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Exploring Optimal Chromatographic Methods for Anthocyanin Extraction and Purification from Food Sources

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Alle mie tre nonne, Ninetta, Caterina e Angela,

che con i vostri piatti mi avete fatto appassionare al mondo degli alimenti.

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Abbreviations:

SPE Solid phase extraction

C3 Carbon in position 3

C7 Carbon in position 7

Cy Cyanidin

Mv Malvidin

Pt Petunidin

Dp Delphinid

BCC Basal cell carcinoma

MAPKs Mitogen-activated protein kinases

UV Ultraviolet

HPLC High-pressure liquid chromatography

UHPLC Ultra-High Performance Liquid Chromatography

GC Gas chromatography

RS Chromatography resolution

PGC Porous Graphitic Carbon

SPP Surface Porous Silica Particles

HCl Hydrochloric Acid

PDA Photodiode array

1M One Molar

BV Bed Volume

Min Minute

1. INTRODUCTION

1.1 POLYPHENOLS

Polyphenols are phytocompounds that are synthesized by plants, they exhibit phenolic structures and have antioxidant properties. They are mainly concentrated in berries, fruits, and vegetables but also found in elaborated products such as wine and tea [1].

These compounds have beneficial effects for both the human who assumes them and the plant that produced them, as these secondary metabolites are synthetized by the plant to protect itself from pathogenic microorganisms while they are functional in humans as they lower the risk of several chronic diseases such as: cardiovascular disease, cancer and chronic type inflammation.

Many studies have shown that the development of cancers or cardiovascular diseases is correlated to oxidative stress caused by reactive oxygen and nitrogen species [2].

The structure of polyphenols, in addition to being highly conjugated, is highly hydroxylated, which allows them to both neutralize free radicals and prevent their generation.

This is possible because polyphenols exert their antioxidant action by releasing an electron or accepting a hydrogen atom; they are also inhibitors of lipid peroxidation [2].

In addition, polyphenols act as chemo-preventive agents by improving the metabolism of the gut microbiota, reducing inflammation at the gastrointestinal level, and also reducing pathogen invasion, this was shown by study conducted on green tea polyphenols which demonstrated have the ability to positively change the gut microbiota by suppressing pathogen-opportunists and restoring its homeostasis [3].

1.1.1 Classification

Under the term of polyphenols exists several classes of compounds with antioxidant power, all these subclasses have phenolic structures. It is however possible to divide polyphenols into two major classes Flavonoids and Non-Flavonoids; this division is made according to the type of structure.

Non-Flavonoids

Among the non-flavonoid polyphenols, the best-known ones with high benefits include Resveratrol, which is found in grapes; and ellagic acid with its derivates which can be found plentifully in nuts and berries.

The benefits of resveratrol are many, it enhances the immune response, slows down the cell aging process and seems to have anti-obesity action. In addition, resveratrol has both preventive action and is capable of alleviating chronic diseases such as diabetes and neurodegenerative diseases.

The anti-inflammatory and antitumour actions of resveratrol are noteworthy, making it useful in all stages of cancer by acting against initiation, promotion, progression, and metastasis.

Due to its very useful characteristics the intake of resveratrol supplements, along with classic therapies, can be suggested to help against tumours, especially for obesity-mediated such as: pancreatic cancer, prostate and colon cancers [4].

Another nonflavonoids very important category is constituted of phenolic acids, which can be divided into two subgroups: benzoic derivatives and cinnamic derivatives. High concentrations of phenolic acids can be found in grains or seeds [2].

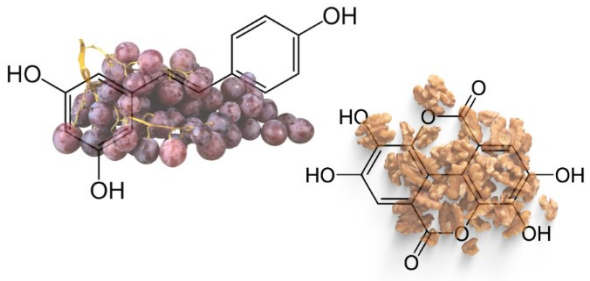

Figure 1. Resveratrol and Ellagic acid

Flavonoids

Flavonoids are the other classification of polyphenols, and all of the subcategories belonging to them have the same basic structure the diphenil-propane (C6-C3-C6), where the phenolic rings are linked by a closed pyran. Molecules belonging to this class vary from each other in different degrees of hydroxylation, as well as the oxidation state of the pyran ring. To the class

of flavonoids belong flavonols, flavanones, flavanols, isoflavones in addition to anthocyanins and anthocyanidins [1].

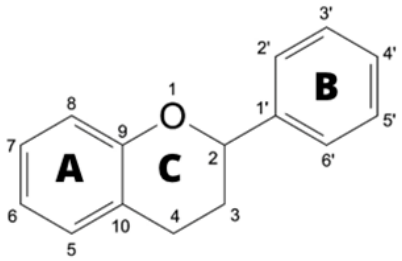

Figure 2. Flavonoid structure base

The structure features a saturated heterocyclic ring is present in the structure. No C2-C3 double bonds, however, a hydroxyl ring is bound at the C3 position, this -OH bound gives rise to a chiral center [1].

Found mainly in citrus fruits, they consist of a saturated chain of three carbon atoms at the C3 position and have different types of substituents. Most flavanones are glycosylated at the C7 position [9]. Among the most common flavanols are kaempferol, quercetin, isorhamnetin, and myricetin. They are present in all plant kingdom except algae and mushrooms. Foods that contain a large amount of them include yellow and red onions that have a high concentration of quercetin-4'-O-glucoside and quercetin-3-4'-O-diglucoside. They are found with more than one glycosylation [5]. While flavones such as apigenin, luteolin, wogonin and baicalein are structurally similar to flavonols, but unlike flavonols they are not oxygenated at the C3 position, they can be methylated, and both O- and C-glycosylated and alkylated. Rooibos tea and coffee contain small amounts of apigenin-8-C-glucoside, apigenin-6-C-glucoside, luteolin-8-C-glucoside [5]. The isoflavonoids constitute a noteworthy third category, in which the B ring is not attached to C2 but to C3, are concentrated in legumes, of the examinations are daidzein and genistein contained in soybeans [5].

1.1.2 Effect on intestine and metabolism

The gastro-intestinal tract consists of a lumen, the walls of this lumen are covered with mucus that has protective action on the gastric tissue. It would appear that polyphenols from various plants act with a positive modulatory action toward the goblet cells, which secerns mucus, by

increasing the mucus secretion. This increase in mucus not only has a mechanical defense action but also reduces the possibility of pathogens adhering to the intestinal tissue [6]. The absorption of polyphenols occurs throughout the intestinal tract, but it does not occur homogeneously, only a fraction between 5 and 10 percent of the polyphenols taken from the diet is absorbed in the small intestine. In this section of the intestine, polyphenols with a monomeric or dimeric structure, namely smaller polyphenols, are absorbed. Polyphenols in this tract undergo deglycosylation. After undergoing this initial metabolism, the resulting phenolic compounds undergo further biotransformations in either phase I or phase II, in fact they can be oxidized, reduced, hydrolyzed or conjugated. These reactions can be either hepatocyte-mediated or enterocyte-mediated, giving rise to highly soluble metabolites that will be distributed to the other organs and will be eliminated later in the urine [7]. In contrast, the metabolism of the remaining more complex polyphenols occurs in the large intestine by the intestinal microbiota, approximately the 90-95 % of the total polyphenols [8]. The microbiota metabolizes polyphenols either by breaking glycosidic bonds or by degrading the skeleton of the phenolic structure. This type of metabolism gives results to the formation of lactones, aromatic acids, and phenolics in varying degrees of glycosylation as well as having different length side chains from the other metabolites. These metabolites can be excreted with the feces or absorbed and distributed in the other organs [9].

1.2 ANTHOCYANINS

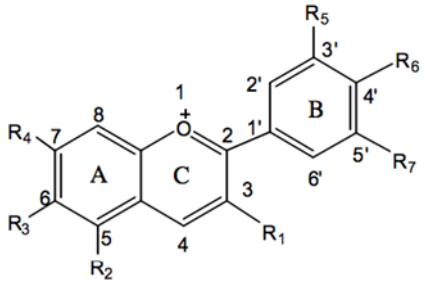

Figure 3. Anthocyanin structure base

Anthocyanins derives from the Greek "*Anthos*" meaning flower and "*Kyanos*" meaning blue, this is due to the bright color that these compounds give to the flowers and fruits in which they are contained. They are mostly responsible for many plants, flowers, seeds and fruits' skin of

red/blue shade as they have a strong variable wavelength of absorption in both visible and ultraviolet spectrum particularly in the 505-550 and 280-320 nm correlated on the functionalization of aromatic ring, the absorption may also vary on the environmental condition as the color can vary depending on pH and temperature. Anthocyanins can be implemented in the diet through fruits like berries, blackcurrants, red grapes, plums and cherries which are rich not only in the skin but also in the flesh. They can also be implemented through some vegetables like radishes or fruit-derived products like wine and juices, although in relatively limited quantities.

Their chemical structure is based on derivatives of 2-phenylbenzopyrylium or flavylium salts in particular polyhydroxy and polymethoxy of which they are glycosides.
Variations among individual anthocyanins stem from differences in several factors, including the quantity of hydroxyl groups, the types and amounts of sugars bonded to the molecule, the positioning of these bonds, and the types and quantities of aliphatic or aromatic acids attached to the sugars within the molecule. Anthocyanins are derivatives of anthocyanidins, with only six anthocyanidins being commonly found in higher plants: pelargonidin (Pg), peonidin (Pn), cyanidin (Cy), malvidin (Mv), petunidin (Pt), and delphinidin (Dp). The glycosides of the three unmethylated anthocyanidins (Cy, Dp, and Pg) are particularly prevalent in nature, being present in significant proportions in pigmented leaves (80%), fruits (69%), and flowers (50%). The average distribution of the six most common anthocyanidins in the edible parts of plants is as follows: cyanidin (50%), pelargonidin (12%), peonidin (12%), delphinidin (12%), petunidin (7%), and malvidin (7%) [10].

1.2.1 The chemistry of anthocyanins

Anthocyanins, derived from anthocyanidins, are compounds synthesized from a skeleton of flavylium cations with hydroxyl groups at different positions, yielding various anthocyanidins. Additionally, anthocyanins commonly feature a sugar moiety attached to the molecule, such as glucose, galactose, or rhamnose.

The flavonoid structure retains its ring nomenclature despite containing an oxonium group, with the oxygen atom carrying a charge on the C ring. These compounds display a range of colors, including red, blue, and purple, depending on their concentration and their absorption of complementary light to chlorophyll. The colors observed in plants, flowers, and fruits are often the result of the combined light absorption of chlorophyll and anthocyanins.

Anthocyanins exist as flavylium cations at low pH levels, while neutral conditions lead to the formation of uncharged quinones. Under normal conditions, anthocyanins are relatively stable but can undergo various degradation pathways, resulting in a loss of color. Anthocyanins serve as natural pH indicators, appearing pink at low pH, purple at neutral pH, and greenish yellow in basic conditions. At highly alkaline pH levels, anthocyanins lose their color.

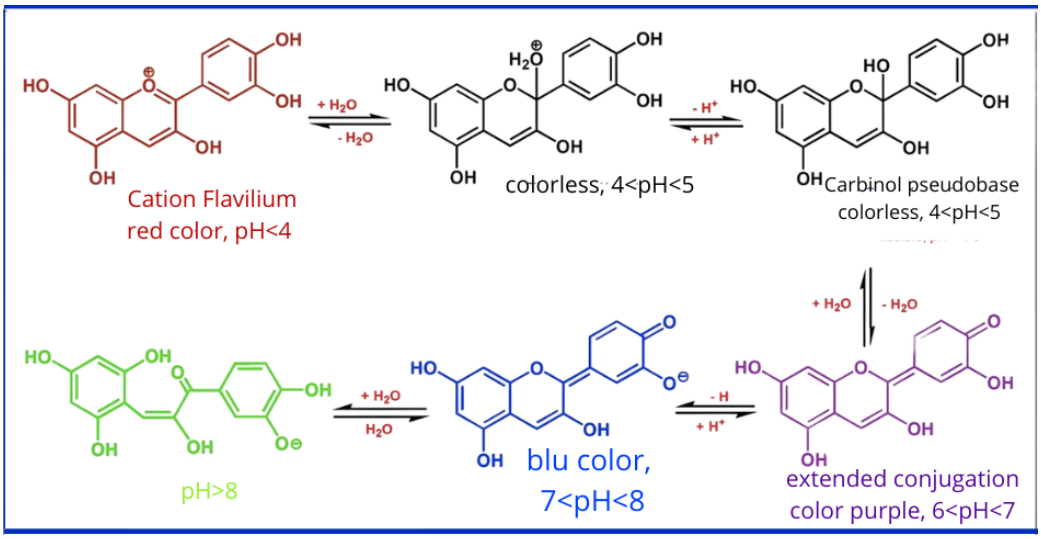

Figure 4. Anthocyanin conformation at different pH

Anthocyanins possess unique characteristics attributed to their flavylium skeleton, which facilitates radical electron delocalization across the sp^2 orbitals of the oxonium group. Antioxidant activity is central to the oxidation of phenolic hydroxyl groups within anthocyanins. Specifically, para- and ortho-phenolic groups are crucial for stabilizing semiquinones, thereby aiding in the stabilization of one-electron oxidation products.

1.2.2 Biosynthesis of Anthocyanins

Anthocyanins represent one of the most widespread classes of secondary metabolites in the human diet. The essential compounds for anthocyanin biosynthesis come from the acetate and shikimate pathways. Starting with phenylalanine, the shikimate pathway produces coumaroyl-coenzyme A (CoA), which will react with three malonyl-CoA molecules to form a

tetrahydroxy chalcone that undergoes further transformation into the flavonoid naringenin. This naringenin molecule is then hydroxylated to eriodyctiol by the flavonoid 3'-hydroxylase enzyme. Both eriodyctiol and naringenin serve as substrates for hydroxylation into the corresponding flavanols, acting as precursors for the subsequent production of flavan-3,4-diols catalyzed by dihydroflavonol-4-reductase.

The activities of two key enzymes, flavonoid-3'-hydroxylase and flavonoid-3',5'-hydroxylase, are crucial for obtaining the chemical structures and subsequent pigmentation of anthocyanins. These enzymes dictate the hydroxylation pattern of the B-ring, thereby conditioning the formation of cyanidin (Cy) and delphinidin (Dp). Anthocyanidin synthase, a 2-oxoglutarate-dependent enzyme, facilitates the synthesis of anthocyanidins from their precursor molecules.

The conversion of anthocyanidins into anthocyanins varies significantly by family or species. Usually, anthocyanidins are 3-O-glucosylated by uridine diphosphate glucose (UDP-glucose) flavonoid 3-glucosyltransferases, followed by C5-glycosylation. In addition, acyltransferases, predominantly belonging to the BAHD family, catalyze the transfer of acylating agents to the hydroxyl group of the bound sugar, further modifying the anthocyanin molecules. After synthesis, the anthocyanins are stored in the vacuole. This process requires several mechanisms, including transport via glutathione S-transferase-like proteins, vesicle-mediated mass transport and flavonoid/H+-antiporter pathways [13].

1.2.3 Function of anthocyanins in the plant

The pathway for the synthesis of anthocyanins is the same as that for flavonols and isoflavonols. The reaction site for anthocyanin production occurs in the cytosol of the smooth endoplasmic reticulum. Once produced, anthocyanins accumulate in the plant's reproductive organs.

The color that anthocyanins give to plant leaves depends on their concentration in the plant, but also on their degree of hydroxylation and the substituents bound to the anthocyanin. The pathway leading to the synthesis of anthocyanins is one of adaptations that plants have undergone over time. Indeed, several studies show that the action of anthocyanins is to protect the chloroplast from light radiation. In this way, the plant has developed resistance to photo-oxidative damage [14].

In addition to their photo-protective action, anthocyanins are useful for attracting animals. In fact, by giving such a bright coloring to flowers, leaves and fruit they increase the attraction of animals, and with this also increases pollination and seed dispersal, of the plant itself [10].

In addition to the photo-protective function and that of attracting animals, anthocyanins have another important action in plants, that of being chelators for metal ions. In fact, if there is a high concentration of metal ions in the vacuole, the anthocyanins, thanks to their 3',4'-O-hydroxyl structure in the B-ring, can bind these ions and reduce the content of free ions circulating in the plant [14].

1.2.4 Beneficial effects of anthocyanins against cardiovascular diseases

Anthocyanins appear to have potential positive influence on reducing the risk of mortality from cardiovascular disease. It would appear that an intake of foods containing a high content of anthocyanins such as strawberries and berries pose up to a 32 percent reduction in both fatal and nonfatal myocardial infarctions. Anthocyanins would also seem to be connected with a positive effect on the prevention of hypertension, as studies have shown a 10% decrease in risk. Although there is no evidence for a connection between beneficial effects of anthocyanins and a decrease in the risk of stroke [16]. A study based on 150 hypercholesterolemic patients who were given purified anthocyanins for 12 weeks showed a positive increase of HDL at the expense of LDL, as well as an improvement in endothelial function. Furthermore, for the portion of these patients who decided to continue the purified anthocyanin supplementation therapy for another 12 weeks after the first study, showed a decrease in inflammatory markers such as high-sensitivity C-reactive protein and plasma IL-1B.This positive action on hypercholesterolemia was also demonstrated by diabetic patients who took blueberries on a daily basis, differences were especially noted between the values of LDL and HDL cholesterol, triglycerides the beginning and the end of therapy, these values improved during treatment with blueberries.

These obtained results show that a daily intake of foods containing a high content of anthocyanins or even pure anthocyanin extracts may reduce the development of cardiovascular disease since these molecules have the ability to reduce arterial stiffness in addition to reducing systolic and diastolic blood pressure. This is because anthocyanins have both anti-inflammatory and antioxidant actions [17].

1.2.5 Beneficial effects of anthocyanins against skin tumours

The skins tumours develop when there is continuous and uncontrolled division of cancerous cells within the affected tissue. Skin cancers can be classified into three different types: basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and finally skin melanoma, of which basal cell carcinoma (BCC) is the most common. In contrast to the other two, melanoma develops from melanocytes that undergo modification and give rise to the tumour. The treatment of this type of cancer with classic chemotherapeutic agents has been superseded by the use of biological agents and biochemistry, in fact today's therapy involves the use of molecules and substances from marine, microbial and botanical sources. Along with this type of therapy, it's very effective the supplementation of antioxidant-rich foods in the diet, like foods that are sources of anthocyanins. In addition to being recommended to patients undergoing therapy, they are also recommended to all those who are exposed to the sun for long periods of the day, so as to reduce the side effects of sun exposure. Furthermore, a study using anthocyanins from bilberry showed the potential of these molecules to promote apoptosis and increase lactate dehydrogenase activity in murine B16-F10 melanoma cells. Anthocyanins can also promote immunosuppression, blocking oxidative DNA damage and acting against UVB-mediated skin photoaging, which occurs with collagen destruction and promotion of inflammation such as MAP kinase activation [18].

1.2.6 Bioavailability of anthocyanins

Anthocyanins have numerous benefits, but before these benefits may be realized in people, the absorption of anthocyanins must be assessed. Anthocyanins have low bioavailability, it's common to observe low plasma concentrations compared to assumption, furthermore the absorption of anthocyanins is not the same for all molecules belonging to this class. As mentioned before, there is a difference in absorption depending on their structure, in fact, anthocyanins with a pelargonidin base structure are more easily absorbed than anthocyanins with a multi-substituted structure, and of the substituted anthocyanins, the acetylated ones are the least absorbed. The absorption of anthocyanins also depends on what food they are ingested with, as it differs when ingested with lipids, proteins or alcoholic substances. Anthocyanins are absorbed in the intestine and undergo processing in the liver, where they are subjected to both phase I and phase II metabolisms, resulting in various metabolites with different biological activities. It is important to note that anthocyanins are metabolized not

only by the liver but also throughout the digestive tract. According to research, the oral microbiota removes the sugary component of anthocyanin, while the stomach may also serve as a site for anthocyanin absorption. However, the majority of anthocyanins are absorbed in the intestine [15].

