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Tailored Polymer Networks for Purification of Polyphenols in Almond Subproducts

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Abstract

The valorization of agro-industrial by-products has aroused great interest nowadays, due to their richness in phenolic compounds, including polyphenols, and their biological activities of high value for various areas. In this work, the recovery and concentration of these compounds, from hydroalcoholic extracts from hull and also blanching water, were studied, being two residues of large volume in the almond industry. The enrichment is given by sorption/desorption processes in columns packed with Molecularly Imprinted Polymers (MIPs), having used the polymers MIP-OA-3, MIP-QUER-2 and MIP-QUER-3, and evaluating the enrichment efficiency against a gradient of several solvents in the elution steps.

The hull extracts were obtained essentially by ultrasound, having worked with a hydroethanolic fraction 50/50 (v/v), due to their yield and equivalent favoring in the extraction of different polyphenolic families, being subsequently submitted to the sorption process. The characterization of the elutions of both by-products was mainly done by determining the total phenolic content (TPC) and the antioxidant activity by the DPPH method (EC50), and the best results were reinforced with chromatographic analyses by HPLC-DAD at 280 and 360 nm.

The results show that the process allowed the selective fractionation and enrichment of phenolic compounds, observing fractions with a high degree of enrichment when compared to the initial extract, both at the TPC and EC50 levels. The intermediate hydroethanolic fractions, as well as the methanolic fractions, present the best results in terms of enrichment, as evidenced by the inverse relationship between TPC and EC50. It was also possible to verify differences in the enrichment trend between the OA3 column and the Q2 Q3 columns, revealing different affinities provided by the polymers. Chromatograms reveal significant changes in the chemical profile, both between fractions, extract and columns, suggesting and proving the enrichment of certain phenolic compounds, particularly flavonoids.

In general, the results obtained indicate that the MIPs used have a high potential for phenolic enrichment from complex extracts, being able to fractionate and enrich, contributing to strategies for sustainable recovery of waste and development of processes

aligned with the principles of green chemistry. Among these results, maximum TPC values of 615.66 ± 28.23 mg GAE/g Extract (hull) and 918.32 ± 34.54 mg GAE/g Extract (blanching water) were observed, together with EC50 values of 0.025 ± 0.000 and 0.028 ± 0.002 mg/mL, respectively. A value of 1135.64 ± 11.55 mg GAE/g Extract and 0.017 ± 0.000 mg/mL was also observed, indicating a high phenolic recovery.

Keywords: Molecularly imprinted polymers; Polyphenols; Almond subproducts; Selective sorption; Antioxidant activity; Total phenolic content; Circular bioeconomy.

Resumo

A valorização de subprodutos agroindustriais tem despertado grande interesse nos dias de hoje, face à sua riqueza em compostos fenólicos, entre eles os polifenóis, e as suas atividades biológicas de alto valor para diversas áreas. Neste trabalho foi estudada a recuperação e concentração destes compostos, provenientes de extratos hidroalcoólicos do cascarão e também a água de branqueamento, sendo dois resíduos de grande volume na indústria da amêndoa. O enriquecimento é dado por processos de sorção/dessorção em colunas empacotadas com Polímeros Molecularmente Impressos (MIPs), tendo sido utilizado os polímeros MIP-OA-3, MIP-QUER-2 e MIP-QUER-3, e avaliando a eficiência de enriquecimento face a um gradiente de diversos solventes nas etapas de eluição.

Os extratos de cascarão foram obtidos essencialmente por ultrassons, tendo se trabalhado com uma fração hidroetanólica 50/50 (v/v), face ao seu rendimento e favorecimento equivalente na extração de diferentes famílias polifenólicas, sendo posteriormente submetido ao processo de sorção. A caracterização, das eluições de ambos os subprodutos, foi feita maioritariamente pela determinação do conteúdo fenólico total (TPC) e a atividade antioxidante pelo método DPPH (EC50), sendo reforçados os melhores resultados com análises cromatográficas por HPLC-DAD a 280 e 360 nm.

Os resultados demonstram que o processo permitiu o fracionamento seletivo e enriquecimento dos compostos fenólicos, observando-se frações com alto grau de enriquecimento quando comparadas com o extrato inicial, tanto a nível de TPC e EC50. As frações hidroetanólicas intermédias, assim como as frações metanólicas, apresentam os melhores resultados em nível de enriquecimento, sendo evidenciado pela relação inversa entre TPC e EC50. Também foi possível verificar diferenças na tendência do enriquecimento entre a coluna OA3 e as colunas Q2 Q3, revelando diferentes afinidades proporcionados pelos polímeros. Os cromatogramas revelam alterações significativas no perfil químico, tanto entre as frações, extrato e colunas, sugerindo e comprovando o enriquecimento de determinados compostos fenólicos, particularmente flavonoides.

De forma geral, os resultados obtidos indicam que os MIPs utilizados apresentam um alto potencial de enriquecimento fenólico partindo de extratos complexos,

conseguindo fracionar e enriquecer, contribuindo para estratégias de valorização sustentável de resíduos e desenvolvimento de processos alinhados com os princípios da química verde. Dentre estes resultados, foram observados valores máximos de TPC de 615,66 ± 28,23 mg GAE/g Extrato (cascarão) e 918,32 ± 34,54 mg GAE/g Extrato (água de branqueamento) juntamente de valores de EC50 de 0,025 ± 0,000 e 0,028 ± 0,002 mg/mL, respetivamente. Também ainda foi observado valores de 1135,64 ± 11,55 mg GAE/g Extrato e 0,017 ± 0,000 mg/mL, indicando uma alta recuperação fenólica.

Palavras-chave: Polímeros molecularmente impressos; Polifenóis; Subprodutos da amêndoa; Sorção seletiva; Atividade antioxidante; Conteúdo total fenólico; Economia biocircular.

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List of abbreviations

4VP – 4-Vinylpyridine

AA – Antioxidant Activity

ACN – Acetonitrile

AcOH – Acetic acid

DMAEMA – 2-(Dimethylamino)ethyl methacrylate

DMSO – Dimethyl sulfoxide

DPPH – 2,2-Diphenyl-1-picrylhydrazyl

EC50 – Half Maximal Effective Concentration

EDF50 – Half Maximal Effective Dilution Factor

EtOAc – Ethyl acetate

EtOH – Ethanol

HPLC – High-Performance Liquid Chromatography

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

LCA – Life Cycle Assessment

MeOH – Methanol

MIP – Molecularly Imprinted Polymer

OA3 – MIP-OA-3

Q2 – MIP-QUER-2

Q3 – MIP-QUER-3

SD – Standard Deviation

SDG – Sustainable Development Goal

SPE – Solid-Phase Extraction

TPC – Total Phenolic Content

UAE – Ultrasound-Assisted Extraction

UV – Ultraviolet

UV-Vis – Ultraviolet–Visible

1. Introduction

The almond tree (*Prunus dulcis*) over the generations has demonstrated great economic, social and cultural relevance, and this value is reflected in the consumption and production of almonds, as well as in the mass production of organic waste, from agricultural and industrial origin [1–5]. This production is a consequence of one of the biggest challenges in the agricultural sector, suppressing customer demand, giving rise to a second challenge, the reduction of environmental impact by crop treatments and waste [6–8].

Estimated at a hundred euros, organic waste tends to have a low economic value, and its treatment is often neglected by farmers due to the lack of buyers, making the labor used for the collection and treatment of waste from the fields monetarily unviable, which results in significant environmental problems [6,7,9,10]. In this way, the valorization of biomass becomes fundamental nowadays, allowing the use of devalued waste and obtaining from it a product of greater economic value, allowing a circular economy. [4,9].

The recovery of almond waste and industrial derivatives focuses mainly on the existence of bioactive compounds, specifically polyphenols [4,11–17]. This family of value-added compounds is known to have antioxidant, anti-inflammatory, antimicrobial and insecticidal properties, which reflects a high interest in the areas of health, cosmetics, food and agriculture [4,11–17]. However, polyphenols are found in complex matrices with low concentration, making it essential to select efficient extraction methods and selective separations, enabling an economically viable and sustainable recovery [4,9,18].

Among the existing techniques, especially in separation, the sorption and desorption processes using engineered polymer networks, in particular molecularly imprinted polymers (MIPs), have gained prominence in the area [18–28]. These molded materials allow a high molecular selectivity, enabling the fractionation and enrichment of certain families and compounds, and all at a low energy cost, operating at room temperature, also preserving thermosensitive compounds [18,20,29]. Thus, the application of MIPs becomes a promising strategy in valorization, bringing flexibility in energy and selective terms [29].

Thus, in this work, hydroethanolic extractions of shell and hull will be carried out, being later enriched in columns packaged with MIPs, as well as the blanching water. This enrichment will be given in the elutions phase, where they will be characterized at the level of TPC, EC50 and HPLC-DAD, presenting only the chromatograms of the best fractions.

2. Dissertation structure

This dissertation is essentially divided into 13 topics, the first being the introduction made previously and the second this section of the structure.

In the third topic, a contextualization of this work is made, introducing the almond and its global production, the by-products addressed (hull, Shell and blanching water) and the problematizations that its large production entails, resulting in the motivation behind this dissertation.

In the fourth topic, what polyphenols are are explained and the families present in the by-products used are elaborated. Phenolic characterization is also carried out.

In the fifth topic, the extraction methods used (ultrasound, soxhlet and adsorption) are addressed, elaborating the method of operation of each one.

In the sixth topic, tailored polymer networks are introduced, specifically MIPs, where it is explained what compounds are, how they work, production methods and advantages in application when compared to typical separation methods.

In the seventh topic, the TPC and AA analysis methods are introduced, briefly explaining how they act in the determination of these parameters.

In the eighth section, this dissertation is contextualized within previous work conducted by this research group in the field, outlining the stages to be undertaken and their sequence in general terms.

In the ninth topic, a framework of the sustainable practices of this work is made, referring to the use of green solvents, low energy consumption methods, avoiding waste generation, comparing equivalent carbon emissions, disadvantages in the approach, principles of green chemistry and framed SDGs.

In the tenth topic, the materials, equipment, reagents and MIPs used are presented.

In the eleventh, the experimental methods and conditions are explained, encompassing the processes of extraction, sorption/desorption, HPLC-DAD chromatography, UV-Vis spectrophotometry, analysis of total phenolic content and antioxidant activity.

In the twelfth topic, the results and discussion are presented, revealing the mass yields, TPC and EC50 of the extractions performed, in addition to showing bar graphs of TPC, EC50/EDF50, TPC and EC50 enrichment and Trolox equivalents, as well as the chromatograms of the best fractions obtained.

In the last topic, the general conclusions are made, evaluating the performance of the MIPs and showing the best results, evaluating the affinity between functional monomers and polyphenols, correlation between TPC and EC50, along with chromatography, and ending with the limitations and future work, as well as possible approaches of interest.

3. Contextualization

3.1. History of almond tree

The almond tree (*Prunus dulcis*), until it is known as it is today and became a global product, has undergone several genetic mutations, due to natural selection and domestication, having its origin more than 10 million years ago [30,31]. According to Watkins, the almond tree originated in Central Asia, where after mountainous upheavals it evolved and spread eastward, in desert and mountainous areas, thus adapting to unfavorable conditions [31].

Wild almonds are mostly bitter, being deadly if ingested in high quantities due to cyanide, while only a tiny amount of almond trees produce sweet almonds, being the ones we consume [30,32]. Given its characteristics, the almond tree was domesticated in order to promote the cultivation of sweet almonds, and this practice began, according to a recent study by Decroocq et al. (2025), in Turkey and began to be disseminated through the silk road, and is now cultivated in several countries [33,34]. Within this dissemination, the almond trees underwent a natural selection in the different regions, establishing the different local varieties that existed [34].

Currently, 48 countries (Figure 1) are almond producers, contributing to a production of 3.52 M (million t) in 2023 [1]. Of this production, 50.84% comes from the USA, followed by Spain (8.45%), Australia (7.38%) and Turkey (4.82%), and with the contribution of Portugal, as the 11th largest producer, representing 1.97% [1].

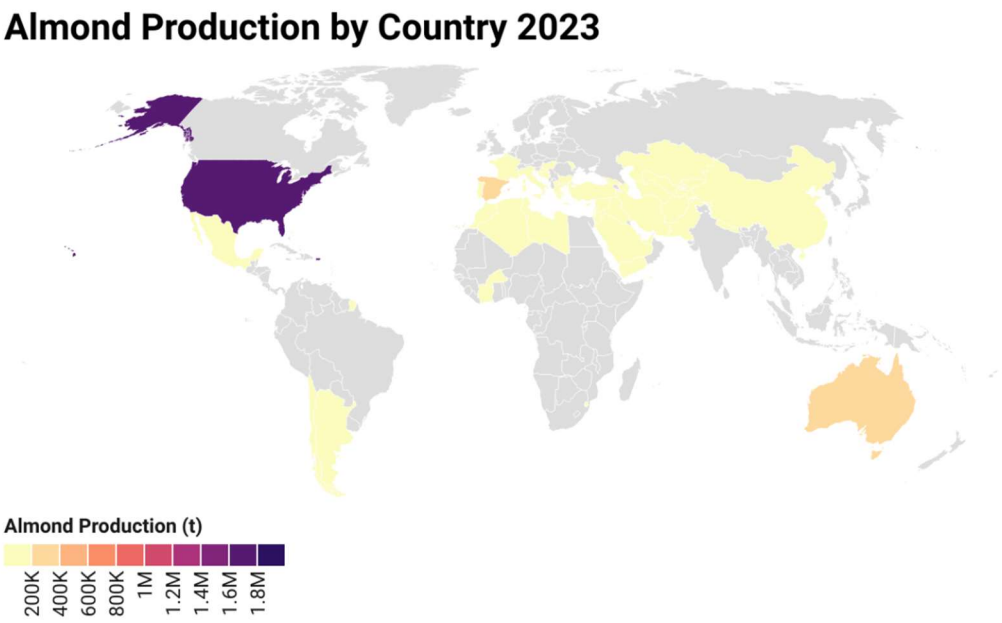


Figure 1 - Almond production by country in 2023 [1].

In Portugal, the almond tree is planted in several places, namely, in Beira Litoral and Interior, Ribatejo, Alentejo, Algarve, Trás-os-Montes, and recently, between Douro and Minho [2]. Among these, Alentejo is currently the largest producer, having surpassed Trás-os-Montes [3].

The production and cultivation area, as well as the consumption, of this nut have been increasing over the years (Figure 2), which, despite revealing its high economic, environmental and cultural value, leads to the accumulation of by-products, both natural and industrial, such as hull, shell, skin and blanching water [3–5].

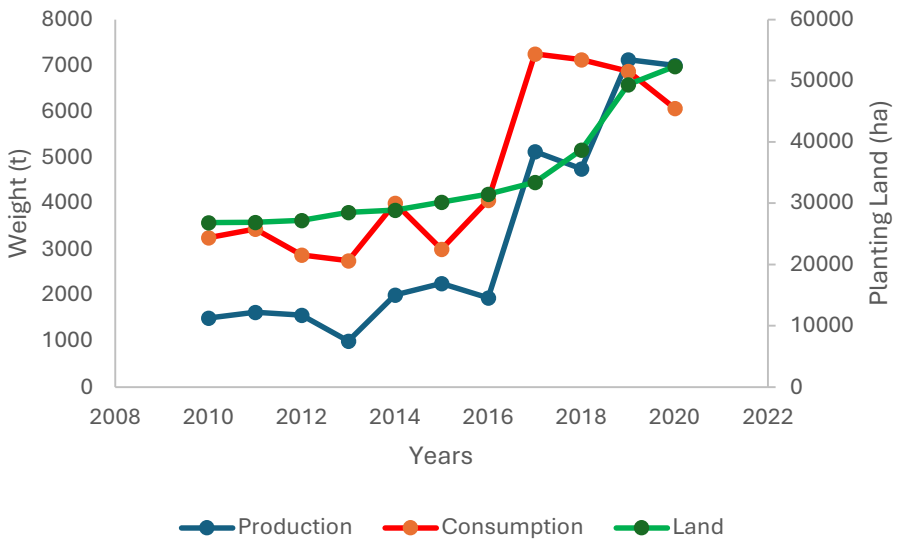


Figure 2 - Profile of production and consumption of almonds (without shell), and planting land, over the years in Portugal [2,35].

3.2. Almond by-products

The almond is botanically classified as a drupe, being separated into 3 main layers: Exocarp (hull), Endocarp (shell) and Endosperm (seed), the latter being also divided into two parts, having the kernel, the seed itself, and a brown wrapping film (skin) (Figure 3), botanically called tegument [36,37].

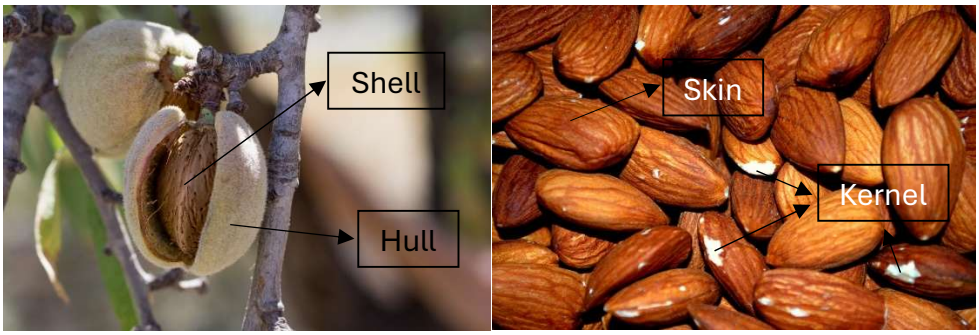


Figure 3 - Almond parts.

Analyzing in mass terms, for the production of 1 kg of isolated almond (kernel + skin) 0.6 kg of shell and 2.5 kg of hull are produced [38].

Considering that in 2023 3.52 M of almonds were produced still in shell, only 2.2 M were actually destined for consumption, with the disposal of 1.32 M of shell and 5.5 M of hull (Figure 4).

In addition to this natural waste, there is also industrial waste, namely blaching water and blanched skin, resulting from the blanching process [4,39]. This process consists of isolating the almond, removing the skin, and for this they are subjected to hot water (85-100ºC) for 2-5 min [39]. It is estimated that 5-10 m^3 of this water is produced for every 10 t of treated almonds (Figure 4) [40].

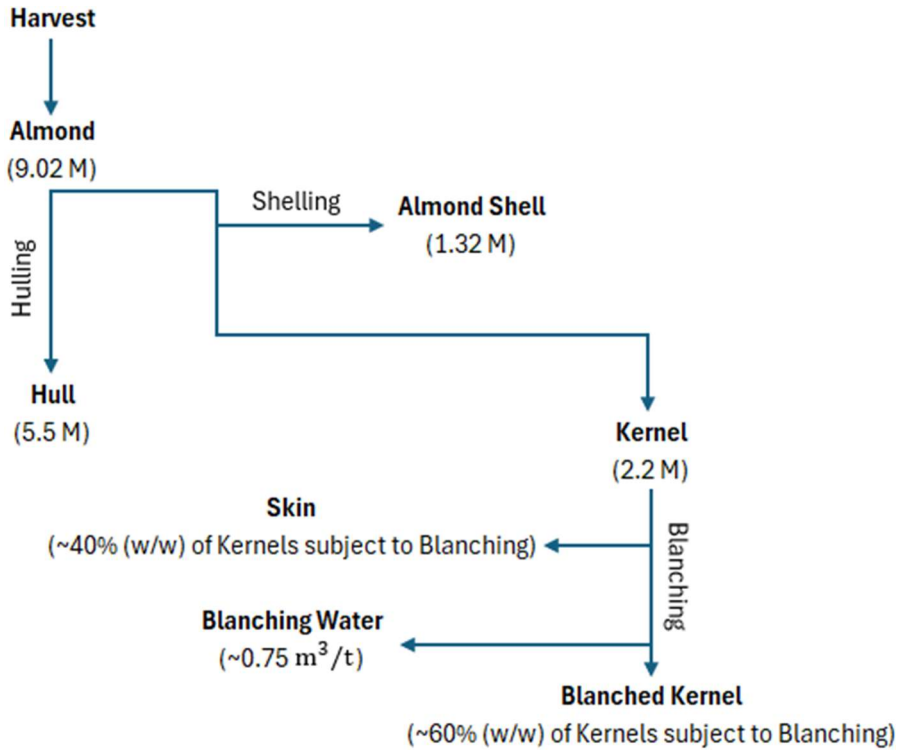


Figure 4 - Almond chain exemplified with 2023 values. Adapted from [4].

With the massive amount of organic waste, not all by-products are treated correctly, which leads to widespread environmental problems, such as soil, water and atmospheric pollution [6–8,41–43].

3.3. Problematization

The excessive accumulation of biomass generates environmental impacts, either directly (pollution caused by biomass) or indirectly (biomass treated by humanity), representing its dangers in a significant way, since about 10-50% of all agricultural products are discarded as waste [6].

Addressing human treatment first, much of the waste has been burned in the open, which causes harmful gases to be released into the atmosphere, such as NO_x, CO/CO_2 and SO_2, and also compromising the soil in which the burning takes place [6,7]. On the other hand, if a treatment is not carried out, the residues will accumulate in the soil, which can prevent sunlight from penetrating to it, making it impossible to grow in the area, as well as structural compounds, such as cellulose and lignin, which are only degraded in the presence of microorganisms, and these may not be able to degrade completely if there is too much mass [8,44].

In addition to the reasons above, one of the biggest problems is the contamination of soil and water [41,44]. Polyphenols are the major metabolites of plants, being present in agricultural residues (namely, in almond by-products), being compounds with variable degradability, depending on the complexity, which makes their retention in soils variable, but can in certain cases be retained for a long time [4,45]. These, in isolation, do not represent a risk, and may even bring benefits to the soil and human health, depending on the interaction with the microorganisms/plants and organisms, respectively, but causing a phytotoxic effect when present in high concentration [4,45,46]. In aqueous matrices there is the same problem, with polyphenols being one of the largest contaminations in the world, having found concentrations around the globe of <0.065-179,000,000 ng/L [41].

Part of the neglect in the treatment is due to the low economic value of the by-products, with hull having a typical cost of 100 $/t (~86 €/t), shell of 0-6 $/t (~0-5 €/t), not being profitable [9,10]. In order to reduce accumulation and increase use, one of the current objectives and challenges is the extraction of profitable compounds from this biomass, namely bioactive compounds (including polyphenols), establishing a circular economy [4,9].

4. Introduction to polyphenols and biomass characterization

4.1. Polyphenols

Polyphenols are characterized by having at least one aromatic ring attached to at least one hydroxyl group [4,11]. As mentioned earlier, they are compounds of natural origin, being present in agricultural products and their by-products, playing an extremely important role in protecting them, being generally in charge of defense against UV rays and other types of pathogens [4,11,18]. These characteristics are due to the antioxidant power of these compounds, serving as a barrier and protecting other compounds from ionizing radiation, or simply protecting from other compounds with reducing power, such as free radicals present in the atmosphere, which would potentially damage biomass [4,11].

Given the vast classification, several compounds are classified as polyphenols, having different characteristic morphologies, which gives rise to the classification by families, and also, consequently, the subdivision by subfamilies, derived from subtle differences in the ramifications to the main structures of each family [4,11,47,48].

Polyphenols are divided into 4 main families: Phenolic Acids, Flavonoids, Stilbenes and Lignans, where flavonoids are the most important group, being the most abundant [4,11,47,48]. In this way, the groups can be divided in a more simplistic way, such as Flavonoids and Non-Flavonoids [4,47]. Among these, phenolic acids, flavonoids and stilbenes will be addressed, with lignans mentioned in the appendix.

The structures of polyphenols directly influence bioavailability, pharmacokinetics, interaction with biomolecules and efficiency in antioxidant power, due to the arrangement of hydroxyl groups, which causes different capacities in different families [18,48]. In general, this bioactive group has a high potential for application in the health area, presenting antihypertensive, anti-obesity, anti-diabetes, immunomodulatory, antimicrobial, antiviral, anti-inflammatory, anti-hypercholesterolemia, antihyperglycemia, anti-hyperlipidemia and anticancer activity [12–15]. They are also promising in the food and cosmetic areas, due to their main characteristic, antioxidant activity, acting as bio-preservatives and photo-protective,

attenuating skin aging, respectively [13,16]. In addition to these applications, polyphenols are also valuable as insecticides, being a more sustainable and healthy option when compared to traditional insecticides [17].

Currently, more than 10,000 phenolic compounds have been reported, about 4000 of which are from the flavonoid family, part of which are soluble in water, another part with solubility in organic solvents, and there are also insoluble compounds [18,49].

4.2. Phenolic acids

Phenolic acids are subdivided into 2 groups: Benzoic Acids and Cinnamic Acids (Figure 5) [48,50]. The characteristic name of the family comes from carboxylic acid, a functional group that, in addition to defining the family, determines whether a compound is a benzoic or cinnamic acid [48,50].

When a carboxylic group is directly attached to the aromatic ring, the compound is a benzoic acid, however, if the group is separated from the ring by a C=C bond, the compound is a cinnamic acid, which is the most abundant of the 2, having very characteristic compounds present in the biomass [11,48,50].

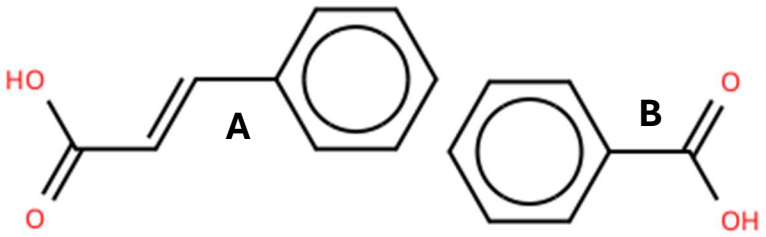


Figure 5 – A: Cinnamic acid; B: Benzoic acid.

Phenolic acids are derived from the shikimate pathway, being responsible for converting pentose phosphate and glycolysis intermediates into 3 aromatic amino acids: Phenylalanine, Tyrosine and Tryptophan [51]. Of these 3 amino acids, phenylalanine is the progenitor of the kinnamic group, while Tyrosine is that of benzoics [51]. This biological process originates different types of compounds, depending on the type and

position of the branches, which can be formed in different positions (Figure 6) [51–54]. Cinnamic acids can have up to 8 branches, 5 of them in the aromatic ring, 2 in the C=C bond, and 1 in the carboxylic group, while benzoic acids have the same possibilities of branches, excluding in the C=C bond, since it does not have it [51–54].

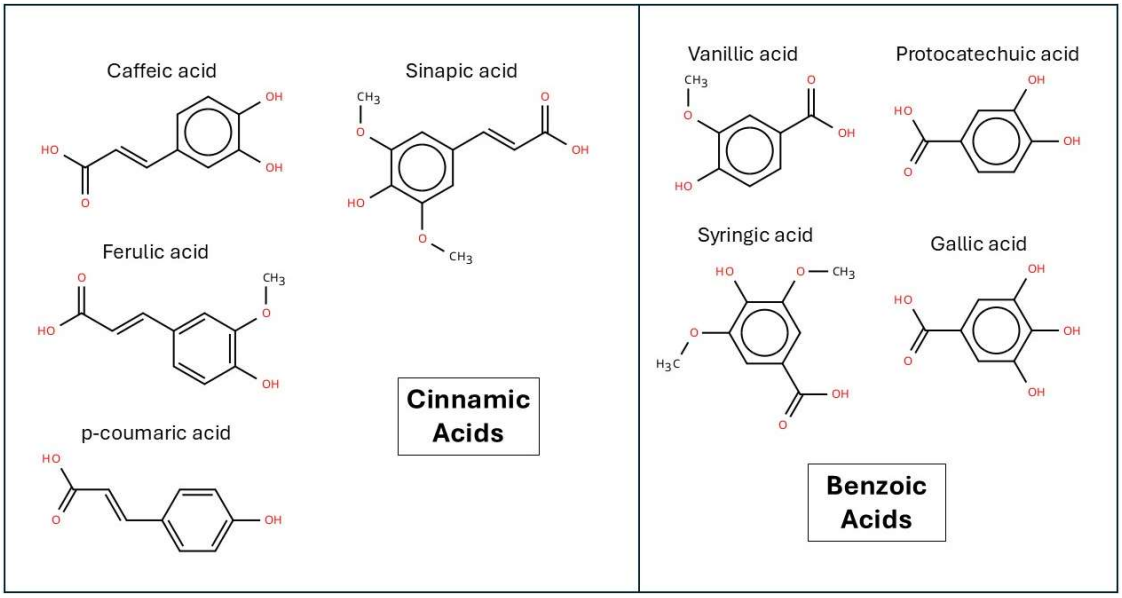


Figure 6 - Typical phenolic acids [50,55].

This class is the second most abundant of the polyphenols, having outstanding biological functions, such as chemopreventive, antimutagenic, cardioprotective, and probiotic effects, as well as great potential in the cosmetic industry, presenting anti-aging, anti-inflammatory, and antimicrobial properties [51,56,57].

These acids, in addition to their individual valuable capacities, are also progenitors of a naturally important class, hydrolyzable tannins [58].

4.3. Flavonoids

The term flavonoids is a generalization of a vast group of natural molecules that exhibit a C_6-C_3-C_6 structure, called phenylbenzopyran [59]. Within this category, we must divide into 3 main classes, depending on the carbon bond between the aromatic ring and

the benzopyran structure: flavonoids (2-phenylbenzopyrans), isoflavonoids (3-benzopyrans), and neoflavonoids (4-benzopyrans) (Figure 7) [59]. Throughout the text, the flavonoids, present in the by-products of the almond, will be detailed, while the remaining groups will be detailed in the appendix.

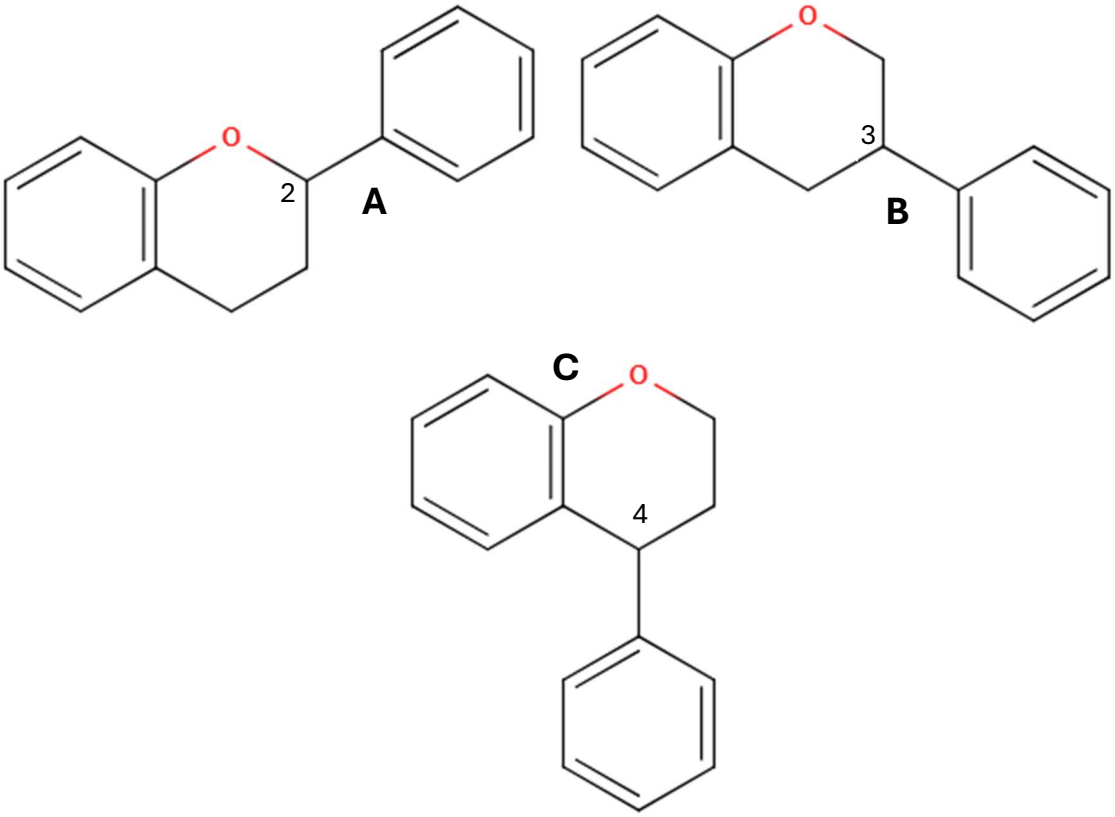


Figure 7 – A: Flavonoids backbone; B: Isoflavonoids backbone; C: Neoflavonoids backbone [59].

