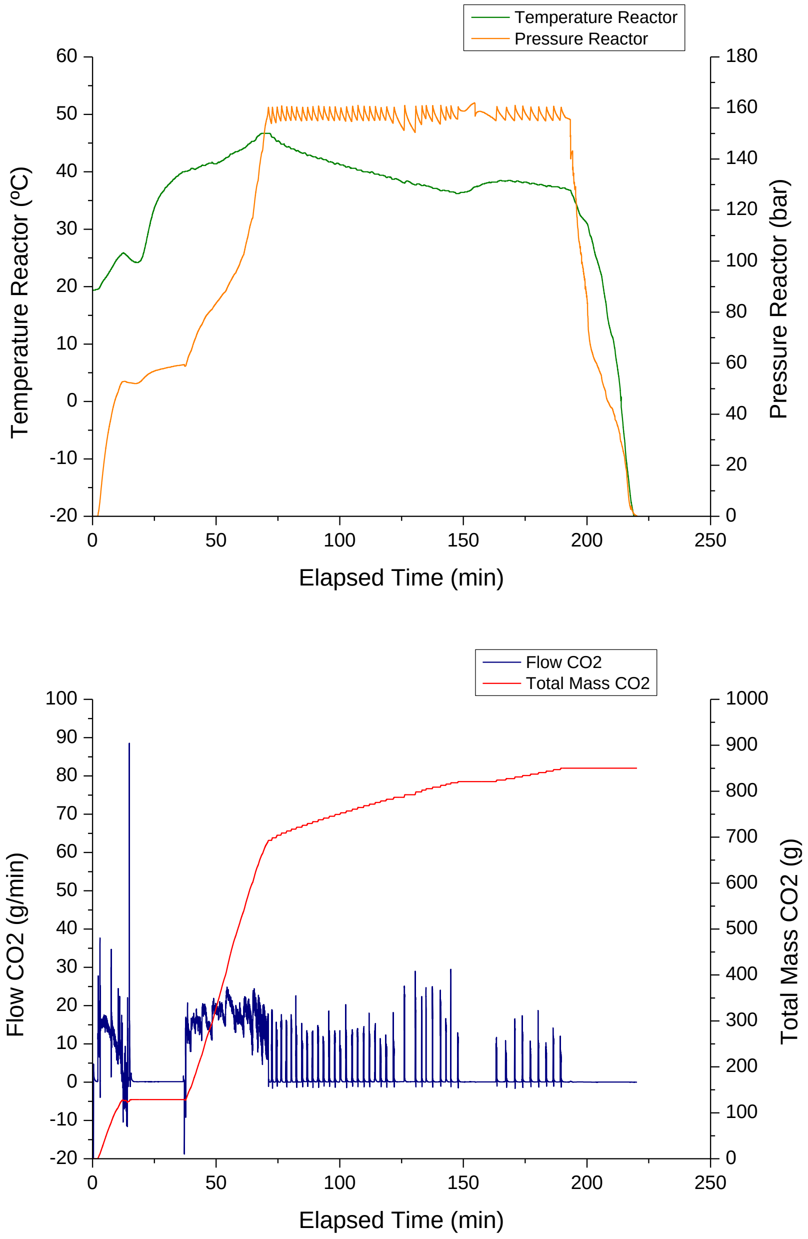

FIGURE 38: Profile of (a) temperature and pressure and (b) flow and total mass of CO_2 correspondent to the olive leaf extraction with supercritical CO_2 at conditions close to T = 40 ºC and P = 160 bar with 5% ethanol as co-solvent.