1.2.7 Anthocyanins in cosmetics

The cosmetics industry is a diverse sector that includes a range of products, from everyday items such as deodorants, body and facial cleansers, and shampoos, to less frequently used products such as nail and hair dyes, and other hair styling products. The industry has experienced continuous growth over the years. While cosmetic products used to contain silicones, ammonia, and synthetic dyes, the market and cosmetic research are now moving towards more natural ingredients. Due to their characteristics in the visible spectrum, anthocyanins are gaining great interest as dyes with the shift from synthetic to natural cosmetics. To overcome the harmful effects of synthetic dye production and application, blackcurrant (*ribes nigrum L.*) peel extracts are being used as a natural alternative as it contains a high content of anthocyanins. This not only produces a natural dye but also repurposes waste from the food supply chain. The peels are washed with acidic water to extract the color, resulting in a completely eco-friendly product. Beside the dye production, anthocyanins are finding uses in products for skin treatment, as antioxidant creams.

Anthocyanins containing products are often mixed with clay minerals to increase stability as the compounds' color changes based on pH and temperature. Another example of the use of anthocyanins are water/oil emulsion from the peel of *malus domestica*, this emulsion contains 3% anthocyanins and has a stability of 90 days. This anthocyanin-enriched emulsion has shown positive effects on both skin erythema and melanin content. Since anthocyanins have the ability to absorb UV rays among their characteristics, sun filters and sun creams containing anthocyanin extracts are being developed. Successful sunscreens have been developed using purple sweet potato TNG73 anthocyanins, or anthocyanins from red wine or elderberry. All sunscreens enriched with different types of anthocyanins showed a good ability to filter UV rays but also reduced the production of reactive oxygen species within keratinocytes and fibroblasts. Furthermore, in addition to presenting the classic characteristics already present in sunscreens, these products enriched with anthocyanins have shown to have an anti-aging effect. The use of cosmetic preparations enriched with anthocyanins from red grapes and

chokeberry as preventive treatment for melanoma have shown positive results, decreasing proliferation, mitochondrial membrane potential and oxidative damage, all this while respecting the healthy cells present on the same tissue [20].

1.2.8 Anthocyanins as food colorant

Anthocyanins, in addition to having a marked bioactivity, are brightly colored molecules, with colors that can change based on the pH, so they are able to cover a large part of the chromatic scale. This characteristic could replace the synthetic dyes used by the food industry. Especially since the food industry makes abundant use of these color even though recent studies seems to have found several adverse effects including neurological toxicity.

Furthermore, the use of anthocyanins instead of synthetic dyes in drinks, jams, jams, jellies and also in industrial pastry making, besides giving the color they would also give all the beneficial characteristics of the compound to the food. To transition from synthetic dyes to anthocyanin-based dyes, addressing the challenge of stabilizing anthocyanins is paramount, as they are sensitive to heat and their stability hinges on pH levels. The starting point should be to choose acetylated anthocyanins, which are more stable compared to other anthocyanins. Furthermore, by creating micro-encapsulated systems it would be possible to increase the stability of these natural molecules. If it was possible to overcome the problem of stability and therefore be able to manufacture a product with a shelf life equal to or similar to that of a food with synthetic colorings, there would still remain an obstacle which today seems insurmountable: the cost, as the finished production of anthocyanins that can be used as colorants in the food industry is several times greater than classic colorants [19].

1.3 CHROMATOGRAPHIC TECNIQUES

Analytical chemistry is a branch of chemistry, it has developed over time in order to determine components, substances and traces from complex matrices. Usually, the matrices on which the research is carried out require pre-treatments before a valid analytical result can be obtained, these pre-treatments require more than half the total time to complete the entire analysis. However, pre-treatments are necessary because they allow to reduce the alterations of the result caused by impurities. On the other hand, however, these treatments, in addition to requiring time, also require high physical and material resources [25].

In chromatography, the components of a matrix are temporary bonded to the stationary phase while the mobile phase separates them from each other base on the strength of the interaction. Factors affecting this separation process include molecular characteristics related to adsorption (liquid-solid), partitioning (liquid-solid) and affinity or differences between their molecular weights [22]. The matrix is composed of various substances, each with its own distinct behavior that sets it apart from others within the same matrix. As a result, there will be differences in retention time in the stationary phase, allowing for each substance in the sample to have its own unique elution time from the stationary phase. This differentiation enables the separation of each substance within the matrix [21].

Every chromatography technique has 3 notable components that influence the separation of the compounds:

- The stationary phase: it can be a “solid” or a solid supported, which is created by absorbing a liquid on a surface.
- The mobile phase: it’s composed by a liquid or gas in which the sample is dissolved, the mobile phase will run through the column.
- The sample: it is the matrix to be separated into its components

The interaction between these three components will determine the separation results. Each matrix has a chromatography method that is more effective than others. For matrices containing amino acids, carbohydrates, and fatty acids, partitioning chromatography is recommended, while affinity chromatography is optimal for separating larger molecules like proteins and nucleic acids.

Gas chromatography is the preferred method for assessing enzyme interactions in separating alcohols, esters, and lipids, while agarose gel chromatography remains the optimal choice for evaluating and purifying DNA, viruses, and RNA [22]. Chromatography separates

components in a matrix using either liquid or gaseous mobile phases. the techniques are classified based on this fundamental difference into: liquid chromatography (LC) and gas chromatography (GC). The mobile phase flows over the solid phase and establishes bonds with the matrix, allowing for correct separation of components [21].

Various types of chromatography exist based on the solid phase: from column chromatography to paper chromatography or thin-state chromatography in addition to gel permeation chromatography; each of these is specific to achieve efficient separation in a reasonable time [21].

Types of chromatography:

Column chromatography
Ion-exchange chromatography
Gel-permeation (molecular sieve) chromatography
Affinity chromatography
Paper chromatography
Thin-layer chromatography
Gas chromatography
Dye-ligand chromatography
Hydrophobic interaction chromatography
Pseudo-affinity chromatography
High-pressure liquid chromatography (HPLC)

1.3.1 Column chromatography

This technique is used for the purification of biomolecules such as proteins; the stationary phase consists of a column, which is located inside a glass substrate, inside the column the sample is first added to be separated and then the mobile phase, each component of the sample is going to accumulate at the bottom of the column at different times, the times depend on the interactions established between matrix and column as well as those between matrix and mobile phase [23].

1.3.2. Ion-exchange chromatography

This Technique is based on the formation of ionic bonds between the components of the sample and the stationary phase; the selected stationary phase has opposite charges with the sample to be separated. Several strategies are available to increase polarity so that bonds are formed, such as: changing the pH, the concentration of salts or the ionic strength of the mobile phase. There are both anionic and cationic stationary phases [21].

1.3.3 Gas chromatography

The Stationary Phase is packed into a column, which is placed inside the instrument. The stationary phase is liquid and is absorbed onto an inert solid. Instead, the mobile phase is made up of He oN2, the mobile phase passes inside the column where the stationary phase is contained at high pressure. The mobile phase acts as a carrier gas allowing the substance that makes up the sample to escape from the stationary phase.

It is a highly sensitive and rapidly applied analytical technique, however it is possible to separate only minimal quantities of analyte [21].

1.3.4 Solid phase extraction chromatography

Among the analytical techniques, one of the most important regarding sample preparation is SPE (solid phase extraction), made up of a solid stationary phase and a liquid mobile phase, this technique is based on the characteristic of the sample to form bonds of different intensity with the mobile phase compared to the stationary phase. SPE allows to eliminate interferences and impurities improving the result. There are different types of SPE, each one has been created to allow precise analysis suitable for the material of interest, such as: cartridge SPE, dispersive SPE (d-SPE) [10], (MSPE) which is the magnetic SPE, in addition to the NTD widely applied in the food and organic sectors, this SPE is the needle trap extraction [26].

The needle trap device (NTD) features an extraction trap equipped with a needle containing a fixed sorbent. NTD is an easy-to-use SPE, which does not require solvents, as was the case with other types of SPE, this is an advantage both in terms of costs and for the environment.

NTD can be used for both passive diffusion and active exhaustive sampling.

Even being an innovative technique, it already has several works that see it used as an SPE; an example would be the determination of volatile nitrosamine, which seems to cause oral and

oesophageal tumours. Nitrosamine is found in matrices such as in cured meat and tobacco [27].

1.3.5 High Performance Liquid Chromatography (HPLC)

High-Performance Liquid Chromatography (HPLC) is an analytical chemistry technique used to separate the components of a mixture. Unlike traditional liquid-liquid separation, this technique employs high pressures to optimize the separation of compounds. A typical HPLC method/instrument is composed of:

•*Solvents:* there are typically at least two solvents with differing polarities. This allows the compounds in the sample to form bonds with one solvent over the other, with ionic-type bonds forming between the solvent and the compounds. The analysis can be conducted under isocratic conditions, where the composition of the solvent remain constant throughout, or under gradient conditions, where the solvent composition changes during the analysis (e.g., the percentage of one solvent decrease while that of another increases).

•*Mixer*: necessary for blending the solvents used in the analysis.

•*Pump*: used to generate a specific flow rate of the mobile phase, with the flow rate expressed in ml/min.

•*Injector*: which may function as a sample management system or an automatic sampler, is responsible for introducing the sample into the continuous flow of the mobile phase.

•*Column*: contains the stationary phase, which is necessary for separation. Van der Waals forces are established between the components of the mixture and the stationary phase, which are polar-polar or nonpolar-nonpolar interactions. The separation of the mixture's components occurs as follows: the sample is injected into the system under pressure, and it is packed at the beginning of the column. As the mobile phase passes between the compounds of the mixture and the stationary phase, ionic bonds are formed, which are 100 times stronger than Van der Waals interaction. This allows compounds that have formed a bond with the eluent to continue the journey through the column until they reach the detector.

Type of column:

Porous Graphitic Carbon (PGC)

Surface Porous Silica Particles (SPP)

Monolithic Silica

Completely Porous Silica Particles
Completely Porous Polymer Particles
Chiral HPLC Columns

How compounds are separated:
Chromatographic resolution (RS) is a term used to describe the degree of compound separation. The factors determining resolution are the mechanical separation force (efficiency), which depends on the column length, particle size, and uniformity of the packed bed, and the chemical separation force (selectivity). Efficiency depends on column length, the uniformity of the column packing, and the size of the particles it contains. Maximum efficiency is achieved with a long column uniformly packed with small particles (around 1-2μ).
Detector: There are different types of detectors based on the type of sample or mixture to be analyzed. Detector types include ultraviolet light (UV), fluorescence, or Evaporative Light Scattering (ELSD). The detector's function is to detect and record the changes in frequencies throughout the analysis, which will compose the chromatogram that can be viewed on the computer connected to the detector. Each peak is characterized by different intensity and retention times [27].

1.3.6 Mass spectrometry (MS-MS)

Mass spectrometry is a fundamental analytical technique utilized across various sectors, from pharmaceutical research to toxicology. Essentially, mass spectrometry enables the identification and characterization of molecules present in a sample based on their mass-to-charge ratio (m/z). This process involves analyzing the ions generated from the sample, allowing for precise determination of molecular composition. An essential component of a mass spectrometer is the ion source. The most common ion sources include electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). These sources are widely used in toxicological screening methods, facilitating the production of ions from compounds present in the sample.

Another component is the analyzer, which determines the mass and charge of the generated ions. The three main types of analyzers are quadrupole (Q), ion trap (IT), and time-of-flight

(TOF). Each type of analyzer has specific advantages. For example, the QqQLIT configuration, which combines quadrupoles and ion traps, offers increased sensitivity and is often employed in screening unknown compounds. Additionally, combining mass spectrometry with other chromatographic techniques, such as liquid chromatography (LC-MS) or gas chromatography (GC-MS), further enhances the capabilities of this analytical technique, allowing for effective separation and detailed characterization of compounds [28].

2. AIM

The focus of this thesis is centered on the objective of developing a unified and efficient method for the isolation of anthocyanins from various plant matrices. This goal stems from research conducted concurrently with the REQUIMTE/LAQV Chemistry and Biochemistry of the Faculty of Sciences at the University of Porto. The group aims to evaluate the absorption and bioaccessibility of anthocyanins at oral, gastric, and intestinal levels, from different edible flowers, including wild pansy and cornflower. These two species are particularly significant as they are increasingly gaining attention in the culinary field, representing an innovation in this environment, as well as for their already observed benefits. Pure anthocyanin extracts are an essential tool to understand the real fate of the anthocyanins present in these edible flowers using in vitro approaches, so as to determine if they are absorbed through oral intake and thus can play their beneficial role. Furthermore, using a pure anthocyanin extract would ensure that any beneficial effects are just mediated by anthocyanins and not potentially confounded by other components present in these two-flower species, yielding a certain result without any doubts. The objective is not easily achievable due to the complexity of hydrophilic substances present in the matrices, where the presence of other polyphenols, such as flavonoid glycosides, may hinder isolation due to their chemical structure similarity to anthocyanins.

Therefore, the primary aim of this research is the development of a standardized and optimized method for anthocyanin extraction, with the goal of obtaining pure and high-quality extracts. This research extends beyond the scientific realm to practical application in various industrial sectors, including food, pharmaceutical, and cosmetic industries. The isolation of anthocyanins will not only promote the development of new high-value-added products but will also contribute to the promotion of sustainable practices in agriculture and food, valorizing the potential of edible flowers as a source of beneficial bioactive substances for human health.

It should be noted that, although the research conducted in parallel primarily focuses on isolating anthocyanins from cornflower and wild pansy, this research has expanded to other plant matrices such as berries, tubers, and vegetables.

This decision was made with the aim of providing a universal method applicable to every plant matrix, ensuring cost-effectiveness, rapidity, and environmental sustainability.

3. MATERIALS AND METHOD

Samples:

The matrices used for the experiments were cornflower (*Centaurea cyanus L.*); wild pansy (*Viola tricolor L.)*, purple basil (*Ocimum basilicum L.)*, purple sweet potato (*Ipomoea batatas)*, purple cabbage (*Brassica oleracea L.)* and Elderberry *(Sambucus nigra L.).*

The selection of such matrices was made to encompass a wide range of sources, including flower, tubers, berries and vegetables that contain structurally different anthocyanins.

Reagents and Instruments:

- Deionized water
- Hydrochloric acid
- Methanol
- Beaker (Simax Czech Republic)
- Freeze dryer (Bench Top Pro with omnitronics™)
- Tweezers
- Flask
- Balance ABT 120-5DM Kern
- Ultrasonic water bath (VWR ultrasonic cleaner)
- Refrigerated Centrifuge (Velocity 14 R refrigerate centrifuge)
- Filter funnel (Fisherbrand™)
- Paper for filtration
- Micropipette

Solvent for SPE:

- Deionized water with HCl 1M
- Methanol with HCl 1M
- Ethyl acetate
- Methanol with NaOH 1M
- Potassium phosphate dibasic HK2O4P
- Ultrapure MilliQ® H_2O 0,1% acid formic
- Phosphate buffer (HK_2O_4P) 10mM pH6

Instruments for SPE:

- Rocker 300C Pump
- HyperSep™ Glass Block Vacuum Manifolds
- Spoon
- Spatula
- Balance (ABT 120-5DM of KERN)
- Vacuum flask 250ml (SIMAX®)
- pH Meter (pH 538 WTW)

- Magnetic stirrer (of DLAB)
- Glass funnel

3.1 Sample preparation

The preparation of the different matrices involves some shared steps, while other vary, in order to achieve the highest level of extraction of the soluble components contained within each matrix.

Cornflower and Wild pansy: for the preparation of samples of the two flowers, the petals were separated from pistils, sepals, receptacles and peduncles, using gloves and tweezers. Subsequently, the water contained in the previously separated petals was removed using freeze drying. The petals underwent this dehydration process for two days and two nights, at temperature of – 85,4°C and pressure of 44mT. Then, 200 mg of dried petals were taken from each flower and 20 ml of deionized water with HCl 1M were added to them. The suspension was placed on a magnetic stirrer for 4 hours to allow the color components in the petals to dissolve into solution. After extracting the soluble components from petals (the petals appear colorless), the soluble par was separated from the insoluble part by using a centrifuge for 10 minutes at 10,000 rpm. This caused the particulate matter to settle at the bottom of the tube, making it easy to separate from the solution, which appeared bright red for cornflower and purple for wild pansy.

The two solutions underwent a final filtration step by using a paper membrane to completely remove the insoluble fractions from the solution. For this filtration, a glass funnel was used, with a paper membrane with pores of 0,44μm placed inside it. This complex was setup on vacuum flask and connected to a pump.

The two solutions obtained were placed on freeze-drying for some days, until the water present in the samples was deleted, and then the powder of cornflower and wild pansy were stored in freeze at -20°C, until use.

Figure 5. Filtration on paper

Purple basil and purple cabbage: The first step performed on these two samples was to reduce their initial size, in order to expedite and enhance the subsequent freeze-drying process. The samples were then freeze-dried for two days and two nights, and subsequently ground to obtain powders - a purple powder for the Purple cabbage and a copper-colored powder for the Purple basil. The grinding process was carried out using the Vorwerk Bimby VM2000. 5g of purple cabbage powder was taken and mixed with 200 mL of deionized water with HCL 1M. 2 g of purple basil powder were added to 100mL of deionized water with HCL 1M. The two suspensions, contained in amber glass jars, were subjected to an overnight ultrasonic bath at room temperature. This process resulted in a purple solution for the Purple cabbage and a red solution for the Purple basil. Following this step, the separation between the particulate matter and the substances in solution was carried out using the same processes mentioned earlier for the cornflower and wild pansy samples: centrifugation and filtration. The two solutions obtained were placed on freeze-drying for some days, until the water present in the samples was deleted, and then the powder of purple basil and purple cabbage were stored in freeze at -20°C, until use.

Black Elderberry: the treatment performed on this matrix involved crushing the berries and subsequently, both the juice and the peels underwent dehydration through freeze-drying. The sample, following the dehydration procedure was ground using a Vorwerk Bimby VM2000, resulting in dark red powder 3 g of powder was them mixed with 200 mL of deionized water

with HCl 1M, and the resulting suspension underwent centrifugation and filtration through a 0.2 µm paper filter. The solution obtained was placed on freeze-drying for some days, until the water present in the berries was deleted, and then the powder was stored in the freeze at -20°C.

Figure 6. Samples within the freeze drying

Purple Sweet Potato: The first step involved cutting the sweet potato into one-centimeter cubes to expedite and enhance the dehydration process. The cubes were then subjected to freeze-drying for two days and two nights at a pressure of 44mT and a temperature of -85.4 °C. Following dehydration, the soluble components were extracted from the matrix by 200ml of deionized water with HCl 1M for 5 g of powder. The suspension was subjected to an ultrasonic bath overnight at room temperature. Since the sweet potato matrix is porous, it was necessary to squeeze the cubes to release the water contained within them before separating the solid part from the solution. The separation of the substances in solution from those in suspension was achieved through centrifugation and filtration, processes already used for the other samples. The solution obtained was placed on freeze-drying for some days, until the water present in was deleted, and then the powder was stored in the freeze at -20°C.

3.2 SPE, solid phase extraction

3.2.1 Liquid-liquid extraction and C18 Silica gel spherical

40 mg of freeze-dried purple cabbage extract was weighed, and 100 mL of deionized water with HCl 1M were added. The solutions obtained were transferred to a separatory funnel. To this solution, 100mL of ethyl acetate were added, previously measured using graduated cylinder. The separatory funnel stopper was closed, and the contents inside were shaken so that solutions components can separate based on their polarity. Occasionally, the funnel's stopcock was opened to release the air that forms inside, and then shaking was resumed. These steps were repeated several times to ensure the best dispersion of the compounds among the organic and the aqueous phase. Finally, the separatory funnel was placed on the stand, the stopper was removed, and a 10-minute waiting period allowed the two phases to properly separate.

Two solutions were obtained: one was aqueous and purple, and above it was an organic straw-yellow solution. The two solutions were collected in two different beakers.