4.3.1. 2-phenylbenzopyrans (Flavonoids)

The flavan structure (Figure 8) is the basis of all flavonoids, having the entire category based on the nomenclature and numbering of the rings and carbons, respectively [59].

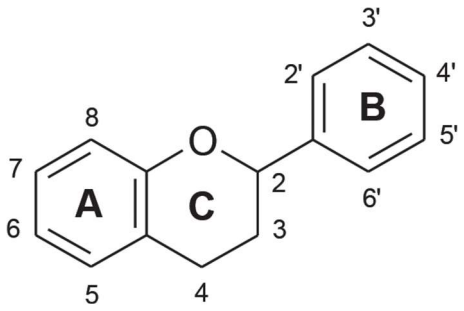


Figure 8 – Flavan [60].

Derived directly from flavan there are 6 base types of families, depending on the degree of saturation and oxidation of the C ring: Flavones, Flavonols, Flavanones, Flanononols (dihydroflavonols), Flavanols and Anthocyanidins (Figure 9) [59–62]. Within these families, namely Flavanols and Anthocyanidins, there are also some branches that, due to their abundance, are named as independent families, called: Flavan-3-ols, Flavan-4-ols, Flavan-3,4-diols (Figure 9) and Anthocyanins, the latter being an anthocyanidin with a glycosidic branch [59–62].

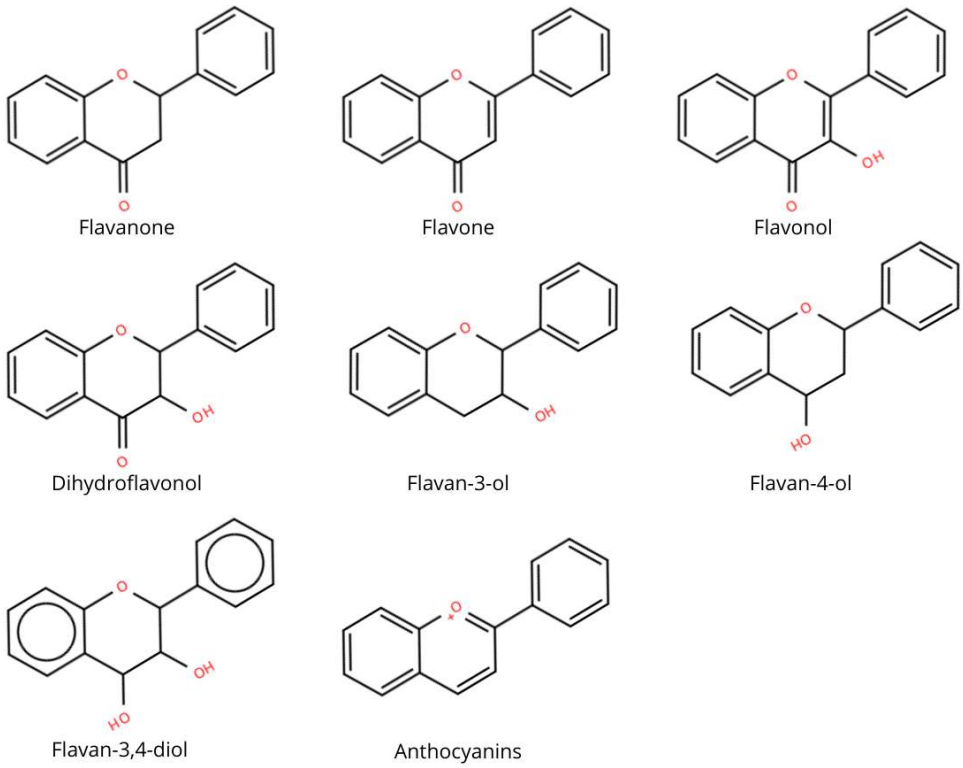


Figure 9 – 2D representation of Flavan-derived flavonoid families [59–62].

In general, flavonoids can have branches at carbons 2, 3, 4, 5, 6, 7, 8, 2', 3', 4', 5' and 6', and these positions are typical of having branches depending on the class of the family (Figure 10) [59,61–65].

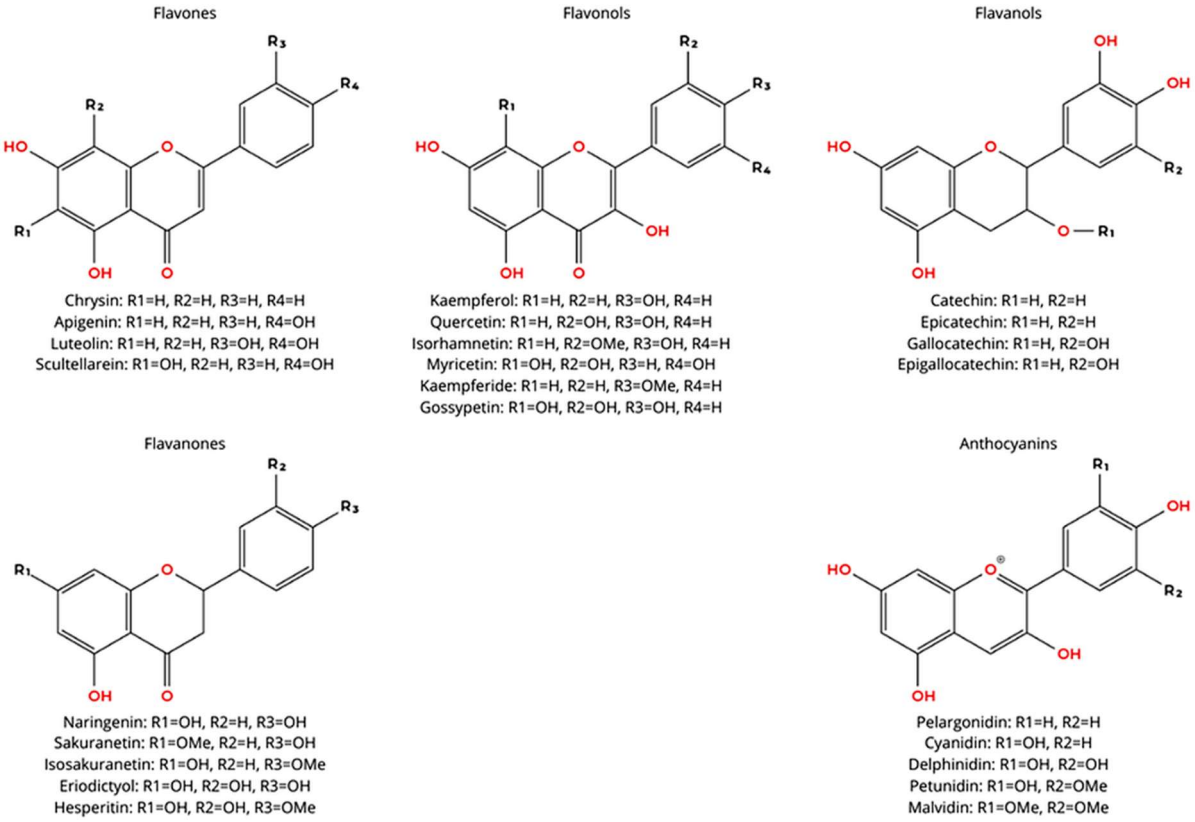
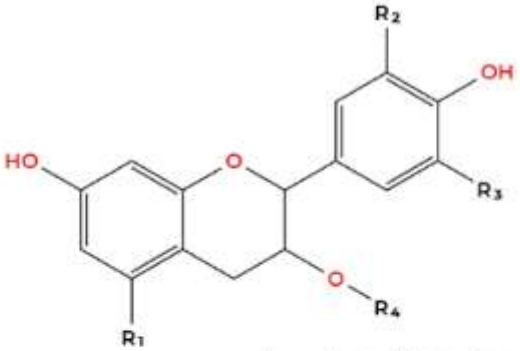


Figure 10 – General structures of the main classes of flavonoids, showing the most common substitution patterns. Adapted from [62,66].

Within the families mentioned, the Flavan-3-ols family has special morphological characteristics, due to the existence of 2 stable chiral carbons (C2 and C3) [66]. This morphology gives rise to the existence of 4 possible diastereoisomers, as well as cis/trans isomeric differentiation and enantiomers [66,67]. Derived from these possibilities, there are 5 prominent groups: Catechins/Epicatechins, Afzelekins/Epiafzelekins, Gallocatechins/Epigallocatechins, Fisetinidol and Robinetinidol (Figure 11) [67].



	R1	R2	R3	R4
Catechin/Epicatechin	OH	OH	H	H
Afzelequins/Epiafzelequins	OH	H	H	H
Gallocatechins/Epigallocatechins	OH	OH	OH	H
Fisetinidol	H	OH	H	H
Robinetinidol	H	OH	OH	H

Figure 11 – 2D representation of Catechins/Epicatechins, Afzelechins/Epiafzelechins, Gallocatechins/Epigallocatechins, Fisetinidol and Robinetinidol. Adapted from [67].

The prefix "Epi" is used when there is a cis configuration structure, referring to ring B and -OH/radical in C3 (Figure 12) [66,67]. In addition to this distinction in nomenclature, another prefix is used, which informs about the geometric arrangement, adding (+) if the -OH/radical in C3 is in the S arrangement, and (-) if it is in the R arrangement (Figure 12) [66,67]. Visually, analyzing the flavan of the representations illustrated so far, it will be a (-)-Catechina when the -OH/radical in C3 is in the background, and it will be (+)-Catechine when it is projected in the upper plane [66,67].

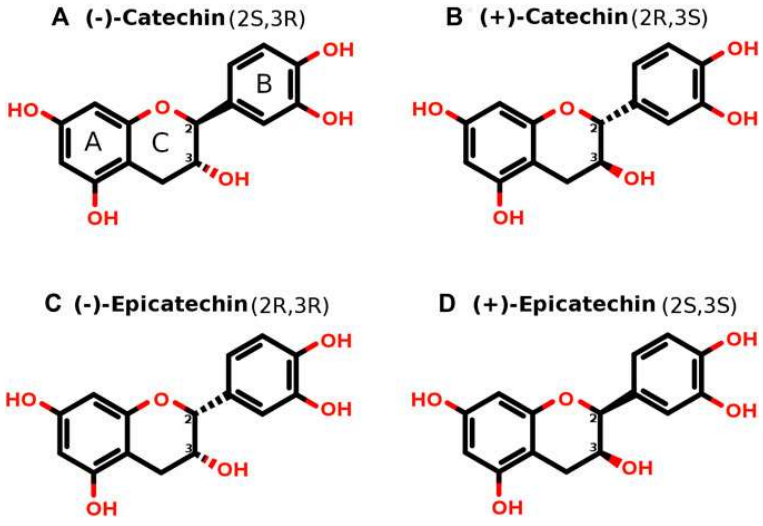


Figure 12 – Differentiation between (+)-Catechin, (-)-Catechin, (+)-Epicatechin and (-)-Epicatechin. From [67].

Within the flavan-3-ols, the catechins/epicatechins are the most abundant group in nature, appearing in the (+)-catechin-and (-)-epicatechin forms [66,68]. This family, as well as a group, are of high significance, both at the individual level, due to their biological activities (Table 1), and at the group level, and can form polymeric chains, giving rise to the class of condensed tannins [57,58,69].

Table 1 – Biological activities of the flavonoid families [57,69].

Flavonoid families	Biological activities
Flavanols	Cardioprotective, antioxidant, anti-inflammatory, antiviral, antimicrobial, cancer treatment adjuvant
Flavones	Chemopreventive, diabetes type II prevention, hypertension prevention, obesity prevention, cardiovascular diseases prevention, antioxidant, cholesterol lowering
Flavonones	Antimicrobial, anti-inflammatory, gut health, antidiabetic
Flavonols	Cardioprotective, anti-inflammatory, antioxidant, cancer prevention, hypolipidemic properties
Anthocyanins	Cardioprotective, prevention of diabetes and obesity, neuroprotective, cancer preventive

4.4. Stilbenes

Stilbenes (Figure 13) are a smaller non-flavonoid family, with about 400 known compounds, most of which are derived from a trans-resveratol structure [70]. They are compounds that have a 14-carbon skeleton, having 2 aromaric rings connected by an ethylene bridge [70].

R3	R5	R2'	R3'	R4'	Stilbene
OH	OH	H	H	OH	Resveratrol
OCH_3	OCH_3	H	H	OH	Pterostilbene
OH	OH	OH	H	OH	Oxyresveratrol
OH	OH	H	OH	OH	Piceatannol
OH	OH	H	H	H	Pinosylvin
OH	OCH_3	H	H	H	Pinosylvin monomethyl ether
OCH_3	OCH_3	H	H	H	Pinosylvin dimethyl ether
OH	OH	H	OCH_3	OH	Isorhapontigenin
OGlu	OH	H	OCH_3	OH	Isorhapontin
OGlu	OH	H	OH	OH	Astringin
OGlu	OH	H	H	OH	Piceid (polyadatin)
OGlu	OH	OH	H	OGlu	Mulberroside A

Figure 13 – Chemical structures of common stilbene monomer derivatives.From [70].

Despite being a less abundant group, stilbenes have important biological properties, such as antibacterial, antifungal, nematocidal, and insecticidal activity [70].

4.5. Polyphenolic characterization of almond by-products

Within the existing by-products, blanching water, hull and shell will be addressed in this work.

4.5.1. Blanching water

According to the article by Smeriglio et al. 2016, having performed extractions in methanol and quantified, complemented with the article by Silvia et al. 2025, the following compounds were reported in Table 2 in blanching water [9,71].

Table 2 – Polyphenols identified in blanching water [9,71].

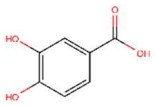
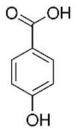
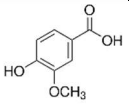
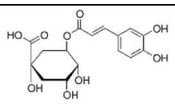
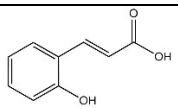
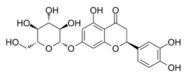
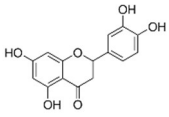
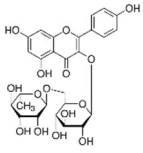
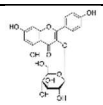
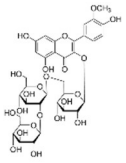
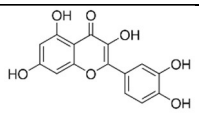
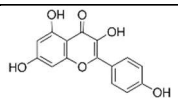
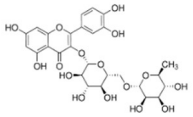
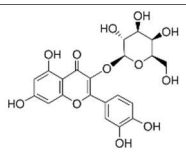
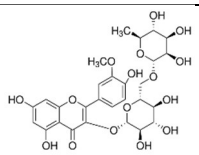
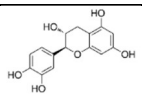
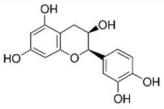
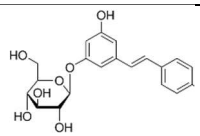
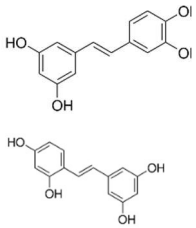
Family	Compound	2D structure	[µg/ 100 g] ± SD	Price	Brand
Hydroxybenzoic acids	Protocatechuic acid		453.86 ± 11.83	22 €/mg	Sigma Aldrich
	4-Hydroxybenzoic acid		97.81 ± 4.96	1.41 €/g	Sigma Aldrich
	Vanillic acid		531.22 ± 12.98	13.60 €/g	Sigma Aldrich
Hydroxycinnamic acids	Chlorogenic acid		4.66 ± 1.94	82.80 €/g	Sigma Aldrich
	Trans-p-cumaric acid		13.90 ± 2.01	1.65 €/mg	Phyproof
Flavanones	Eriodictyol-7-O-glucoside		124.67 ± 5.15	39.60 €/mg	Med Chem Express
	Eriodictyol		216.94 ± 11.37	22.60 €/mg	Sigma Aldrich
Flavonols	Kaempferol-3-O-rutinoside		6,247.21 ± 10.16	105.40 €/mg	Sigma Aldrich

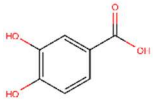
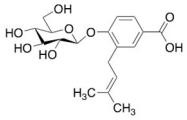
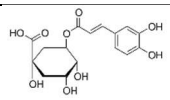
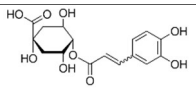
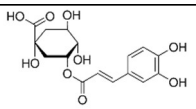
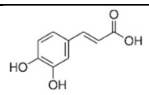
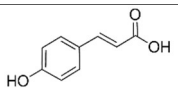
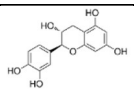
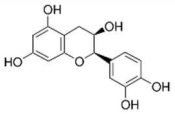
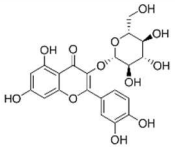
Table 2 – Continuation.

Flavonols	Kaempferol-3-O-glucoside		2,080.24 ± 9.30	29.20 €/mg	Sigma Aldrich
	Isorhamnetin-3-O-glucoside		56.57 ± 8.73	65.70 €/mg	Sigma Aldrich
	Quercetin		31.04 ± 4.9	3.21 €/g	Sigma Aldrich
	Kaempferol		2,392.44 ± 4.587	27.10 €/mg	Sigma Aldrich
	Rutin		11.32 ± 0.74	3.32 €/mg	Sigma Aldrich
	Quercetin-3-O-galactoside		34.47 ± 2.06	93.40 €/mg	Sigma Aldrich
	Isorhamnetin-3-O-rutinoside		333.12 ± 4.79	36.60 €/mg	Sigma Aldrich
Flavanols	(+)-Catechin		47.76 ± 7.33	6.60 €/mg	Sigma Aldrich
	(-)-Epicatechin		20.82 ± 3.17	30.90 €/mg	Sigma Aldrich
Resveratrol	Polydatin		6.33–8.43	8.28 €/g	Sigma Aldrich
	Piceatannol + oxyresveratrol		0.91–2.55	52 €/mg 2.80 €/mg	Sigma Aldrich

4.5.2. Almond hull

According to the articles by Meshkini 2016, Kahlaoui et al. 2019, Sang et al. 2002 and Takeoka et al. 2003, the compounds in Table 3 were identified in hull [72–75].

Table 3 – Polyphenols identified in almond hull [72–75].

Family	Compound	2D structure	Price	Brand
Hydroxybenzoic acids	Protocatechuic acid		22 €/mg	Sigma Aldrich
	Malaxinic acid		26.48 €/mg	Fisher Scientific
Hydroxycinnamic acids	Chlorogenic acid		82.80 €/g	Sigma Aldrich
	Cryptochlorogenic acid		40.60 €/mg	Sigma Aldrich
Hydroxycinnamic acids	Neochlorogenic acid		14.24 €/mg	Sigma Aldrich
	Caffeic acid		4.88 €/g	Sigma Aldrich
	p-coumaric acid		4.88 €/g	Sigma Aldrich
Flavanols	(+)-Catechin		6.60 €/mg	Sigma Aldrich
	(-)-Epicatechin		30.90 €/mg	Sigma Aldrich
Flavonols	Quercetin-3-glucoside		27.30 €/mg	Sigma Aldrich

Among the identified compounds, the majority is chlorogenic acid, followed by (+)-Catechin, with the others having a residual presence when compared [73].

4.5.3. Almond shell

According to the article by Khadher et al. 2023, based on extractions of cyclohexane, dichloromethane, ethyl acetate, and methanol, the compounds in Table 4 were identified in Almond shell [76].

Table 4 – Polyphenols identified in almond shell [76].

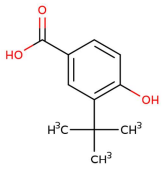
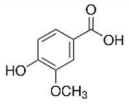
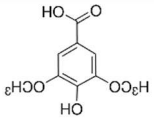
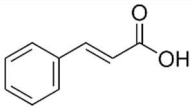
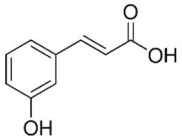
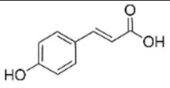
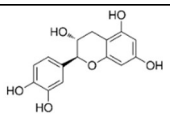
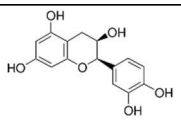
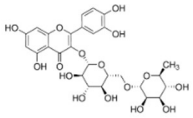
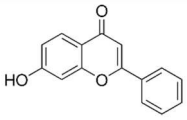
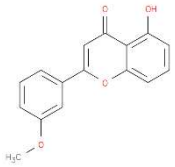
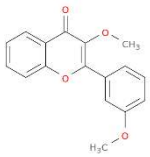
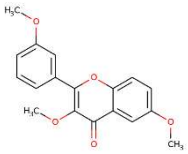
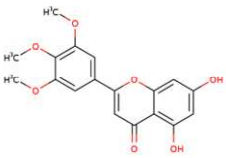
Family	Compound	2D structure	Price	Brand
Hydroxybenzoic acids	3-tert-butyl-4-hydroxybenzoic acid		80.60 €/g	Cymit Química
	Vanillic acid		13.60 €/g	Sigma Aldrich
	Syringic acid		0.62 €/mg	Sigma Aldrich
Hydroxycinnamic acids	trans-Cinnamic acid		9.08 €/g	Sigma Aldrich
	trans-3-hydroxycinnamic acid		6.66 €/g	Sigma Aldrich
	p-coumaric acid		4.88 €/g	Sigma Aldrich
Flavanols	(+)-Catechin		6.60 €/mg	Sigma Aldrich
	(-)-Epicatechin		30.90 €/mg	Sigma Aldrich

Table 4 – Continuation.

Flavonols	Rutin		3.32 €/mg	Sigma Aldrich
Flavones	7-Hydroxyflavone		2.06 €/mg	Sigma Aldrich
	5-hydroxy-3′-methoxyflavone		~1.54 €/mg	A2B Chem
	3,3′-dimethoxyflavone		~1.37 €/mg	A2B Chem
	3,6,3′-trimethoxyflavone		1.24 €/mg	Biosynth
	5,7-dihydroxy-3′,4′,5′-trimethoxyflavone		16.48 €/mg	Biosynth

Among the identified compounds, when the ethyl acetate extraction is performed, the major compound is rutin, with a concentration of 13.06 mg/g, while in the methanol extraction the major compound is (-)-epicatechin, with a concentration of 4.42 mg/g [76].

5. Extraction methods

The process of isolation/enrichment of the polyphenols in this work consists of two fundamental steps: Solid-Liquid Extraction and Sorption. In the case of hull and shell, when it comes to solid waste, the first step is to extract the polyphenols into a liquid medium, using methods such as Ultrasound and Soxhlet. After extraction and treatment (filtration and freeze-drying), the extract can be applied in a column adsorption process, previously packaged with a Molecularly Imprinted Polymer (MIP), being a type of Tailored Polymer Network, which will be used as an adsorbent. In the case of blanching water, the procedure is the same, but without the need for solid-liquid extraction, since it is already an industrial liquid waste.

5.1. Ultrasound

The ultrasound-assisted extraction (UAE) method (Figure 14) is based on the propagation of sound waves in a liquid medium, causing cavitation around the solid particles and thus breaking their cell walls, resulting in the release of the bioactive compounds into the solvent [77].

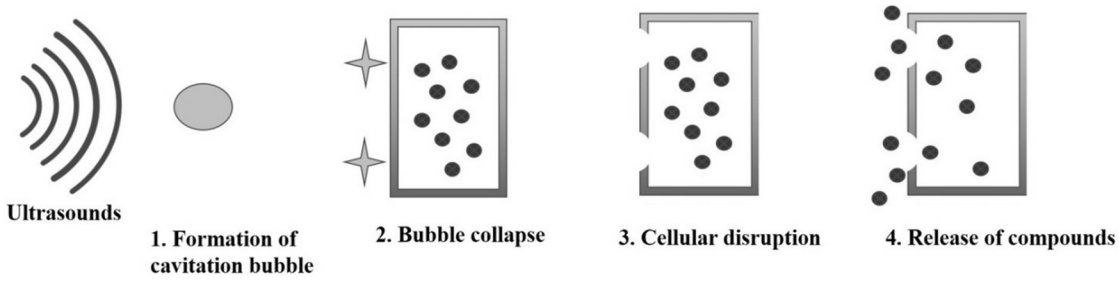


Figure 14 – Basic principle of ultrasound-assisted extraction. From [77].

UAE is widely used at the laboratory level, as it is a highly efficient method, increasing dispersion and promoting mass transfer between the solid phase and the liquid phase, resulting in shorter extraction times [78]. In addition to its efficiency, the use of

low-intensity wave UAE allows a safer extraction of bioactive compounds, being less aggressive to unstable compounds, promoting only physical convection, not interfering with the chemical stability of the compounds in the system [4,78].

5.2. Soxhlet

The soxhlet (Figure 15) is a piece of equipment that applies several engineering concepts, applying evaporation, condensation, mass transfer via convection and siphon effect [79,80].

In a central container, the soxhlet extractor, a highly porous cartridge filled with ground biomass is placed, where the extraction will be carried out [79,80]. This extractor is opened from above, with a condenser being coupled that works based on the passage of a cold-water stream, and also having two side connections, both connected to the evaporator [79,80]. One of the connections is a passage for the steam, which must be above the solvent level, while the other is a system for emptying the extractor via siphon effect [79,80].

Thus, the extraction via soxhlet happens on the basis of cycles, where the solvent is placed in the evaporator balloon, and entering a gaseous state it goes through the passage for the steam to the condenser [79,80]. With the condensation given in it, the solvent begins to accumulate in the extractor, until it reaches the level of turning of the siphon, thus emptying the extractor and repeating the cycle [79,80]. In this way, the extraction is not limited to a single mass balance, the pure solvent is evaporated/condensed and the extracted waste is left behind, managing to consistently extract the bioactive compounds, until the biomass is exhausted.

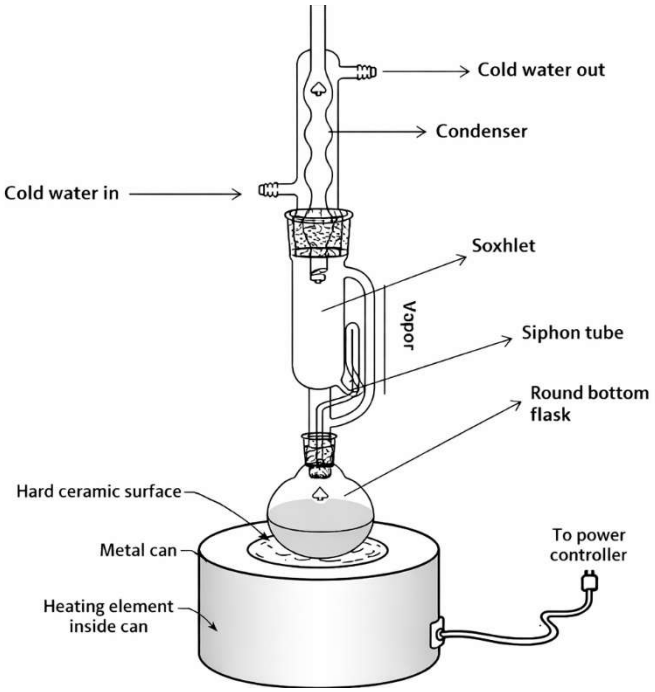


Figure 15 – Soxhlet schematization. From [80], with improved resolution through AI.

Although advantageous, being able to obtain high concentrations of extract, this method has a high energy demand, due to evaporation, as well as requiring an operation at high temperatures for it to occur, which can lead to the degradation of more sensitive bioactive compounds [4,79].

5.3. Adsorption

Adsorption is a mass transfer phenomenon, where the migration of matter present in a fluid to the surface of a porous solid occurs, and matter can be a single/multicomponent system of molecules, atoms or ions [81]. This process is essentially given in two ways: Physical (Physisorption) and Chemical (Chemisorption) [81]. In physical adsorption, the target compounds (adsorbate) interact with the adsorbent from weak interactions, such as van der Waals forces and hydrogen bonds, generating enthalpies between 5-40 kJ/mol, which results in a reversible accumulation, and the reverse process can be carried out, desorption, which consists of the passage of a pure solvent through the system. extracting the compounds present on the surface of the solid

[81]. In chemical adsorption, the adsorbate forms covalent bonds with the adsorbent, changing the structure of both and making the process irreversible, generating enthalpies between 40-800 kJ/mol [81]. To occur chemisorption it is necessary to heat the system to high temperatures, given its enthalpy, resulting in the physisorption being the usual process at room temperature [81].

This spontaneous process goes through 3 essential phases, with diffusion in the film formed between the particles and the bulk, diffusion in the pores of the particles and surface reactions, which allows a molecular selection by the adsorbents, and the pores may have more affinity with a certain type of molecules in view of their morphology/type of interactions, which favors these compounds, or certain compounds have a slower diffusion, causing the compounds with faster diffusion to lodge in the adsorbent first [4,81]. Within these differentiations, different types of adsorbents, such as MIPs, are explored, creating an artificial selection of adsorbates.

Adsorption is a versatile process, and can be applied both in batch and columns, which provides variety to the method and operating conditions. In this work a column system will be used, operating both in open and closed cycle, and previously used the Solid-Phase Extraction method to test the adsorbent on a small scale.

5.3.1. Solid-phase extraction

Solid-Phase Extraction (SPE) (Figure 16) is comparable to a small-scale, open-loop column operation, where the adsorbent is placed in a cylindrical container connected to a vacuum chamber, functioning as a pump [82]. In the operation of this equipment, 4 essential steps are carried out, namely conditioning, loading, washing and elution [82]. In the conditioning, the adsorbent is subject to the solvent that will be used in the next step, adapting it to the solvent that will be used, as well as not contaminating the post-extraction solution with another solvent [82,83]. In loading is applied to the sample, containing the target compounds and impurities [83]. In washing, a selective solvent is used, essentially removing unwanted compounds from the matrix and preferably leaving the analytes in it [82]. Finally, in the elution, a solvent is used that the analytes have an affinity for, removing them from the column and thus managing to enrich them, bordering on isolation [83].