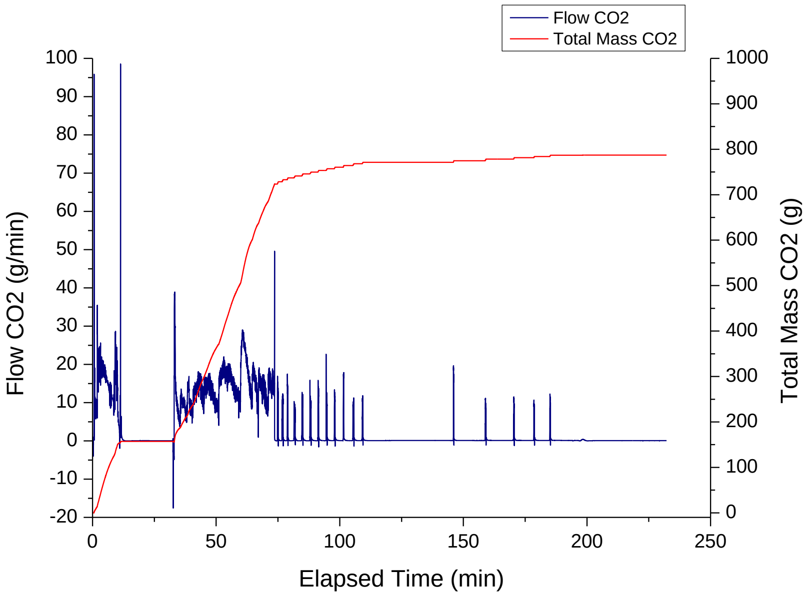

FIGURE 39: Profile of flow and total mass of CO_2 correspondent to the onion shell extraction with supercritical CO_2 at conditions close to T = 40 ºC and P = 160 bar with 5% ethanol as co-solvent.

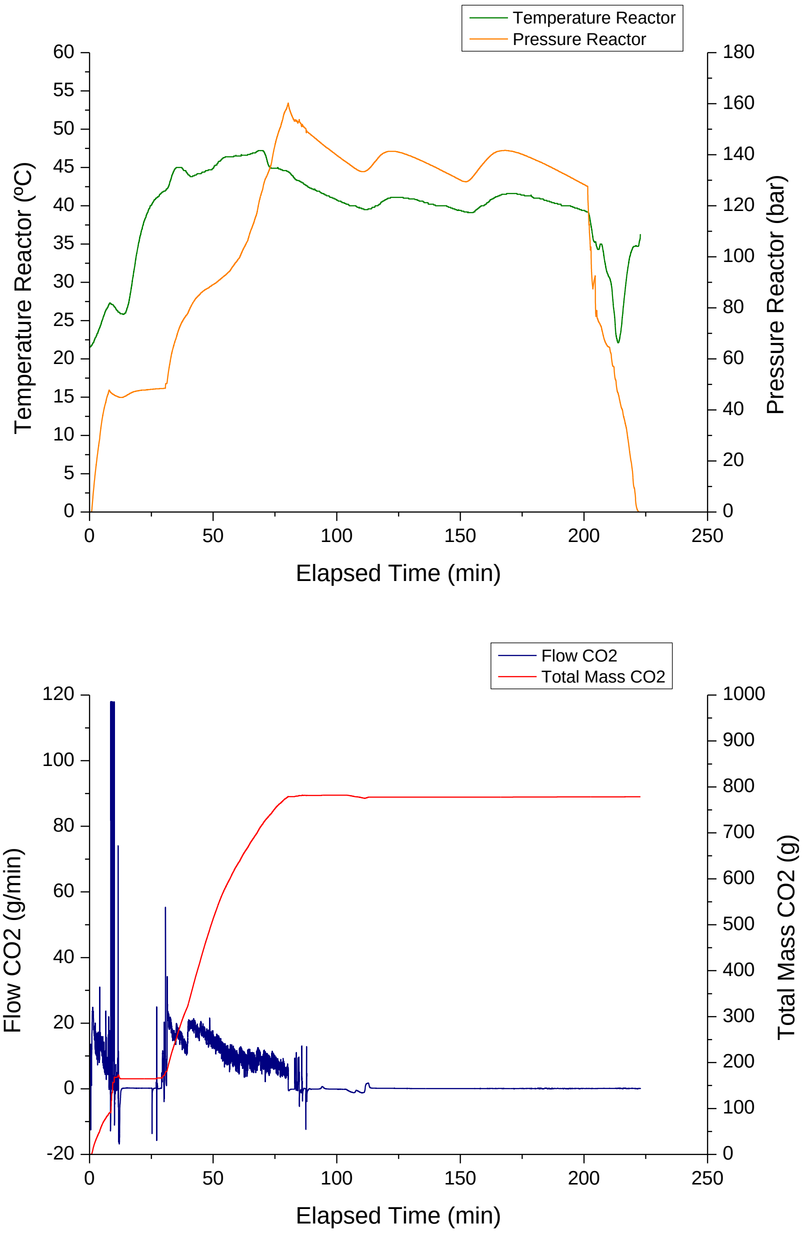

FIGURE 40: Profile of (a) temperature and pressure and (b) flow and total mass of CO_2 correspondent to the onion shell extraction with supercritical CO_2 at conditions close to T = 40 ºC and P = 160 bar with 10% ethanol as co-solvent.