20 g of C18 Silica gel spherical were weighed and transferred to a funnel with a membrane. Everything was setup on a vacuum flask connected to a pump to create vacuum. Before introducing the sample to be purified, the activation and packaging of the solid phase was necessary, so three washing phases with methanol with HCl 1M and three with deionized water with HCl 1M are performed. Then, the aqueous purple solution obtained previously was added. During the step, it is better to work with the vacuum pump off so that C18 Silica gel spherical becomes impregnated with solutions and changes from white to pink. The water contained in the solutions was collected in the coded flask. To remove sugar contained in the matrix, a washing step with 500 mL of deionized water with HCl 1M was performed.

Finally, to elute the matrix from the powder, washes with methanol with HCl 1M were performed until the powder appear colorless. To the obtained solution, 10 mL of water was added, and subsequently a step by using a rotary evaporator to remove the methanol was performed. 100µl of the resulting solution is taken and examined with HPLC.

Figure 7. Purple cabbage liquid-liquid extraction

3.2.2 C18 Silica gel spherical

Twenty grams of C18 Silica gel spherical were weighed and transferred into a funnel with a membrane. The setup was placed on vacuum flask connected to a pump to a vacuum. Packing and activation were then carried out using three washing phases of methanol with HCl 1M and three of deionized water with HCl 1M.

Then, 3 mg of dried purple cabbage powder obtained powder obtained post-lyophilization were weighed and diluted in 10 mL of deionized water with HCl 1M. The resulting solution was added to the funnel containing the C18 Silica gel Spherical, with the pump disconnected. After the matrix has been absorbed by the powder, the purification and treatment proceeds as follows:

- 40 bed volumes (BV) of ethyl acetate to recover phenolic acids.
- 40BV of deionized water with HCl 1M to remove sugars.

Subsequently, the water and ethyl acetate volumes were evaporated. To the ethyl acetate volumes, 2 ml of water were added. Of the resulting solutions 100 µL were analyzed by HPLC. To extract the matrix containing the anthocyanins, methanol with HCl 1M elution was performed until the powder is colorless. Two milliliters of water were added to the methanol solution, which was evaporated using rotary evaporator until reaching a volume of 2 mL (to

ensure complete removal of methanol). Finally, 100µL were injected into the HPLC to evaluate the degree of purification achieved.

Figure 8. Purple cabbage purification on C18 Silica gel spherical

3.2.3 Amberlite XAD 7HP

20 g of Amberlite XAD 7HP were weighed and transferred into a beaker. 50 mL of methanol were added to the beaker and a magnet stir bar, and the beaker was covered with parafilm. The beaker was placed on a magnetic stirrer for 1 hour at a speed that allows all the material to come into contact with the methanol. After an hour, the excess methanol was removed, and an aqueous solution containing 3 mg of free-dried powder of purple cabbage was added to the beaker. The beaker was placed back on the magnetic stirrer for one day. After a day, the 20 g of Amberlite XAD 7HP which had inside the sample were packed into the membrane funnel.

The purification treatment consisted:

- 40BV of ethyl acetate are used to recover phenolic acids.
- 40BV of deionized water with HCl 1M are used to eliminate sugars.

The volumes of water and ethyl acetate were evaporated, and 2 mL of water were added to the volumes of ethyl acetate obtained. 100 µl of the resulting solutions were analyzed using HPLC. To recover the anthocyanins, elution with acidic methanol was performed until the

powder is no longer colored. 2 mL of water were added to the methanol solution, and an evaporating step using a rotary evaporator until a volume of 2 mL was reached (to ensure complete removal of the methanol). Finally, 100 µl were injected into the HPLC to assess the degree of purification achieved.

Repetition 2 of Amberlite XAD 7HP

7,5 g of Amberlite XAD 7HP were weighed and transferred into a beaker. 50 mL of methanol were added to the beaker and a magnet stir bar, and the beaker was covered with parafilm. The beaker was placed on a magnetic stirrer for 1 hour at a speed that allows all the material to come into contact with the methanol. After an hour, the excess methanol is removed, and an aqueous solution containing 3mg of free-dried powder of purple cabbage is added to the beaker. The beaker was placed back on the magnetic stirrer for one day. After a day, the 7,5 g of Amberlite XAD 7HP which had inside the sample were packed into the membrane funnel.

The purification treatment consisted:

- 40BV of ethyl acetate are used to recover phenolic acids.
- 40BV of deionized water with HCl 1M are used to eliminate sugars.

The volumes of water and ethyl acetate were evaporated. 2 mL of water are added to the volumes of ethyl acetate obtained. 100µl of the resulting solutions were analyzed using HPLC. To extract the matrix containing the anthocyanins, elution with acidic methanol is performed until the powder is no longer colored. 2 mL of water were added to the methanol solution, and an evaporating step using a rotary evaporator until a volume of 2 mL was performed (to ensure complete removal of the methanol). Finally, 100 µl were injected into the HPLC to assess the degree of purification achieved.

3.2.4 Toyopearl HW65-S

30 g of Toyopearl HW65-S were weighed and transferred into a funnel with membrane. The setup was then placed on a vacuum flask connected to a pump to create a vacuum. It was compacted and activated through three washing phases with methanol with HCl 1M and three with water with HCl 1M.

3 mg of purple cabbage powder obtained after lyophilization were weighed and diluted in 10mL of deionized water. The obtained solution was added to the funnel containing Toyopearl HW65-S, with the pump disconnected. After the matrix has been absorbed by the powder, the purification treatment consisted:

- 40BV of ethyl acetate to recover phenolic acid.
- 40BV of deionized water with HCl 1M to remove the sugars.

The volumes of water and ethyl acetate were evaporated. 2 mL of water are added to the volumes of ethyl acetate obtained. 100µl of the resulting solutions were analyzed using HPLC. To extract the matrix containing the anthocyanins, elution with acidic methanol is performed until the powder is no longer colored. 2 mL of water were added to the methanol solution, and an evaporating step using a rotary evaporator until volume of 2 mL was performed (to ensure complete removal of the methanol). Finally, 100 µl were injected into the HPLC to assess the degree of purification achieved.

3.2.5 Sephadex LH-20

20 g of Sephadex LH-20 were carefully weighed and swollen in methanol for one hour. Subsequently, the membrane funnel is packed with the swollen powder and methanol. Once the SPE column was prepared, the aqueous solution containing 3 mg of lyophilized purple cabbage extract was added.

The purification treatment consisted:

- 40BV of ethyl acetate to recover phenolic acid.
- 40BV of deionized water with HCl 1M to remove the sugars.

The volumes of water and ethyl acetate were evaporated. 2 mL of water are added to the volumes of ethyl acetate obtained. 100µl of the resulting solutions were analyzed using HPLC. To extract the matrix containing the anthocyanins, elution with acidic methanol is performed until the powder is no longer colored. 2 mL of water were added to the methanol solution, and an evaporating step using a rotary evaporator until volume of 2 mL was performed (to ensure

complete removal of the methanol). Finally, 100µl were injected into the HPLC to assess the degree of purification achieved.

3.2.6 Oasis® HLB 3cc Vac cartridge

Repetition 1: Oasis® HLB 3cc Vac cartridge

Oasis® HLB 3cc Vac cartridge: 0.5 mg of lyophilized purple cabbage powder were weighed and dissolved it in acidified water. Place an Oasis® HLB 3cc Vac cartridge on a HyperSep™ Glass Block Vacuum Manifold and connect the system to a pump to create a vacuum. Before turning on the pump, the Oasis® HLB 3cc Vac cartridge was activated with acidic methanol. After activation, was add the solution containing the sample to be purified.

Subsequently, the purification treatment consisted:

- 40BV of ethyl acetate to recover phenolic acid.
- 40BV of deionized water with HCl 1M to remove the sugars.

The volumes of water and ethyl acetate were evaporated. 2 mL of water are added to the volumes of ethyl acetate obtained. 100µl of the resulting solutions were analyzed using HPLC. To extract the matrix containing the anthocyanins, elution with acidic methanol is performed until the powder is no longer colored. 2 mL of water were added to the methanol solution, and an evaporating step using a rotary evaporator until volume of 2mL was performed (to ensure complete removal of the methanol). Finally, 100 µl were injected into the HPLC to assess the degree of purification achieved.

Repetition 2: Oasis® HLB 3cc Vac cartridge, determining how many BV (bed volumes) to use of acidic water and ethyl acetate

0.6 mg of freeze-dried purple cabbage powder were weighed and dissolved it in acidic water. Place the Oasis® HLB 3cc Vac cartridge on the HyperSep™ Glass Block Vacuum Manifolds and connected the system to a pump to create a vacuum. Before starting the pump, the Oasis® HLB 3cc Vac cartridge was activated with methanol with HCl 1m, then was added the solution containing the sample to be purified. To determine how many BV were needed to purify the sample, it was necessary to collect various fractions in different containers. Four fractions of ethyl acetate corresponding to 8BV (8BV corresponds to a full cartridge), 16BV, 24BV, and 32 BV were collected. The same procedure was carried out with acidic water, introducing 8,

16, 24, 32, and 40BV. All fractions were evaporated using a rotavapor, brought to a volume of 2 mL, and of these, 100 µl were injected into an HPLC to determine the necessary volumes of the two solvents for purification.

Repetition 3: purification with pre-established BV

0,6 mg of lyophilized purple cabbage powder were weighed and dissolved in water. Place the Oasis® HLB 3cc Vac cartridge on a HyperSep™ Glass Block Vacuum Manifold and connect the system to a pump to create a vacuum. Before starting the pump, activate the Oasis® HLB 3cc Vac cartridge with methanol with HCl 1M, then add the solution containing the sample to be purified.

Subsequently, the purification process consisted:

- 24 BV of ethyl acetate to recover phenolic acids.
- 40BV of water with HCl 1M to delete sugars.

After evaporating the volumes of water and ethyl acetate, add 2 mL of water to the volumes of ethyl acetate. Of the obtained solutions, 100 µL were examined using HPLC. To extract the matrix containing the anthocyanins, proceed with elution using acidic methanol until the powder no longer shows any color. Then add 2 mL of water to the methanol solution and evaporate it using a rotary evaporator until the volume reaches 2 mL to ensure complete removal of the methanol. Finally, was injected 100 µL into the HPLC to assess the achieved purity level.

Repetition 4: Oasis® HLB 3cc Vac cartridge, solvent exchange for purification, in this case deionized water with HCl 1M was used first followed by ethyl acetate.

0,6 mg of purple cabbage powder was weighed and dissolved in deionized water. The cartridge of Oasis® HLB 3cc Vac cartridge was placed on the HyperSep™ Glass Block Manifolds, and system was connected with a pump. The cartridge was activated with methanol with HCl 1M, and after that the solution was loaded inside the cartridge.

Subsequently, the purification treatment consisted:

- 40BV of deionized water with HCl 1M, to remove sugars
- 24BV of ethyl acetate to remove phenolic acids

After evaporating the volumes of water and ethyl acetate, add 2 mL of water to the volumes of ethyl acetate. Of the obtained solutions, 100 µl were examined using HPLC. To extract the matrix containing the anthocyanins, proceed with elution using acidic methanol until the powder no longer shows any color. Then add 2 mL of water to the methanol solution and evaporate it using a rotary evaporator until the volume reaches 2 mL to ensure complete removal of the methanol. Finally, was injected 100 µL into the HPLC to assess the achieved purity level.

Figure 9. Purple cabbage purification on Oasis® HLB 3cc Vac cartridge

3.2.7 Oasis® HLB 3cc Vac cartridge and Discovery® DSC-MCAX SPE 52788-U

Repetition 4: for all sample, application of this method for the other matrix:

Purple basil, Black Elderberry, Centaurea cyanus, wild pansy and sweet potato.

Purple Basil: 20 mg;1,003 mM of anthocyanins

Black Elderberry: 0,909 mM of anthocyanins

Centaurea cyanus:10,27 mg; 1,469 mM of anthocyanins

Wild pansy: 9,49 mg; 0,637 mM of anthocyanins

Sweet potato: 7,575 mM of anthocyanins

The molar concentration of anthocyanins was determined using the calibration curve with reference to Cyanidin-3-O-glucoside (Cy-3-glc). An aqueous solution of the different matrices was prepared, which was then loaded into Oasis® HLB 3cc Vac cartridge for purification. For each matrix, an Oasis® HLB 3cc Vac cartridge is used to avoid contamination and alterations to the results. The cartridges were placed on HyperSep™ Glass Block Vacuum Manifolds, connected to a pump. The pump is turned on until a pressure of 5 atmospheres was reached, and the cartridges are activated with different volumes of methanol with HCL 1M. Subsequently, the samples are loaded. This step is performed with the pump turned off to promote interactions between the matrices and the powder contained in the cartridge.

Subsequently, the purification treatment consisted:

- 40BV of deionized water with HCl 1M, to remove sugars
- 24BV of ethyl acetate to remove phenolic acids

After evaporating the volumes of water and ethyl acetate, add 2 mL of water to the volumes of ethyl acetate. Of the obtained solutions, 100 µL were examined using HPLC. To extract the matrix containing the anthocyanins, proceed with elution using acidic methanol until the powder no longer shows any color. Then add 2 mL of water to the methanol solution and evaporate it using a rotary evaporator until the volume reaches 2 mL to ensure complete removal of the methanol. Finally, was injected 100 µL into the HPLC to assess the achieved purity level.

Discovery® DSC-MCAX SPE 52788-U.

The Discovery® DSC-MCAX SPE 52788-U cartridges were arranged on HyperSep™ Glass Block Vacuum Manifolds, with each matrix having its own cartridge to avoid altering the results. They were connected to a pump set at 5 atm. The cartridge is activated with 15 mL of methanol (1M HCl) and equilibrated with 15 mL of 0.1% formic acid in water. Samples from the various matrices obtained from purifications with: Oasis® HLB 3cc Vac cartridge are added to 10 mL of 0.1% formic acid in water and inserted into the matrix with the pump turned off to promote interactions between the sample and the matrix components. To remove the non-anthocyanin fraction, 100 mL of methanol is used, while a 1:1 solution of methanol and pH 6 buffer is used to elute the anthocyanins from the cartridge, with 100 mL of this solution being used.

The Anthocyanins fraction contained a salt to delete it, it was necessary load the anthocyanins fraction for all sample on the Silica C-18SPE™ cartridges. The salt was deleted with 500mL of deionized water, and then to take out the anthocyanins from the cartridges some wash with methanol with HCl 1M. Both the anthocyanin and non-anthocyanin fractions were evaporated using a rotary evaporator, dried, and finally brought to a volume of 2 mL. 100 µl of both solutions were injected into the HPLC to determine the degree of purity.

3.2.8. The Discovery® DSC-MCAX SPE 52788-U

Cartridges were arranged on HyperSep™ Glass Block Vacuum Manifolds, with each matrix having its own cartridge to avoid altering the results. They were connected to a pump set at 5 atm. The cartridge is activated with 15 mL of methanol (HCl 1M) and equilibrated with 15 mL of 0.1% formic acid in water. Samples from the various matrices obtained from purifications with: Oasis® HLB 3cc Vac cartridge are added to 10 mL of 0.1% formic acid in water and inserted into the matrix with the pump turned off to promote interactions between the sample and the matrix components. To remove the non-anthocyanin fraction, 100 mL of methanol is used, while a 1:1 solution of methanol and pH 6 buffer is used to elute the anthocyanins from the cartridge, with 100 mL of this solution being used.

The Anthocyanins fraction contained a salt to delete it, it was necessary load the anthocyanins fraction for all sample on the Silica C-18SPE™ cartridges. The salt was deleted with 500mL of deionized water, and then to take out the anthocyanins from the cartridges some wash with methanol with HCl 1M. Both the anthocyanin and non-anthocyanin fractions were evaporated using a rotary evaporator, dried, and finally brought to a volume of 2 mL. 100µL of both solutions were injected into the HPLC to determine the degree of purity.

3.3 UHPLC-DAD Analysis

The instrument was used to determinate the purity of the anthocyanin fractions is the Thermo Scientific® Dionex Ultimate UHPLC 3000 ultimate, which is a high-performance liquid chromatography HPLC system. An aliquot of 20 µL of all fraction was injected and the method was carried out in a Thermo Scientific® Dionex Ultimate UHPLC 3000 ultimate.

For the chromatographic separation, conditions were as follows: of 5% formic acid as solvent A and 5 % of formic acid, 30 % acetonitrile as solvent B. The solvent gradient started with

5,5 % B up to 36 % B (0-7min), and then up to 80 % B (7-10min), and then up to A solution of 100 % B was then applied (10-15min), followed by the equilibration step under the initial conditions. The stationary phase utilized was a reversed-phase C18 Hypersil GOLD™ 50 × 2.1 mm with 5 µm size column with a steady flux of 0.5 mL min-^{1}.

For the chromatographic separation, conditions were as follows: use of 5 % formic acid as solvent A and 1 % formic acid, acetonitrile as solvent B. The solvent gradient started with 1,0 % B up to 15 % B (0–3min), and then up to 18 % B (3–10 min), and then up to A solution of 100 % B was then applied (10–20 min), followed by the equilibration step under the initial conditions (20–25 min). The stationary phase utilized was a reversed-phase C18 Hypersil GOLD™ VANQUISH 150 × 2.1 mm with 1.9 µm particle size column with a steady flux of 0.3 mL min^{-1}.

4. RESULTS AND DISCUSSION

The research findings are centered around two parameters: the purity and recovery of anthocyanins in the methanol fraction, a solvent aimed at extracting only the anthocyanins from the stationary phases where they are located, following purification washes. The primary objective remains obtaining a pure extract, which can yield specific results regarding the potential of the matrices from that sample. The aim is to avoid other substances, always belonging to that matrix but of a different nature from the anthocyanins, from altering the result. Recovery remains an important parameter, especially at the time when one wants to obtain a purified extract of anthocyanins from matrices such as cornflower or wild pansy, given the high cost of edible flowers. High cost given that there are only two companies in Portugal involved in the cultivation of edible flowers.

The first studies are based on purple cabbage, since it is the cheapest matrix in addition to being the matrix that has a higher number of anthocyanins than the other matrices examined, so by going to analyze the behavior of anthocyanins contained in this matrix it could be possible to treat the other matrices with the purification method obtained for purple cabbage.

Both purity and recover values were obtained by using Thermo Scientific® Dionex Ultimate UHPLC 3000 ultimate, where the peak area was integrated and calibrated with a standard.

The first step for this analysis was to optimize a method that would allow both the elution of all soluble components extracted from the matrix in the time interval of the run and also allow for good peak definition, all of which were possible using Chromeleon® software. The following chromatograms and PDA were set at two wavelengths: 280 nm and 520 nm. At the first wavelength all the components of the different matrices absorb light, while at 520 nm only the anthocyanins contained in the matrix absorb the light intensity.

Therefore, a fraction is defined as pure when its chromatogram shows the same peaks at both a wavelength of 280 nm and 520 nm. Calculation of anthocyanin recovery, on the other hand, is based on the ratio of the total area obtained at 520 nm for that specific fraction, divided by the total area of the original sample without purification processes. This calculation is performed by keeping the intensity of light at 520 nm constant for all analyses.