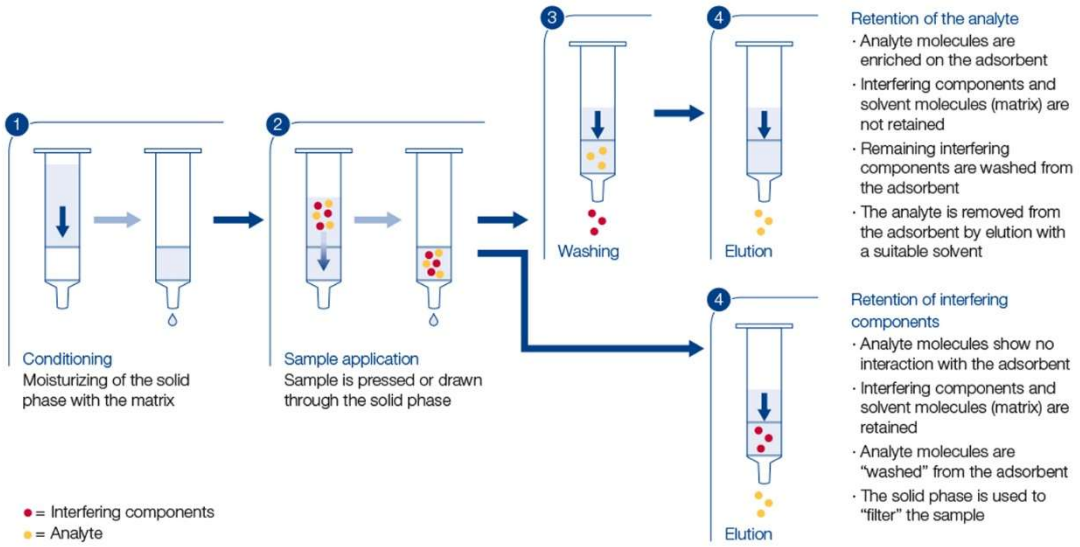


Figure 16 – Diagram of the different stages of the SPE. From [84].

This method, despite its simple and practical execution, has several advantages of use, such as high selectivity, resulting in a high experimental quality, and allowing several tests to be carried out at the same time [83]. On the other hand, it is necessary to make a good selection of solvents, both in nature and in their order of passage, since it is necessary to have a solvent that removes the impurities and does not remove the analyte from the adsorbent, or removes it in a residual way, as well as a solvent that has a good affinity with the analyte, removing it from the adsorbent [82]. Normally, for desorption, it is necessary to resort to organic solvents, which are often toxic in nature [85].

5.3.2. Comumn adsorption

As mentioned in the description of the SPE, given its similarity, a column operation is essentially given by 4 steps: conditioning, loading, washing, and elution. The process can be open-loop or closed-loop, leading to different outcomes. In an open-loop operation, the adsorbent will be constantly fed with a solution of known concentration, reaching a solid-liquid equilibrium state, making it possible to determine the maximum adsorption capacity, both at the general mass level, and for each of the target compounds [86,87]. In a closed-loop operation, the system can reach solid-liquid equilibrium at lower

concentrations, depending on variables such as adsorbent mass, concentration, and solution volume, not by saturating the column, but by decreasing the concentration of the supply solution as a whole [86,87].

After the adsorption process, washing and elution begin, which are desorption processes, being an open cycle process.

6. Tailored polymer networks

Tailored polymer networks are polymer networks intentionally designed to have specific properties, whether physical or chemical [88]. Within the possible categories of networks, the MIPs aim to increase selectivity for certain compounds/families, allowing a higher enrichment/isolation than the standard.

6.1. Molecularly imprinted polymers

MIPs are a technology first introduced by Wulf and Sarhan in 1972, and later gaining fame through the efforts of Mosbach and his colleagues in the 1980s [89]. This polymeric adsorbent works on the basis of molding, where, in its synthesis, a desired model compound is added to the reaction, so that polymeric chains form around it, and thus obtaining a cavity [90].

In more detail, MIPs are copolymers that need 3 main compounds for their formation: Monomer, Cross-Linker and Template [18,26]. Given the beginning of the reaction, the Cross-Linker will polymerize with each other, forming polymeric chains, where, at some specific points, there will be branches of Monomer that are arranged in a certain way due to interactions that it has with the Template (Figures 17 and 18) [18,89]. The solvent used in the production of the MIPs has an important and significant impact on the properties resulting from the polymer, such as the intensity between the bonds between the Template – MIP and the type of bonds itself, being an extremely important factor [91].

There are two main types of bonds that can occur between the Template – MIP, which are covalent and non-covalent bonds [18,90]. In covalent bonds there is great stability in the desired polymerization, due to the intensity, and the cavity intended to adsorb Template becomes more selective, but the removal of the Template becomes complicated, requiring drastic conditions [18,90]. On the other hand, non-covalent bonds, such as hydrogen bonds, van der Waals forces and π-π stacking, make it much easier to remove the template, and it is only necessary to pass a solvent for the desorption of it, which makes it the most used method [18,90].

There is also an intermediate method, called semi-covalent, where the Template establishes covalent bonding with certain monomers, but establishing only non-covalent interactions with others [18,90].

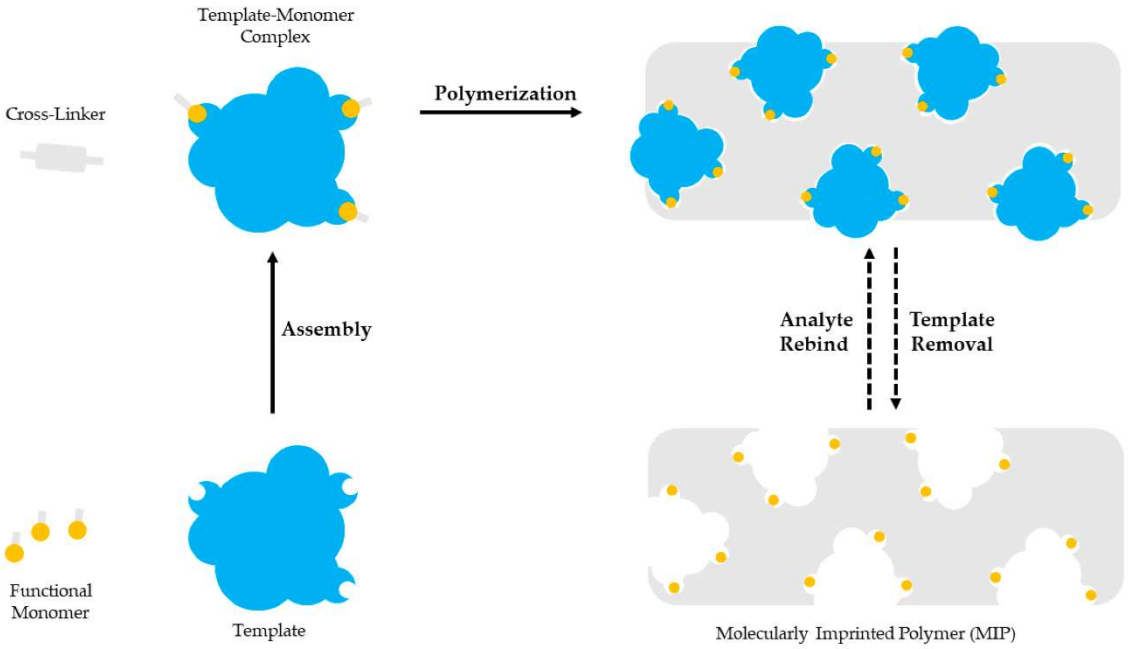


Figure 17 – Schematization of the MIP formation process. From [92].

Figure 18 – Chemical Schematization of Quercertin MIP Functioning with Different Compounds. From [29], licensed under CC BY 4.0.

Just as there are different MIPs depending on the bonds, there are also different types of polymerizations that can be applied to the process [18].

- **Free Radical Polymerization** – It is a simple and scalable process, and it is well known, taking place by the formation of a first free radical in front of an initiator, which will cause a chain reaction until there are no more free radicals to react, thus forming polymeric chains [18]. The disadvantage of this process is that the polydispersion index diverges from 1, since the reaction happens in an unpredictable way, and can give rise to both long and small chains [18]. Derived from the polymerization of free radicals there are subtypes of polymerization, such as in Bulk and Suspended.
  - ❖ **Bulk Polymerization** – It is a process distinct by its simplicity, and it is only necessary to add all the reagents in their stoichiometric proportion in a given solvent, in order to originate a homogeneous solution [18]. Despite the easy execution, the final product tends to have varied sizes and to have a coating, the latter of which makes it difficult to remove the template [18].
  - ❖ **Suspended Polymerization** – It is a heterogeneous process considered relatively simple, characterized by the dispersion of solid particles in a liquid phase [18]. It begins with liquid-liquid dispersion and ends with solid-liquid dispersion, and later a separation is made between the organic and aqueous phases, with the ratio between them generally being from 0.1 to 0.5 or higher [18]. The organic phase includes monomers, primers, cross-linking agents, and diluents, while the aqueous phase contains surfactants and stabilizers dissolved in water [18].

Methods such as crystallization and membrane filtration are usually used to concentrate or purify solutions similar to those involved in the valorization of bioactive compounds, but when compared with adsorption/desorption, it is possible to identify some disadvantages [29]. Crystallization involves a large amount of energy, as it needs to cool the solutions to melting temperatures, as well as proving to be difficult in its purpose when working with complex solutions [29]. Membranes, on the other hand, have little selectivity, also working with complex solutions [29].

Given these disadvantages, sorption/desorption with MIPs consumes little energy, preserves temperature-sensitive compounds, has operational flexibility and is a cyclic process, and, as mentioned earlier, is receptive to intensification, which leads to its feasibility for industrial application [29]. One of the disadvantages of this process is a possible difficulty in isolating a compound, depending on the composition of the solution that will go through the process, since, although the MIPs are molded, smaller compounds can enter the pores of the polymer and stay there, if the Template is of a larger compound, which makes it necessary to even if it is with the use of another MIP, for the highest efficiency of isolation/enrichment [18].

Mixed technologies, between sorption/desorption and membrane filtration, are already gaining ground and fame in this type of applications [29].

7. Evaluation of biological activity of phenolic extracts and application

After enrichment and obtaining of different extracts, due to the selectivity of the MIPs and affinity of the adsorbates with the different desorption solvents, fractions of different phenolic profiles will be obtained, resulting in different biological activities and capacities [93]. Within the existing methods, the determination of the Total Phenol Content (TPC) and the Antioxidant Activity (AA) are widely used options, being simple analyses, giving a general notion of the concentration of phenols in solution, typically expressed by gallic acid equivalents, and the reducing/antioxidant power of the sample, serving as a comparison with the initial extract and giving an enrichment factor of the family of compounds and biological activity [94].

Both methods work essentially on the basis of reduction, and in TPC it is determined by the ability of the sample to reduce a stable compound, using Folin-Ciocalteu as a target, while AA is determined by the reduction of free radicals, typically using 2,2-diphenyl-1-picrylhydrazyl (DPPH) [94,95].

The determination of these parameters quickly clarifies whether enrichment has occurred [93]. For various applications it is not necessary to obtain purified compounds, but only to identify certain families and characteristics of the compounds, which makes this approach sufficient in some cases, such as in the determination of the insecticidal capacity [93]. According to studies, already having a practical confirmation, enriched polyphenolic extracts have shown insecticidal potential, given their toxicity when concentrated and oxidative stress in insects, in addition to other prominent mechanisms, making it a sustainable solution, compared to traditional insecticides, being biodegradable compounds and of low environmental toxicity [96].

8. General framework and background

The methodology of the operation was previously established and elaborated by the research group of Professor Rolando Dias, and this master's thesis is a continuation work, and not a work to establish the bases of the operation [4,18–25,27–29,97–100].

The first stage of the process begins with the extraction of selected residues from the almond tree, where aqueous and hydroalcoholic solvents will be used, carrying out an ultrasonic infusion process. Then, after a previous filtration of the suspended material from the extraction, the sorption process is carried out in columns packed with MIPs. Different columns are packaged with different MIPs, which, in addition to the difference in templates, have different functional groups, essentially using 4-Vinylpyridine (4VP) or 2-(Dimethylamino)ethyl methacrylate (DMAEMA), conferring different selection properties, differentiating in the type of interaction between the adsorbate and the polymer. 4VP functional adsorbents show selectivity against acid-base interactions, π–π stacking and hydrogen bonding, while DMAEMA functional groups interact electrostatically, pKa and ionic affinity [29,97].

After the adsorption process, desorption processes are carried out at 45ºC, being separated in washing and elution. Water is used for washing, while ethanol/water fractions are used for elution in addition to pure ethanol, as well as more intense organic solvents in the final passes, such as methanol.

Given the obtaining of the different solutions, they are evaporated until the solid waste is obtained, using a rotavapor, at 45ºC, where the pure solvent is also obtained, and then the material is dried in an oven between 40-45ºC.

Obtaining the enriched extracts, they are characterized in different ways, using HPLC and UV-Vis, in addition to the TPC and AA analyses, aiming at the comparison of the bioactivity of the enriched fractions, which in this work are aimed at a practical application, intending to study the insecticidal capacity.

9. Sustainable practices involved in the process

This methodology adopts several sustainable practices, being notorious in most stages.

In the extraction of phenolic compounds from biomass, green solvents are used, which are biodegradable and of low toxicity, in addition to the application of ultrasound, being an extraction method with low energy demand. In the desorption stage, mostly green solvents are also used, using toxic solvents, such as methanol, to a lesser extent. In the isolation of the solid residue dissolved in the elutions, the process allows the obtaining of pure solvents, which allows their reuse in the process, avoiding unnecessary disposal of solvents and avoiding to a certain extent the introduction of more solvent in the process.

By analysing factors external to the technical process, it is also possible to identify other positive aspects in the valuation, with a reduction in carbon emissions compared to the replacement of certain practices. As mentioned in the subchapter of the problematization, when poorly managed, the by-products are burned in the open, generating 0.445 tons of CO2 equivalent per ton of biomass, and considering that they are generated in the millions, the emission becomes immense [101]. In view of these emissions, thinking about industrialization, the transport of waste reduces this problem, when compared. The transport has an emission factor of 62.1 g CO2 equivalents/km.t, and considering a live-bottom trailer with a capacity of 80.3 m^3, it is possible to transport 18.16 t of whole hull/shell or 22.7 t ground, which results in emissions of approximately 1.128 kg CO2 equivalents/km and 1.41 kg CO2 equivalents/km, respectively [102,103].

A disadvantage of the procedure is that it has high localized energy costs, particularly in the drying stages. Considering rotavapor, being the most energetic stage, with a capacity of 50 L, the equipment requires 6300 W of power, which results in an equivalent emission of 295.8 kg_{CO2} in approximately 7 days of operation [101,104]. These types of procedures tend to present this difficulty, and a study was carried out by Carpentieri et al. 2025, where they analyze the Life Cycle Assessment (LCA) of 3 agro-industrial waste recovery processes, with a general structure similar to that of this work, in all of which a greater environmental impact from energy expenditure is notorious, when compared to the other stages, which leads to one of the challenges of improving these approaches [105]. On the other hand, this treatment, in addition to obtaining

fractions enriched in polyphenols, when applied in blanching water, it is possible to treat it, resulting in a possible more appropriate disposal of water, and avoiding an emission equivalent to 0.708 kg CO2 for each m^3 treated [101]. This value does not compensate for the energy expenditure, also considering the amount of water produced in the blanching process, but it is also a compensation parameter, which allows the visualization of a positive perspective on the development of the valorizations.

The approach of this work adopts some of the 12 principles of green chemistry, in full or in part, namely [106]:

- **1) Waste prevention** – The recovery and reuse of solvents in rotavapor avoids unnecessary disposal, preventing waste generation;
- **5) Use of safer solvents and auxiliaries** – Most of the solvents used are hydroalcoholic ethanol/water, which are green solvents;
- **6) Energy efficiency** – Despite the more energetic stages, in the concentration and drying phase, the operation method proposes the fractionation of polyphenols by selective adsorption at room temperature, being an approach of low energy expenditure;
- **7) Use of renewable raw materials** – Use of by-products of almond growth, being a renewable agro-industrial residue;
- **8) Reduction of derivatives** – As it is only a physical treatment, the process does not present the formation of by-products or intermediates;
- **10) Degradation project** – In the case of enriched fractions from a natural source, the dissolved solids will be biodegradable, resulting in a more benign life cycle;
- **12) Safer inherent chemistry** – As it is a controlled process with a reduced use of toxic solvents, it becomes a positive replacement for the problems of sloppiness in waste treatment.

Similarly, this methodology falls primarily under two Sustainable Development Goals (SDGs) (Figure 19) [107].

- **SDG 12) Responsible production and consumption** – This is the main SDG of the work, involving the valorization of agro-industrial waste and the minimization of waste, both agricultural and laboratory/industrial process;
- **SDG 9) Industry, innovation and infrastructure** – The study approach intends to scale up to an industrial level, bringing innovation to the recovery of waste.

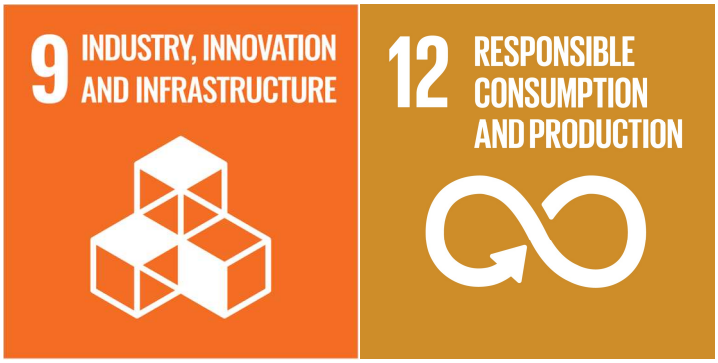


Figure 19 – SDGs of the present work.

10. Materials and equipment

10.1. Reagents

In this work several solvents and reagents were used, which are mentioned in the following table (Table 5).

Table 5 – Reagents used in this work.

Reagents	Brand	Purity	Application
2,2-diphenyl-1-picrylhydrazyl (DPPH)	Thermo Scientific	≥95%	Antioxidant activity assay
Acetic acid glacial (AcOH)	Fisher Scientific	≥ 99.7%	Mobile phase pH adjustment and desorption solvent
Acetonitrile (ACN)	Fisher Scientific	≥ 99.8%	HPLC mobile phase
Chlorogenic acid	ThermoFisher	≥ 95%	Standard for HPLC
Dimethyl sulfoxide (DMSO)	Fisher Scientific	≥ 99.9%	TPC calibration curve solvent
Ethanol (EtOH)	Fisher Scientific	≥ 99.8%	Extraction, desorption and TPC calibration curve solvent
Ethyl acetate (EtOAc)	Fisher Scientific	≥ 99.5%	Desorption solvent
Folin-Ciocalteu reagent	Sigma-Aldrich	---	TPC determination
Gallic acid	Sigma-Aldrich	≥ 97.5%	TPC calibration standard

Table 5 – Continuation.

Methanol (MeOH)	Fisher Scientific	≥ 99.8%	Extraction, desorption and TPC calibration curve solvent
Sodium carbonate	Sigma-Aldrich	≥ 99.5	TPC analysis
Trolox	Sigma-Aldrich	≥ 98%	Antioxidant standard

10.2. Equipments

The following table (Table 6) shows all the equipment used in the work.

Table 6 – Equipment used in this work.

Equipment	Model	Brand	Software
Analytical balance	AC 220/C/2	RADWAG®	---
Centrifuge	CompactarStar CS 4	VWR	---
	Centrifuge 5810 R	Eppendorf	---
Heating/stirring plate	VMS-C7	VWR Advanced	---
HPLC-DAD	---	Knauer	ClarityChrom
Microplate reader	Epoch 2	Biotek	Gen5
pH meter	Level 1	InoLab	---
Pump	P4.1S	KNAUER	---
Rotary evaporator	RE 300	Stuart	---
Soxhlet	HM01 series	Labbox	---
SPE	Visiprep	Supleco	---
Ultrasound bath	Ultrassons-H	P SELECTA	---
	SW1	Sono Swiss	---
UV-Vis spectrophotometer	V-530	JASCO	VWS-580 Spectra
Vacuum oven	VUS-B2V-M-VU 55	VACUCELL	---
Vacuum pump	R20 series	Ibx instruments	---
	RE30022C	Stuart	---

10.3. MIPs

Table 7 shows the MIPs used in this study, highlighting the most important parameters in their synthesis. These polymers were previously prepared, outside the scope of this work.

Table 7 – MIPs used in this work.

Adsorbent	Template	Polymerization technique	Functional monomers	Solvent	Y_M	Y_I	Y_{CL}	$Y_{FM/T}$
MIP-QUER-2 (Q2)	Quercetin	Inverse Suspension	4VP	ACN/DMF (85/15 v/v)	30.43	3.97	51.48	15.69
MIP-QUER-3 (Q3)	Quercetin	Precipitation	4VP	ACN/DMF (85/15 v/v)	6.49	10	51.48	10
MIP-OA-3 (OA3)	Oleanolic Acid	Inverse Suspension	DMAEMA	MeOH	18.68	1.25	20.01	206.56

Where:

Y_M – Mass fraction of functional monomer (FM) and crosslinker in the solution (%);

Y_I – Mole fraction of initiator relative to the FM and the crosslinker (%);

Y_{CL} – Mole fraction of the crosslinker in the mixture FMs + crosslinker (%);

$Y_{FM/T}$ – Mole ratio of the FM to the template molecule.

11. Experimental methods

11.1. Extraction process

In the extractions, different approaches were made, depending on the extraction method and by-product. For hull and shell, when ultrasonic extraction was performed, the proportion of 40 g of ground biomass per 200 mL of solvent was used. In soxhlet, having only been made in the hull, the cartridge was filled with ground biomass (having weighed 84.2083 g) and extracted in 720 mL of methanol. Table 8 shows the main extraction conditions used in this study.

Table 8 – Hull and shell extraction conditions.

Location	Biomass	Solvent	Solvent percentage (v/v)	Extraction method	Extraction time (min)	Concentration (mg/mL)	Identifier
Bragança	Hull	$EtOH/H_2O$	30/70	Ultrasound	30	200	Extraction 1
			30/70		60		Extraction 2
			50/50		30		Extraction 3
			50/50		60		Extraction 4
			80/20		30		Extraction 5
			80/20		60		Extraction 6
		H_2O	100		60		Extraction 7
		MeOH	100	Shoxlet	180	116.96	Extraction 8
	Shell	$EtOH/H_2O$	80/20	Ultrasound	30	200	Extraction 9
Macedo de Cavaleiros	Hull	$EtOH/H_2O$	80/20	Ultrasound	30		Extraction 10
Moncorvo							Extraction 11
Mirandela							Extraction 12

Table 8 – Continuation.

Planalto Mirandês	Hull	EtOH/H_2O	80/20	Ultrasound	30	200	Extraction 13

In addition to the above extractions, in an exploratory phase, two extractions were carried out in shell UAE, in a proportion of 6.25 g ground biomass – 50 mL solvent for 30 min, using ACN and EtOH/H2O 80/20 (v/v). The main extractions, namely shell and localities other than Bragança, were carried out considering the experimental conditions of this initial phase, being limited exclusively to the use of the solvent EtOH/H2O 80/20 (v/v).

After extractions, the mixtures are filtered in paper filters and then centrifuged, until the largest possible amount of suspended solids are removed.

11.2. Sorption/desorption process

In this work, 4 experiments will be carried out, generically represented by figures 20 and 21, using the polymers MIP OA3, Q2 and Q3, with the 3 being packaged in columns, using the SPE, with dimensions L×D = 250 mm × 20 mm. Together, the Q2 and Q3 columns have approximately 50g of adsorbent, while the OA3 column has approximately 22g, resulting in a system with a mass of more than 70g. Table 9 shows the operating conditions of the system, which are given until mass equilibrium is reached. Desorptions are done 3 times for each solvent set and columns, passing 100 mL in each cycle. After each experiment the columns are cleaned with MeOH/AcOH 90/10 (v/v) until the mixture coming out of the columns has an absorbance of less than 0.1 AU.

Table 9 – Operating conditions of the experiments.

Extract	Concentration (mg/mL)	Flow rate (mL/min)	Sorption		Desorption		Identifier
			Cycle	Temperature	Cycle	Temperature	
Hull 50/50 EtOH/H_2O (v/v)	10	1	Open	Ambient temperature	Open	45ºC	Experience 1
			Close				Experience 2
			Open				Experience 3
Blanching water	7.95	1	Open	Ambient temperature	Open	45ºC	Experience 4

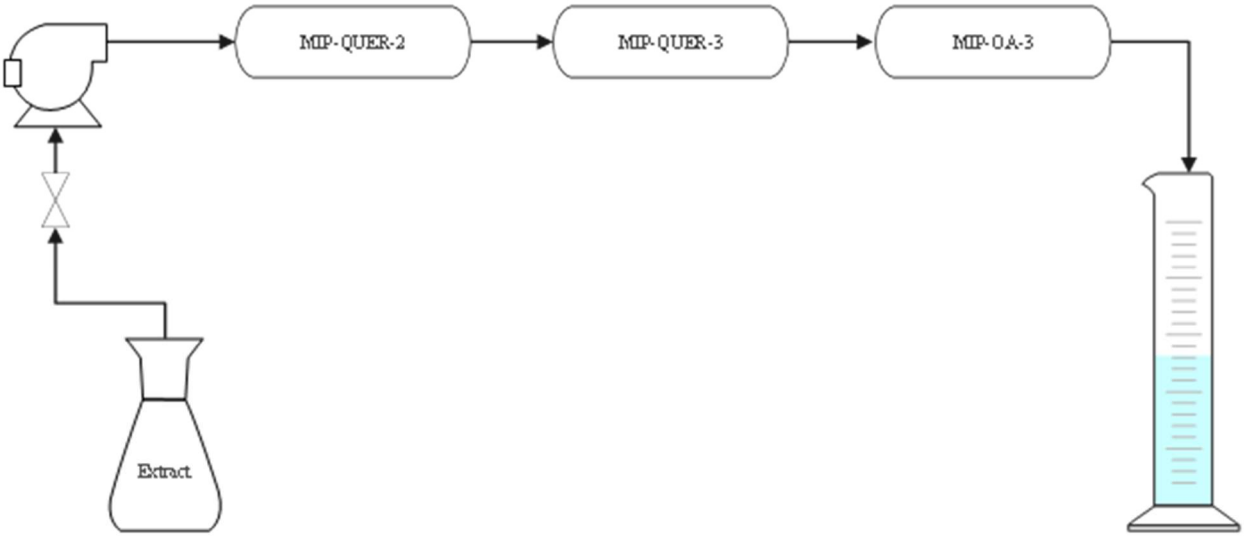


Figure 20 – Generic sorption scheme.

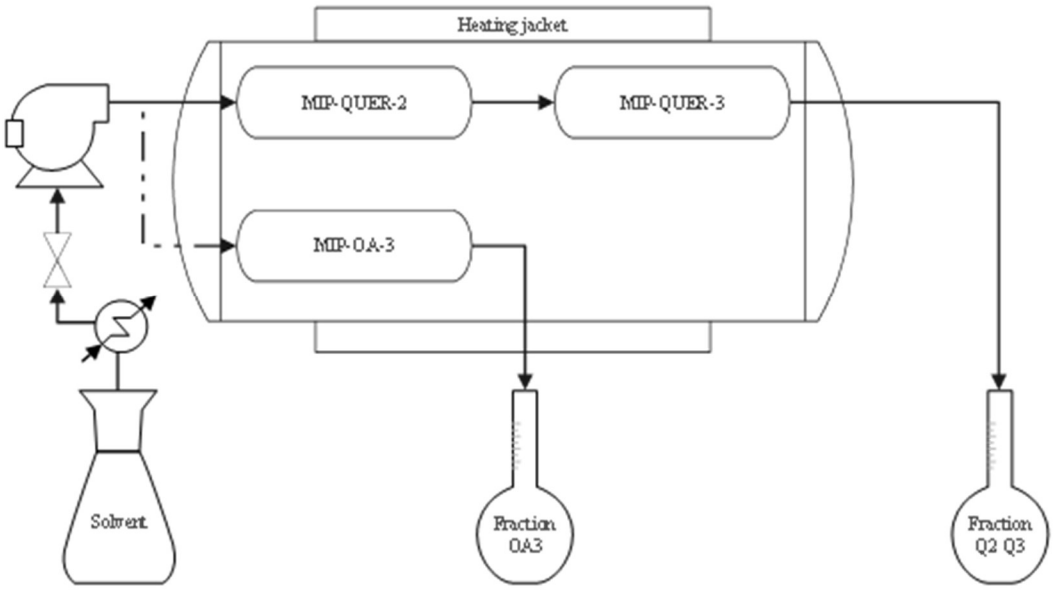


Figure 21 – Generic desorption scheme.

11.3. High-performance liquid chromatography with diode array detection (HPLC-DAD)

HPLC-DAD was used for the identification and quantification of compounds throughout the experimental stages, including the solutions resulting from the desorption process as well as the extractions performed.

The equipment used was produced by Knauer, consisting of a gradient pump (P6.1 L), a column thermostat (CT2.1), an autosampler (6.1 L), and a diode array detector (DAD 6.1 L). The system operates using the ClarityChrom system and analyte separation is performed on a C18 reverse-phase column (Ascentis®, Supelco; 25 cm × 4.6 mm, 5 μm particle size).

For the analysis of the polyphenols, a linear gradient elution method was used using two mobile phases: solvent A (water pH=3/acetonitrile 90:10, v/v) and solvent B (water pH=3/acetonitrile 10:90, v/v). The gradient transitions from pure solvent A to pure solvent B in a period of 30 minutes at a flow rate of 1 mL/min, at a temperature of 45ºC.

The detection of analytes was done at two wavelengths: 280 nm, analyzing polyphenols in general, and 360 nm, visualizing almost exclusively flavonoids. All samples are filtered through 0.45 μm nylon syringe filters prior to injection.

11.4. UV-Vis ultraviolet visible

Ultraviolet-visible (UV-Vis) spectroscopy was used during the sorption and desorption processes in columns for rapid analysis, mainly to monitor when the system comes into equilibrium. The readings were performed using a VWR P9 Double Beam spectrophotometer with 1 cm path length quartz cuvettes. The scan was carried out between 200 – 600 nm and the quantification was performed at 280 nm.

11.5. Total phenolic content analysis

The method of quantification of the total phenolic content applied in this work is based on the article by Gomes et al. 2024, being expressed in gallic acid equivalents per gram of extract (mg GAE/g Extract) [28].

The first step of the procedure is the construction of gallic acid curves, and solutions with the following concentrations are prepared: 0.5, 0.25, 0.1, 0.05, 0.01 and 0.005 mg/mL. In graduated centrifuge tubes, 250 µL of each of the gallic solutions and a solvent control are added, followed by the addition of 1.25 mL of Folin-Ciocalteu reagent (1:10 v/v distilled water) and 1 mL of sodium carbonate solution (75 g/L). The tubes are stirred in vortex for 15 seconds and then placed in an oven at 40ºC for 30 min, in the dark. After the reaction the tubes are centrifuged for 2 minutes at 6500 RPM and 250 – 300 µL of the reaction solutions are transferred 3 times to 96 wells microplate and the absorbance is read on a microplate reader (Epoch 2, BioTek Instruments, USA) at 765 nm.

For extracts and fractions, the same procedure is carried out, in triplicate, and the concentration of the respective fractions is known, and dilutions can be made if necessary. Throughout the work, different approaches were taken in the determination of concentrations:

1. The extracts are evaporated in a vacuum (Stuart RE 300 rotary evaporator) and freeze-dried, then redissolved to a known concentration;
2. The fractions are evaporated in a vacuum until the solid is obtained, and then redissolved to a known concentration;
3. A portion of the extract/fraction to be used is evaporated in a vacuum until only solid is obtained, obtaining the concentration of the solution.