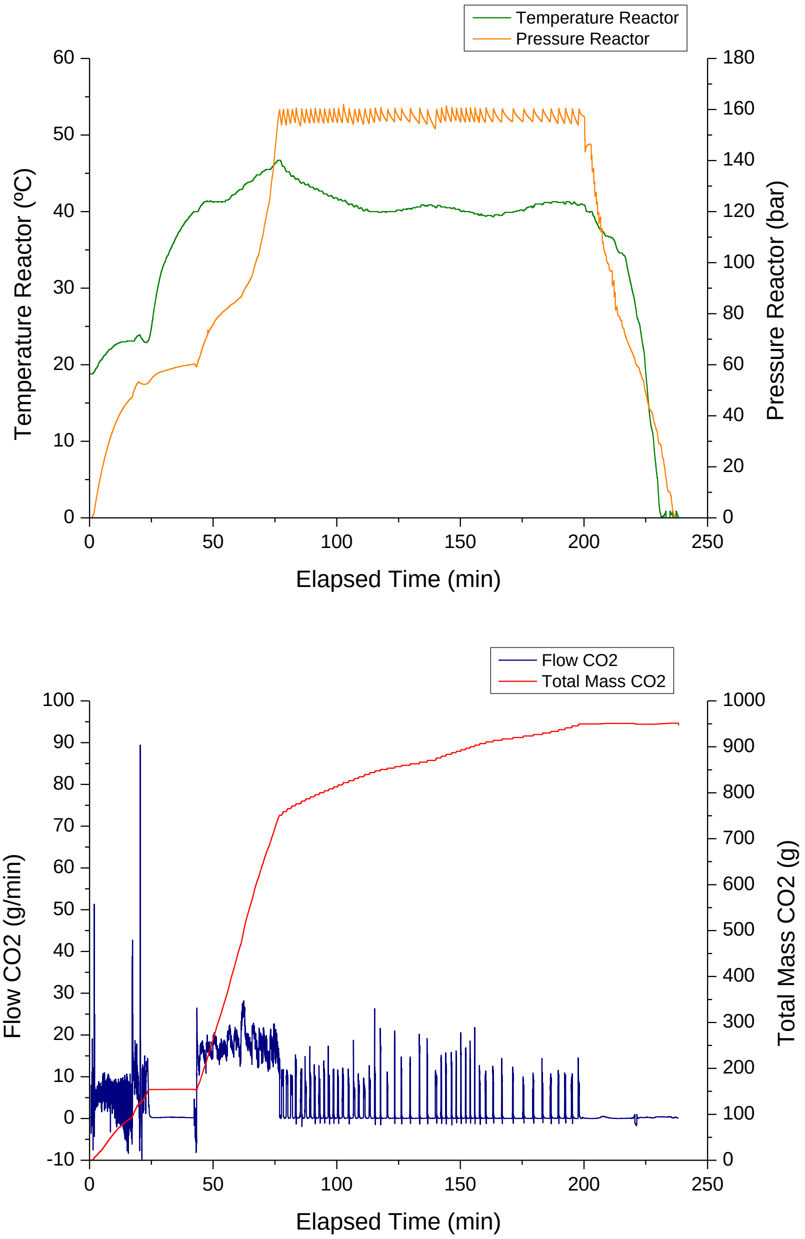

FIGURE 41: Profile of (a) temperature and pressure and (b) flow and total mass of CO2 correspondent to the walnut shell extraction with supercritical CO2 at conditions close to T = 40 ºC and P = 160 bar with 5% ethanol as co-solvent.