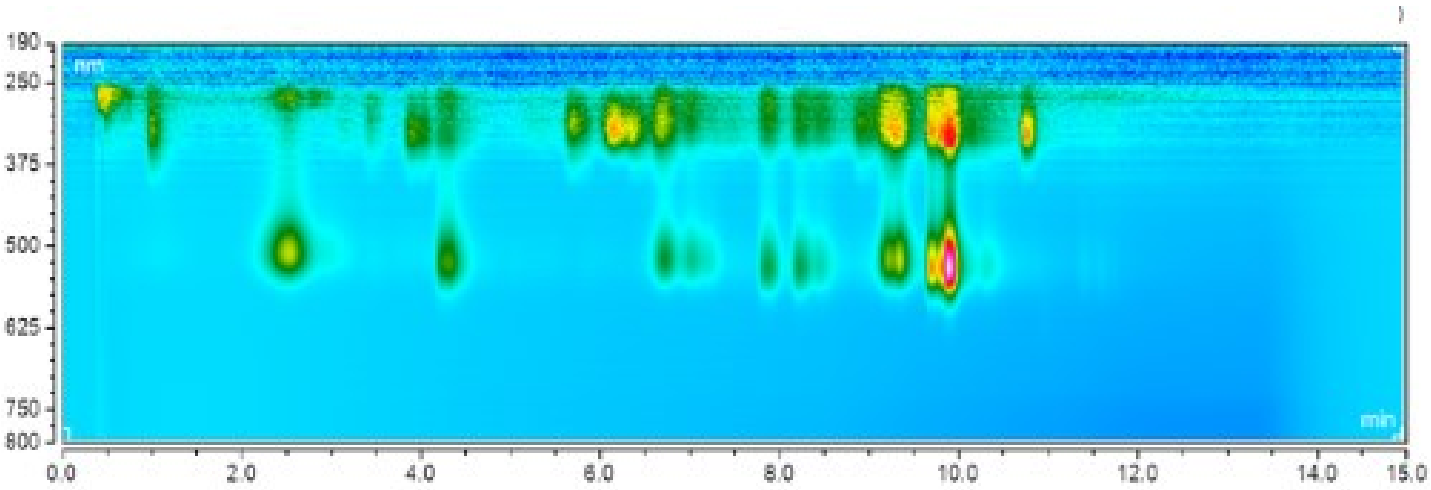

Figure 10. PDA 1Extract of purple cabbage after ultrasounds

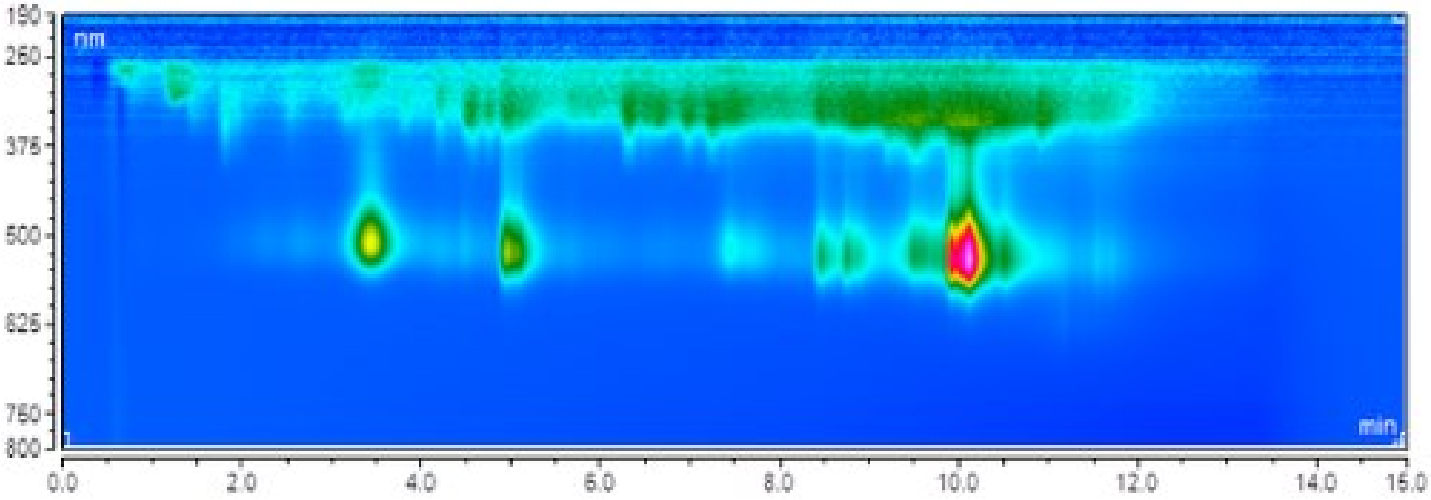

Figure 11. PDA 2 Liquid-liquid extraction and after C18 Silica gel Spherical

Figure 10PDA 1 shows the chromatogram of purple cabbage after ultrasound, it can be seen that the injection method chosen is optimal because in the range of the chromatographic run both anthocyanins and other soluble components of the matrix elute, moreover, the peaks show up with good definition. The colors range from green to red, where green indicates low concentrations and red indicates high concentrations.

The soluble extract, which was prepared after extraction with water, contains 7.5 % of anthocyanins. This assumes that it is necessary to find a purification method that can concentrate all the anthocyanins contained in purple cabbage powder.

Instead, Figure 11 PDA 2 shows the chromatogram obtained after purification process based on two steps: the first is a liquid-liquid extraction with an organic solvent (ethyl acetate) while the solvent in which the anthocyanins were concentrated is water. The aqueous solution from the liquid-liquid extraction was introduced into the packed and activated C18 column. The solutes (anthocyanins, sugars, proteins) that were contained in the aqueous solution were bound to the stationary phase.

To obtain the purified anthocyanins, water was washed with HCL 1M to remove sugars and proteins. This purification process in addition to not presenting a pure anthocyanin extract is also time-consuming, thus after observing the outcome and the amount of time needed to get there it was preferred to proceed and use other stationary steps, to obtain a pure anthocyanin extract.

4.1. Purification treatment with: Amberlite XAD 7HP

It is a macroporous, non-ionic, aliphatic acrylic resin supplied in the form of insoluble beads. Its macroporous structure, consisting of a continuous polymer phase and a continuous spore phase, together with its high surface area and aliphatic nature, contribute to its adsorptive properties. Amberlite XAD 7HP is able to adsorb non-polar compounds from aqueous systems and polar compounds from non-polar solvents due to its aliphatic nature. The resin microspheres are white, translucent and spherical, with a surface area of 500m^2/g. The pore size is generally around 550Å and the particle diameter varies from 430 to 690 µm.

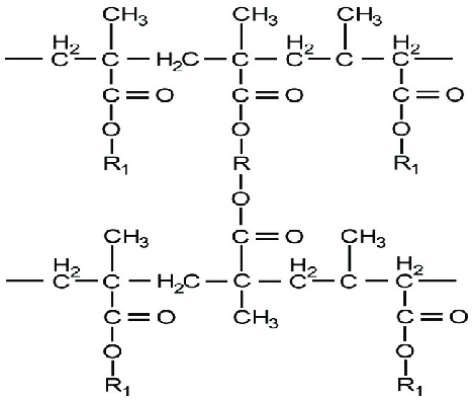

Figure 12. Polymer from which Amberlite XAD 7HP is composed

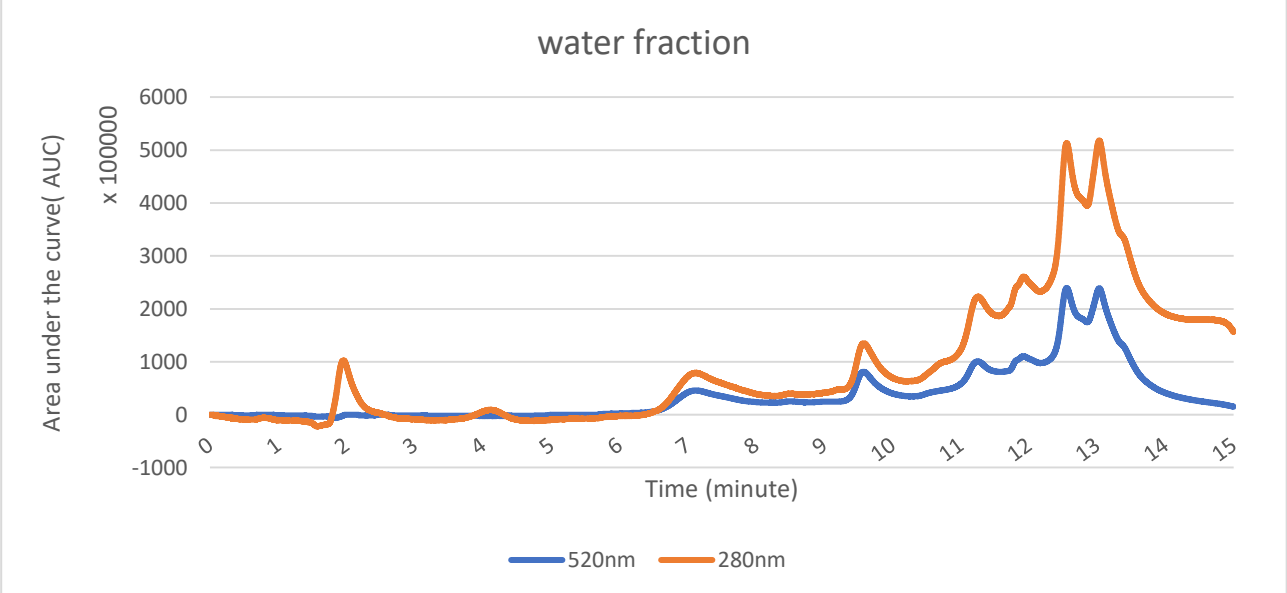

Figure 13. water fraction obtained from the stationary phase Amberlite XAD 7HP

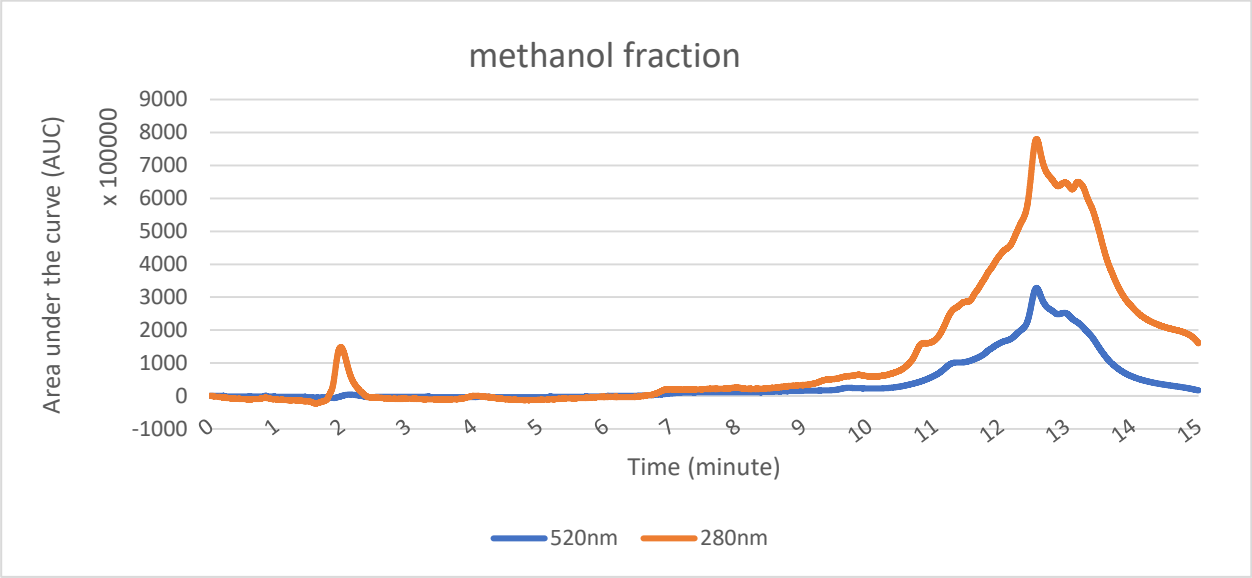

Figure 14. methanol fraction obtained from the stationary phase Amberlite XAD 7HP

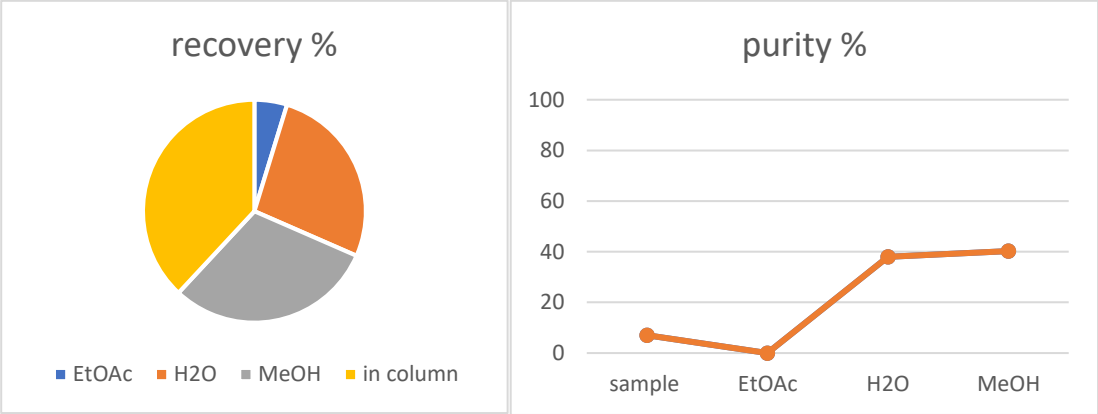

Table 1. recovery and purity after purification with Amberlite XAD 7HP

Figures 13 and 14 show the result of the first treatment (rep1) with Amberlite XAD 7HP.For this treatment, 20 g Amberlite XAD 7HP and 3mg purple cabbage powder were used. The purification process involves a first treatment with 40BV ethyl acetate followed by 40BV water with HCl 1M, figure 13 shows the results obtained from this second wash. Figure 14, on the other hand, is the result of the extraction of the anthocyanins plus the other purple cabbage substances that were still in the Amberlite XAD 7HP stationary phase. The elution of these was done with methanol with HCl 1M until the stationary phase was colorless. Assessing the results obtained, several problems were encountered, the first of which

concerned the stationary phase as it retained 38 % of the anthocyanins contained in the sample (a fairly predictable result given that during the purification process, the powder remained a pale pink color, even after undergoing several methanol washes). The result would be even more problematic if, the purification process was carried out on high-cost matrices. The second problem encountered is that another 27 % anthocyanins elute in the aqueous fraction, further reducing the methanol recovery. Instead, the last problem, which is the most serious since the objective of the research is to obtain a pure extract, the methanol fraction not only has a low recover of anthocyanins but is no more than 40% pure. It was not possible to add the aqueous fraction to the methanol extract or to collect the anthocyanins in the two fractions as the aim of the research remains to find a solution that can be adopted on all matrices. Since other vegetables such as tubers, fruits and berries contain a higher amount of sugars than purple cabbage, this would not allow a pure anthocyanin extract to be obtained, and carbohydrates and proteins would also have to be collected. Assessing the anthocyanin distribution in the aqueous fraction and in the methanol fraction, and this can be accomplished by examining the chromatogram at a wavelength of 520 nm (blue line). In fact, the distribution of anthocyanins according to their polarity is not homogeneous. Thus, the number of anthocyanins in the aqueous fraction is greater than the number of anthocyanins in the methanol fraction.

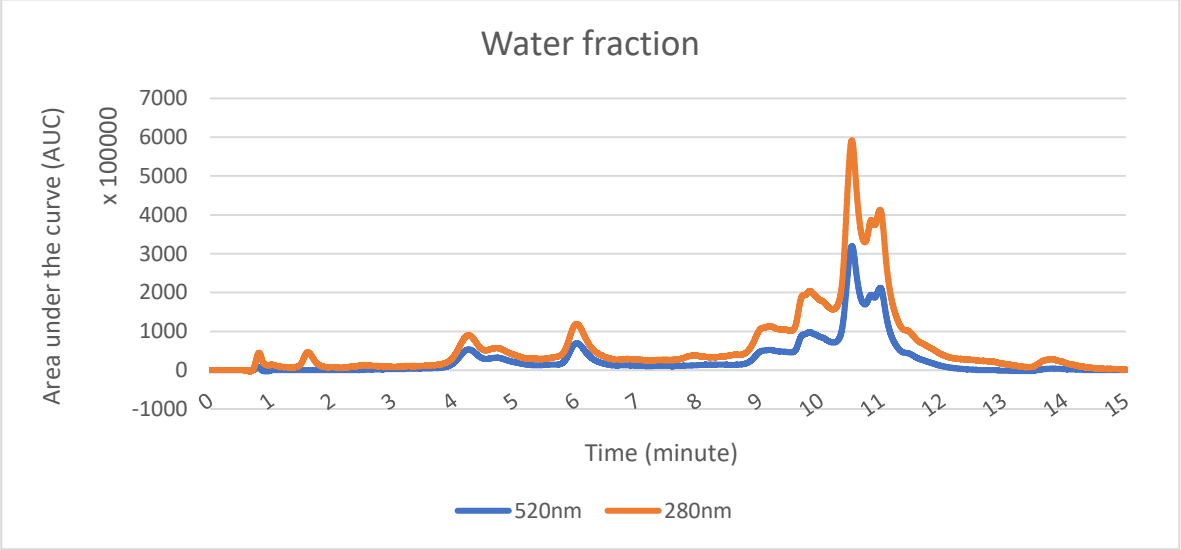

Figure 15. aqueous fraction obtained from the stationary phase Amberlite XAD 7HP

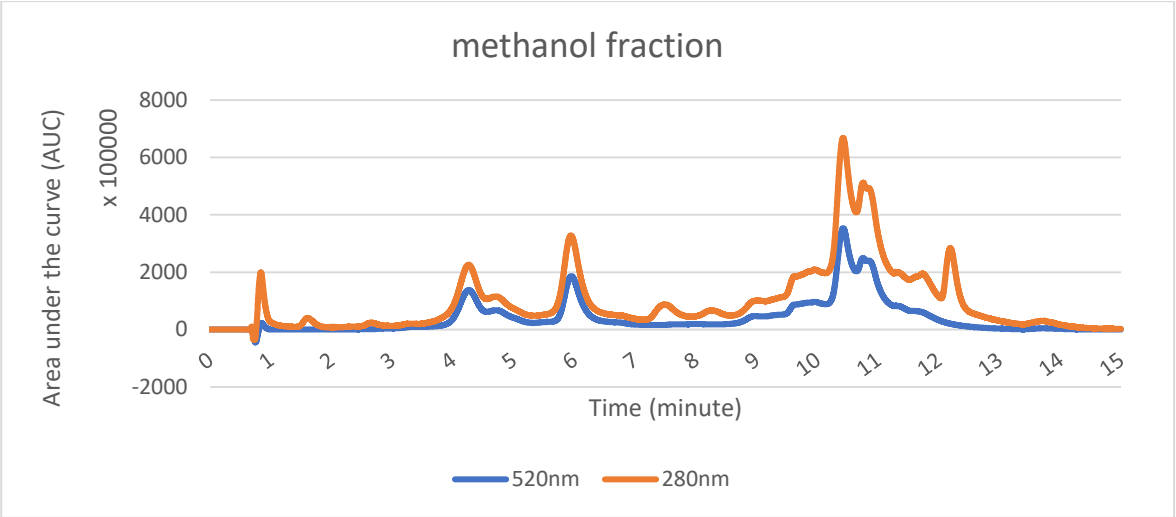

Figure 16. methanol fraction obtained from the stationary phase Amberlite XAD 7HP

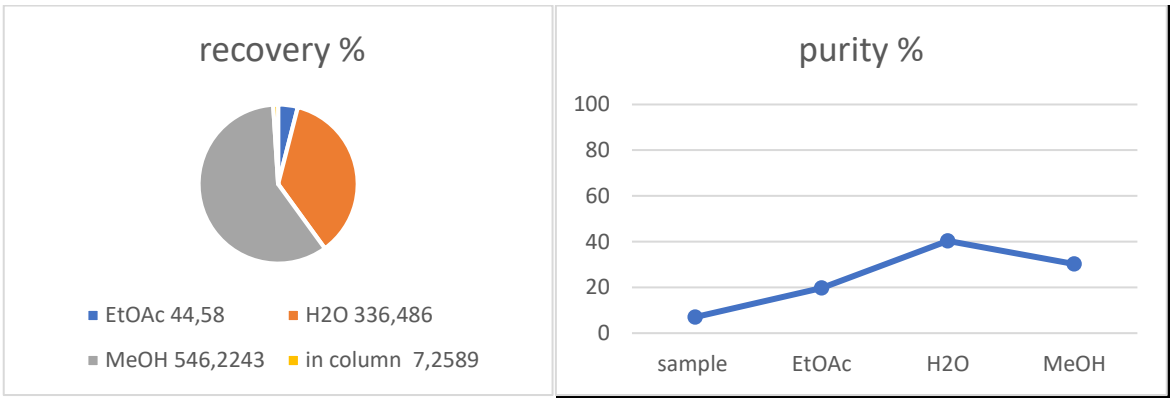

Table 2. recovery and purity after purification with Amberlite XAD 7HP

Figures 15 and 16 report the result of the first treatment (rep2) with Amberlite XAD 7HP. For this treatment, 7,5 g Amberlite XAD 7HP and 3mg purple cabbage powder were used. The purification process involves a first treatment with 40BV ethyl acetate followed by 40BV water with 1M HCl, figure 15 shows the results obtained from this second wash. Figure 16, on the other hand, is the result of the extraction of the anthocyanins plus the other purple cabbage substances that were still in the Amberlite XAD 7HP stationary phase, the elution of these was done with methanol with HCl 1M until the stationary phase was colorless.