After reading the absorbances of the samples and moving them to an equivalent concentration of gallic acid, the conversion to gallic acid equivalents per gram of extract is carried out according to the following formula.

$$\left[\frac{mg\ GAE}{g\ Extract}\right] = \frac{\left[\frac{mg\ GAE}{mL}\right]}{\left[\frac{g\ Extract}{mL}\right]} \quad (1)$$

During the work, it was noted that, even changing the hydroethanol and methanolic fractions, when compared to the same volume analyzed in the plates, the calibration curve had no significant changes, only fluctuations in the face of experimental uncertainty, and were thus performed, for the optimization of the procedure, in the following solvents: H_2O, EtOH/H_2O 30/70 (v/v), EtOH/H_2O 50/50 (v/v), EtOH/H_2O 80/20 (v/v), MeOH, DMSO.

11.6. Antioxidant activity analysis

The method of antioxidant activity applied in this work is based on the work carried out by Gomes et al. 2024, and in the end the concentration of extract that neutralizes 50% of the DPPH free radicals (EC50) was obtained, under the experimental conditions carried out [28].

In the performance of the procedure, 3 equal sequences of dilutions are made for each extract/fraction analyzed. Once the dilutions are made, 30 µL of each, as well as one blank, are transferred 3 times to 96 wells microplate and then 270 µL of DPPH solution 6×10^{-5} M is added to each sample well. After rapid addition of the reagent, the microplate is placed in the dark at room temperature for 1 hour, and the absorbance reading is quickly taken at 517 nm after the reaction time. As a term of comparison, the analysis with Trolox, a strong commercial antioxidant, is also made.

This test adopts the same approach in the determination of concentrations as in the TPC analysis, but also in terms of dilution factors. Thus, analyses can be separated into 3 main types:

1. Reduce the fractions to solid and only then proceed with the analysis, performing serial dilutions based on concentrations;
2. Concentrate the fractions and determine EC50 in terms of dilution factor (EDF50);
3. Perform the analysis with the original fraction based on dilution factors while, in parallel, a part of the fraction is reduced to solid, in order to determine the concentration of the fraction and the dilutions made.

The type of approach adopted depends on the solubility and antioxidant capacity of the fractions, and in the tests carried out the turning point between a radical uptake greater than 50% and a lower one.

The percentage of Free Radical Scavaging (FRS%) is calculated from the difference in the absorbance of the white and the sample, and is expressed by the following formula.

$$FRS\% = \frac{A_b - A_s}{A_b} \times 100 \quad (2)$$

Where:

A_b – Control absorbance;

A_s – Absorbance of the sample.

After obtaining the percentages, a linear interpolation is made with the two closest values obtained from 50%. The calculation is given by the following expression:

$$EC50 = [Upper\ Limit] - \frac{([Upper\ Limit] - [Lower\ Limit])(Upper\ FRS\% - 50)}{Upper\ FRS\% - Lower\ FRS\%} \quad (3)$$

This method allows linear interpolation since, as proven in studies of the area and in the experimental results obtained during the work, in the representation of the FRS% as a function of the concentration of the sample it is possible to verify a constant zone at higher concentrations, followed by a linear decline until reaching another constant zone at lower concentrations [108].

With the correlation $C_f = \frac{C_i}{DF}$, where DF is the dilution factor, it is possible to express equation 3 as a function of the DF without influence of concentration and still maintaining the linear correlation with FRS%, since the concentration is linearly

correlated with the inverse of the dilution factor. The equation after transformation is as follows:

$$EDF50 = \frac{1}{\frac{1}{DF_{Upper\ Limit}} - \frac{(\frac{1}{DF_{Upper\ Limit}} - \frac{1}{DF_{Lower\ Limit}})(Upper\ FRS\% - 50)}{Upper\ FRS\% - Lower\ FRS\%}} \quad (4)$$

12. Results and discussion

12.1. Extractions

Initially, shell extractions were carried out, using ACN and EtOH/H_2O 80/20 (v/v) as solvents, in order to compare the efficiency in the use of a typical organic solvent in this practice and hydroethanolic fractions in general. The solutions obtained were analyzed via HPLC-DAD, resulting in the spectra of figures 22 and 23.

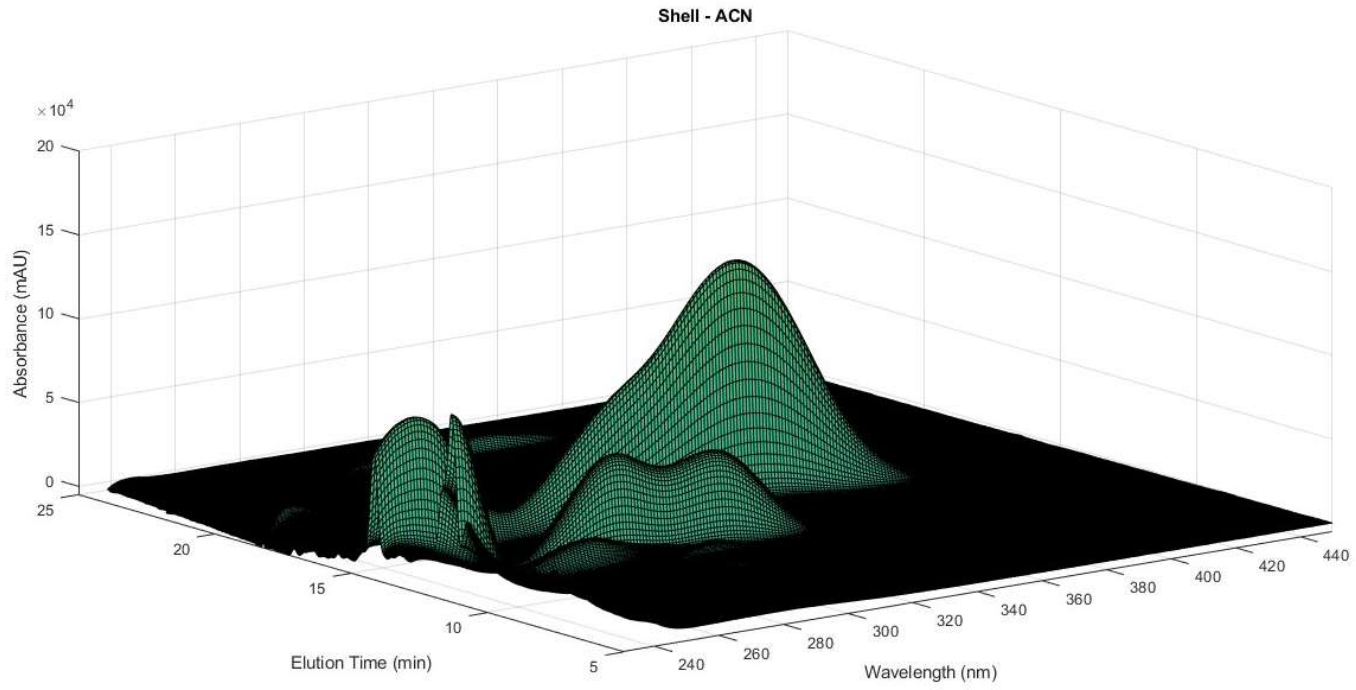


Figure 22 – HPLC-DAD analysis of shell extraction in ACN.

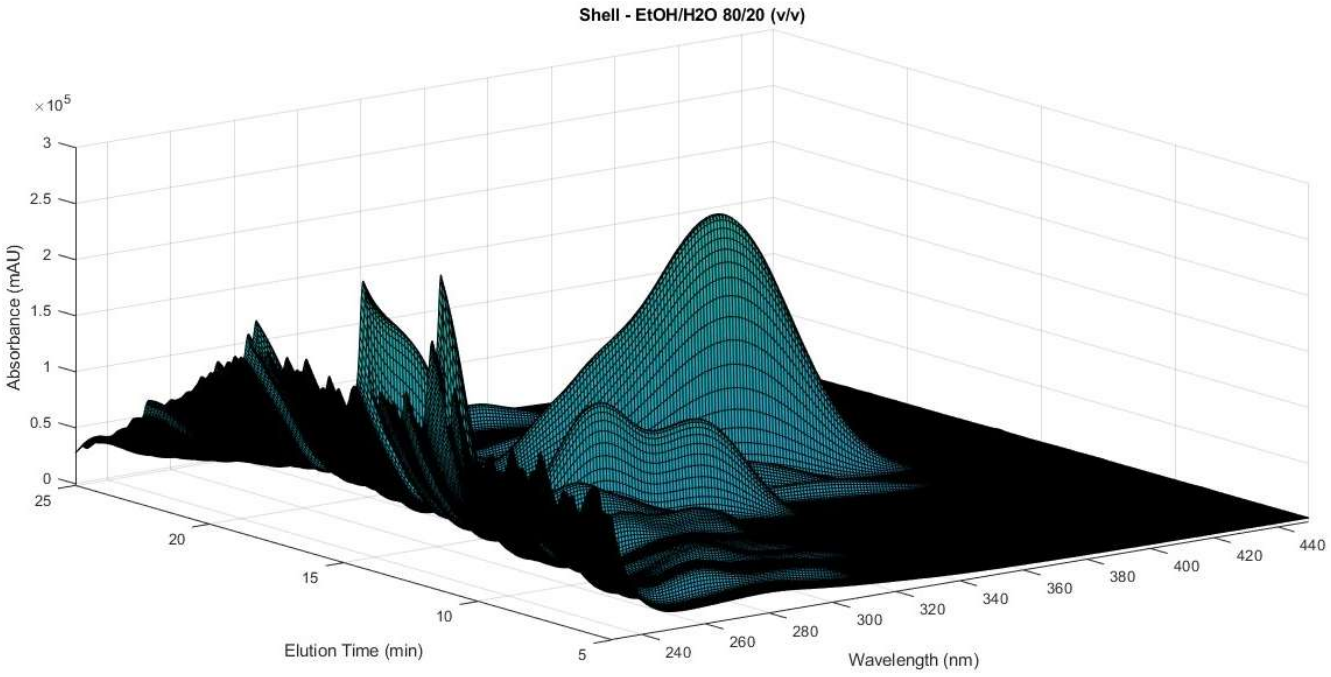


Figure 23 – HPLC-DAD analysis of shell extraction in EtOH/H_2O 80/20 (v/v).

Comparing the two analyses, it is possible to verify that the hydroethanolic extraction was superior, with a difference of up to twice the concentration. Based on this result, in line with the purpose of the work, the following extractions were performed using the solvent mixture, in different fractions, as well as using MeOH in soxhlet, to compare with another method/organic solvent typical in this type of procedures. The mass income obtained, as well as the TPC, are shown in table 10, following the identifiers in table 8.

Table 10 – Mass yields of extractions.

Identifier	Yield (%)	TPC (mg GAE/g Extract)
Extraction 1	11.91	135.16
Extraction 2	8.38	152.14
Extraction 3	17.94	151.28
Extraction 4	12.62	188.74
Extraction 5	5.86	176.93

Table 10 – Continuation.

Extraction 6	6.11	193.97
Extraction 7	9.00	49.27
Extraction 8	8.88	76.11
Extraction 9	---	---
Extraction 10	1.85	209.49
Extraction 11	3.19	164.65
Extraction 12	3.20	155.31
Extraction 13	6.11	136.62

Analyzing the yields first, it can be seen that when doubling the extraction time the value decreases, with the exception of the 80/20 solvent (Extraction 5 and 6), which had a slight increase. However, the TPC increased, but not linearly with the extraction time, with a maximum increase of 25% in the 50/50 solvent (Extraction 3 and 4). This correlation indicates that, although the phenolic mass balance has not yet been reached at 30 min, it is not far from being achieved, and it is not advantageous to increase the time and energy consumption in extraction.

Analyzing the remaining TPCs, it is verified that the extraction in water and in soxhlet (Extraction 7 and 8) have low phenolic concentration, revealing that the addition of ethanol is a crucial factor for a more efficient extraction of polyphenols, due to its amphiphilic character, and that soxhlet is not an efficient method for this purpose, even using methanol.

After shell extraction, no quantifiable mass was obtained, and the hypothesis of analysis in this work was discarded.

Among the analyzes performed, the AA of some specific extracts was also analyzed, namely: Extraction 3 (higher yield), Extraction 10 (higher TPC), Extraction 12 (TPC comparable to Extraction 3, in the same extraction time) and Extraction 8 (Soxhlet).

- Extraction 3 – EC50 = 0.341 mg/mL;
- Extraction 10 – EC50 = 0.157 mg/mL;
- Extraction 12 – EC50 = 0.763 mg/mL;

- Extraction 8 – EC50 = 0.476 mg/mL.

Making a comparison between Extraction 3 and 12 shows that, despite the similar value of TPC, EC50 varies more than double, which indicates that the qualitative composition of the extracted compounds varies between them. Extraction 10 was performed under the same conditions as Extraction 12, but has the highest TPC and lowest EC50, indicating that the marked variation is due to the location and not to the solvent used. The use of soxhlet resulted in a high EC50, which is consistent with the TPC value obtained.

In view of the results obtained, in the following experiments Extraction 3 will be used, being the one with the highest yield and also, being an equivolumetric fraction of EtOH/H2O, it presents both phenolic acids and flavonoids in significant quantities, having TPC values = 151.28 ± 4.34 mg GAE/g Extract and EC50 = 0.341 ± 0.039 mg/mL.

12.2. Experience 1

In this experiment, desorptions were made with H2O, EtOH/H_2O 50/50 (v/v), EtOH/H_2O 75/25 (v/v), EtOH, MeOH, MeOH/AcOH 90/10 (v/v), Acetone and Ethyl Acetate, all of which were done individually except for the fractions of H2O, Acetone and Ethyl Acetate, being performed with a set of 3 columns. The order of the solvents follows the one listed above, but only the following ones (Figures 24 and 25) with the highest expected phenolic potential are analyzed at the TPC and AA levels, in addition to the sorption output.

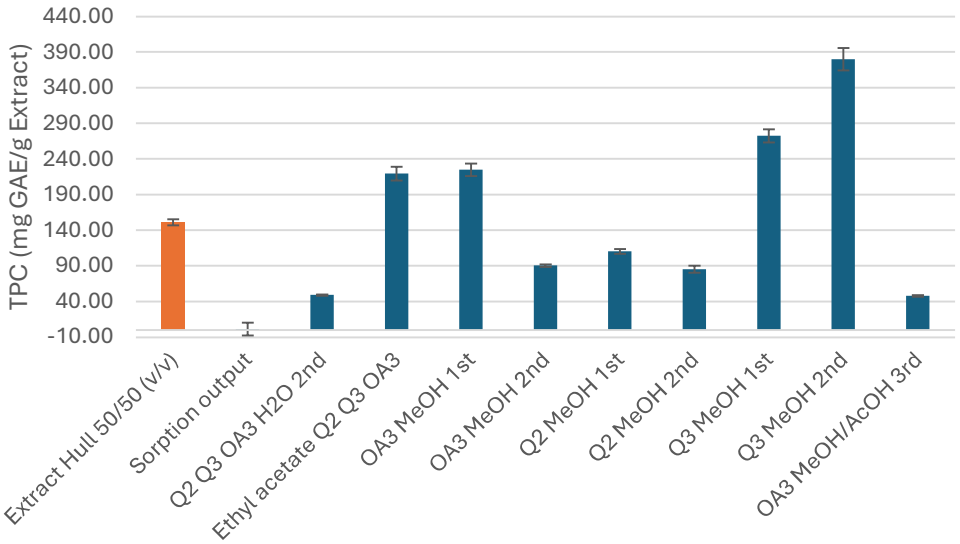


Figure 24 – TPCs (n=3) of experience 1.

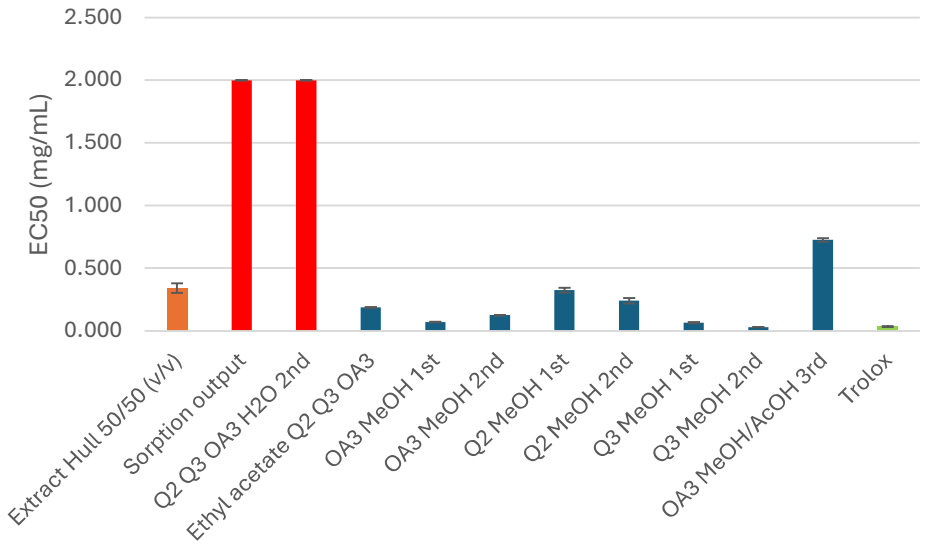


Figure 25 – EC50 (n=3) of experience 1.

Analyzing Figure 24, it can be seen that only four fractions presented phenolic concentration higher than the initial extract: ethyl acetate, OA3 MeOH 1st, Q3 MeOH 1st and Q3 MeOH 2nd, the latter being the fraction with the highest TPC. Observing the desorption profile, it is noted that in the OA3 and Q2 columns, the second methanol fraction presented lower values than the first, which may be related to a more efficient removal of phenolic compounds during the first pass of the solvent. In addition, the

residual presence of the previous solvent in the column may also have influenced the results obtained, especially if it still contained phenolic compounds in significant concentration. However, in column Q3 the opposite trend was observed, with the second methanolic fraction showing higher TPC compared to the first. In this case, it is possible that the residual presence of the previous solvent contributed to a partial dilution of the first fraction collected. This discrepancy suggests differences in the affinity and/or composition of phenolic compounds retained and subsequently desorbed in each system. In the final passage (ethyl acetate) there is still an increase in phenolic content, which suggests the removal of compounds with lower polarity and greater affinity with the MIPs that are not eluted in the previous solvents, being solvents of significant polarity. Also considering the sorption output, it is strongly concluded that the polymers preferentially retained the polyphenols, observing a negligible TPC value. These differences, which will be notorious throughout the remaining experiments, are a decisive indicator of selectivity in the MIPs, revealing a strong connection to polyphenols and varying according to their structure.

Figure 25 also shows an inverse proportion between TPC and EC50, but not linear, verifying that there are fractions that had a TPC higher than that of extract and a lower EC50. This discrepancy in linearity may be due to the possible nature of the phenolics present in the sample, having isolated compounds that, at the current concentration, represent an equivalent low amount of gallic acid, but that present high antioxidant activity, due to the greater presence of hydroxyl groups (flavonoids).

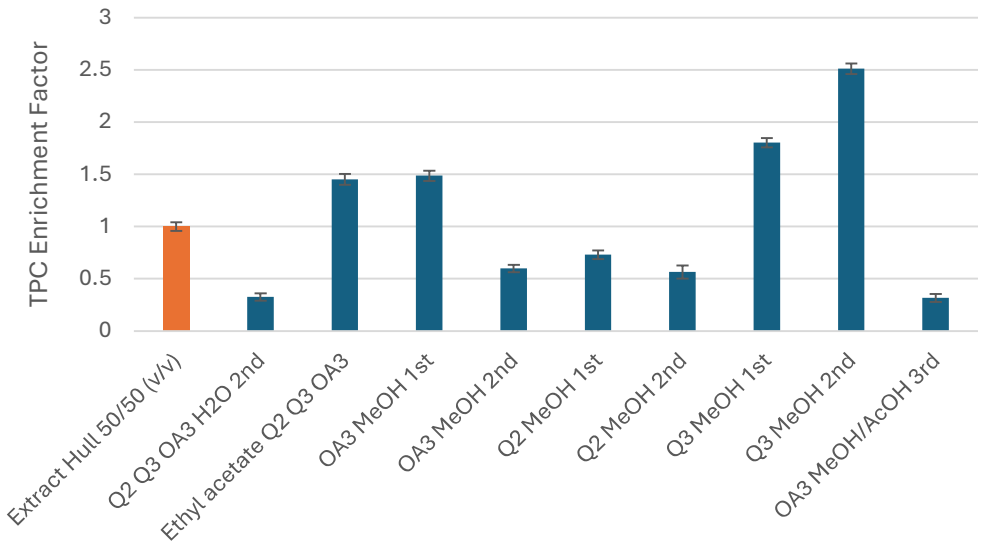


Figure 26 –TPC enrichment factor relative to the initial extract (n=3) of experience 1.

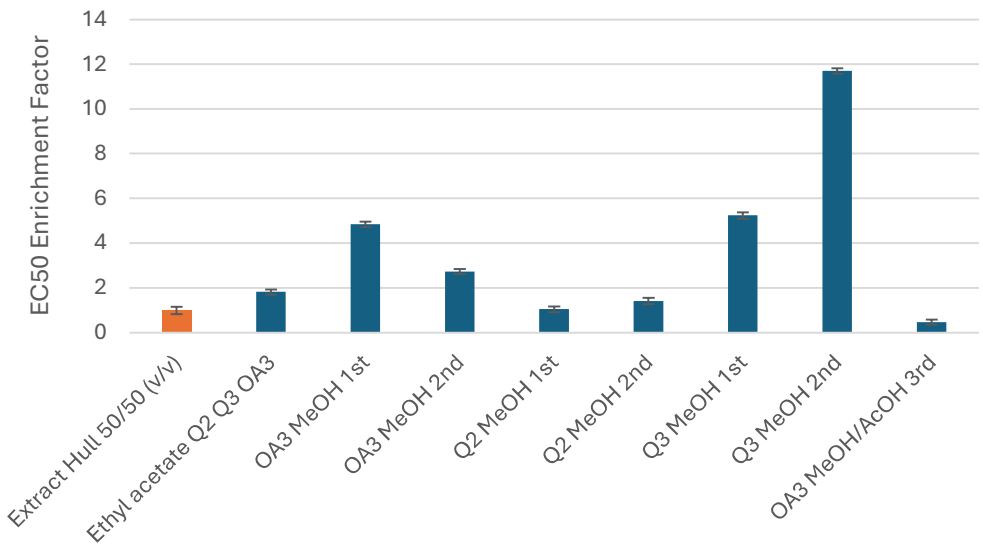


Figure 27 –EC50 enrichment relative to the initial extract factor (n=3) of experience 1.

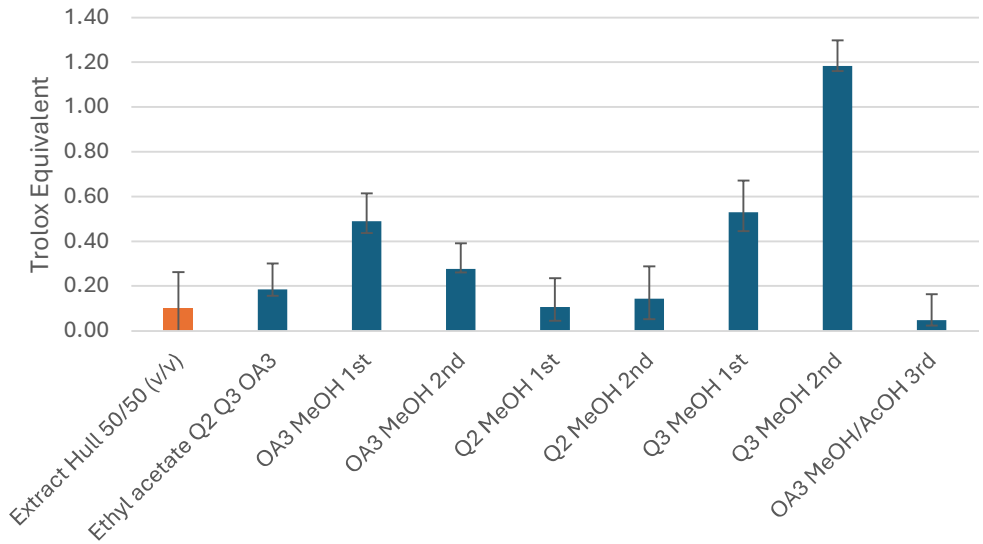


Figure 28 –Trolox equivalents (n=3) of experience 1.

Figures 26, 27 and 28 help to have a better perception of what was previously commented, observing a significant enrichment of TPC and EC50 in the Q3 MeOH 2nd fraction, in addition to being the only fraction that exceeds an equivalent unit of trolox. This performance can be explained by the following chromatogram (Figure 29).

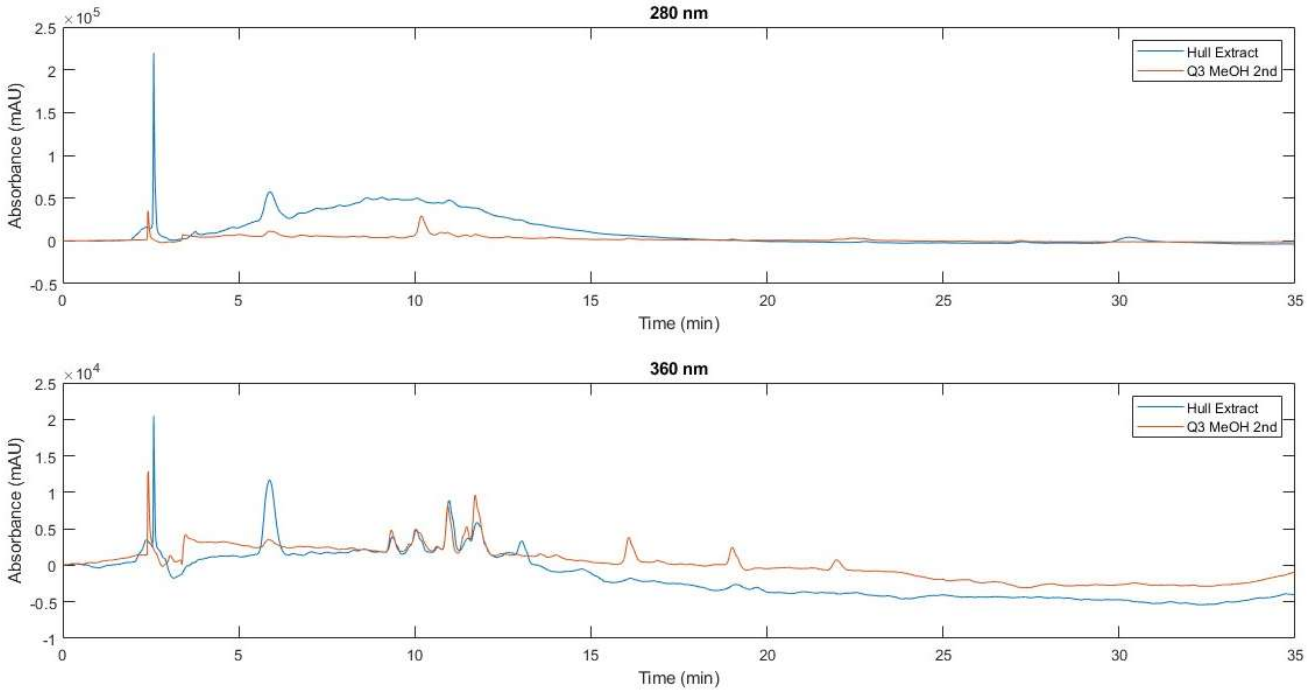


Figure 29 – Chromatogram of the initial extract at 5 mg/mL and Q3 MeOH 2nd fraction from experiment 1 at 280 and 360 nm.

As observed in the chromatogram, at 280 nm, being a general wavelength for polyphenols, it is notorious that the fraction is less concentrated in phenolic content, but when analyzed at 360 nm there is a higher concentration of some compounds, which are flavonoids. This selective enrichment caused the generalized phenolic load to be compressed preferentially to flavonoids, a class of compounds that by its nature has a high antioxidant capacity, tending to be higher than the capacity of phenolic acids, with hull being rich in this type of compounds.

12.3. Experience 2

In this experiment, unlike the others, only one desorption was performed per column for each solvent, thus having only one fraction per solvent-column set. The order of passage of solvents in the OA3 and Q2 columns individually, after a first cleaning with 100 mL of cold H_2O in the set of 3 columns, was as follows: H_2O, EtOH/H_2O 50/50 (v/v), EtOH, MeOH/EtOAc 50/50 (v/v), MeOH, MeOH/AcOH 90/10 (v/v). During the

operation of this experiment, the Q2 column presented fractions with solubility and low concentration problems, and thus only the fraction of MeOH/AcOH 90/10 (v/v) was analyzed. All the fractions mentioned were analyzed, both at the level of TPC and AA (Figures 30 and 31).

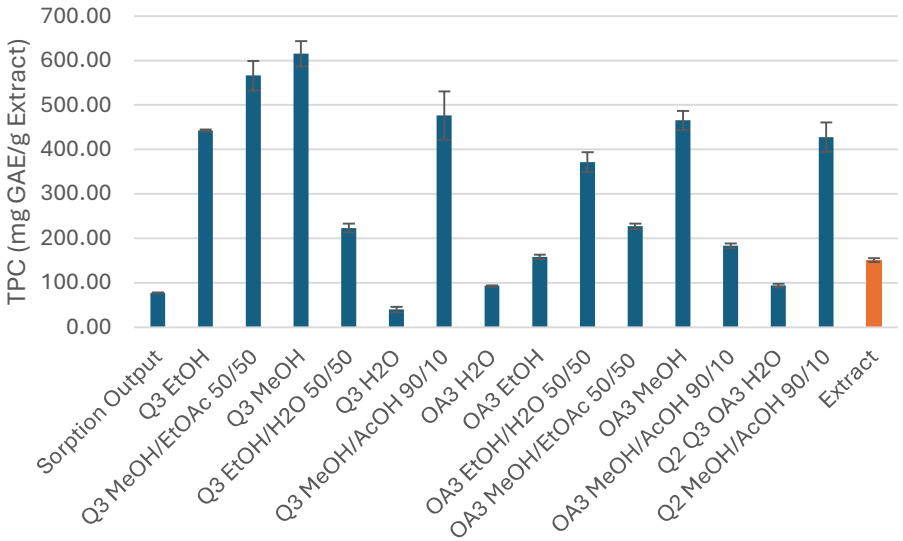


Figure 30 – TPCs (n=3) of experience 2.

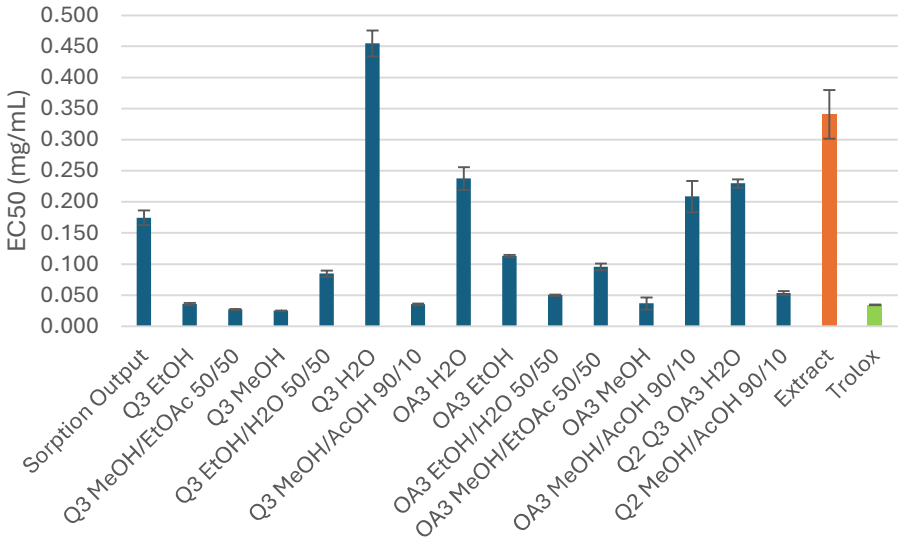


Figure 31 – EC50 (n=3) of experience 2.