By reducing the grams of the stationary phase, the recovery of the methanol fraction increased from 30 % of (rep1) to 59 % of (rep2), which is a remarkable result given that the recovery from the prior treatment is doubled, even though 36 % of anthocyanins still elute in the

aqueous phase. As in the previous treatment, the methanol fraction does not exceed 40% purity, demonstrating that the methanol fraction does elute anthocyanins, but also other compounds belonging to purple cabbage. For this reason, research with additional stationary phases was conducted. The distribution of anthocyanins in the water and methanol fractions is very similar. The peaks are separated according to their polarity, with the less polar components eluted first and the anthocyanins more polar, over time. This separation is achieved through a gradient chromatographic run, with water as the initial solvent and acetonitrile as the final solvent. Interactions between the solvent and matrix components occur through ionic bonds, influencing elution according to their affinity for the solvent. On the other hand, the interactions between the sample substances and the column are polar in nature, allowing the substances to elute and not become trapped in the column.

4.2. Purification treatment with: Toyopearl HW65-S

Toyopearl HW65-S is a hydroxylated methacrylic polymer bead designed for size chromatography of proteins between 40.000-5.000.000 Da. It is white and slurry with 30µm of dimension and pore size 100 nm. It works in a range of pH 2-12.

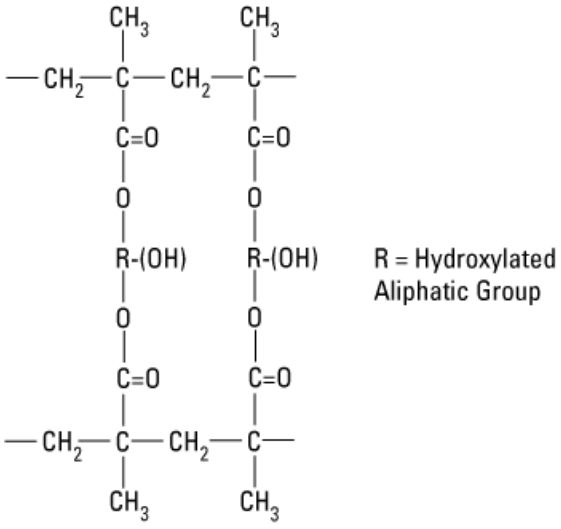

Figure 17. polymer from which Amberlite Toyopearl HW65-S is composed

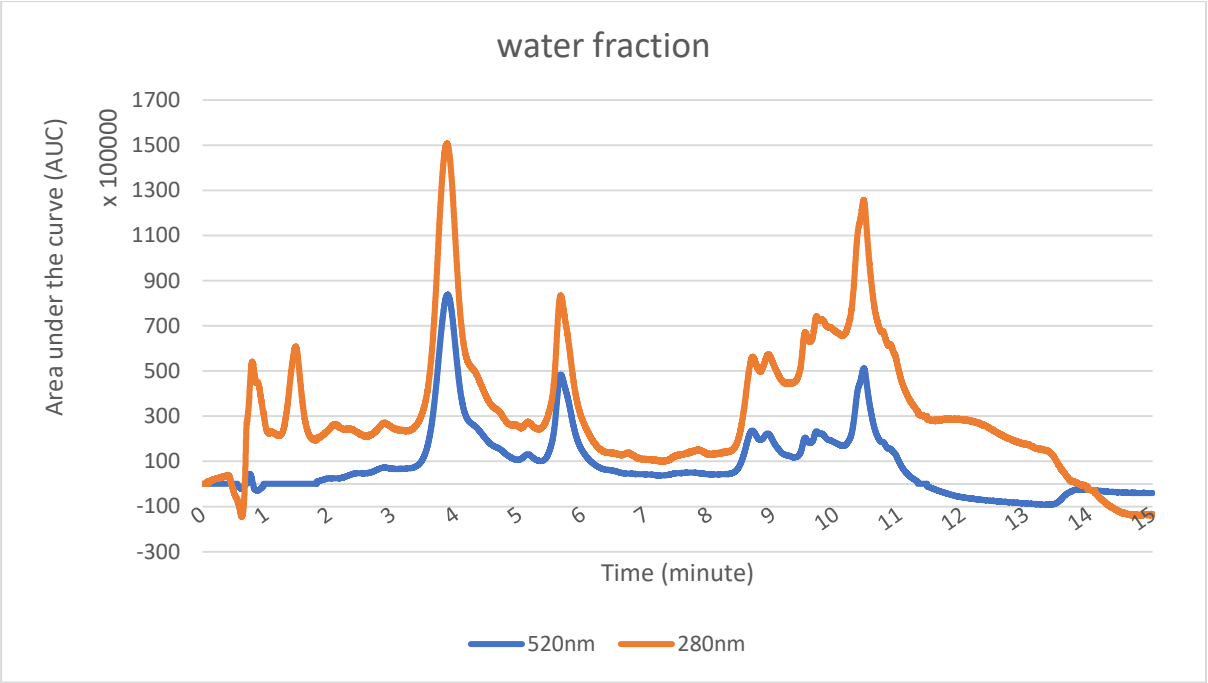

Figure 18. aqueous fraction obtained from the stationary phase Toyopearl HW65-S

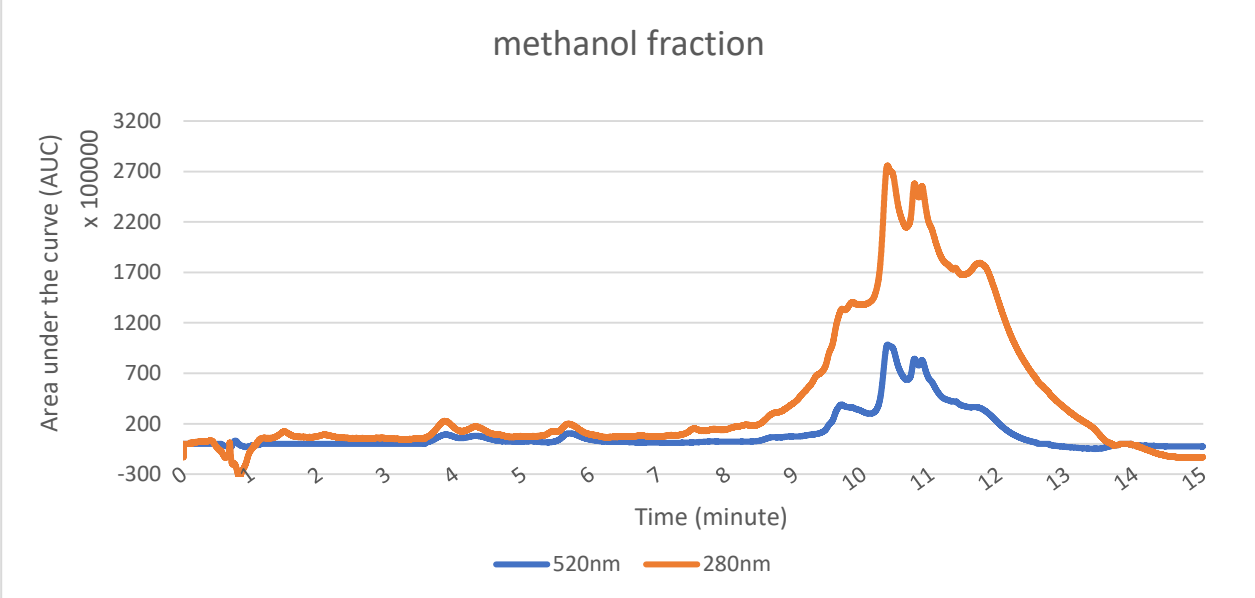

Figure 19. methanol fraction obtained from the stationary phase Toyopearl HW65-S

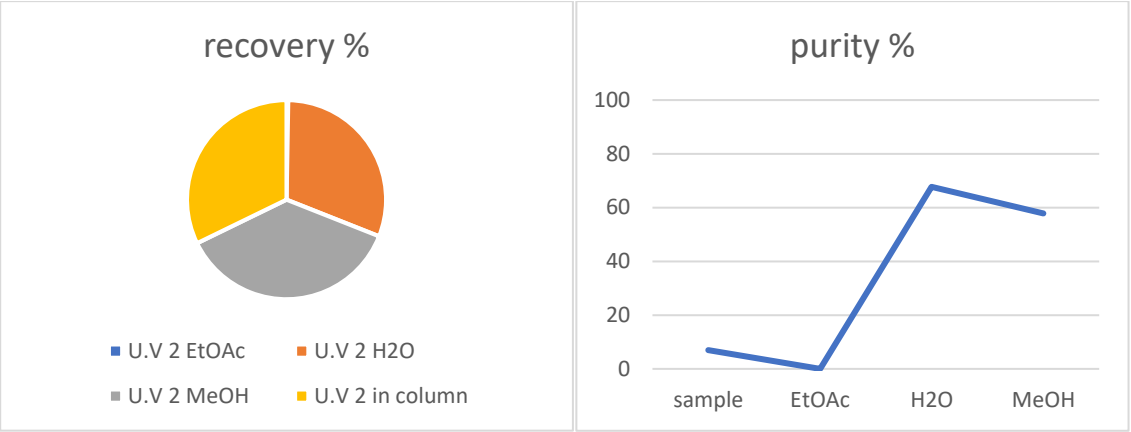

Table 3. recovery and purity after purification with Toyopearl HW65-S

Figures 18 and 19 report the result of purification with Toyopearl HW65-S. For this treatment, 30 g of Toyopearl HW65-S and 3mg purple cabbage powder were used. The purification process involves a first treatment with 40BV ethyl acetate followed by 40BV water with 1M HCl, figure 18 shows the results obtained from this second wash. Figure 19, on the other hand, is the result of the extraction of the anthocyanins plus the other purple cabbage substances that were still in the Toyopearl HW65-S stationary phase, the elution of these was done with methanol with HCl 1M until the stationary phase was colorless. The issues that had previously occurred during the purifying process with this stationary phase were revealed. The stationary phase retained 32 % anthocyanins even after several methanol washes with HCl 1M.The stationary phase appeared colorless on the outside, so it might have seemed as if all the contents had been extracted from it, but this was not the case, because Toyopearl HW65-S is a highly porous stationary phase, as it was designed to purify proteins, and the anthocyanins being small in comparison to the proteins, once absorbed by the stationary phase, it became impossible to elute them completely. No further attempts were made as had previously been the case with Amberlite XAD 7HP, as the fraction with the highest purity is the aqueous fraction, in fact it has a purity of 67 % compared to methanol which has a purity of 57 %. In this case, the distribution of anthocyanins is not homogeneous. However, an uneven distribution is observed, with the water fraction showing a higher concentration of anthocyanins with lower polarity, while the opposite behavior occurs with the methanol fraction.

4.3 Purification treatment with: Sephadex LH-20

Sephadex LH-20 is a crosslinked dextran-based resin used with organic solvent mobile phases for gel permeation, normal phase partition and adsorption chromatography of lipids, steroids, fatty acids, hormones, vitamins). It is also used in gel filtration chromatography, protein chromatography, gel filtration media, resin and separation media. Sephadex LH-20 has been used to isolate bioactive polyphenolic content from blackberry genotypes and tryptophan from the aqueous extract of lotus rhizomes. It comes in the form of a white agarose slurry polymer, where the dimensions of the spheres are 25-100 µm. Working with this gel is feasible in the pH range of 2-13.

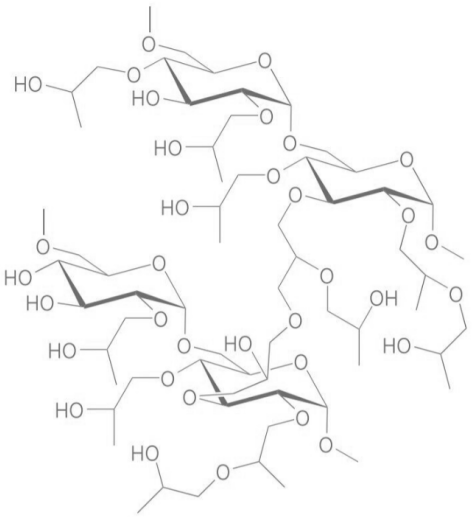

Figure 20. polymer from which Sephadex LH-20 is composed

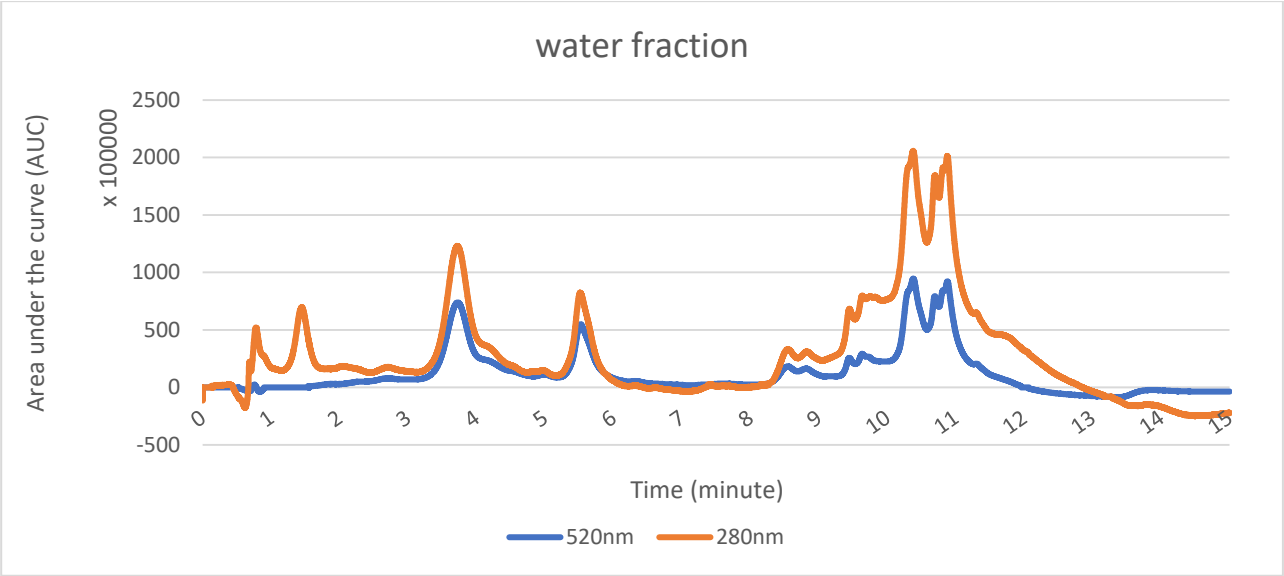

Figure 21. aqueous fraction obtained from the stationary phase Sephadex LH-20

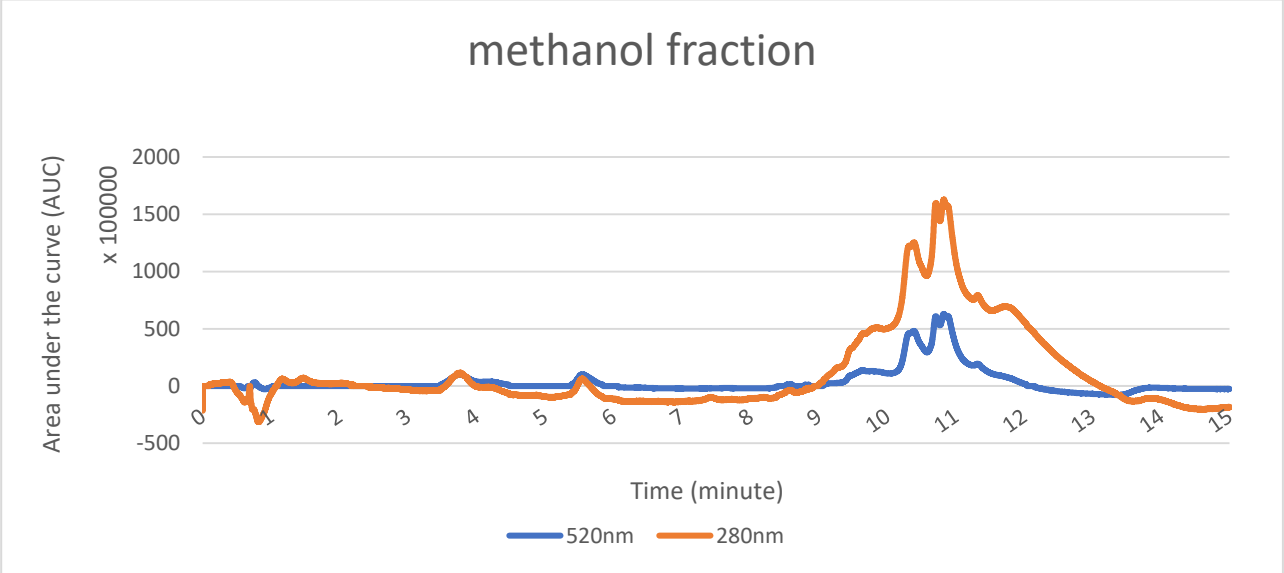

Figure 22. methanol fraction obtained from the stationary phase Sephadex LH-20

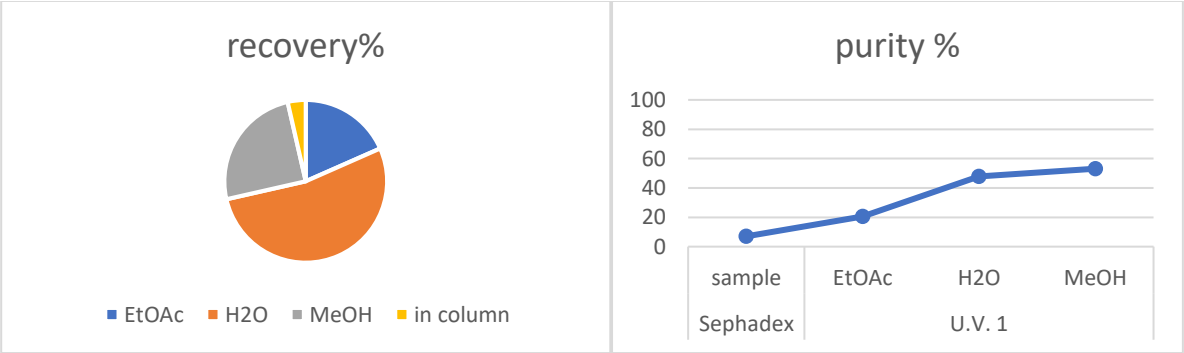

Table 4. recovery and purity after purification with Sephadex LH-20

Figures 21and 22 report the result of purification with Sephadex LH-20. For this treatment, 20 g of Sephadex LH-20 and 3 mg purple cabbage powder were used. The purification process involves a first treatment with 40BV ethyl acetate followed by 40BV water with 1M HCl, figure 21 shows the results obtained from this second wash. Figure 22, on the other hand, is the result of the extraction of the anthocyanins plus the other purple cabbage substances that were still in the Sephadex LH-20 stationary phase, the elution of these was done with methanol with HCl 1M until the stationary phase was colorless. From the point of view of the stationary phase, the result is positive in fact unlike the other two stationary phases Amberlite XAD 7HP and Toyopearl HW65-S only 4 % of the anthocyanins contained in purple cabbage remain trapped in the stationary phase, but this does not guarantee that the fraction with the highest recovery is the methanol fraction. In fact, the ethyl acetate fraction collects 18 % of the total anthocyanins and the aqueous fraction contains 53 % of the anthocyanins; this means that only 25% of anthocyanins are in solution in the methanol fraction. On the other hand, as far as purity is concerned, the methanol fraction is the purest fraction with a result of 53.09 %. Even being the purest methanol fraction compared to all other methanol fractions from the other stationary phases. the research continued with the study of other types of stationary phases. The distribution of anthocyanins is also not homogeneous, mainly due to a question of recovery, rather than a division of polarity between anthocyanins, in fact the aqueous fraction contains more anthocyanins.