Analyzing Figure 30, it can be seen that the Q3 column, until it reaches the MeOH solvent, always has an increase in the TPC value, which indicates a constant and accentuated removal of phenolic compounds, increasing the concentration and/or changing the composition along the fractions. This increase is also a reflection of the decrease in the polarity of the solvents, in addition to the possible hydrophobic character of the compounds to be removed and more intense interactions that they have with the adsorbent, requiring solvents that penetrate better into the pores for a more accentuated removal. The fraction of MeOH/AcOH decreasing, relative to that of MeOH, means that there were possibly not many compounds that required an acid medium for their desorption, being a factor that had no influence on it, or that the amount of compounds retained more strongly did not have a significant mass to influence the gallic acid equivalents. In the OA3 column, two trends are mainly observed: high enrichment in hydroalcoholic desorption and in methanolic desorption. Unlike column Q3, which increased TPC with a certain pattern, indicating a gradual removal, in this column large point enrichments are observed, which suggests a marked removal of different families/compounds. These results are a reflection of the nature of the functional monomers of the polymers, and the MIP OA3, having DMAEMA as functional monomers, is absorbed by interactions more susceptible to the composition of the solvent, and may have point releases due to the great influence of the solvent, being typically associated with phenolic acids and/or hydrophilic compounds. On the other hand, the Q2 and Q3 MIPs, having 4VP as a functional monomer, establish more specific and stable interactions with the target compounds, allowing the retention of different compounds belonging to the same phenolic subclass, which are fractionated in the different elutions.

Figure 31 shows the evolution of EC50 in the different fractions, and comparing it with Figure 30 shows that it remains inversely proportional to the TPC. This standard is proof that there is a real enrichment of polyphenols, and there are no contaminant compounds of a reducing nature that influence the TPC and AA tests, resulting in discrepancies.

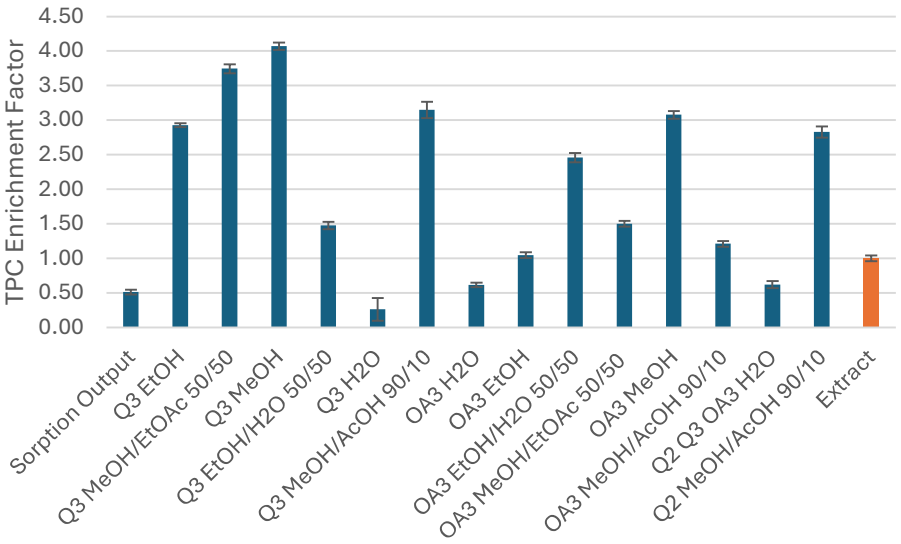


Figure 32 –TPC enrichment factor relative to the initial extract (n=3) of experience 2.

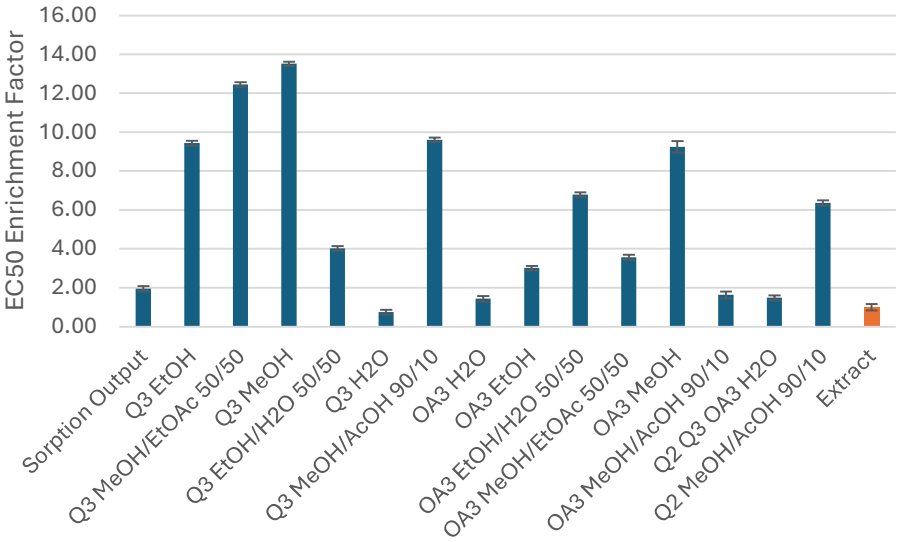


Figure 33 –EC50 enrichment factor relative to the initial extract (n=3) of experience 2.

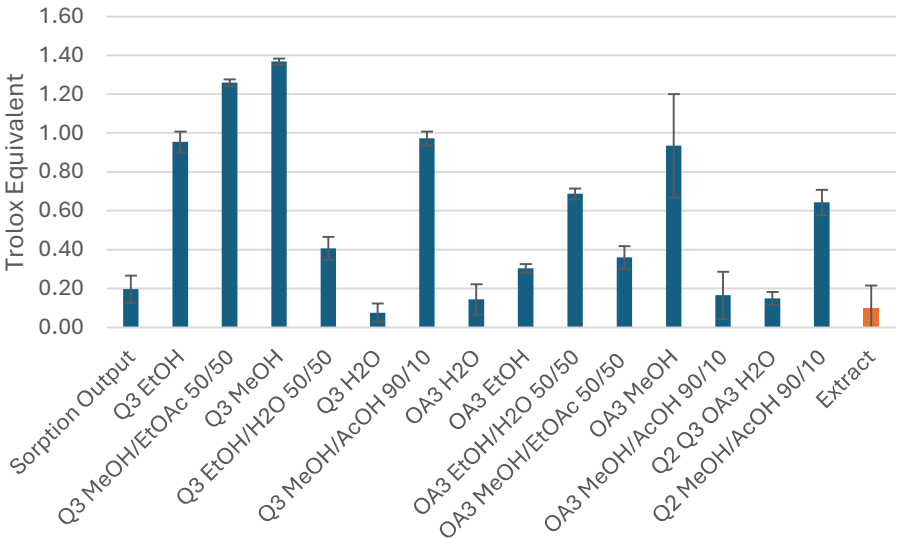


Figure 34 –Trolox equivalents (n=3) of experience 2.

Figures 32, 33 and 34 help to understand the data obtained, showing how much the fractions were enriched when compared with the initial extract, in addition to comparing with the trolox, revealing high values of enrichment. Within the fractions obtained, only two proved to be higher than an equivalent unit of trolox, being the fraction of MeOH/EtOAc and MeOH of Q3, but values very close to those of the trolox were also observed.

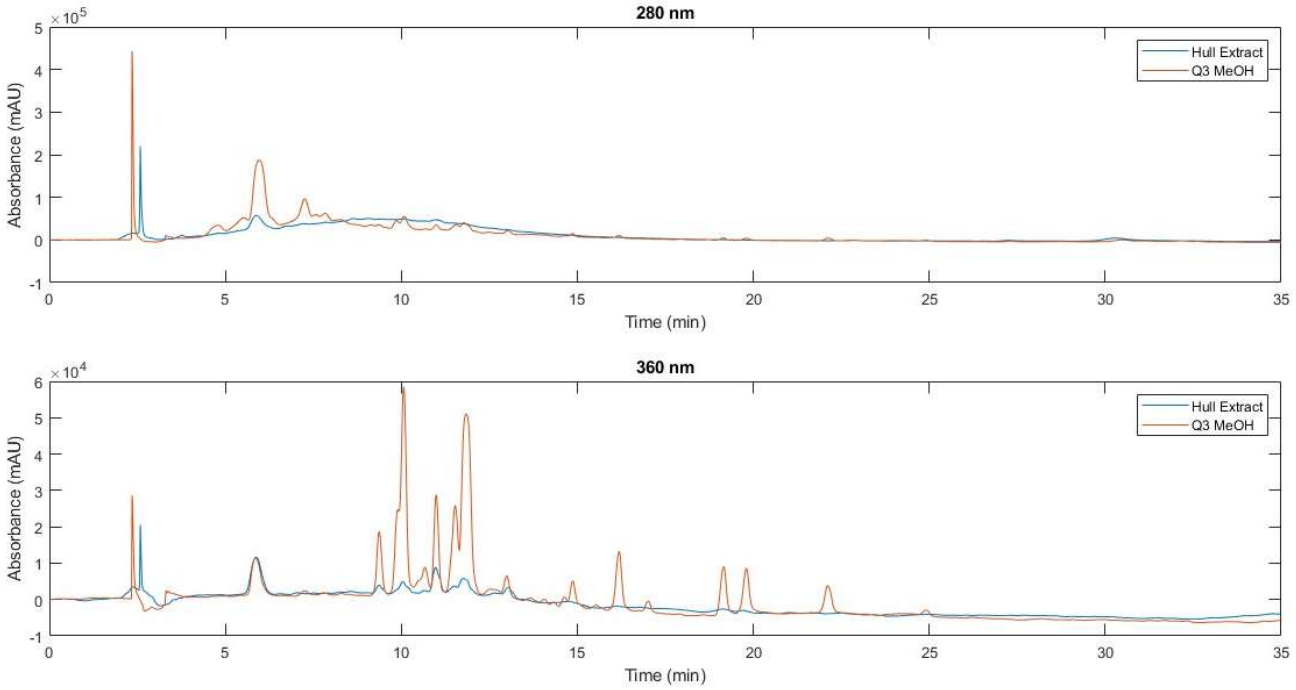


Figure 35 – Chromatogram of the initial extract at 5 mg/mL and Q3 MeOH fraction from experiment 2 at 280 and 360 nm.

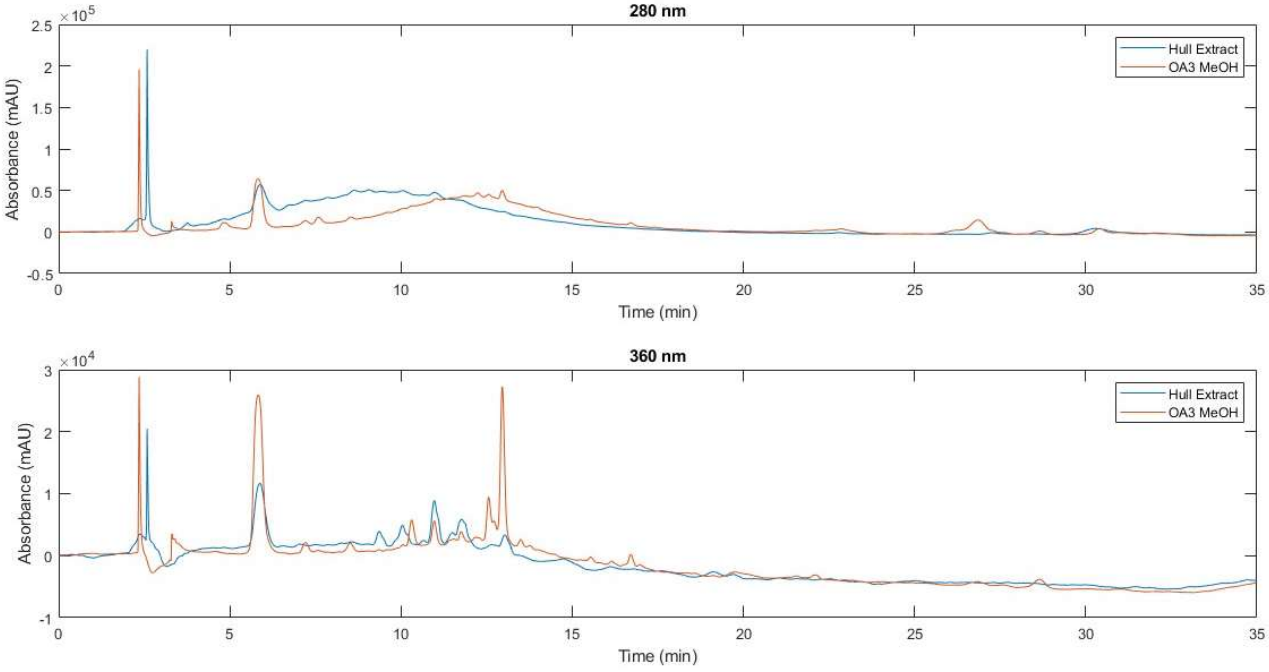


Figure 36 – Chromatogram of the initial extract at 5 mg/mL and OA3 MeOH fraction from experiment 2 at 280 and 360 nm.

Through the chromatogram of the best fractions of each column (Figures 35 and 36) it is possible to draw some about the reasons why the fractions present such significant enrichments. Analyzing the Q3 chromatogram first, there is a high enrichment of flavonoids in the fraction, conferring a high phenolic content of compounds with great antioxidant potential. In the OA3 fraction, there is also an enrichment of flavonoids, but above all there is a high concentration of chlorogenic acid (peak that appears around 6 min). This same peak appears in the Q3 analysis, but as is observable, it was not the most concentrated compound in the fraction, which resulted in the difference in the representation of gallic acid equivalents and antioxidant activity. Based on the chlorogenic present in the methanolic fraction of OA3, it is expected that the ethanolic fraction will be highly concentrated of this phenolic acid, due to its high compatibility with hydroalcoholic systems, which would have given this fraction a high TPC value. This difference in chlorogenic between the columns also allows to establish a relationship between the functional monomers and the type of compounds, verifying that DMAEMA preferentially absorbs compounds such as chlorogenic and similar in nature, while the results suggest greater retention of flavonoids by MIPs containing 4VP, being compounds with several hydroxyl groups, which allows them to form several hydrogen bonds, and π–π stacking in relation to the aromatic structure.

12.4. Experience 3

In this experiment, after passing 100 mL of cold H2O through the 3 columns, the following order of solvents was used in the desorptions: H_2O, EtOH/H_2O 50/50 (v/v), EtOH, EtOH/AcOH 90/10 (v/v), MeOH, MeOH/AcOH 90/10 (v/v). The purpose of this experiment, unlike the others, is to determine the enrichment of the fractions without necessarily obtaining a solid first, thus aiming at a more applicable approach at an industrial level in obtaining solutions rich in polyphenols applicable at the insecticidal and antimicrobial level. Thus, the 100 mL solutions were reduced until a 5 mL solution was obtained, and after filtration, AA tests were performed, obtaining EDF50 values and the final total volume of each fraction necessary to reach EC50 (Figures 37 and 38). Among all the fractions obtained, only the most promising of each column/solvent were analyzed.

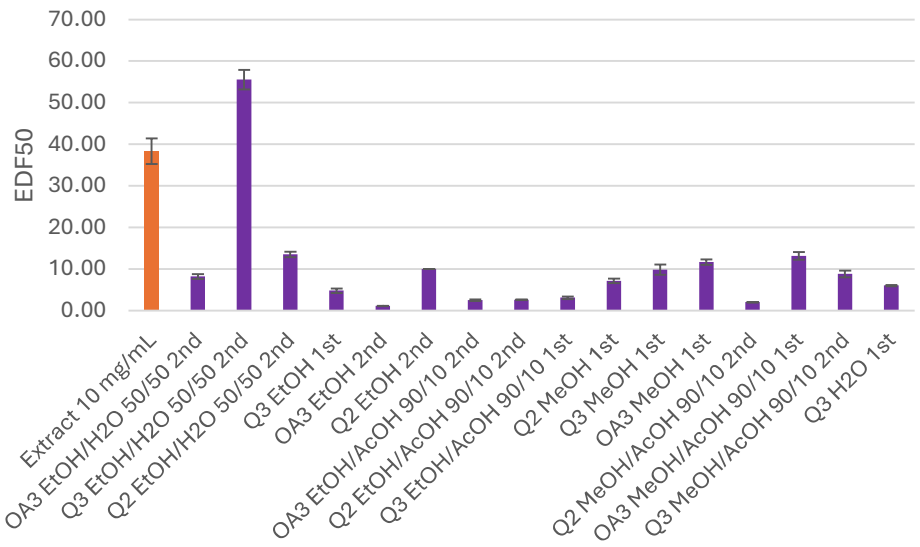


Figure 37 – EDF50 (n=3) of experience 3.

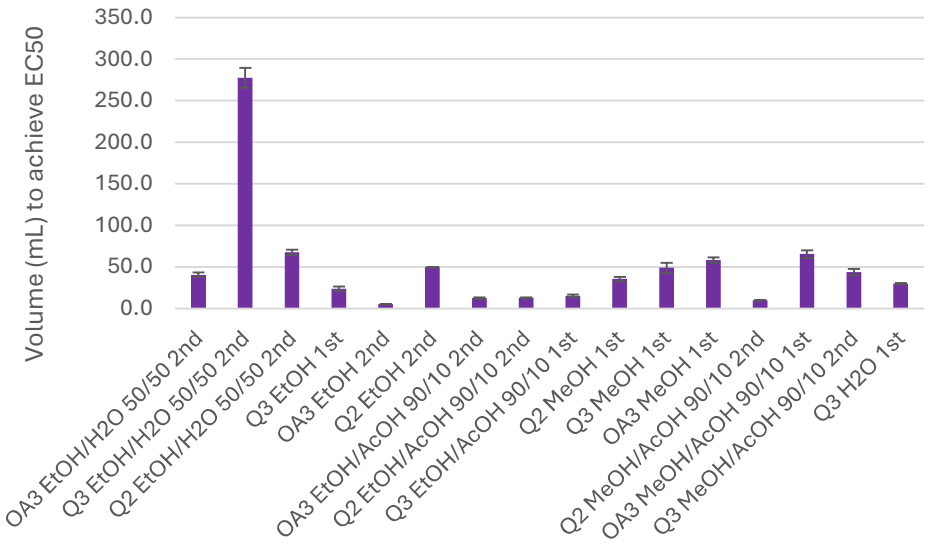


Figure 38 – Final volume to achieve EC50 (n=3) of experience 3.

Within the results obtained, it is notorious that the fraction of Q3 EtOH/H_2O 50/50 2nd is the one that stands out the most in antioxidant power, being the only one that needs to be diluted by more than two times to reach EC50, while the others need to be concentrated to reach the same potential. This reveals a high affinity of the target compounds with the Q3 column, as well as with the hydroethanolic solvent. Analyzing the other fractions, it is notorious that the EtOH/AcOH solutions of Q2 and Q3 have lower

power than those of EtOH, which means that not many molecules that require acid medium for removal were retained, but the opposite happens in the OA3 column, reflecting once again the influence of the medium (pKa) and the interactions of the functional monomer (DMAEMA). The remaining fractions follow a similar profile to the previous experiments, observing good results in the methanol solutions, when compared to the others.

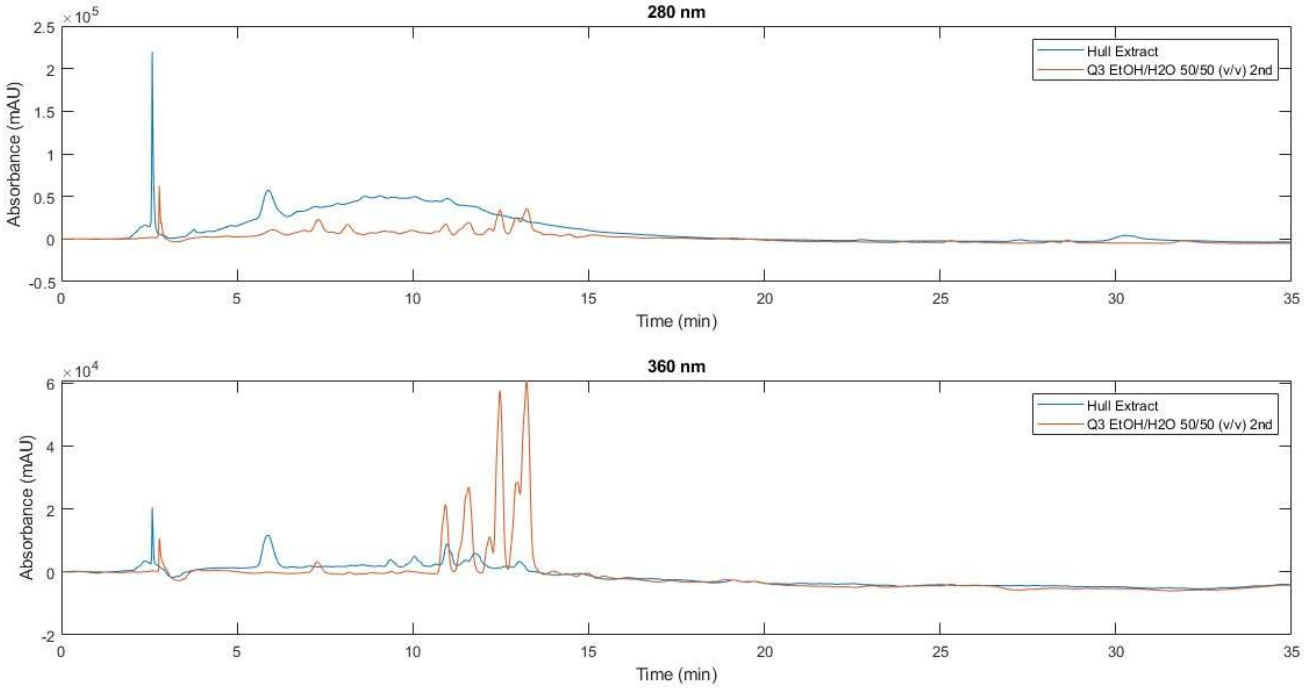


Figure 39 – Chromatogram of the initial extract at 5 mg/mL and Q3 EtOH/H_2O 50/50 2nd 1 mg/mL fraction from experiment 3 at 280 and 360 nm.

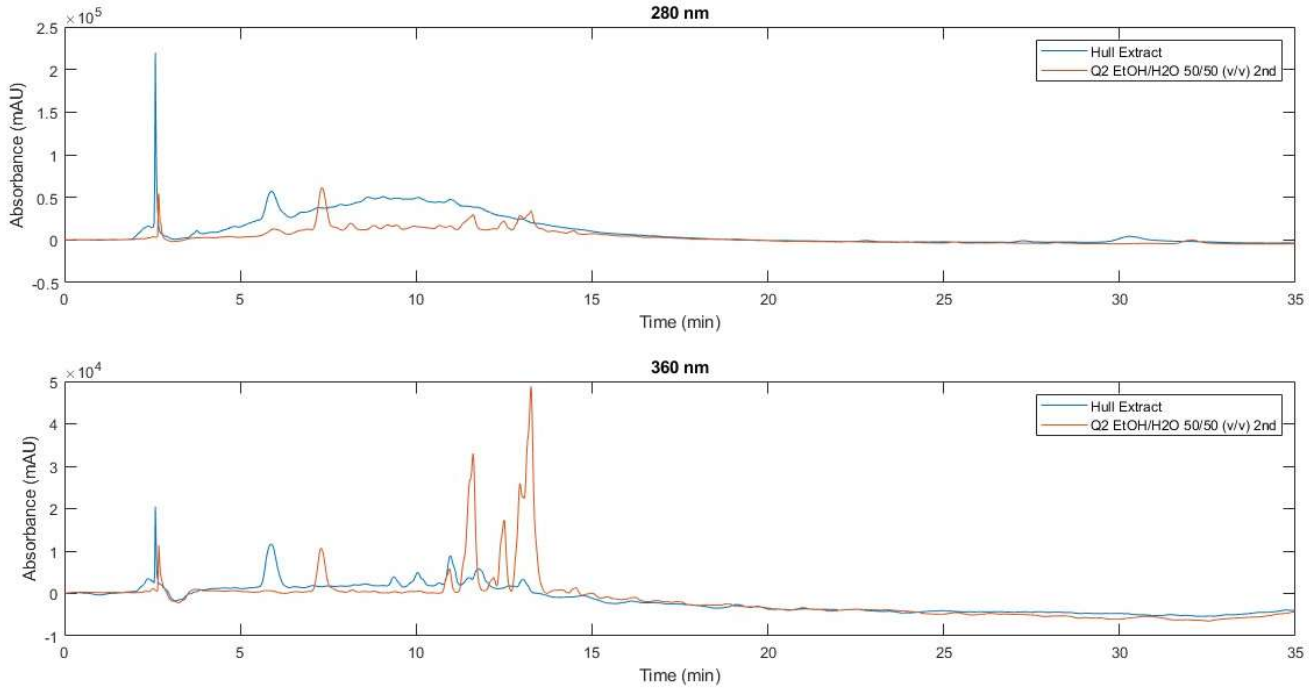


Figure 40 – Chromatogram of the initial extract at 5 mg/mL and Q2 EtOH/H_2O 50/50 2nd 1 mg/mL fraction from experiment 3 at 280 and 360 nm.

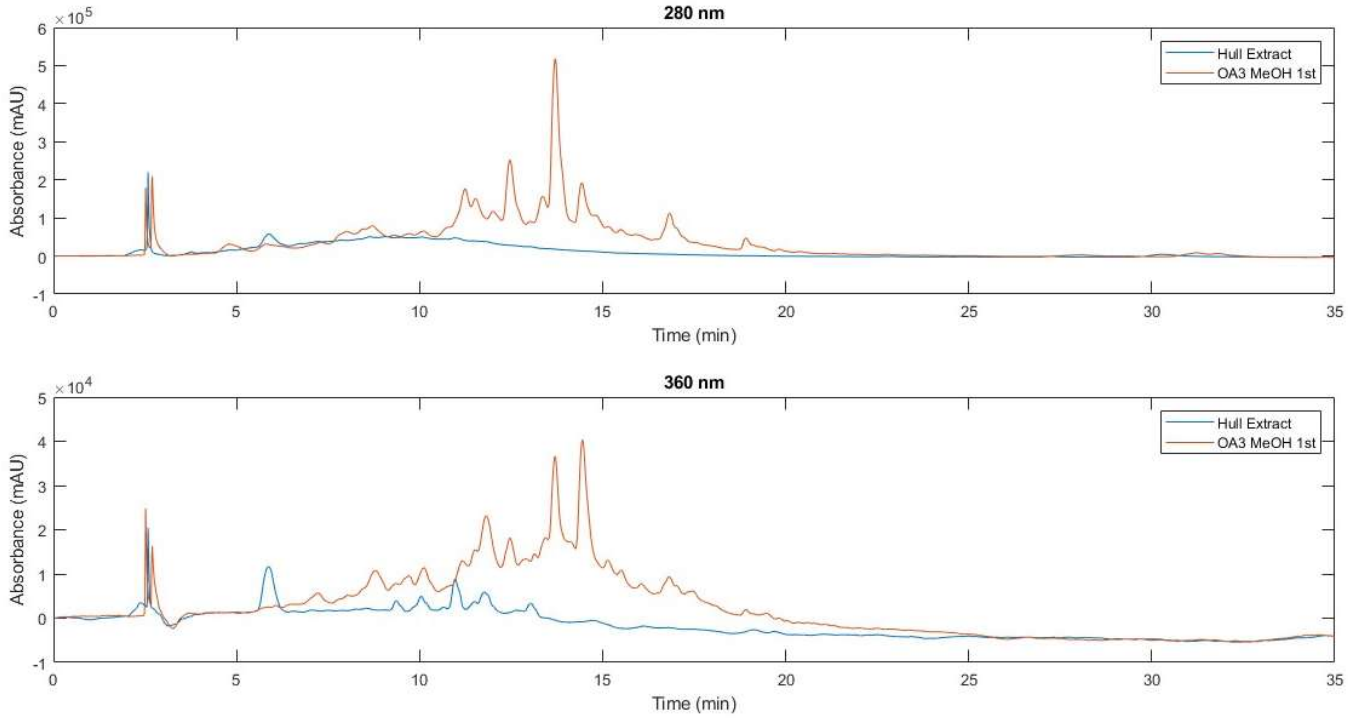


Figure 41 – Chromatogram of the initial extract at 5 mg/mL and OA3 MeOH concentrated fraction from experiment 3 at 280 and 360 nm.

Analyzing the chromatograms (Figures 39, 40 and 41) there is a high and punctual enrichment of flavonoids in the Q2 and Q3 columns, mainly in Q3, while in the OA3 column a more generic enrichment in its MeOH fraction is seen. But, despite the more varied profile, the column still strongly enriched the phenolic compounds, but there was not only an accentuated fractionation.

12.5. Experience 4

In this experiment, an industrial blanching was used, supplied by the company DAMENDOA, with an estimated concentration of dissolved solids (by the difference between total and suspended solids) of 7.95 mg/mL. Unlike previous experiments, which obtained a solid/concentrated fraction for the estimation of TPC and AA, in this case the analysis was done directly with all fractions, and only a part was evaporated for the determination of concentration. This approach is possible because the initial extract has a high phenolic load (TPC = 276.55 ± 1.02 mg GAE/g Extract; EC50 = 0.073 ± 0.004 mg/mL), which is reflected in a more pronounced enrichment in the fractions. In addition, by preserving the original solutions, any physical-chemical change that may occur in the drying/redissolving process is avoided, in addition to resulting in a lower energy expenditure. The solvent gradient in this time was made in a smoother way, in order to achieve a higher fractionation and study the affinity, thus using the following: H_2O, EtOH/H_2O 10/90 (v/v), EtOH/H_2O 20/80 (v/v), EtOH/H_2O 30/70 (v/v), EtOH/H_2O 40/60 (v/v), EtOH/H_2O 50/50 (v/v), EtOH/H_2O 60/40 (v/v), EtOH/H_2O 80/20 (v/v), EtOH, MeOH, MeOH/AcOH 90/10 (v/v). Before the elutions, a cold cleaning of 100 mL of H_2O was performed in the set of 3 columns, which were later separated, analyzing the OA3 separately and the Q2 and Q3 columns together.

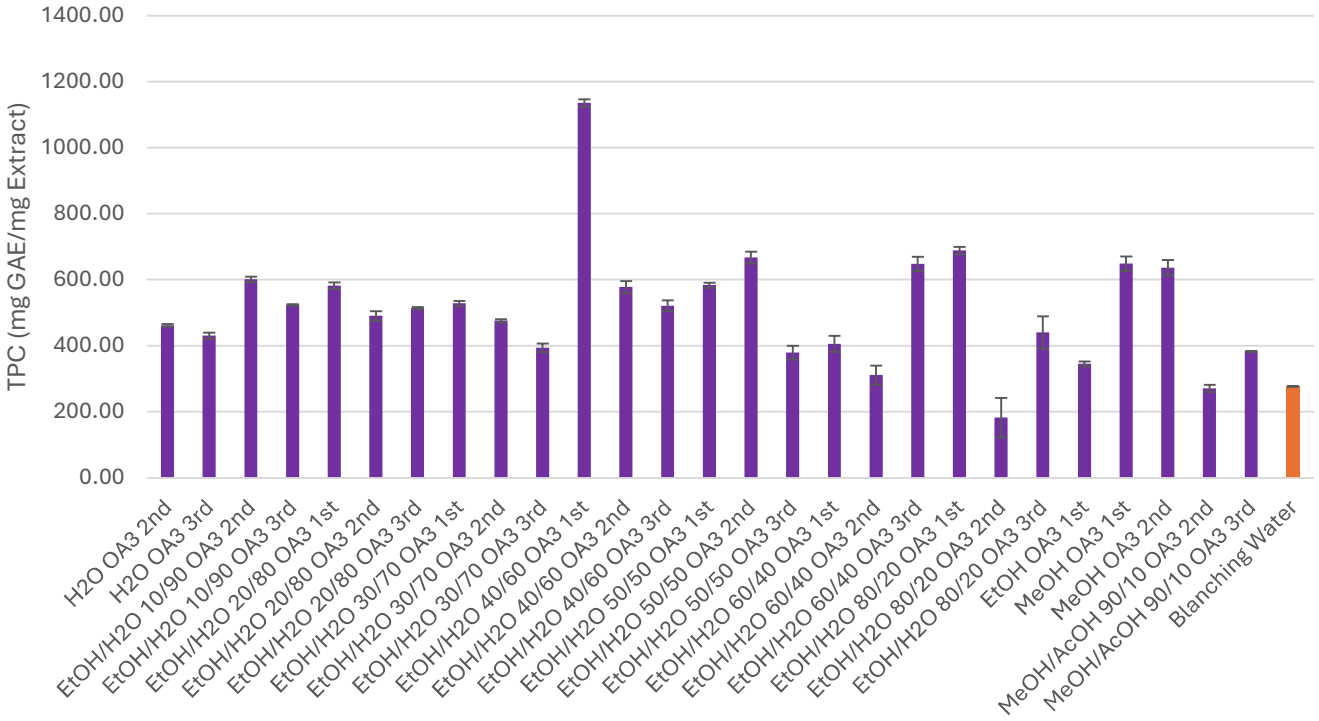


Figure 42 – TPCs (n=3) of OA3 column, experience 4.

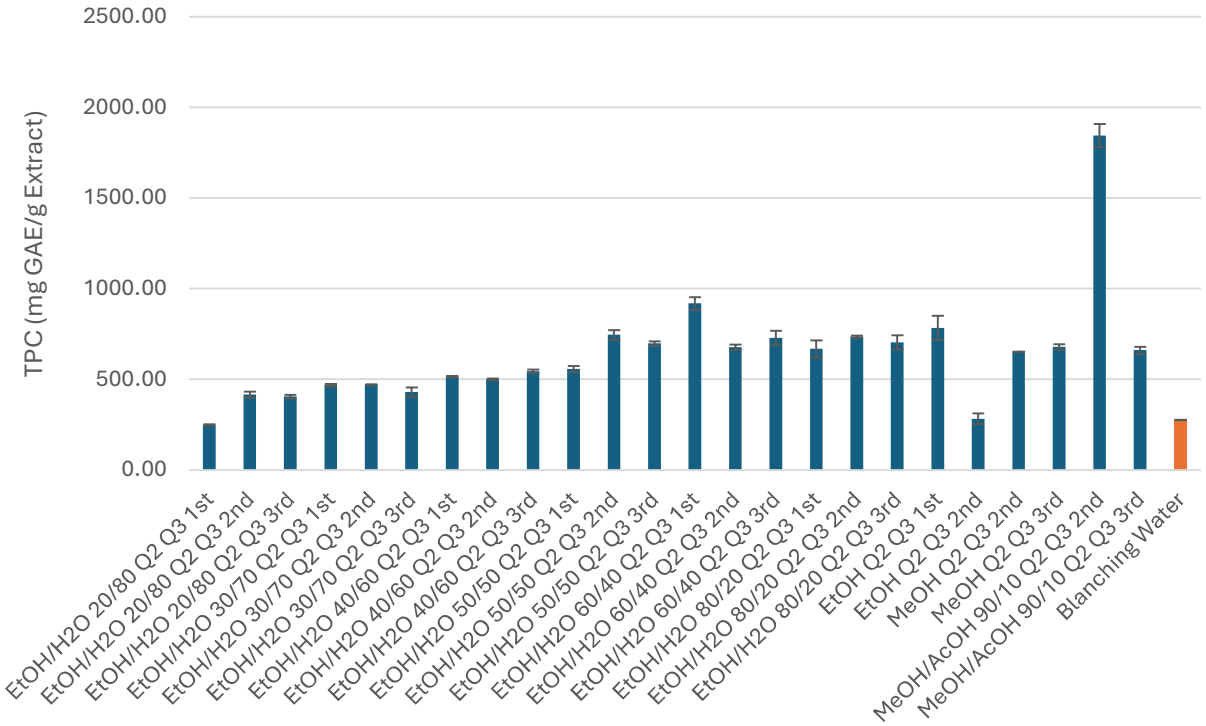


Figure 43 – TPCs (n=3) of Q2 Q3 column, experience 4.

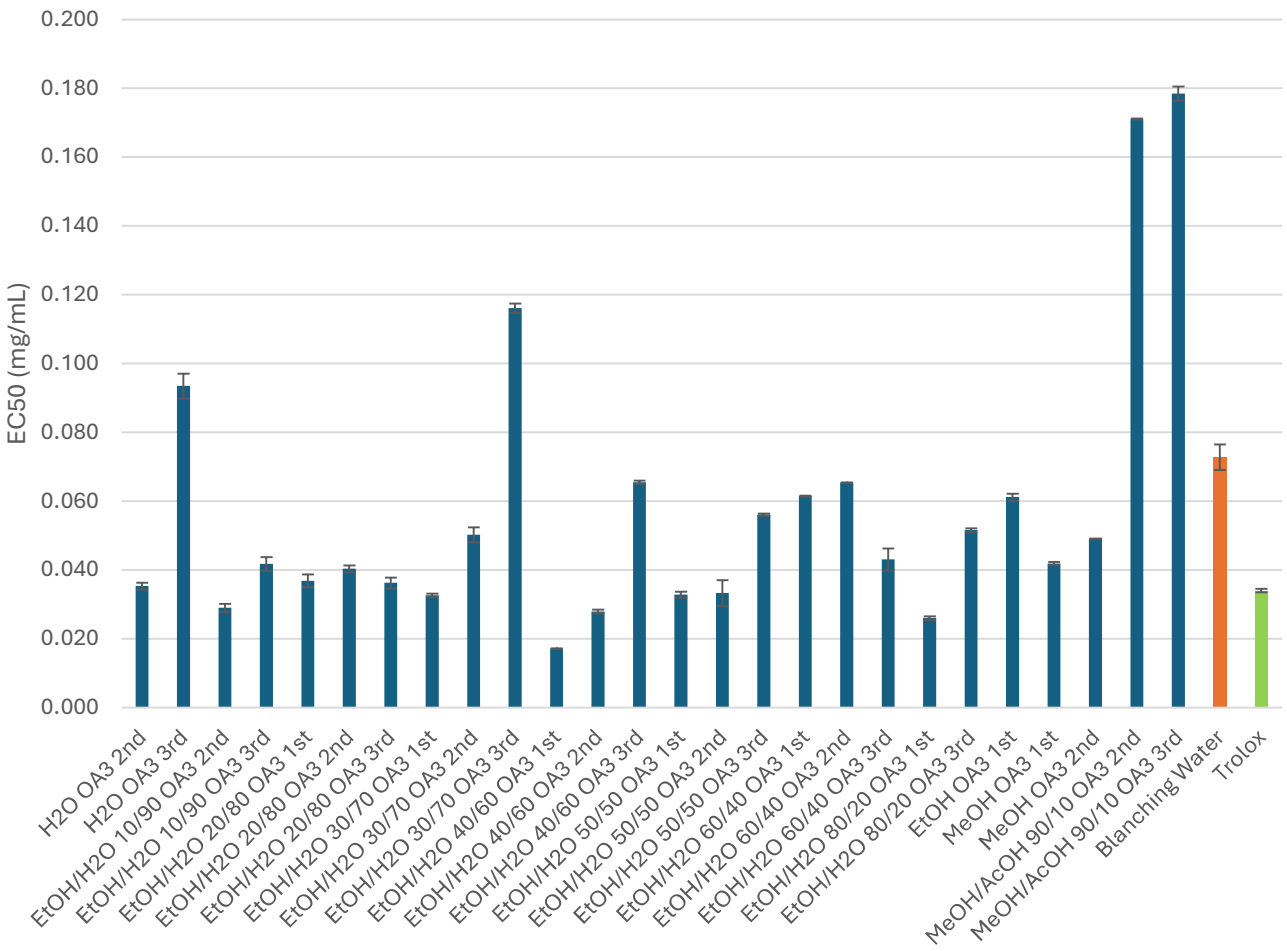


Figure 44 – EC50 (n=3) of OA3 column, experience 4.

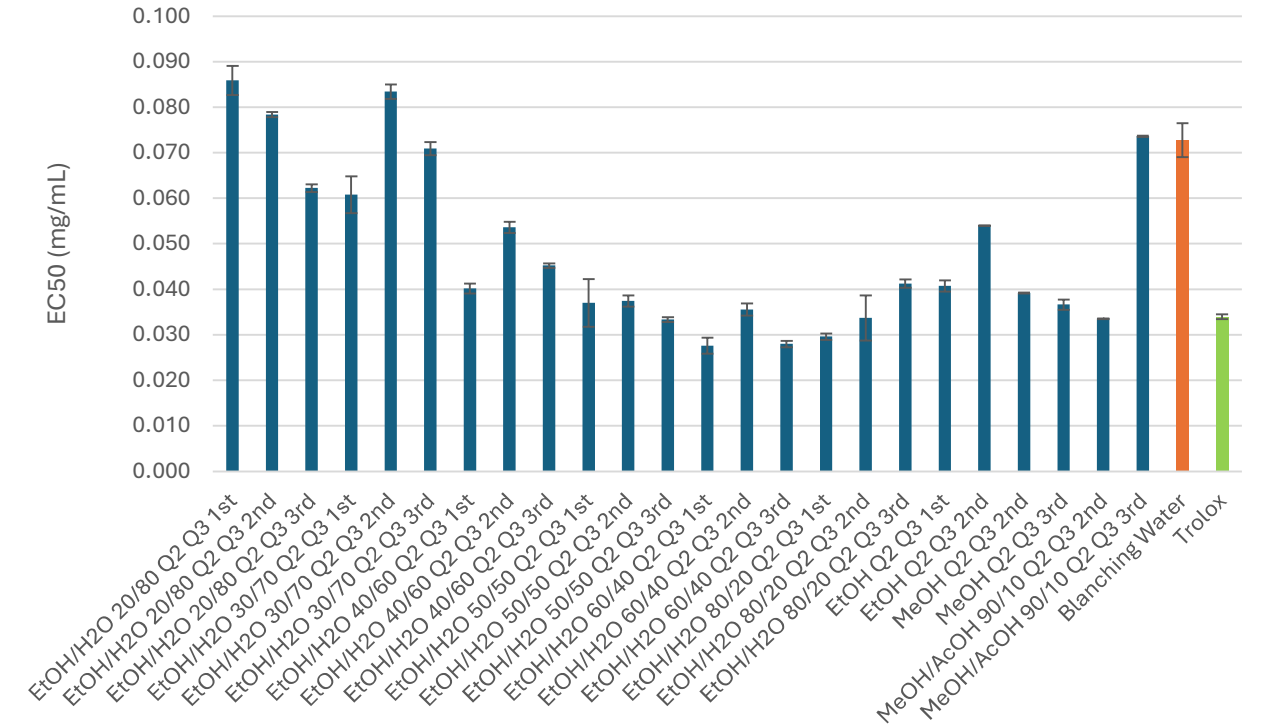


Figure 45 – EC50 (n=3) of Q2 Q3 column, experience 4.

In Figures 42, 43, 44 and 45 it is possible to see the evolution profile of the TPC of column OA3 and Q2 Q3 and EC50 of the same columns, respectively. Analyzing Figure 42, despite the existence of variation, mainly up to the 50/50 solvent, a relatively constant TPC is observed, resulting from a possible wide range of polyphenols of different affinities sorbed in the polymer, which when analyzed at the level of concentration and reducing power (compared to the TPC test), results in similar gallic equivalents. This trend is also a possible indicator that, even sipping polyphenols more generally, when compared to 4VP columns, it is possible to fractionate the compounds in the face of the change in polarity of the solvent. A corroborator of this possibility is the OA3 EtOH/H_2O 40/60 1st fraction, which is highly enriched when compared to the others, obtaining a TPC higher than 1000 mg GAE/g Extract. Although it is a value that does not represent physical sense, due to numerical limitations in the conversion of volumetric to mass concentration, it indicates that a fraction containing almost exclusively polyphenols was recovered in gallic equivalents. After the hydroethanolic fraction 50/50, a significant increase in TPC is still observed, especially in the EtOH/H_2O 60/40 3rd, EtOH/H_2O 80/20 1st and in the first and second MeOH fractions. Also analyzing with Figure 44, although the correlation between TPC and EC50 is not perfect, it is notable that with the increase in TPC the EC50 decreases, being another indication of the real enrichment/fractionation of polyphenols.

Analyzing Figure 43, referring to the Q2 and Q3 columns, it can be seen that with the increase in the amount of ethanol, TPC increases, indicating the preferential sorption of hydrophobic/amphiphilic compounds. It is also noteworthy, among the ethanolic fractions, a maximum in the solvent EtOH/H_2O 60/40 1st, indicating a preferential/superior retention of compounds in this polarity range, this being due to its higher concentration in the initial extract or affinity with the cavity (Quercetin template), given its structure, and/or functional monomer. High values are also obtained in the methanol fractions, possibly resulting from molecules that have made stronger and deeper interactions with the MIPs, reaching a TPC value higher than 1800 mg GAE/g Extract, which would indicate a pure sample in phenolics. However, analyzing Figure 45, the EC50 intensity of this sample does not correspond to its phenolic content, when compared to the other samples, and these results may be influenced by non-target eluted compounds, bringing a great influence on the TPC test but not an equivalent influence on AA.

In the following Figures (46, 47 and 48) the enrichment factors and equivalents in Trolox are presented, in order to visualize the efficacy of the process in a more direct way.

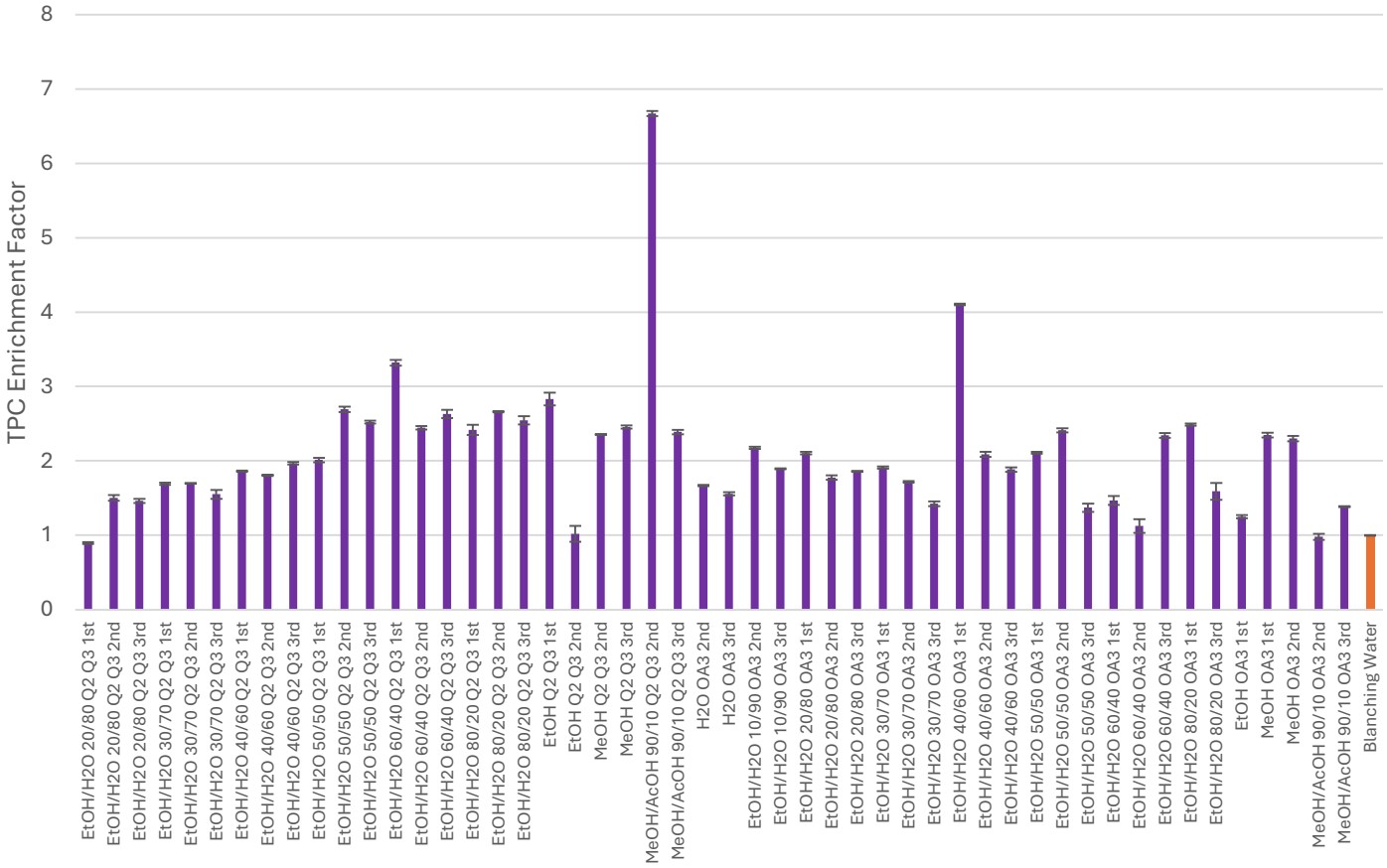


Figure 46 – TPC enrichment factor relative to the initial extract (n=3) of experience 4.

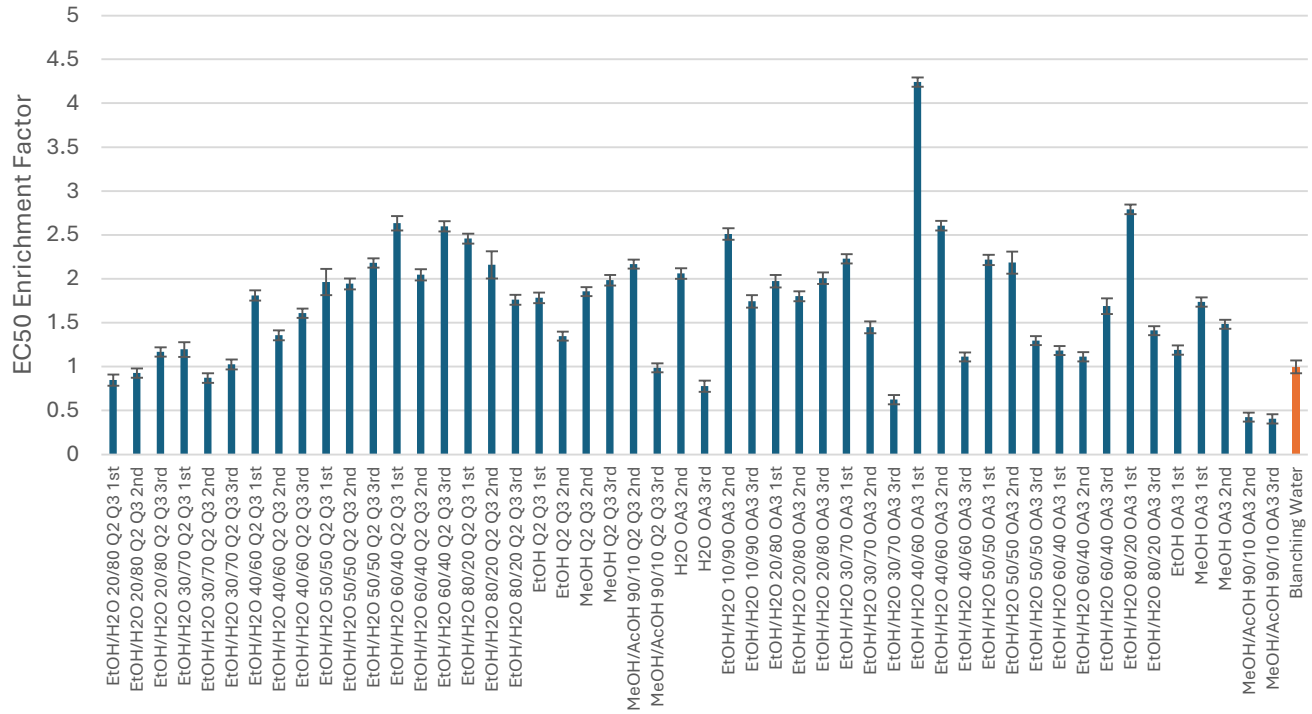


Figure 47 – EC50 enrichment factor relative to the initial extract (n=3) of experience 4.

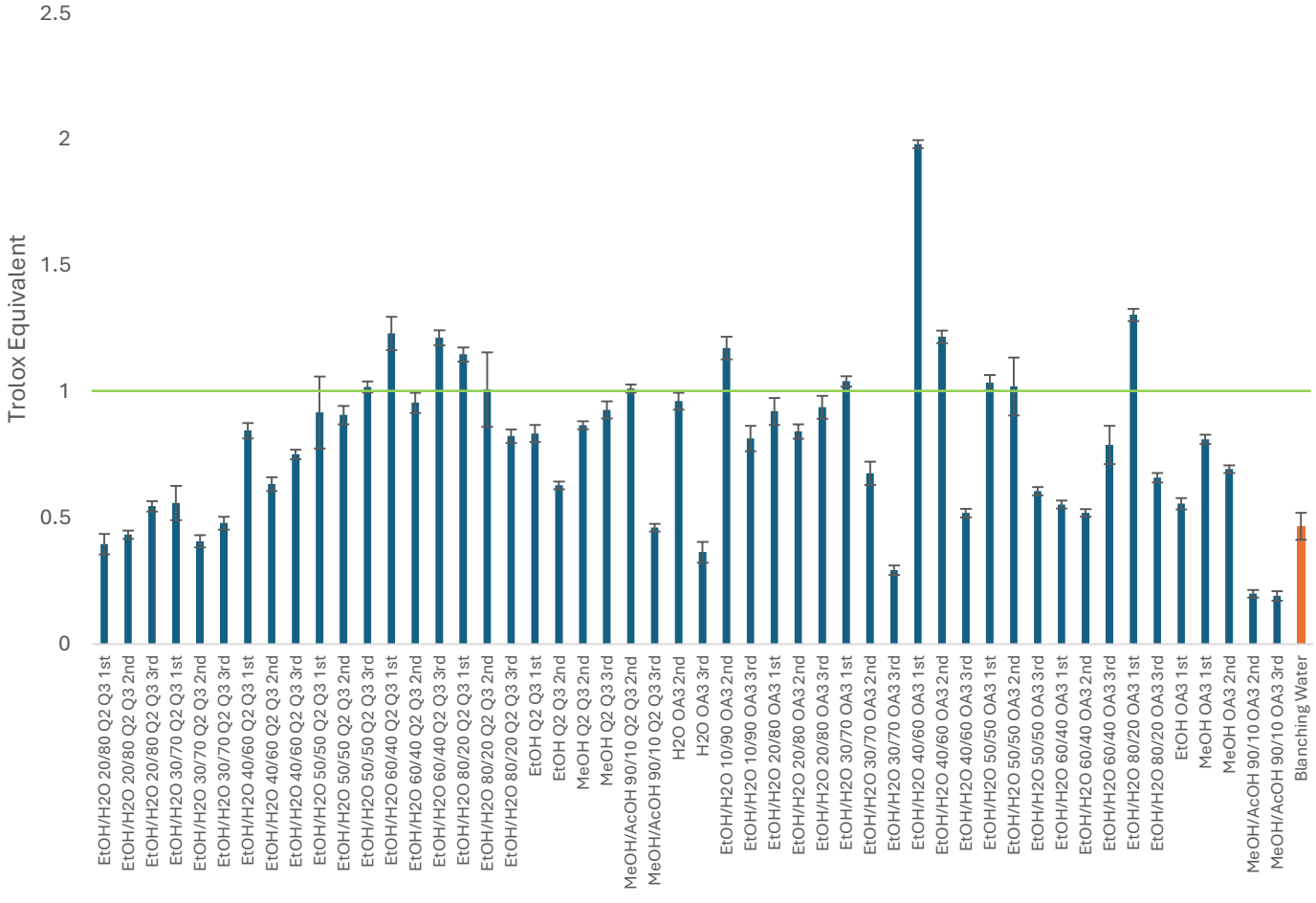


Figure 48 – Trolox equivalent (n=3) of experience 4.

Within the fractions obtained, the vast majority were enriched compared to the initial extract, both at the level of TPC and AA, with the best performance being OA3 EtOH/H_2O 40/60 (v/v) 1st and Q2 Q3 EtOH/H2O 60/40 (v/v) 1st, with TPC enrichments higher than 4 and 3, and EC50 higher than 4 and 2.5, respectively. In trolox equivalents, 12 fractions showed an activity higher than one trolox unit, with OA3 EtOH/H_2O 40/60 (v/v) 1st being the most relevant, equivalent to about twice the activity of the same.

In the following figures (49, 50 and 51) the chromatograms of the 3 best performing fractions are shown.

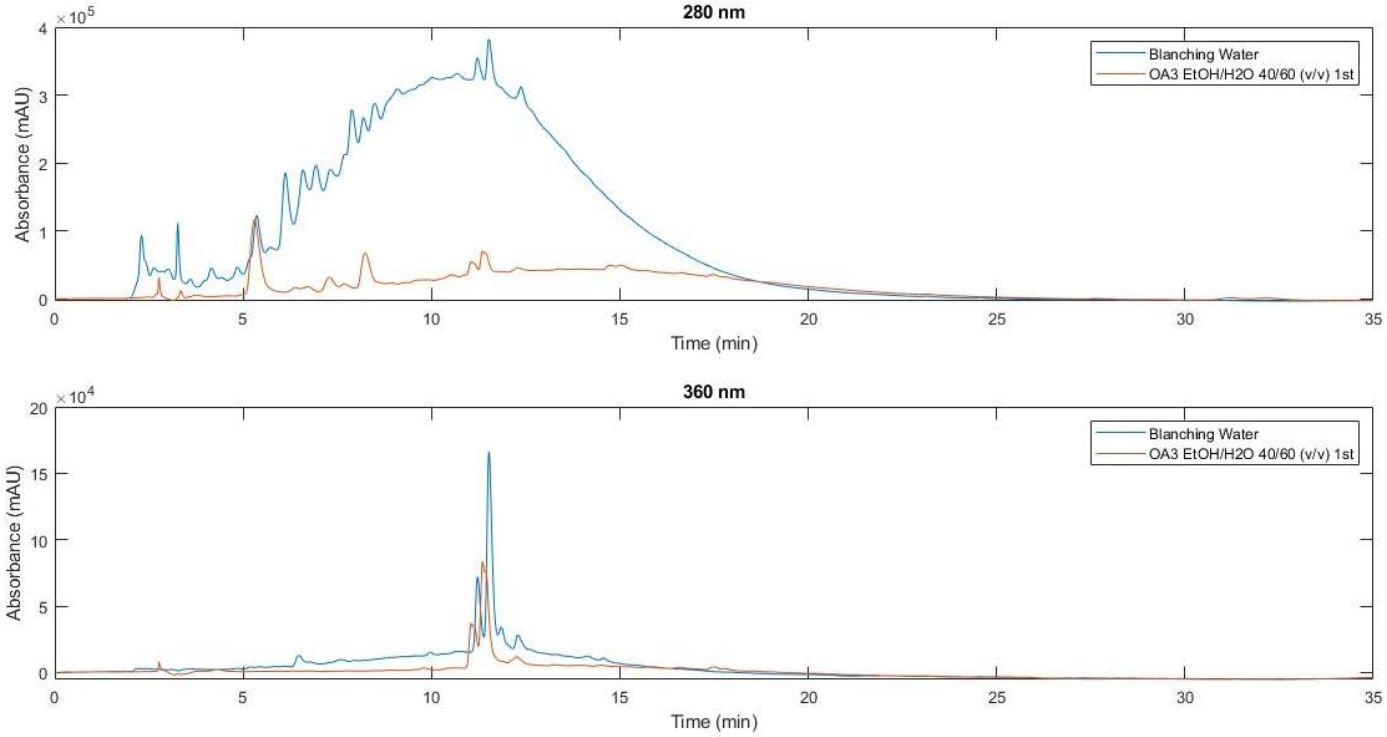


Figure 49 – Chromatogram of the blanching water and OA3 EtOH/H_2O 40/60 (v/v) 1st fraction at 1.50 mg/mL from experiment 4 at 280 and 360 nm.

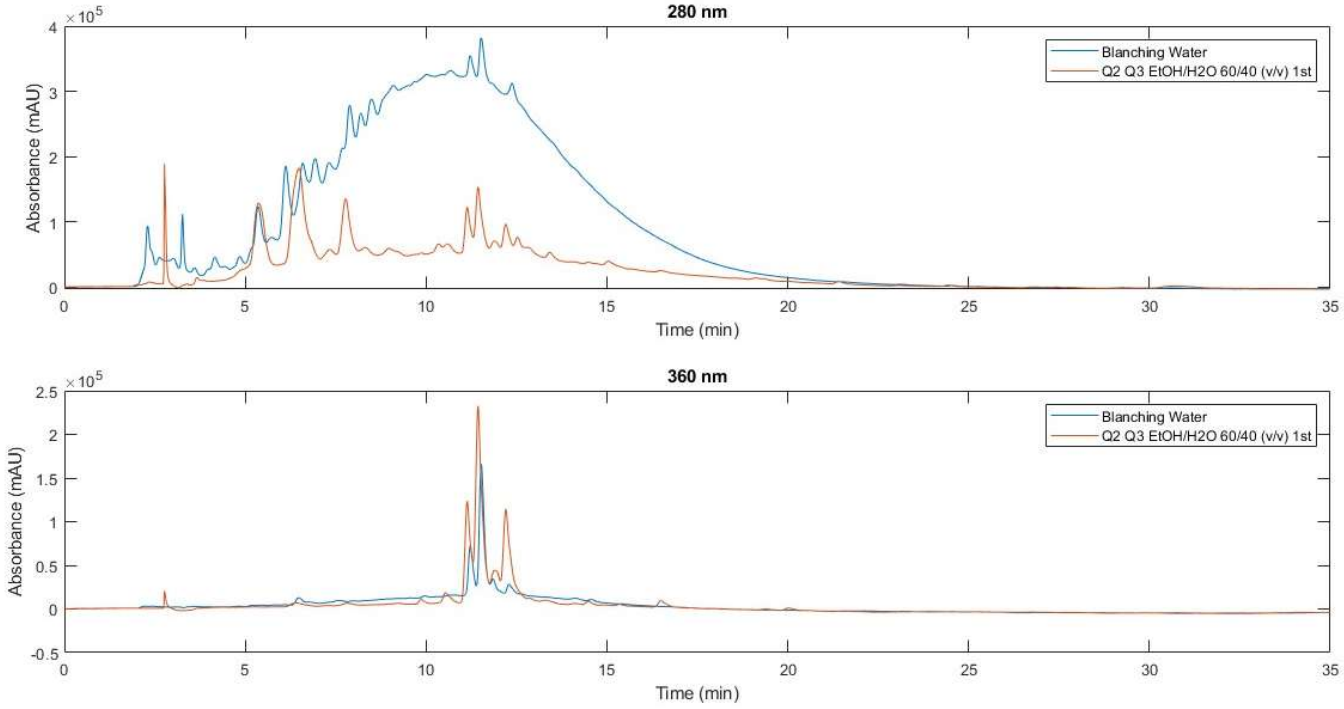


Figure 50 – Chromatogram of the blanching water and Q2 Q3 EtOH/H_2O 60/40 (v/v) 1st fraction at 1.53 mg/mL from experiment 4 at 280 and 360 nm.