4.4 Purification treatment with: C18 Silica gel spherical

This is a nonpolar powder, capable of forming hydrophobic interactions with a wide variety of organic compounds. It is commonly used for the separations of nonpolar to moderately polar compounds such as fatty acids, glycerides, polycyclic aromatics, esters, vitamins and steroids. It is white in color with an irregular shape, and the particles that constitute it have a size of 5µm with 300 Å pores.

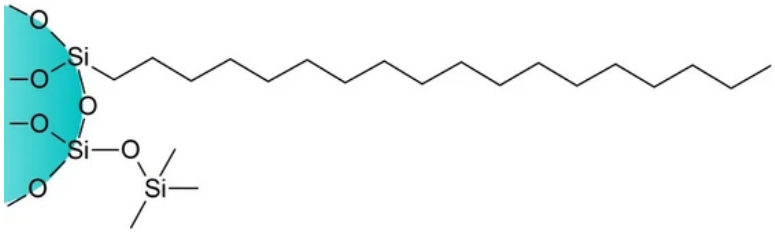

Figure 23. polymer from which C18 Silica gel spherical is composed

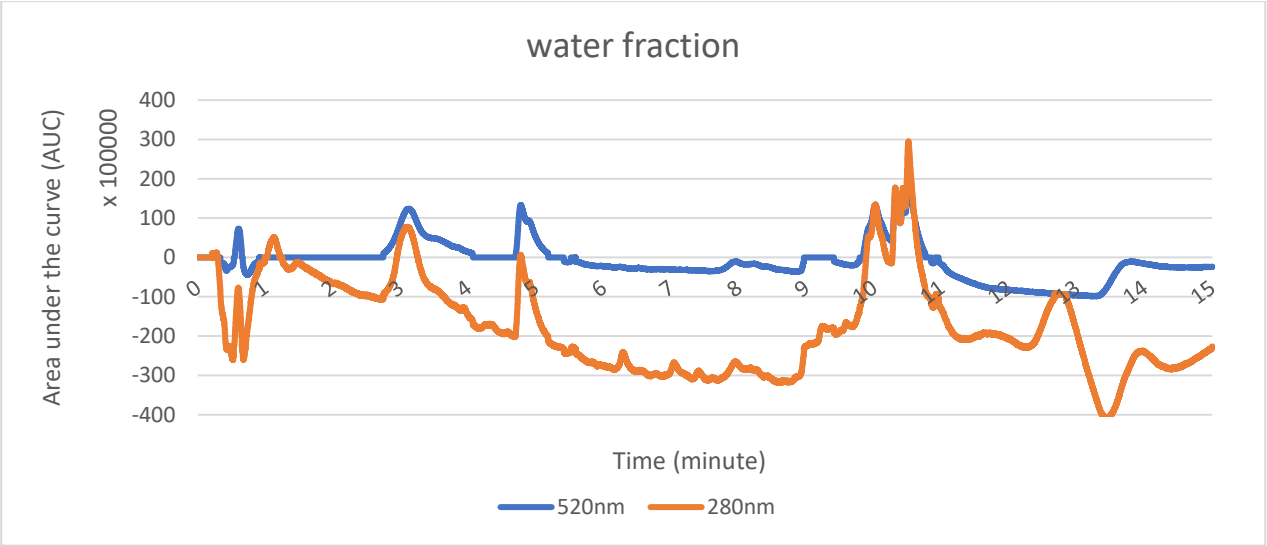

Figure 24. aqueous fraction obtained from the stationary phase C18 Silica gel spherical

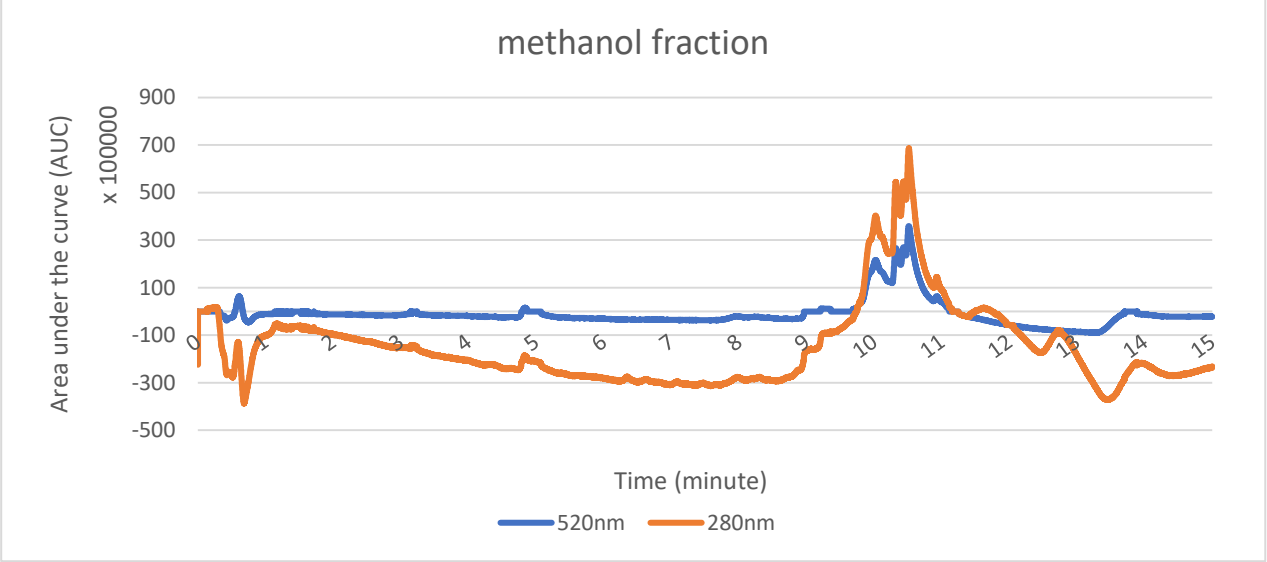

Figure 25. methanol fraction obtained from the stationary phase C18 Silica gel spherical

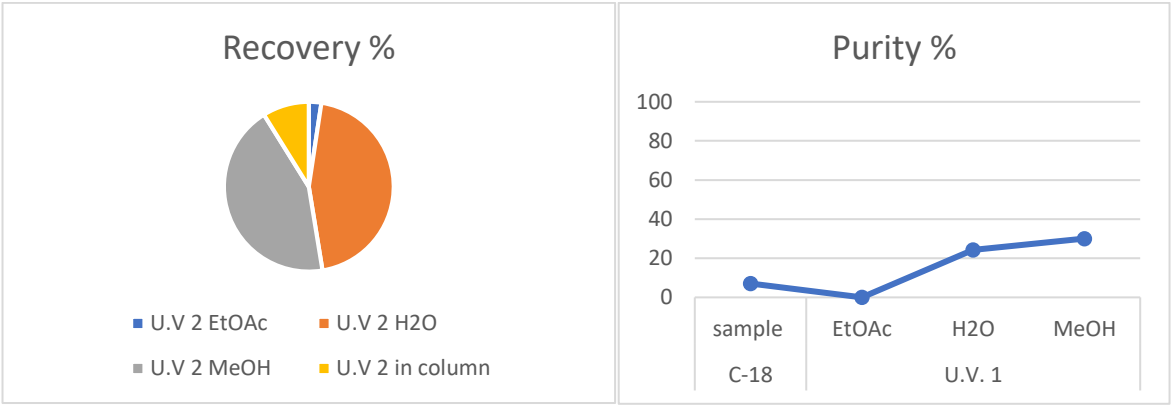

Table 5. recovery and purity after purification with C18 Silica gel spherical

Figures 24 and 25 show the result of purification with C18 Silica gel spherical. For this treatment, 20 g of C18 Silica gel spherical and 3 mg purple cabbage powder were used. The purification process involves a first treatment with 40BV ethyl acetate followed by 40BV water with 1M HCl, figure 24 shows the results obtained from this second wash. Figure 25, on the other hand, is the result of the extraction of the anthocyanins plus the other purple cabbage substances that were still in the C18 Silica gel spherical phase, the elution of these was done with methanol with HCl 1M until the stationary phase was colorless. C18 Silica gel spherical had been used before, when the aqueous fraction from the liquid-liquid extraction was added to this stationary phase, in this case no liquid-liquid extraction took place, but a single-step purification was tested. The aim was to test this stationary phase again, in order to understand whether the failure to purify was due to an inadequate stationary phase (C18 Silica gel spherical) or due to an inefficient purification method. Evaluating the results obtained with regard to both recover and purity, it is possible to state that the aqueous fraction and the methanol fraction are very similar to each other, with both having a recover of 45 % and a purity below 40 %. These findings allowed for the conclusion that the lack of purity issue was not related to the technique used. The stationary phase C18 Silica gel spherical is not appropriate for the objective of this research.

4.5 Purification treatment with: Oasis® HLB 3cc Vac cartridge

Oasis® HLB 3cc Vac cartridge: used in the experiment are 3 cc in size and are designed with a Hydrophilic-Lipophilic Balanced (HLB) reversed-phase sorbent, suitable for acids, bases, and neutrals. These cartridges are Vac cartridges with low polarity and contain 60 mg of 1-(2-(4-(sec-butyl) phenyl) loctan-4-y) pyrrolidine-2-one copolymer.

The copolymer powder has a particle size of 30 µm and pore sizes of 80 Å, making it suitable for use in a pH range of 0-14. The cartridge itself, is made of polypropylene.

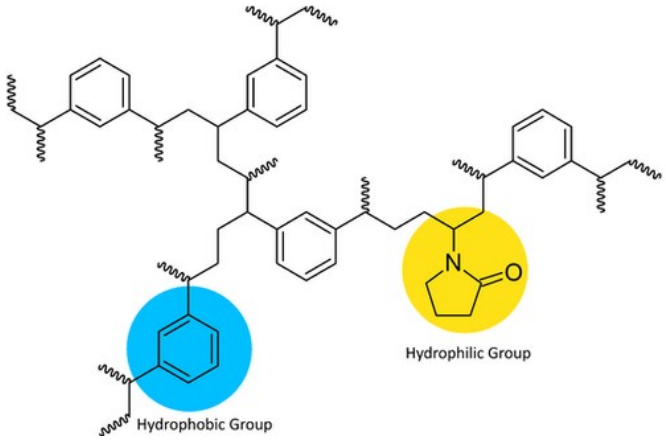

Figure 26. polymer from which Oasis® HLB 3cc Vac cartridge is composed

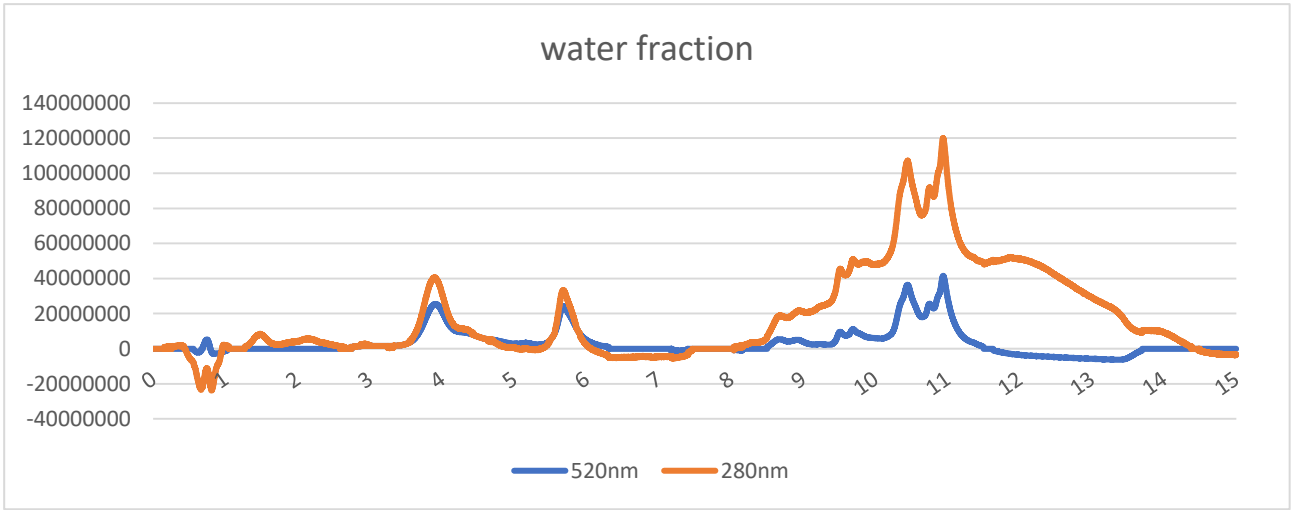

Figure 27. aqueous fraction obtained from the stationary phase Oasis® HLB 3ccVac cartridge

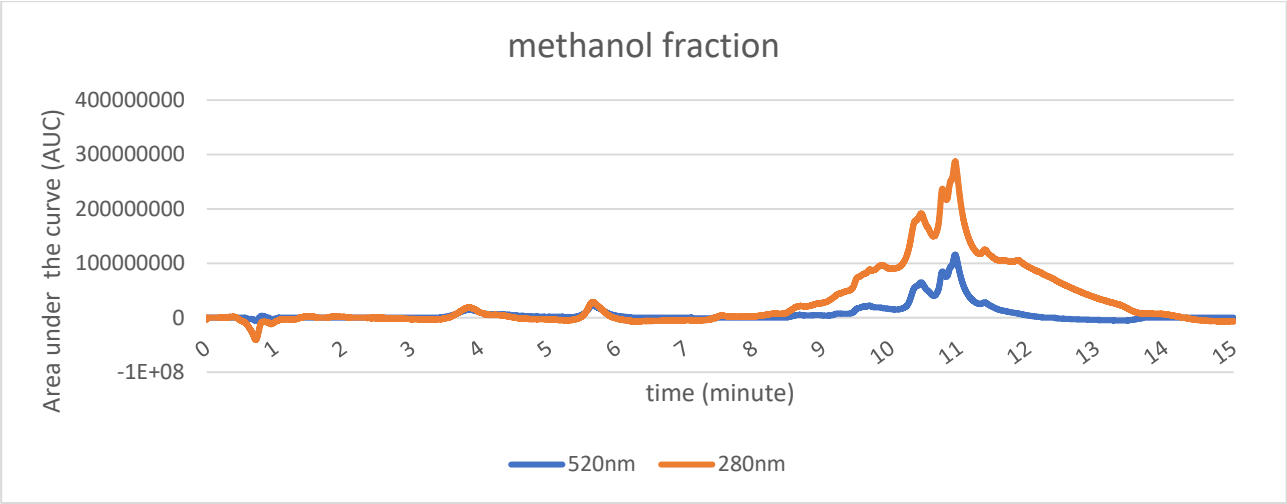

Figure 28. methanol fraction obtained from the stationary phase Oasis® HLB 3ccVac cartridge

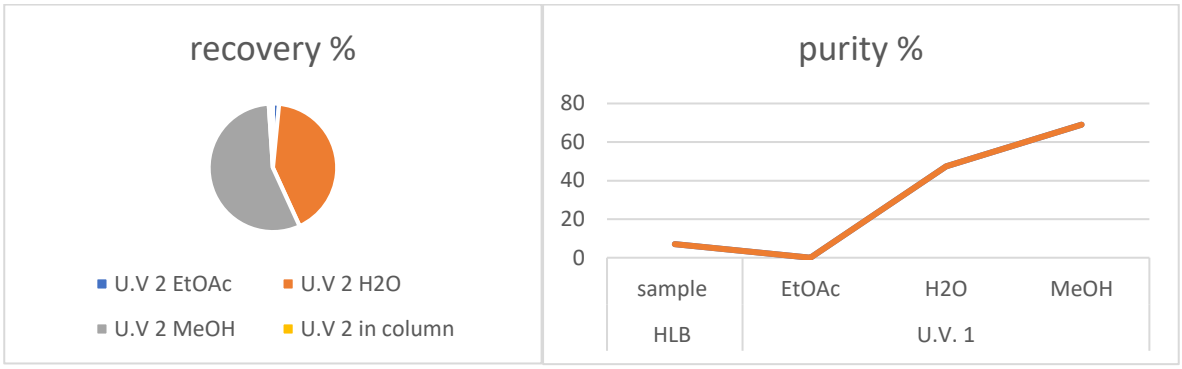

Table 6 *recovery and purity after purification with Oasis® HLB 3ccVac cartridge*

Figure 27 and 28 report the purification results obtained with Oasis® HLB 3cc Vac cartridge. 0.5mg of purple cabbage to be purified was introduced into the Oasis® HLB 3cc Vac cartridge after the cartridge was activated. For purification, 40BV of ethyl acetate was used to eliminate phenolic acids present in the matrix, and then a water wash with HCl 1M was performed, 40BV was used to eliminate all polar substances other than anthocyanins so this wash was necessary to eliminate amino acids, proteins and sugars present in the starting matrix.

Figure 27 shows the fraction of water, while figure 28 is the result of purification, obtained by eluting the compounds bound to the stationary phase using methanol. Compared to all prior stationary phases, the Oasis® HLB 3cc VAC cartridge provided superior purity and recover values. In fact, the methanol fraction shows a recover of 56 % and a purity of 70 %, while only 1 % of the anthocyanins remain in the stationary phase after washing with methanol. On

the other hand, the aqueous fraction shows a recovery of 42 % and a purity of 50 %. The aim of the others with this stationary phase will be to reduce the recovery of the aqueous fraction and increase the purity of the methanol fraction. Analyzing the two chromatograms it is possible to see that the distribution of anthocyanins between the two chromatograms are not homogeneous, in fact in the aqueous fraction the anthocyanins with lower polarity have a higher area than those in the methanol fraction.

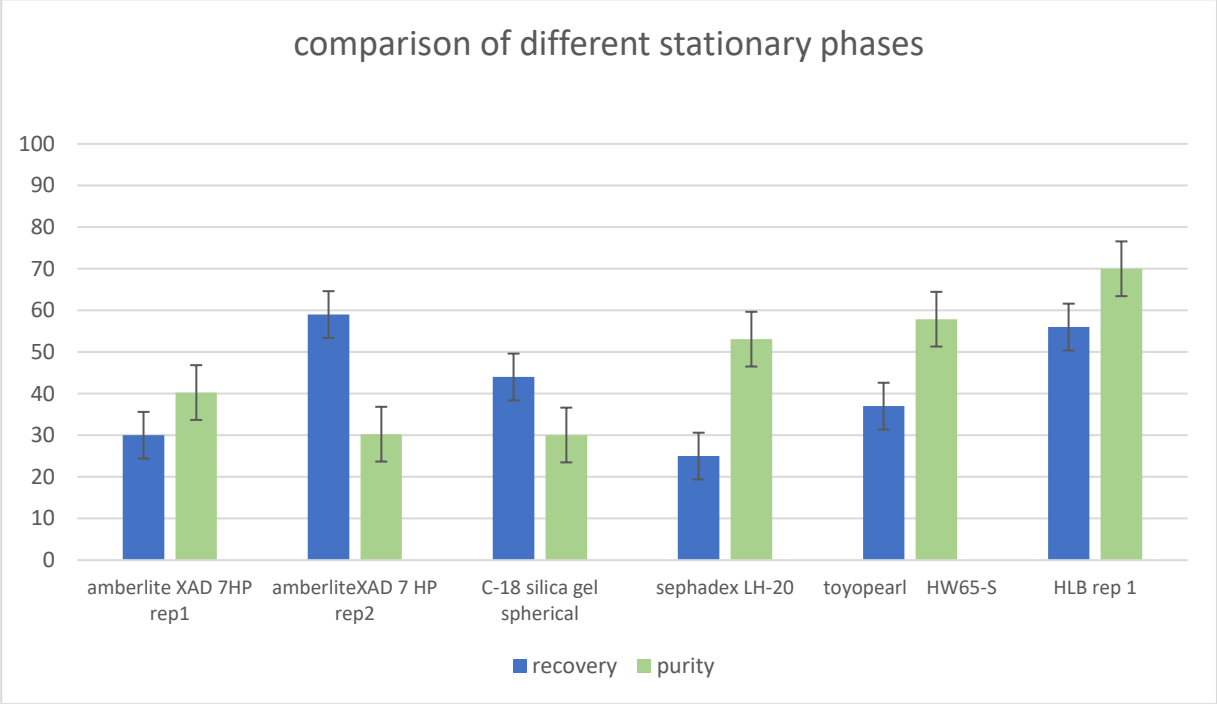

Table 7. comparison of different stationary phases

Table 7 summarizes the recovery and purity values obtained through the purification process with the different matrices, the results refer only to the methanol fraction. The methanol fractions from: Amberlite XAD 7HP (rep2) and Oasis® HLB 3cc Vac cartridge are the ones with the highest recover compared to all the others, instead regarding the degree of purity again Oasis® HLB 3cc Vac cartridge presents the highest value and next we find the result obtained with Toyopearl HW65-S. In this first part of the experiment, none of the stationary phases gave the possibility of obtaining a pure anthocyanin extract, however, it was decided to invest more time in Oasis® HLB 3cc Vac cartridge because it appears to be the most efficient stationary phase. The research to find a purification method that would be suitable

for the purple cabbage and that could be applied to the other matrices chosen for this research continued using the Oasis® HLB 3cc Vac cartridge as the stationary phase, since it was the stationary phase that already from the first experiments proved to be the best in terms of both recovery and purity. The first objective was to increase the recovery of the methanol fraction, so the BVs really needed to achieve the purity value previously obtained were calculated. This was achieved by dividing the ethyl acetate and water washes with HCl 1M into smaller units than the 40BV previously used for both solvents.