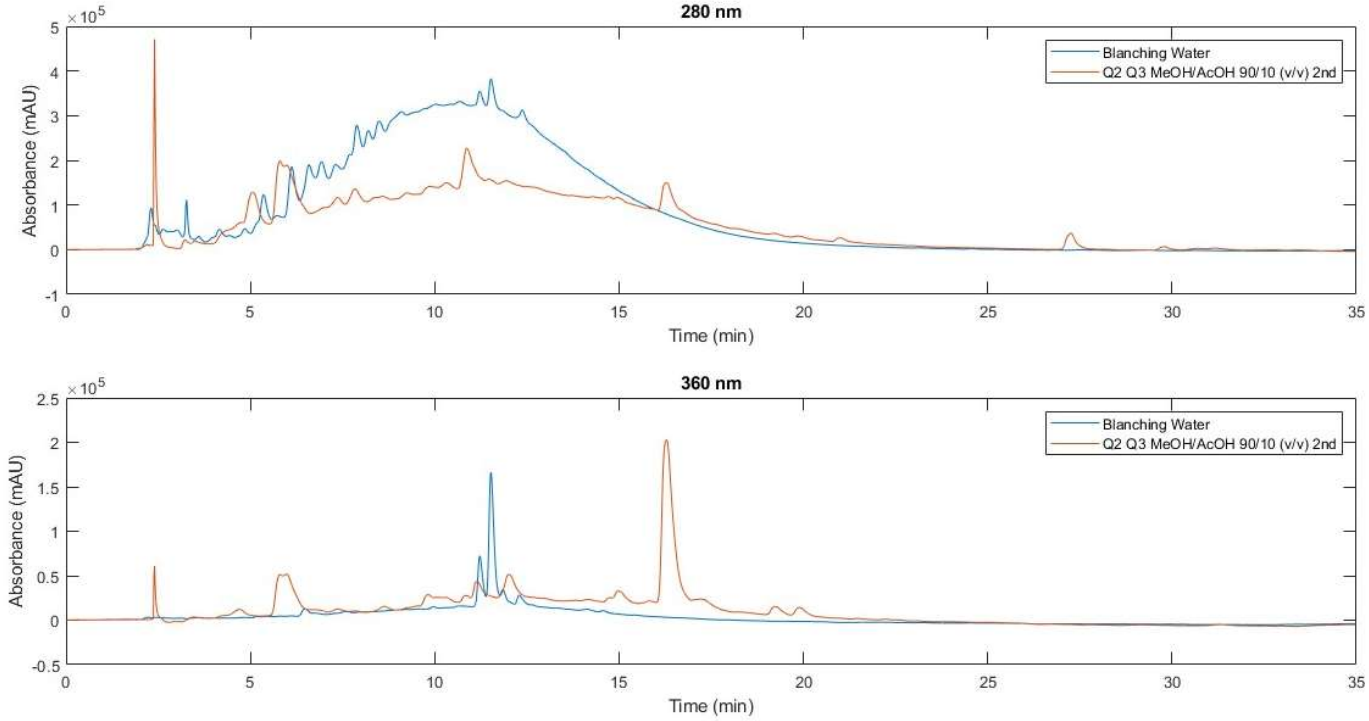


Figure 51 – Chromatogram of the blanching water and Q2 Q3 MeOH/AcOH 60/40 (v/v) 2nd fraction at 1 mg/mL from experiment 4 at 280 and 360 nm.

Comparing the first two chromatograms, it can be seen that the Q2 column Q3 enriched more phenolic compounds, while the OA3 column partially fractionates the same compounds, but in a more subtle way. However, observing the baseline, it is notorious that in the fraction of OA3 there is less interference, when compared to that of Q2 Q3, which indicates a higher phenolic purity. This difference in purity, despite the difference in profile, results in a higher TPC, being a mass concentration. But in the same way, the TPC of the Q2 Q3 column is around 900 mg GAE/g Extract, which reflects an intense phenolic load in compounds with high reducing power.

Analyzing the chromatogram of Figure 51, mainly at 360 nm, it can be seen that this fraction markedly enriched compounds that were in lower concentration in blanching. However, comparing the two wavelengths, it is verified that the baseline at 280 nm undergoes a large change that does not happen at 360 nm, possibly being an interference resulting from non-target compounds with reducing power, having contributed in a strongly positive way in the TPC analysis, but not demonstrating an equivalent performance in AA.

13. Conclusion

In this work it was possible to evaluate the application of Molecularly Imprinted Polymers (MIPs) in the concentration, recovery and fractionation of polyphenols present in the agro-industrial by-products of almonds. The results obtained demonstrate that the sorption/desorption processes applied effectively enriched the target compounds present in the extracts used, thus obtaining fractions with potential application in the agricultural area. Among these results, a maximum TPC of 615.66 ± 28.23 mg GAE/g Extract and EC50 of 0.025 ± 0.000 mg/mL were obtained in the experiment of hull extraction, in addition to values in the blanching water experiment of 918.32 ± 34.54 mg GAE/g Extract and EC50 of 0.028 ± 0.002 mg/mL. In this same last experiment, a fraction of 1135.64 ± 11.55 mg GAE/g Extract and 0.017 ± 0.000 mg/mL was obtained, having no physical significance due to numerical limitations, but indicating the recovery of a pure fraction in phenolics.

The use of different solvents showed a strong influence on the desorption efficiency and phenolic profile in each fraction, having observed significant enrichments in the intermediate hydroethanolic fractions, as well as in some methanolic elutions, being proven with the increase of TPC and EC50, as well as by the inverse correlation of these two parameters. These results indicate that intermediate-polarity phenolics have a high affinity with the system, either in view of their initial concentration in the extract and/or the affinity provided by the MIPs for their set of Functional Monomer – Template.

In addition to the relationship between TPC and EC50, which allows verifying the concentration of phenolics by 2 distinct factors, being able to confirm whether there was a real enrichment/fractionation of families, the chromatograms obtained by HPLC-DAD demonstrate significant differences between the initial extracts and the fractions, proving more rigorously the selective fractionation, especially of the flavonoids, and revealing the influence of the phenolic purity of the fractions obtained in the tests performed.

Despite the promising results, this work has some limitations that can be overcome in the future, such as the individual identification of the desorbed compounds and performing mass balances to the process, which was not one of the objectives of this work. In this way, future work may include a greater amount of analysis via HPLC, or include analyses such as LC-MS/MS. These and other fractions are currently being

investigated for their antimicrobial and insecticidal properties within ongoing doctoral research in the “Tecnologia e Produtos de Base Natural” PhD program at the Instituto Politécnico de Bragança.

In conclusion, the results obtained demonstrate a high potential of MIPs as selective materials in the recovery of polyphenols present in organic matrices, in this case agro-industrial extracts of almonds, thus contributing to the sustainable valorization of these same by-products and aligning with the principles of green chemistry and biocircular economy.

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Appendix

I. TPC calibration curves

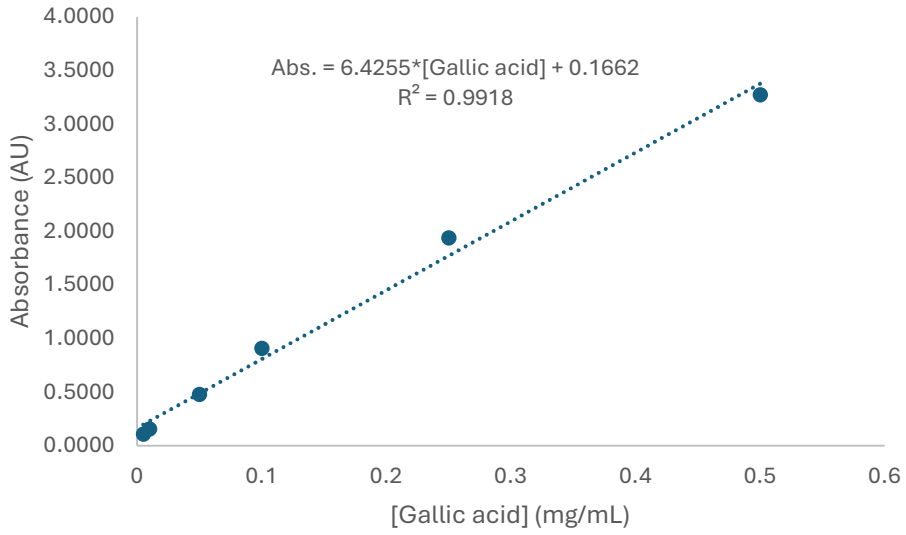


Figure 52 – TPC calibration curve on EtOH/H_2O 50/50 (v/v), 250 µL.

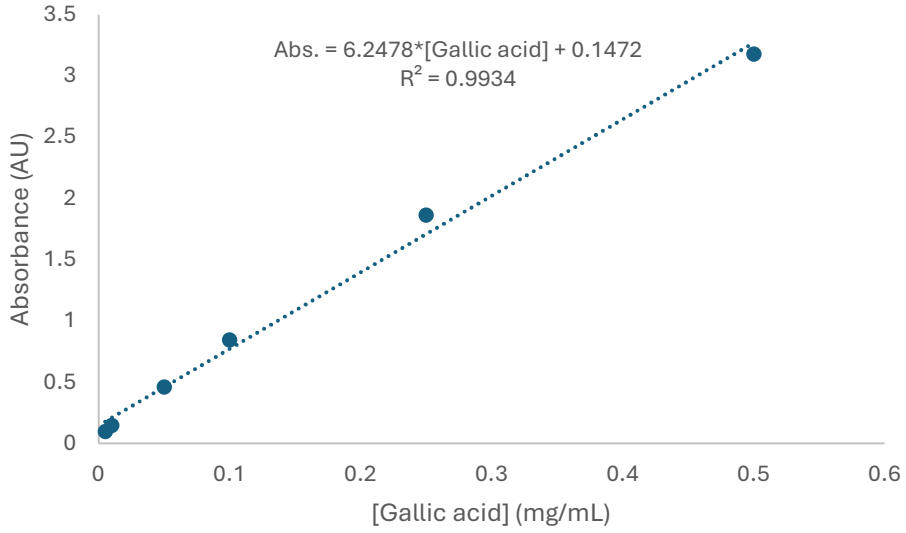


Figure 53 – TPC calibration curve on H_2O, 250 µL.

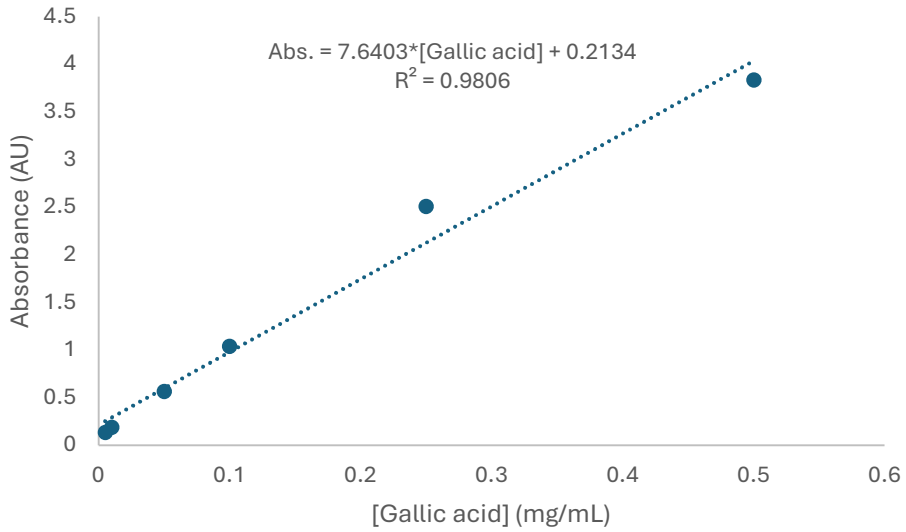


Figure 54 – TPC calibration curve on EtOH/H_2O 80/20 (v/v), 300 µL.

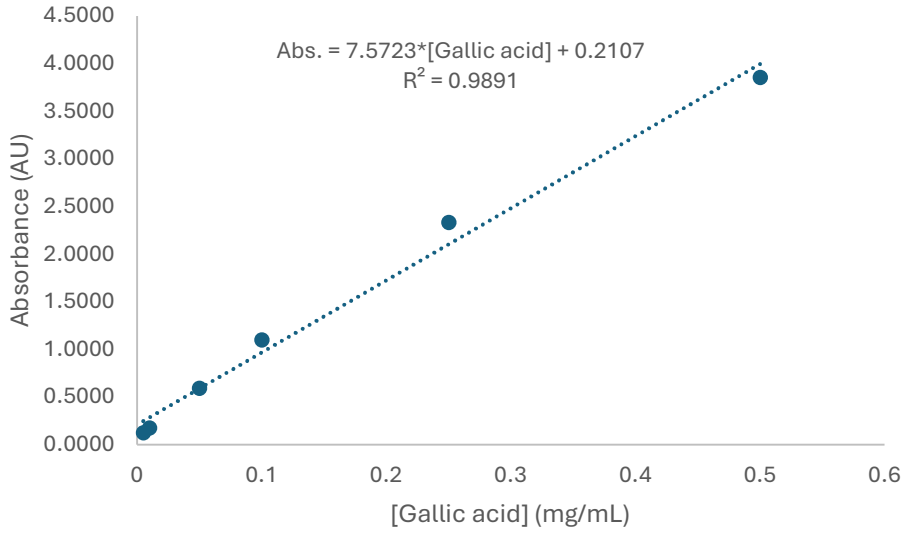


Figure 55 – TPC calibration curve on EtOH/H_2O 30/70 (v/v), 300 µL.

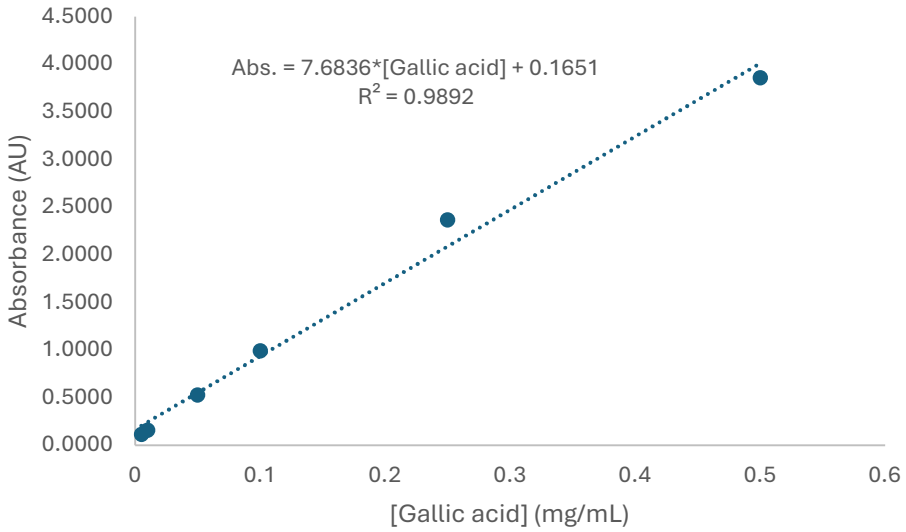


Figure 56 – TPC calibration curve on MeOH, 300 µL.

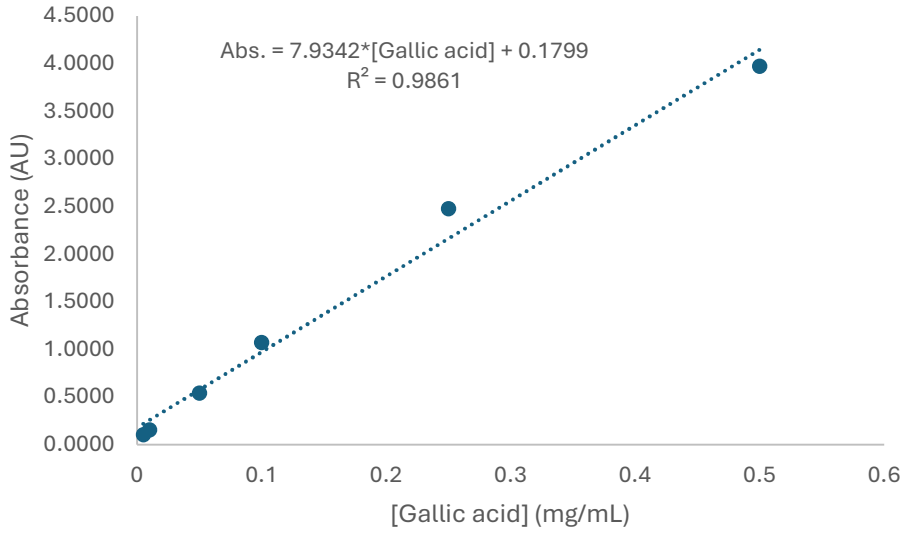


Figure 57 – TPC calibration curve on DMSO, 300 µL.

II. TPC, EC50, enrichment factors and trolox equivalent values of experience 1

Table 11 – TPC and EC50 values (n=3) of experience 1.

	TPC (mg GAE/g Extract)	SD (mg GAE/g Extract)	EC50 (mg/mL)	SD (mg/mL)
Extract Hull 50/50 (v/v)	151.28	4.34	0.341	0.039
Sorption output	1.29	9.00	> 2	---
Q2 Q3 OA3 H_2O 2nd	49.27	0.91	> 2	---
EtOAc Q2 Q3 OA3	219.49	9.47	0.187	0.004
OA3 MeOH 1st	224.82	8.73	0.070	0.004
OA3 MeOH 2nd	90.48	1.80	0.125	0.000
Q2 MeOH 1st	110.37	3.31	0.324	0.019
Q2 MeOH 2nd	85.44	4.74	0.241	0.021
Q3 MeOH 1st	272.58	9.29	0.065	0.005
Q3 MeOH 2nd	380.06	15.91	0.029	0.000
OA3 MeOH/AcOH 3rd	47.84	1.13	0.726	0.014
Trolox	---	---	0.034	0.001

Table 12 – TPC and EC50 enrichment factor and Trolox equivalent (n=3) of experience 1.

	Enrichment Factor TPC	SD	Enrichment Factor EC50	SD	Trolox Equivalent	SD
Extract Hull 50/50 (v/v)	1	0.04	1	0.16	0.10	0.12
Sorption output	0.01	7.00	---	---	---	---
Q2 Q3 OA3 H_2O 2nd	0.33	0.03	---	---	---	---
EtOAc Q2 Q3 OA3	1.45	0.05	1.82	0.12	0.18	0.03
OA3 MeOH 1st	1.49	0.05	4.84	0.12	0.49	0.05
OA3 MeOH 2nd	0.60	0.03	2.73	0.11	0.28	0.01
Q2 MeOH 1st	0.73	0.04	1.05	0.13	0.11	0.06
Q2 MeOH 2nd	0.56	0.06	1.42	0.15	0.14	0.09
Q3 MeOH 1st	1.80	0.04	5.24	0.14	0.53	0.08
Q3 MeOH 2nd	2.51	0.05	11.70	0.12	1.18	0.02
OA3 MeOH/AcOH 3rd	0.32	0.04	0.47	0.12	0.05	0.02
Trolox	---	---	---	---	1.00	0.02

III. TPC, EC50, enrichment factors and trolox equivalent values of experience 2

Table 13 – TPC and EC50 values (n=3) of experience 2.

	TPC (mg GAE/g Extract)	SD (mg GAE/g Extract)	EC50 (mg/mL)	SD (mg/mL)
Sorption Output	77.52	1.18	0.175	0.012
Q3 EtOH	442.91	1.93	0.036	0.002
Q3 MeOH/EtOAc 50/50	566.29	33.27	0.027	0.000
Q3 MeOH	615.66	28.23	0.025	0.000
Q3 EtOH/H_2O 50/50	223.13	10.18	0.085	0.005
Q3 H_2O	39.70	6.42	0.455	0.021
Q3 MeOH/AcOH 90/10	476.19	54.85	0.035	0.001
OA3 H_2O	93.09	1.36	0.238	0.018
OA3 EtOH	158.31	4.90	0.113	0.002
OA3 EtOH/H_2O 50/50	371.71	22.38	0.050	0.001
OA3 MeOH/EtOAc 50/50	227.04	6.52	0.096	0.005
OA3 MeOH	465.47	21.72	0.037	0.010
OA3 MeOH/AcOH 90/10	183.10	5.29	0.209	0.025
Q2 Q3 OA3 H_2O	93.86	3.82	0.230	0.007
Q2 MeOH/AcOH 90/10	427.90	32.77	0.054	0.003
Extract	151.28	4.34	0.341	0.039
Trolox	---	---	0.034	0.001

Table 14 – TPC and EC50 enrichment factor and Trolox equivalent (n=3) of experience 2.

	Enrichment Factor TPC	SD	Enrichment Factor EC50	SD	Trolox Equivalent	SD
Sorption Output	0.51	0.03	1.95	0.13	0.20	0.07
Q3 EtOH	2.93	0.03	9.44	0.13	0.96	0.05
Q3 MeOH/EtOAc 50/50	3.74	0.07	12.45	0.11	1.26	0.02
Q3 MeOH	4.07	0.05	13.52	0.11	1.37	0.02
Q3 EtOH/H2O 50/50	1.47	0.05	4.02	0.13	0.41	0.06
Q3 H2O	0.26	0.16	0.75	0.12	0.08	0.05
Q3 MeOH/AcOH 90/10	3.15	0.12	9.61	0.12	0.97	0.04

Table 14 – Continuation.

OA3 H2O	0.62	0.03	1.43	0.14	0.14	0.08
OA3 EtOH	1.05	0.04	3.01	0.12	0.30	0.02
OA3 EtOH/H2O 50/50	2.46	0.07	6.79	0.12	0.69	0.03
OA3 MeOH/EtOAc 50/50	1.50	0.04	3.56	0.13	0.36	0.06
OA3 MeOH	3.08	0.05	9.25	0.29	0.94	0.27
OA3 MeOH/AcOH 90/10	1.21	0.04	1.63	0.17	0.17	0.12
Q2 Q3 OA3 H2O	0.62	0.05	1.48	0.12	0.15	0.03
Q2 MeOH/AcOH 90/10	2.83	0.08	6.36	0.13	0.64	0.07
Extract	1	0.04	1	0.16	0.10	0.12
Trolox	---	---	---	---	1.00	0.02

IV. EDF50 and final volume to achieve EC50 values of experience 3

Table 15 – EDF50 and final volume to achieve EC50 values (n=3) of experience 3.

	EDF50	SD	Volume (mL) to achieve EC50	SD (mL)
Extract 10 mg/mL	38.30	3.07	---	---
OA3 EtOH/H_2O 50/50 2nd	8.20	0.55	41.0	2.7
Q3 EtOH/ H_2O 50/50 2nd	55.53	2.35	277.7	11.7
Q2 EtOH/ H_2O 50/50 2nd	13.53	0.67	67.6	3.3
Q3 EtOH 1st	4.78	0.53	23.9	2.7
OA3 EtOH 2nd	1.10	0.05	5.5	0.2
Q2 EtOH 2nd	10.00	0.00	50.0	0.0
OA3 EtOH/AcOH 90/10 2nd	2.51	0.19	12.5	1.0
Q2 EtOH/AcOH 90/10 2nd	2.61	0.09	13.1	0.4
Q3 EtOH/AcOH 90/10 1st	3.10	0.29	15.5	1.5
Q2 MeOH 1st	7.14	0.54	35.7	2.7
Q3 MeOH 1st	9.82	1.23	49.1	6.2
OA3 MeOH 1st	11.67	0.65	58.3	3.3
Q2 MeOH/AcOH 90/10 2nd	2.03	0.09	10.1	0.5
OA3 MeOH/AcOH 90/10 1st	13.14	0.92	65.7	4.6
Q3 MeOH/AcOH 90/10 2nd	8.81	0.78	44.0	3.9
Q3 H_2O 1st	6.02	0.16	30.1	0.8

V. Concentration of total, dissolved and suspended solids in blanching water, TPC, EC50, enrichment factors and trolox equivalent values of experience 4

Table 16 – Concentration of total, dissolved and suspended solids in blanching water (n=1).

Total solids (mg/mL)	Dissolved solids (mg/mL)	Suspended solids (mg/mL)
14.94	7.95	6.99

Table 17 – TPC and EC50 values (n=3) of experience 4.

	TPC (mg GAE/g Extract)	SD (mg GAE/g Extract)	EC50 (mg/mL)	SD (mg/mL)
EtOH/H_2O 20/80 Q2 Q3 1st	247.98	3.37	0.086	0.003
EtOH/H_2O 20/80 Q2 Q3 2nd	415.50	16.15	0.078	0.001
EtOH/H_2O 20/80 Q2 Q3 3rd	404.62	10.56	0.062	0.001
EtOH/H_2O 30/70 Q2 Q3 1st	468.44	6.59	0.061	0.004
EtOH/H_2O 30/70 Q2 Q3 2nd	470.19	2.16	0.083	0.002
EtOH/H_2O 30/70 Q2 Q3 3rd	428.91	25.66	0.071	0.001
EtOH/H_2O 40/60 Q2 Q3 1st	515.28	4.02	0.040	0.001
EtOH/H_2O 40/60 Q2 Q3 2nd	500.09	4.13	0.054	0.001
EtOH/H_2O 40/60 Q2 Q3 3rd	544.68	10.05	0.045	0.001
EtOH/H_2O 50/50 Q2 Q3 1st	556.59	16.66	0.037	0.005
EtOH/H_2O 50/50 Q2 Q3 2nd	745.06	25.89	0.037	0.001
EtOH/H_2O 50/50 Q2 Q3 3rd	697.84	12.11	0.033	0.001
EtOH/H_2O 60/40 Q2 Q3 1st	918.32	34.54	0.028	0.002
EtOH/H_2O 60/40 Q2 Q3 2nd	676.65	15.52	0.036	0.001
EtOH/H_2O 60/40 Q2 Q3 3rd	727.67	39.46	0.028	0.001
EtOH/H_2O 80/20 Q2 Q3 1st	668.55	46.48	0.030	0.001
EtOH/H_2O 80/20 Q2 Q3 2nd	735.89	6.31	0.034	0.005
EtOH/H_2O 80/20 Q2 Q3 3rd	704.26	39.64	0.041	0.001
EtOH Q2 Q3 1st	783.18	66.87	0.041	0.001
EtOH Q2 Q3 2nd	281.81	30.23	0.054	0.000
MeOH Q2 Q3 2nd	651.66	1.88	0.039	0.000
MeOH Q2 Q3 3rd	679.46	14.96	0.037	0.001

Table 17 – Continuation.

MeOH/AcOH 90/10 Q2 Q3 2nd	1844.91	63.94	0.034	0.000
MeOH/AcOH 90/10 Q2 Q3 3rd	660.67	19.48	0.074	0.000
H_2O OA3 2nd	461.84	4.37	0.035	0.001
H_2O OA3 3rd	430.66	8.95	0.093	0.004
EtOH/H_2O 10/90 OA3 2nd	601.65	8.37	0.029	0.001
EtOH/H_2O 10/90 OA3 3rd	524.01	1.90	0.042	0.002
EtOH/H_2O 20/80 OA3 1st	582.14	9.75	0.037	0.002
EtOH/H_2O 20/80 OA3 2nd	491.19	14.05	0.040	0.001
EtOH/H_2O 20/80 OA3 3rd	514.45	3.52	0.036	0.002
EtOH/H_2O 30/70 OA3 1st	528.32	7.95	0.033	0.000
EtOH/H_2O 40/60 OA3 1st	1135.64	11.55	0.017	0.000
EtOH/H_2O 40/60 OA3 2nd	577.68	18.76	0.028	0.001
EtOH/H_2O 40/60 OA3 3rd	521.10	16.30	0.065	0.001
EtOH/H_2O 50/50 OA3 1st	583.72	7.55	0.033	0.001
EtOH/H_2O 50/50 OA3 2nd	667.33	18.09	0.033	0.004
EtOH/H_2O 50/50 OA3 3rd	379.44	21.02	0.056	0.000
EtOH/H_2O 60/40 OA3 2nd	311.56	28.54	0.065	0.000
EtOH/H_2O 60/40 OA3 3rd	648.10	21.49	0.043	0.003
EtOH/H_2O 80/20 OA3 1st	688.36	11.20	0.026	0.001
EtOH/H_2O 80/20 OA3 3rd	440.02	48.91	0.052	0.001
EtOH OA3 1st	345.79	7.31	0.061	0.001
MeOH OA3 1st	649.13	21.64	0.042	0.000
MeOH OA3 2nd	636.50	23.25	0.049	0.000
MeOH/AcOH 90/10 OA3 2nd	270.90	10.87	0.171	0.000
MeOH/AcOH 90/10 OA3 3rd	383.00	2.02	0.178	0.002
Blanching water	276.55	1.02	0.073	0.004
Trolox	---	---	0.034	0.001

Table 18 – TPC and EC50 enrichment factor and Trolox equivalent (n=3) of experience 4.

	Enrichment factor TPC	SD	Enrichment factor EC50	SD	Trolox equivalent	SD
EtOH/H_2O 20/80 Q2 Q3 1st	0.90	0.01	0.85	0.06	0.40	0.04
EtOH/H_2O 20/80 Q2 Q3 2nd	1.50	0.04	0.93	0.05	0.43	0.02
EtOH/H_2O 20/80 Q2 Q3 3rd	1.46	0.03	1.17	0.05	0.55	0.02

Table 18 – Continuation.

EtOH/H_2O 30/70 Q2 Q3 1st	1.69	0.01	1.20	0.08	0.56	0.07
EtOH/H_2O 30/70 Q2 Q3 2nd	1.70	0.01	0.87	0.05	0.41	0.02
EtOH/H_2O 30/70 Q2 Q3 3rd	1.55	0.06	1.03	0.06	0.48	0.03
EtOH/H_2O 40/60 Q2 Q3 1st	1.86	0.01	1.81	0.06	0.85	0.03
EtOH/H_2O 40/60 Q2 Q3 2nd	1.81	0.01	1.36	0.06	0.63	0.03
EtOH/H_2O 40/60 Q2 Q3 3rd	1.97	0.02	1.61	0.05	0.75	0.02
EtOH/H_2O 50/50 Q2 Q3 1st	2.01	0.03	1.96	0.15	0.92	0.14
EtOH/H_2O 50/50 Q2 Q3 2nd	2.69	0.03	1.94	0.06	0.91	0.04
EtOH/H_2O 50/50 Q2 Q3 3rd	2.52	0.02	2.18	0.05	1.02	0.02
EtOH/H_2O 60/40 Q2 Q3 1st	3.32	0.04	2.63	0.08	1.23	0.07
EtOH/H_2O 60/40 Q2 Q3 2nd	2.45	0.02	2.05	0.06	0.96	0.04
EtOH/H_2O 60/40 Q2 Q3 3rd	2.63	0.05	2.60	0.06	1.21	0.03
EtOH/H_2O 80/20 Q2 Q3 1st	2.42	0.07	2.46	0.06	1.15	0.03
EtOH/H_2O 80/20 Q2 Q3 2nd	2.66	0.01	2.16	0.16	1.01	0.15
EtOH/H_2O 80/20 Q2 Q3 3rd	2.55	0.06	1.76	0.06	0.82	0.03
EtOH Q2 Q3 1st	2.83	0.09	1.79	0.06	0.83	0.03
EtOH/H_2O 20/80 OA3 1st	2.11	0.02	1.97	0.07	0.92	0.05
EtOH/H_2O 20/80 OA3 2nd	1.78	0.03	1.80	0.06	0.84	0.03
EtOH/H_2O 20/80 OA3 3rd	1.86	0.01	2.01	0.07	0.94	0.05
EtOH/H_2O 30/70 OA3 1st	1.91	0.02	2.23	0.05	1.04	0.02
EtOH/H_2O 30/70 OA3 2nd	1.72	0.01	1.45	0.07	0.68	0.05
EtOH/H_2O 30/70 OA3 3rd	1.43	0.03	0.63	0.05	0.29	0.02
EtOH/H_2O 40/60 OA3 1st	4.11	0.01	4.24	0.05	1.98	0.02
EtOH/H_2O 40/60 OA3 2nd	2.09	0.03	2.61	0.06	1.22	0.02
EtOH/H_2O 40/60 OA3 3rd	1.88	0.03	1.11	0.05	0.52	0.02
EtOH/H_2O 50/50 OA3 1st	2.11	0.01	2.22	0.06	1.04	0.03
EtOH/H_2O 50/50 OA3 2nd	2.41	0.03	2.19	0.12	1.02	0.11
EtOH/H_2O 50/50 OA3 3rd	1.37	0.06	1.30	0.05	0.61	0.02
EtOH/H_2O 60/40 OA3 1st	1.47	0.06	1.18	0.05	0.55	0.02
EtOH/H_2O 60/40 OA3 2nd	1.13	0.09	1.11	0.05	0.52	0.02
EtOH/H_2O 60/40 OA3 3rd	2.34	0.03	1.69	0.09	0.79	0.08
EtOH/H_2O 80/20 OA3 1st	2.49	0.02	2.79	0.05	1.31	0.02
EtOH/H_2O 80/20 OA3 3rd	1.59	0.11	1.41	0.05	0.66	0.02
EtOH OA3 1st	1.25	0.02	1.19	0.05	0.56	0.02

Table 18 – Continuation.

MeOH OA3 1st	2.35	0.03	1.74	0.05	0.81	0.02
MeOH OA3 2nd	2.30	0.04	1.48	0.05	0.69	0.02
MeOH/AcOH 90/10 OA3 2nd	0.98	0.04	0.43	0.05	0.20	0.02
MeOH/AcOH 90/10 OA3 3rd	1.38	0.01	0.41	0.05	0.19	0.02
Blanching water	1.00	0.01	1.00	0.07	0.47	0.05
Trolox	----	----	----	----	1.00	0.02

VI. Isoflavonoids

The isoflavan structure (Figure 58) is the basis of the isoflavonoid family, being biologically derived from a migration of 1,2-aryl into a 2-phenylchroman precursor [59].

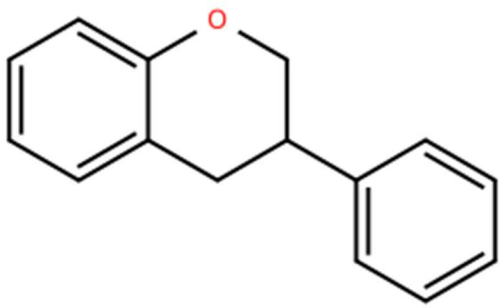


Figure 58 – Isoflavan.

Despite having a limited presence in nature, isoflavonoids present several variations (Figure 59), varying in the level of oxidation and saturation in the C ring (Figure 8) as well as in the formation of cyclic structures between the B and C rings (Figure 8) [59].

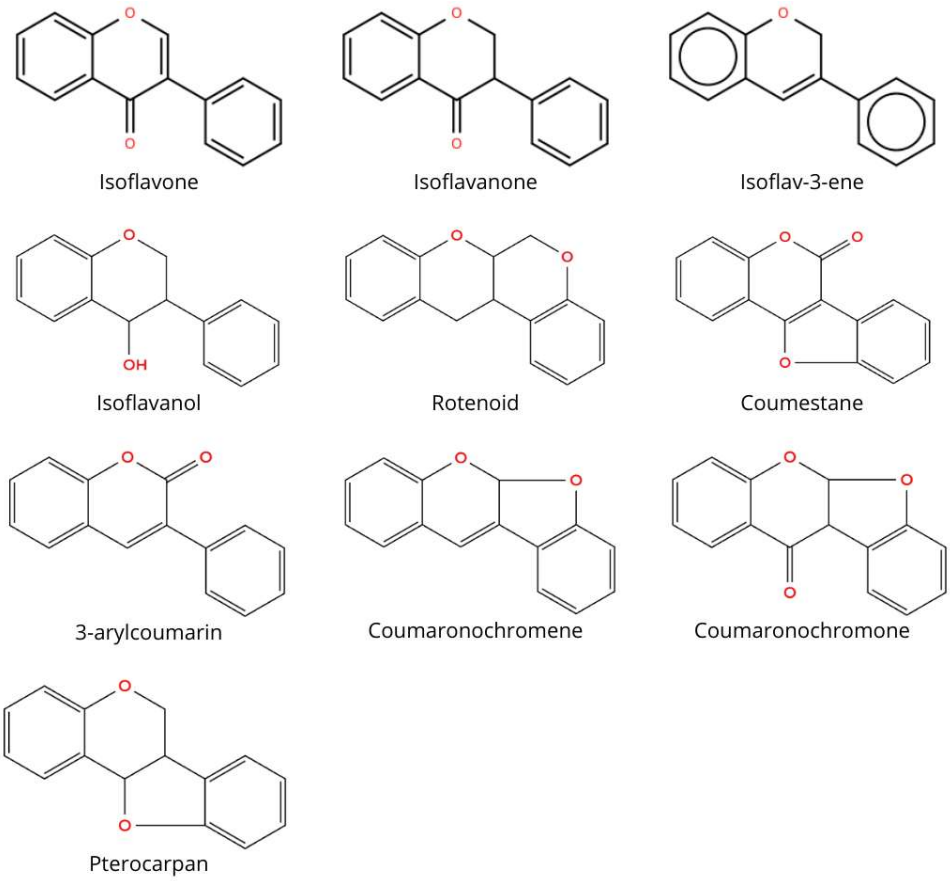
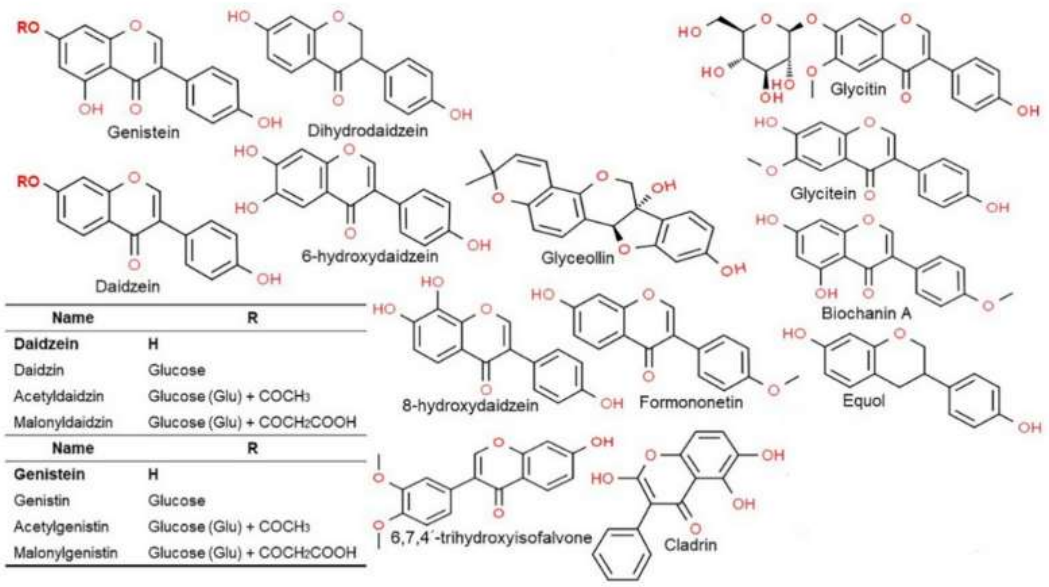


Figure 59 – Isoflavan-derived flavonoid families. Adapted from [59,109].

In general, isoflavonoids can have the same number of branches as flavonoids, but there are more typical positions (Figure 60) [110–112].



Name	R
Daidzein	**H**
Daidzin	Glucose
Acetyldaidzin	Glucose (Glu) + $COCH_3$
Malonyldaidzin	Glucose (Glu) + $COCH_2COOH$

Name	R
Genistein	**H**
Genistin	Glucose
Acetylgenistin	Glucose (Glu) + $COCH_3$
Malonylgenistin	Glucose (Glu) + $COCH_2COOH$

Figure 60 – Examples of isoflavonoids and branches. Taken and modified from [111].

In addition to these direct derivations of the isoflavan, there is a variation of this base structure, giving rise to the homoisoflavonoid category (Figures 61 and 62), which consists of the existence of an additional carbon in the isoflavan structure, usually occurring between the bond of the aromatic ring with the benzopyran, but not being a rule [109,110].

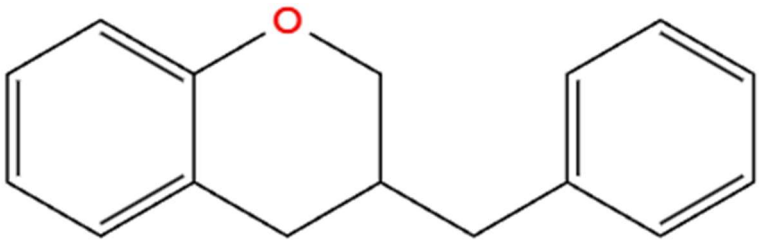


Figure 61 – Example of homoisoflavan.

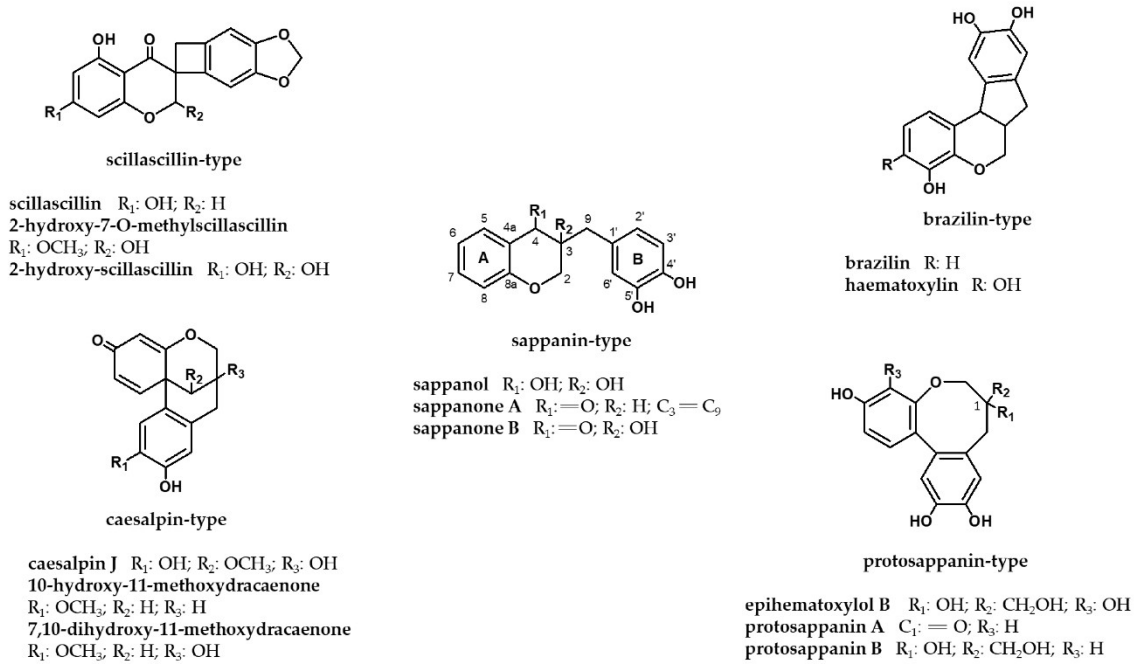


Figure 62 – Homoisoflavonoid classification with some representatives. From [110].

As a whole, the class of isoflavonoids has valuable biological activities, such as chemopreventive and bone health, weight loss adjuvant, cholesterol lowering, antibacterial, hepatoprotective, estrogenic activity, prevent atherosclerosis, antioxidant, prevent/treat osteoporosis [57,69,113].

VII. Neoflavonoids

Neoflavan (Figure 63) is the basic structure of the neoflavonoid family, with all compounds in this category being derived from it [59].

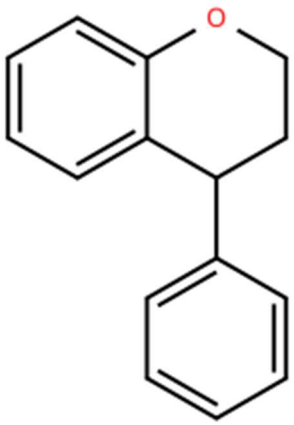


Figure 63 – Neoflavan.

Within this category of flavonoids there are some prominent families, which can be divided into two characteristic groups: Dalbergin and Latifolin (Figure 64) [114]. The differentiation between the groups is mainly due to the level of oxidation and carbons in the C ring, not being a complete ring in the latifolin [114].

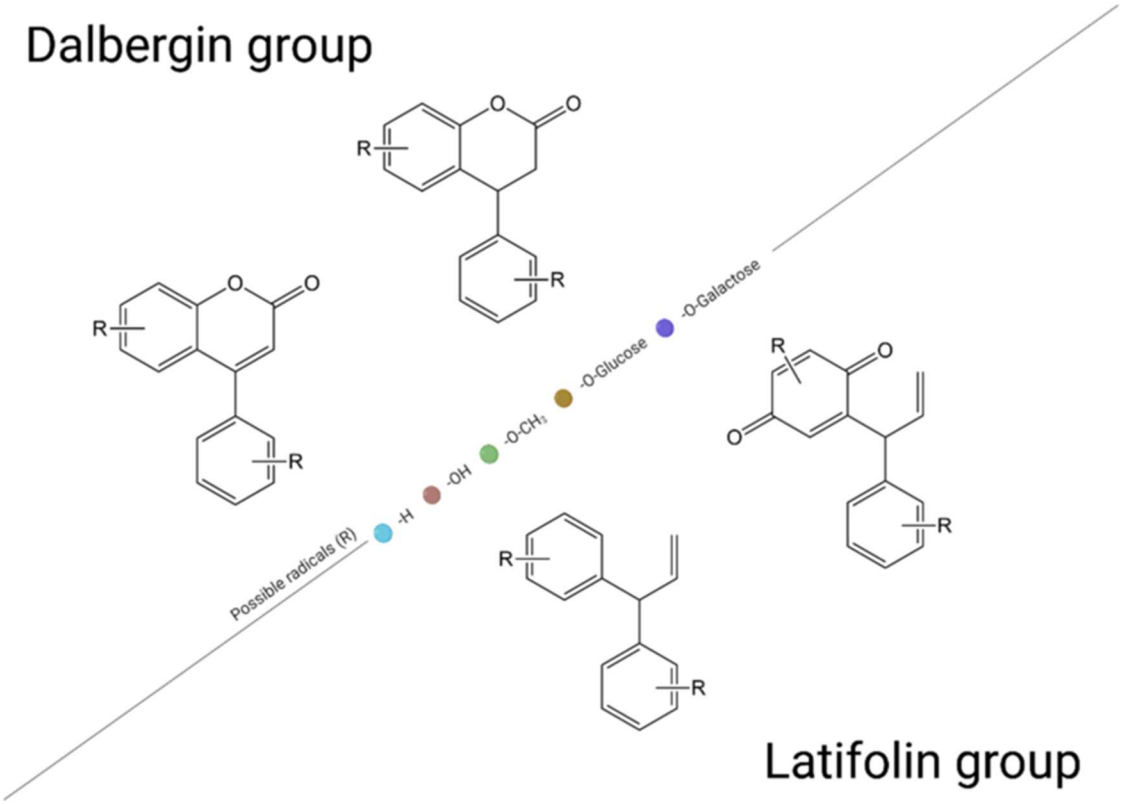


Figure 64 – Neoflavonoids classification. From [114].

Derived from these 2 groups there are essentially 8 families, also varying in the level of oxidation and saturation (Figure 65).

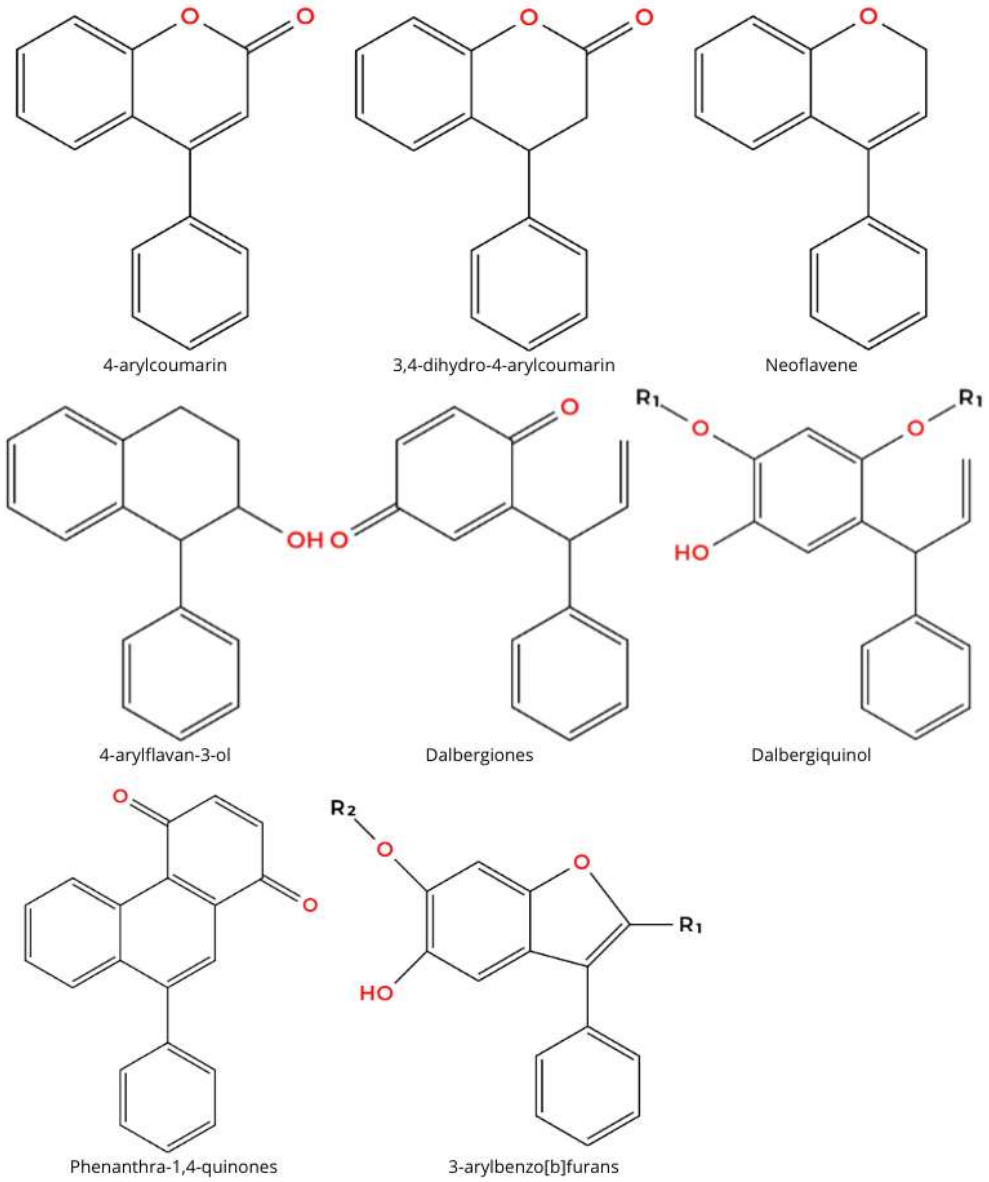


Figure 65 – 2D representation of neoflavonoids families [59,115–119].

In addition to the 8 families, each of which has a well-defined base structure, there is a ninth called 1,1-Diarylpropanoids, which consists of having two aromatic rings

attached to the first carbon of a propane, being a biosynthetic derivation of the dalbergin group and the latifolin group, but not presenting the pyran ring [116].

Globally, neoflavonoids have significant biological activities, such as cytotoxic, cardiovascular, antidiabetic, antioxidant, antiplasmodial, anti-allergic, anti-inflammatory, anti-melanogenic, antimicrobial, antileishmanial, urease inhibitor and anti-osteoporosis [120].

VIII. Minor flavonoids

In addition to the above classes (Flavonoids, Isoflavonoids and Neoflavonoids), there are 2 other families that have a C6-C3-C6 structure, but having a slightly different base structure, namely the Chalcones and the Aurones (Figure 66) [59,61].

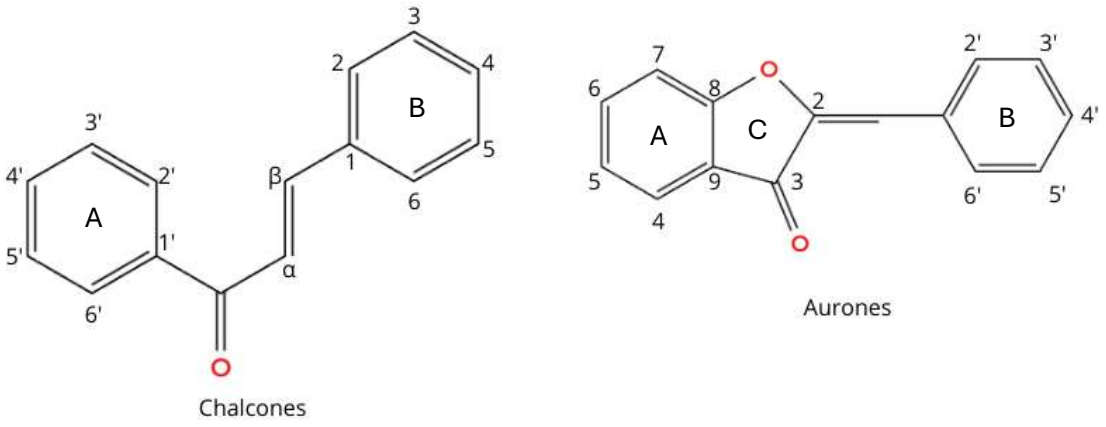


Figure 66 – Chalcones e Aurones backbone [61].

These compounds have prominent derivatives, such as 2'-OH-chalcone, 2'-OH-dihydrochalcone, 2'-OH-retro-chalcone, auronols (Figure 67) [59].

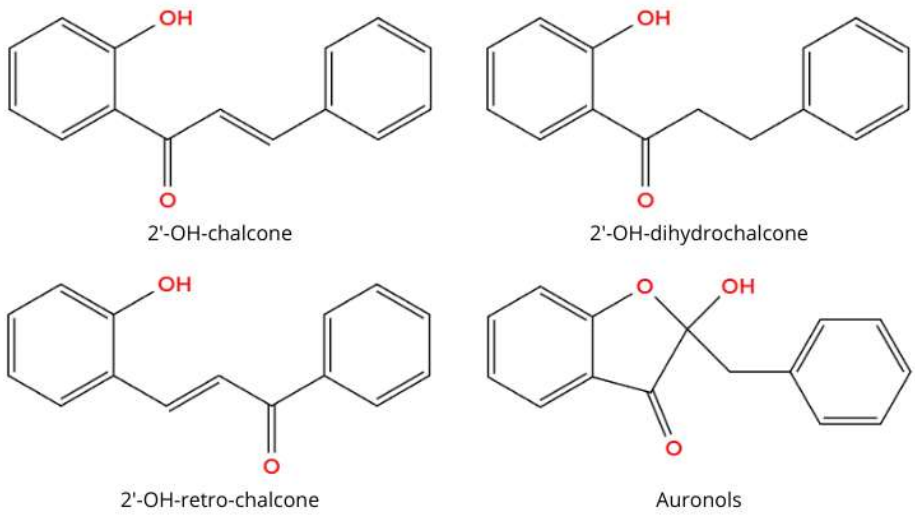


Figure 67 – Minor flavonoids subclasses [59].

Both families provide valuable biological activities (Table 19), making it a class of medical interest [121,122].

Table 19 – Biological activities of the minor flavonoid families [121,122].

Minor flavonoid families	Biological activities
Chalcones	Antioxidant, anti-inflammatory, anticancer, antimalarial, antiviral, antibacterial, antidiabetic, antihypertension, antihyperlipidemia
Aurones	Antioxidant, antiparasitic, antitumor, antiviral, antibacterial, antiinflammatory, anti-SARS-CoV-2, neuropharmacological

IX. Tannins

Unlike typical low molecular weight polyphenols (monomers), the most abundant tannins in nature are oligomers with a molecular weight between 500–3000 Daltons and containing up to 20 hydroxyl groups [123]. With their phenolic nature, tannins become very reactive groups, typically by hydrogen bonds, and can interact with and precipitate larger molecules, such as proteins [123]. They are divided into 4 categories (the first two being the main ones), varying in formation and structure: Hydrolyzable Tannins and Condensed Tannins, Complex Tannins, and Phlorotanins [123].

Hydrolyzable tannins are based on the aggregation of phenolic acids around a sugar, dividing into 2 types depending on the bonds: Gallotannins and Ellagitannins [123,124]. Gallotannins consist of derivatives of gallic acid, and when subjected to hydrolysis sugar and gallic acid are released [123,124]. Ellagitannins, on the other hand, in addition to having a central sugar linked to at least two gallic acids that are linked to each other by a C-C bond, giving rise to the monomer hexahydroxydiphenoyl (HHDP), causing the formation of hydrolysis, in addition to sugar and gallic acid, ellagic acid (derived from HHDP after lactonization) [123,124]. This second group is the most abundant of the hydrolyzable tannins, with more than 1000 natural compounds [124].

Condensed tannins, also called proanthocyanidins, are the most common group to occur naturally within tannins, being oligomers of flavan-3-ol structures, more specifically catechin, and/or flavan-3,4-diol (leukoanthocyanidin), typically by C-C bonds at carbons 4-8 or 6-8, and occasionally by C-O-C bonds [123,124].

Complex tannins are a junction of hydrolyzable and condensed tannins, presenting flavonoid structures and phenolic acids together glycosidically, with flavan-ellagitannins, procyanidin-ellagitannin, and flavonoellagitannins bonds being typical [124]. Finally, phlorotannins are the least abundant group, being almost exclusively produced by brown seaweeds, with phloroglucinol monomers and C–C and/or C-O-C interconnections in their structure [124]. Figure 68 presents a schematized summary of tannins.

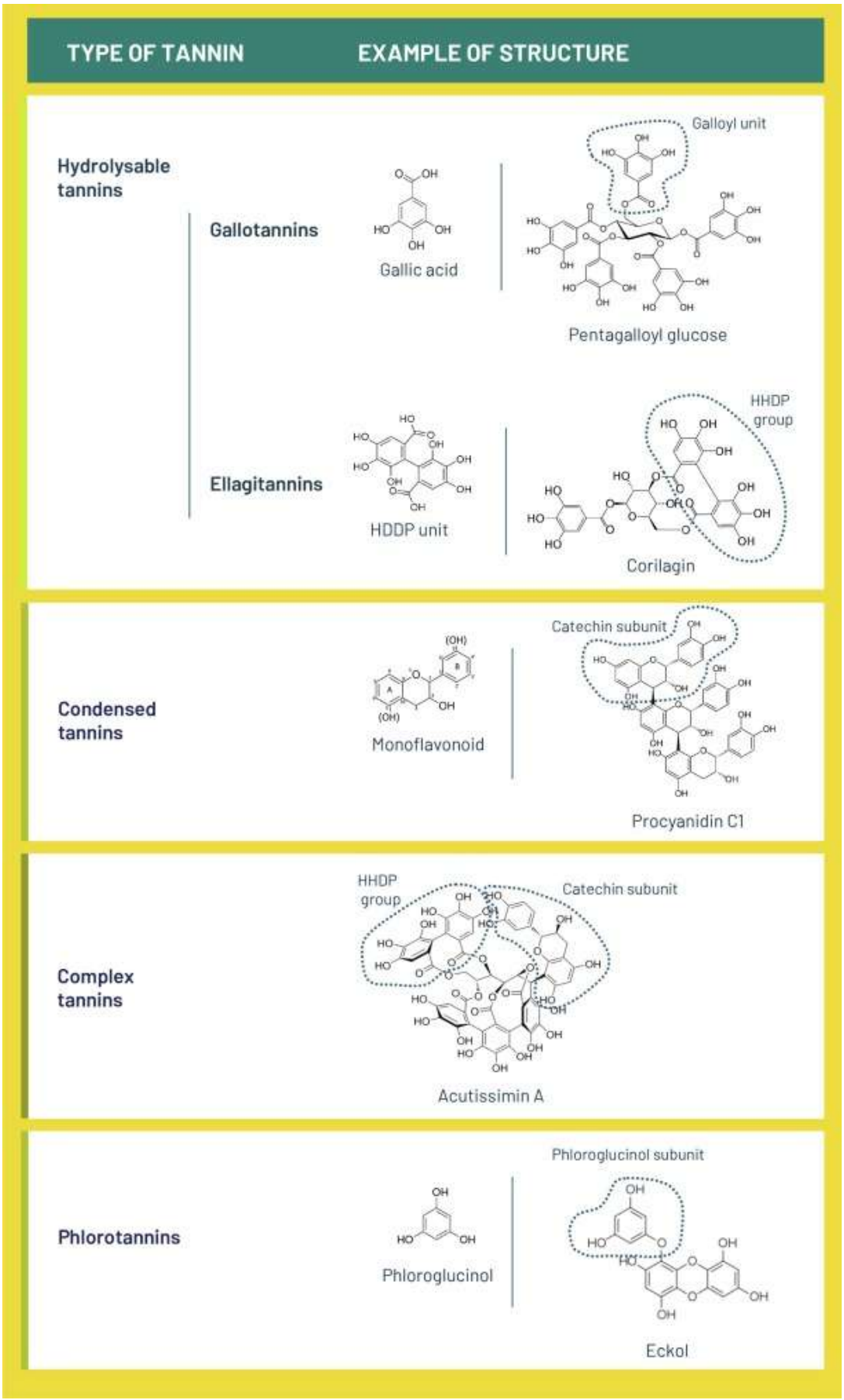


Figure 68 – Structures of tannins. From [124], licensed under CC BY-NC-ND 4.0.

X. Lignans

Lignans (Figure 69) are compounds that have two phenylpropanoid units connected, resulting in an 18-carbon base compound [125]. It is a well-distributed group throughout nature, being an intermediate/by-product in the pathway of lignin formation [125].

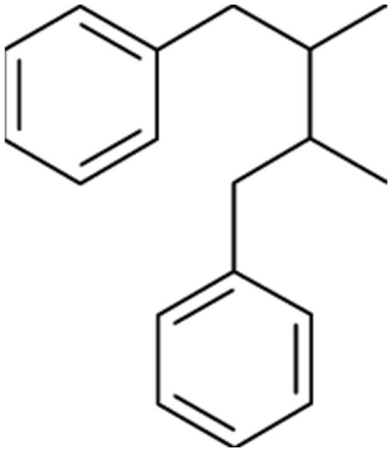


Figure 69 – Lignan structure. From [126].

Like most polyphenols, lignans have biological activities such as antifungal, antimicrobial, antiviral and insecticidal [125].