4.5.1 Ethyl acetate washes divided by BV

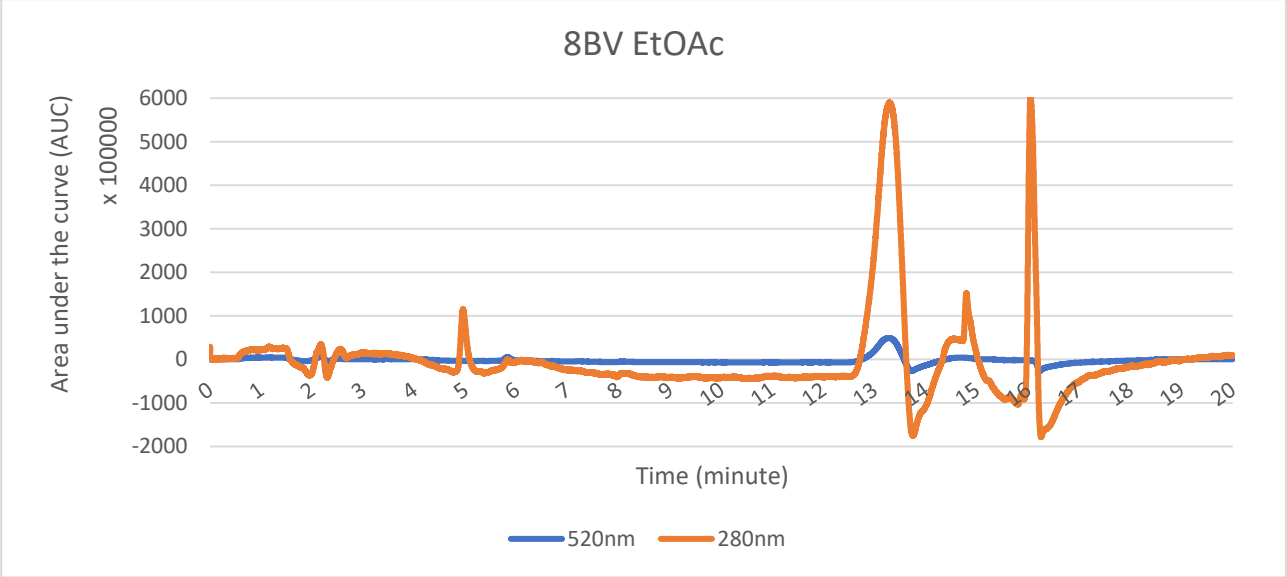

Figure 29. 8BV of ethyl acetate with stationary phase Oasis® HLB 3cc Vac cartridge

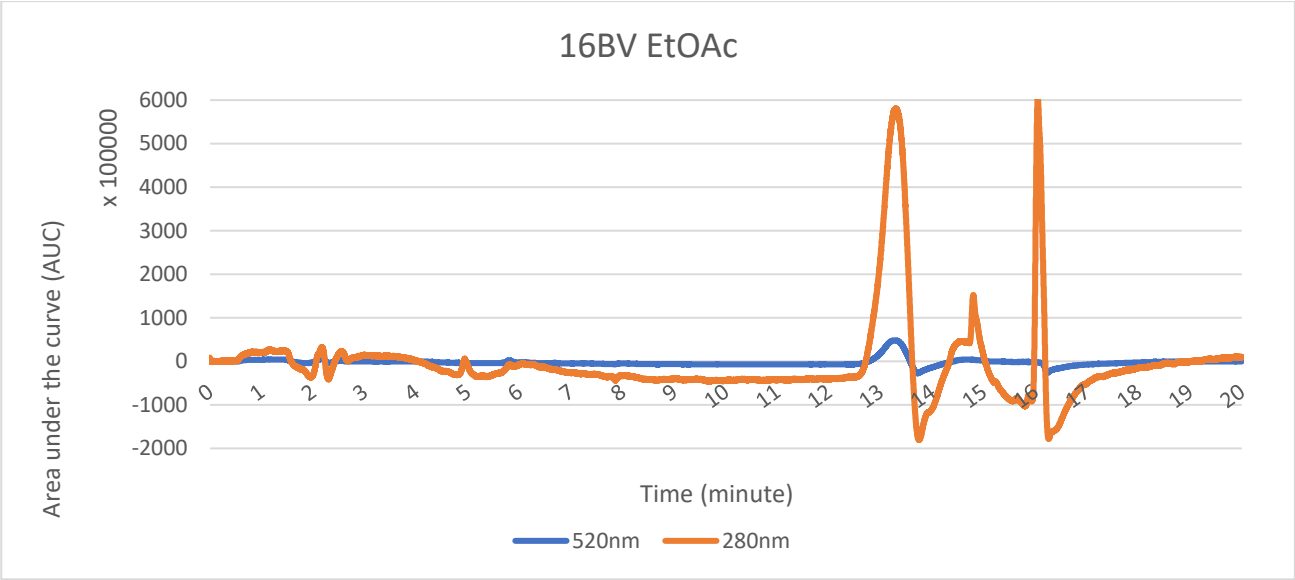

Figure 30. 16BV of ethyl acetate with stationary phase Oasis® HLB 3cc Vac cartridge

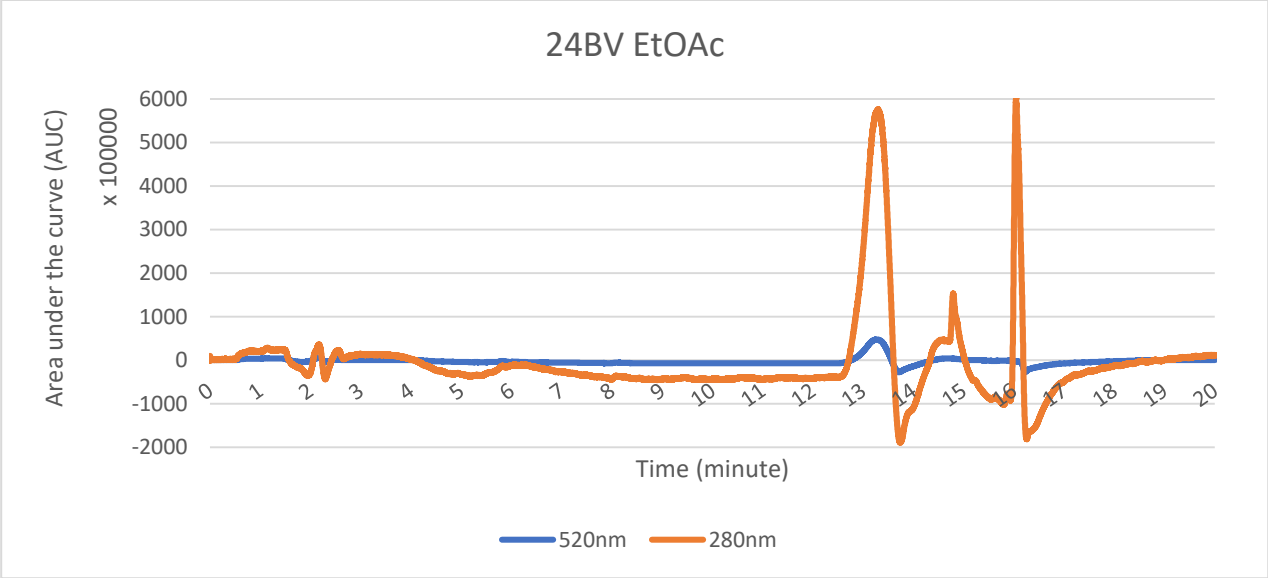

Figure 31. 24BV of ethyl acetate with stationary phase Oasis® HLB 3cc Vac cartridge

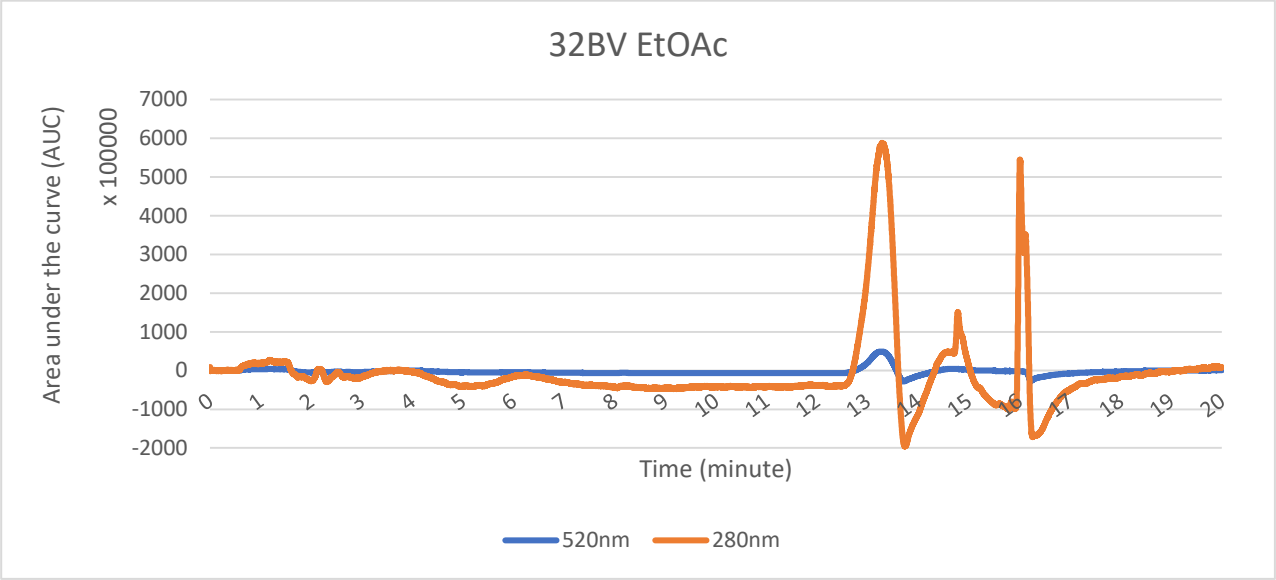

Figure 32. 32BV of ethyl acetate with stationary phase Oasis® HLB 3cc Vac cartridge

Evaluating figures 29-32, 24BV of ethyl acetate is sufficient to eliminate phenolic acids; in fact, in the following purification processes, 40BV will no longer be used, but only 24BV, which should increase the recovery of anthocyanins in the methanol fraction as well as reduce the waste of ethyl acetate.

4.5.2 Water with HCl 1M washes divided by BV

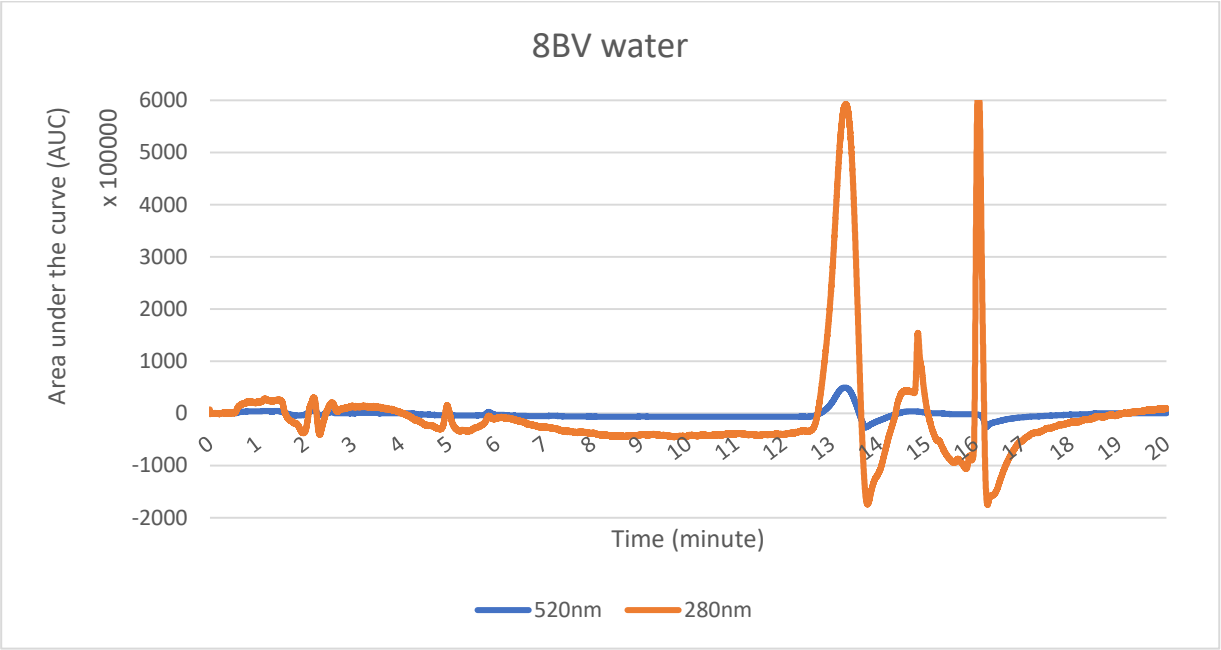

Figure 33. 8BV of water with HCl 1M with stationary phase Oasis® HLB 3cc Vac cartridge

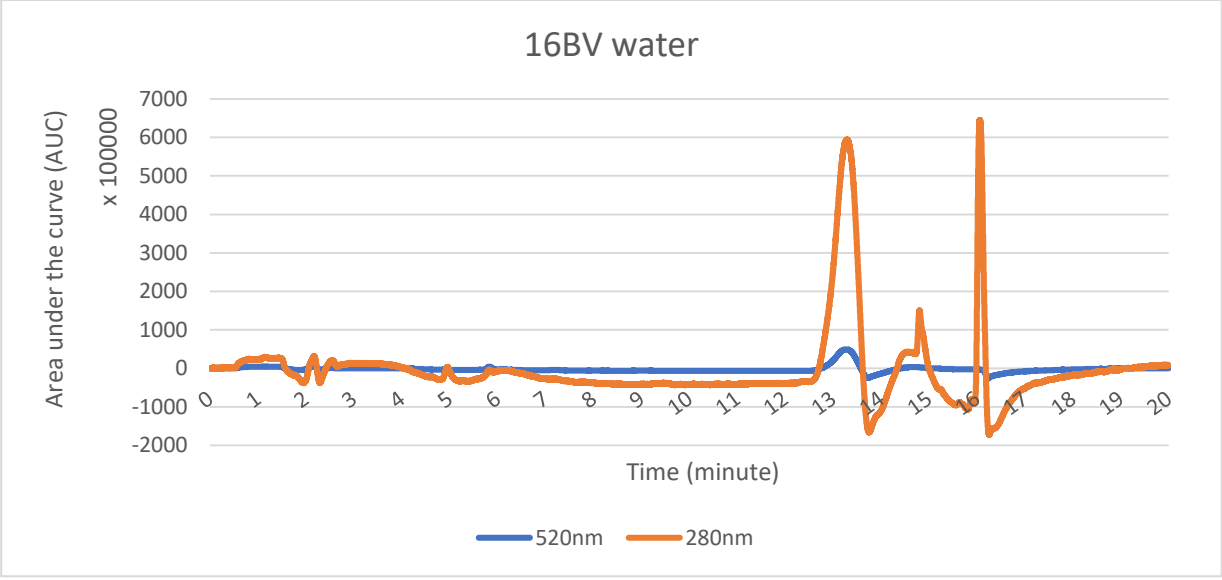

Figure 34. 16BV of water with HCl 1M with stationary phase Oasis® HLB 3cc Vac cartridge

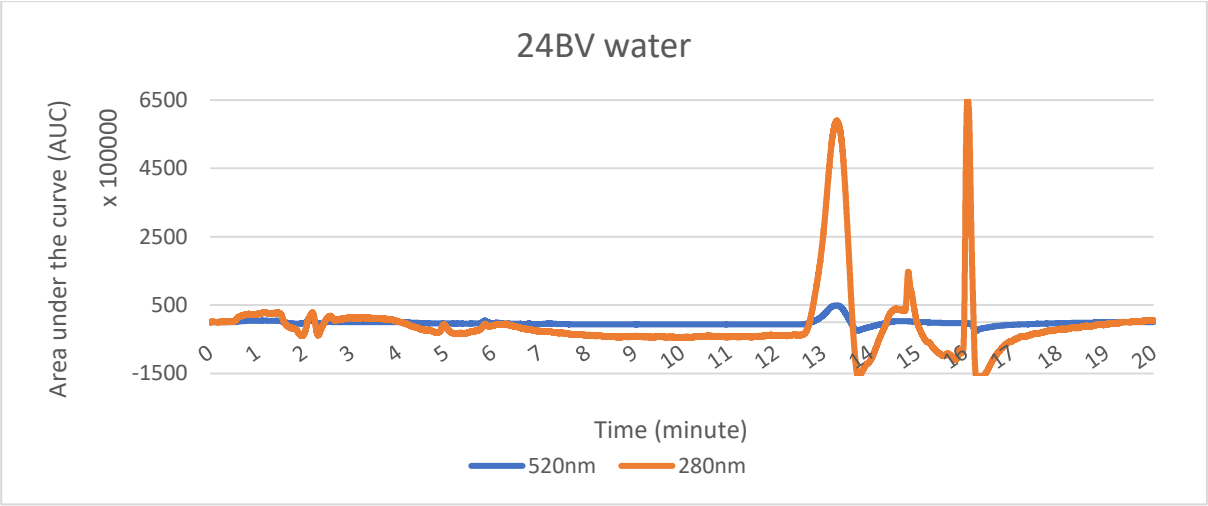

Figure 35. 24BV of water with HCl 1M with stationary phase Oasis® HLB 3cc Vac cartridge

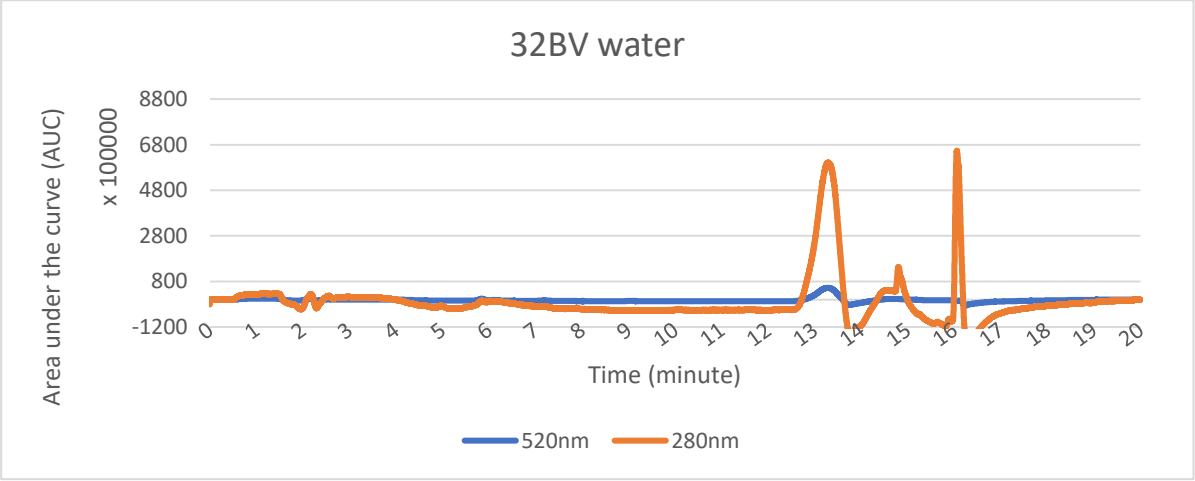

Figure 36. 32BV of water with HCl 1M with stationary phase Oasis® HLB 3cc Vac cartridge

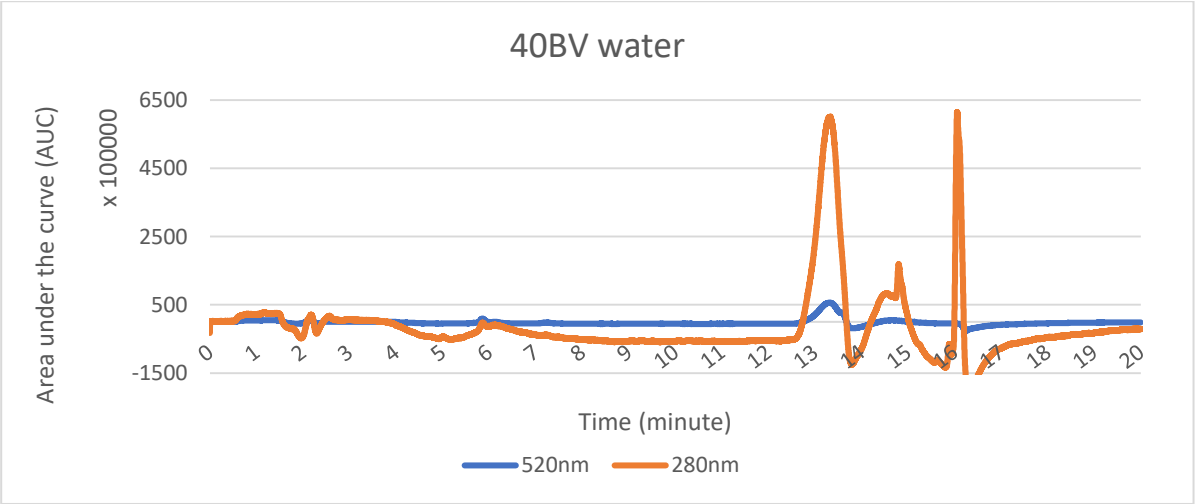

Figure 37. 40BV of water with HCl 1M with stationary phase Oasis® HLB 3cc Vac cartridge

On the other hand, evaluating figures (33-37), the BVs of water required still remain 40, so 24 BVs of ethyl acetate and 40BVs of water with HCl 1M will be used for the next purification processes.

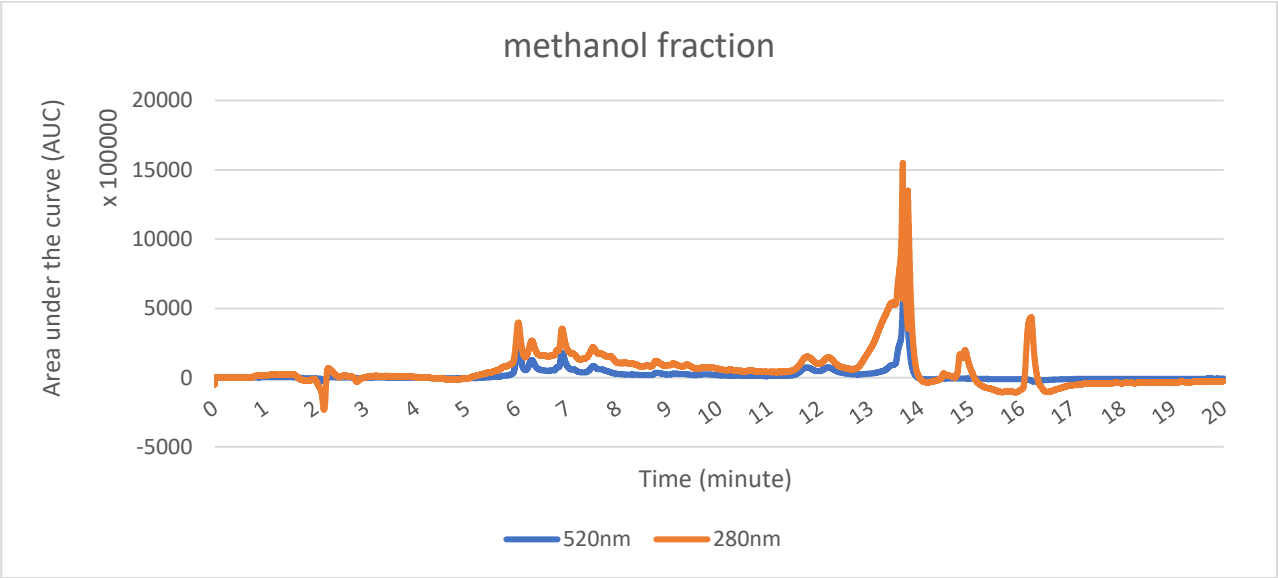

Figure 38. methanol fraction with stationary phase Oasis® HLB 3cc Vac cartridge

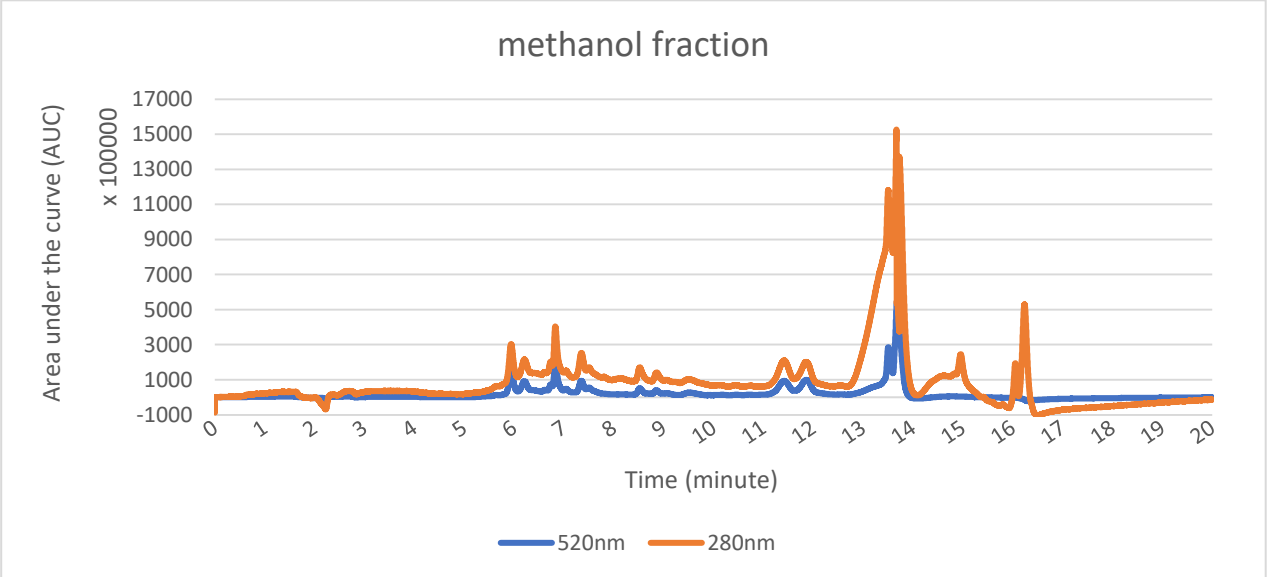

Figure 39. methanol fraction with stationary phase Oasis® HLB 3cc Vac cartridge

Figure 38 shows the results of the BVs needed to purify 24 BVs of ethyl acetate and 40BV of water with HCl 1M, which increased the recover from 57 % to 70 %, and also increased the degree of purity from 70 % to 80 %. Figure 39 shows the results obtained from changing the order of the solvents used for purification. Even using the determined BV, it was noted that between washing with ethyl acetate and washing with water, some red drops eluted from the Oasis® HLB 3cc Vac cartridge. This meant that some anthocyanins were still present in the aqueous fraction and therefore, even with a determined BV, it would have been impossible to achieve a recovery of more than 76 %. With this change in solvent purification, a "stress" condition for anthocyanins was created. Previously, the anthocyanins went from a polar solvent of 4.4 for ethyl acetate to water with a polarity of 9.0, allowing them to enter the solution and leave the stationary phase. By changing the order, the polarity changes from 9.0 for water to 4.4 for ethyl acetate and then to the methanol polarity of 5.1. This change in solvent purification resulted in a 90 % recovery rate and high purity. Therefore, the method used to obtain figure 39 has not been applied to the other matrices. The method constructed on purple cabbage with the Oasis® HLB 3cc Vac cartridge stationary phase, which resulted in a purity of 93 % together with a recover of 90 %, has been applied on the other matrices: elderberry, purple sweet potato, purple basil, cornflower and wild pansy.

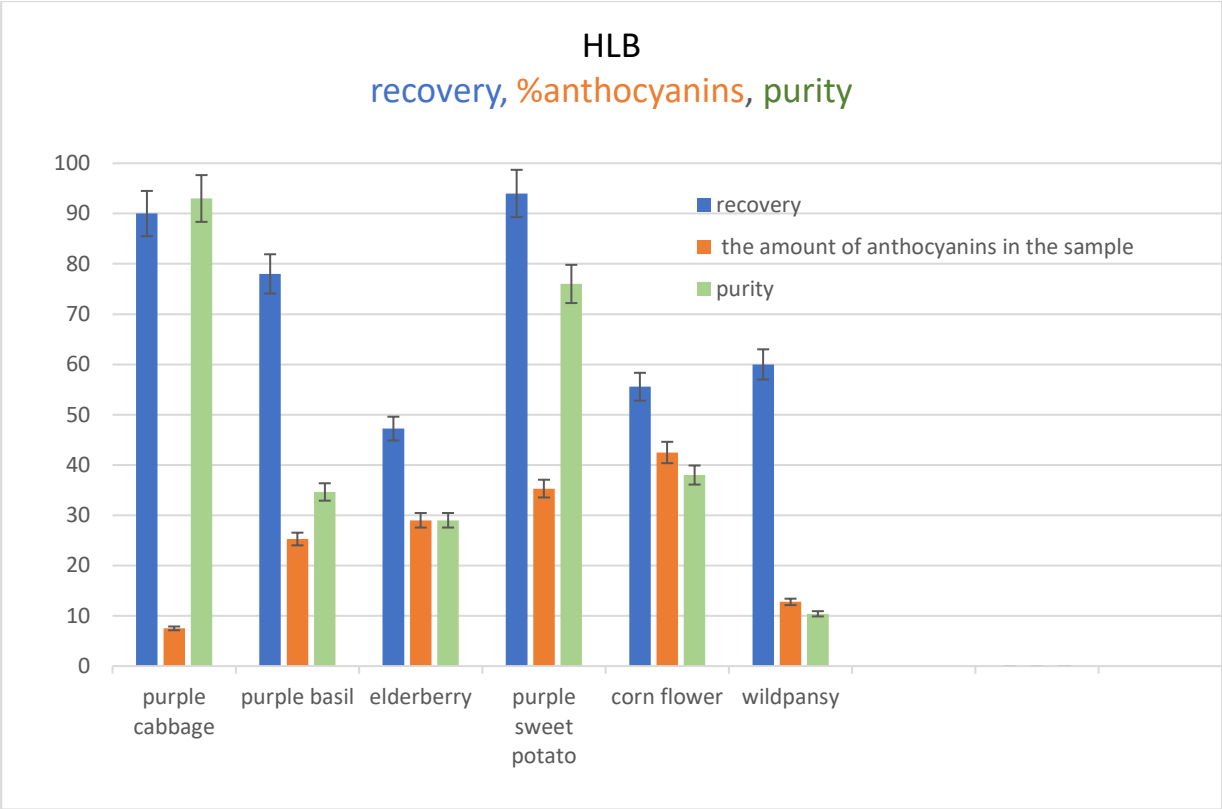

Table 8. comparison of different samples with stationary phase the Oasis® HLB 3cc Vac cartridge

Table 8 reports the results obtained from treating other matrices with the method derived from purple cabbage. Although it was thought that finding a method on this matrix would be applicable to matrices with a low diversity of anthocyanins, this did not happen. In fact, there are no samples reporting the purity and recovery that were obtained with purple cabbage. The only outcomes that can be deemed promising are for purple sweet potato and purple basil, as far as recover is concerned, whereas for elderflower, cornflower and pansy, the recovery is around 50 %, which is particularly problematic for the latter two flowers, as their cost is around 50 EUR/g. This cost is due to the difficulty of producing these matrices, since being edible flowers, they cannot be treated with pesticides and herbicides and currently only two companies in Portugal produce these flowers. However, when analyzing the purity value for the purple sweet potato alone, it has increased significantly. Starting from an initial purity level of 35.3 %, a final purity of 76 % was reached. Thus, for the purple sweet potato, it shows a recovery of 94 % of the total amount of anthocyanins contained in the sample and a purity of 76 %. It can therefore be stated that the method obtained for purple cabbage gives acceptable results, at least for purple sweet potato. However, for cornflower and wild pansy,

less purity is obtained after the purification treatment than initially. This means that other substances contained in these two flowers have been concentrated as opposed to anthocyanins. Similarly, no significant purity results were obtained for purple basil and elderberry. Therefore, although the process developed for purple cabbage works on this matrix and gives optimal results for purple sweet potato. Further studies will be necessary to adapt the method to a wider range of food matrices.

4.6 Purification treatment with: Discovery® DSC-MCAX SPE 52788-U

Discovery® DSC-MCAX SPE 52788-U: is a mixed-mode Cation Exchange (MCAX) SPE product, featuring a combination of reversed-phase and cation-exchange properties. It is compatible with aqueous solutions, including biological fluids and water. This dual retention mechanism broadens retention capabilities for a range of neutral, basic, acidic, and zwitterionic compounds. It has been developed to provide superior selectivity and sample clean-up when isolating basic compounds from biological fluids, offering a greater ion-exchange capacity for isolating polar basic and zwitterionic compounds. It can also be used to fractionate zwitterionic compounds from acidic and neutral compounds. Chemically, the packed bed contains octyl (C8) and benzene sulfonic acid (SCX) bondings. Physically, it is end-capped with a bed weight of 1 g. The composition is based on irregularly shaped, acid-washed silica gel beads with a size of 50 µm and pores of 70 Å.

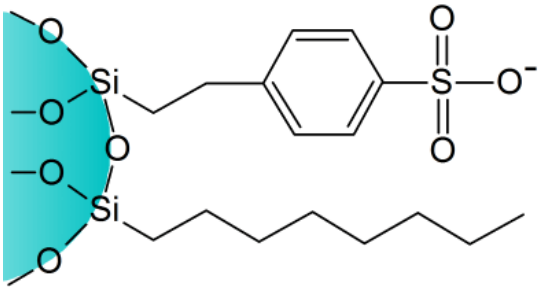

Figure 40. polymer from which Discovery® DSC-MCAX SPE 52788-U is composed

The applications on this matrix were twofold. One was to take the methanol fraction from the Oasis® HLB 3cc Vac cartridge and introduce it onto this ion exchange matrix to eliminate substances that could not be removed with the previous stationary phase. The other application

was to directly load the original solution from various matrices. Analyzing the "non-anthocyanin" fraction revealed that this fraction from the original sample contained both substances that couldn't be eliminated previously and compounds that were removed during treatment with the Oasis® HLB 3cc Vac cartridge. For this reason, it was decided not to subject the matrices to any pre-treatment but to purify them directly with Discovery® DSC-MCAX SPE 52788-U. The purity of all matrices is above 90 %, similar to the purity values achieved for purple cabbage treated with HLB. However, the difference lies in the percentage of anthocyanins lost. A recovery rate below 50 % is obtained for all matrices except for purple sweet potato, which had already shown good results with the purification process using the Oasis® HLB 3cc Vac cartridge.

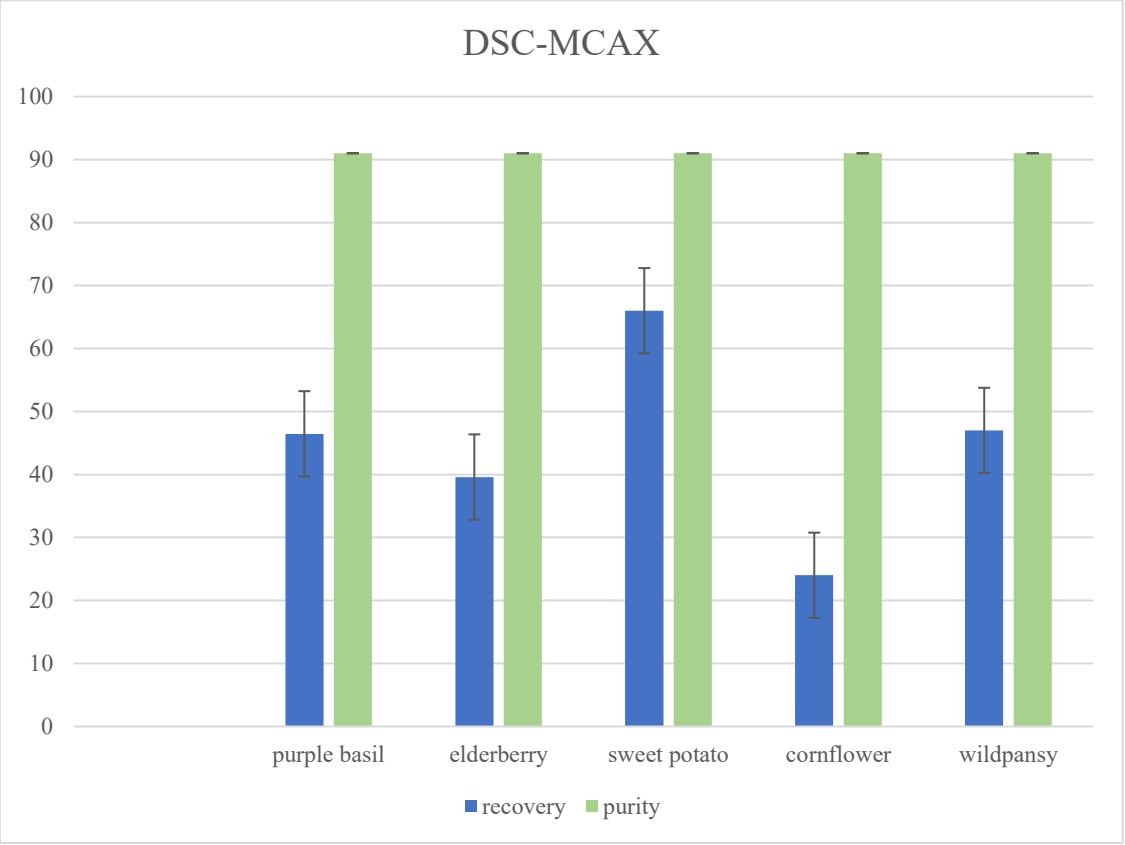

Table 9. comparison of different samples with stationary phase the Discovery® DSC-MCAX SPE 52788-U

The following figures (41-44) reported related chromatograms: in blue those obtained after purification with the Discovery® DSC-MCAX SPE 52788-U stationary phase of elderberry, purple sweet potato, cornflower, and wild pansy samples. Meanwhile, in orange and gray are the chromatograms obtained after extraction with water with HCl 1M from the original matrix. The blue chromatogram is set at a wavelength of 280 nm, at which all components in solution of the various samples are visible. Each blue chromatogram of the different samples reflects the orange chromatogram of the same samples. The orange chromatogram is set at a wavelength of 520 nm, at which only anthocyanins absorb. Since the orange and blue chromatograms are the same in shape but different in intensity. This result indicates that the purification process with this stationary phase was efficient, therefore the anthocyanin extracts contain only anthocyanins and no other compounds. Being the blue chromatograms of lower intensity compared to the orange ones, it means that some of the anthocyanins contained in the starting sample remained bound to the stationary phase or were eluted during the purification process. Instead, the gray chromatogram represents all substances in solution for each sample, including anthocyanins and others. In fact, these chromatograms are set at a light intensity of 280 nm.

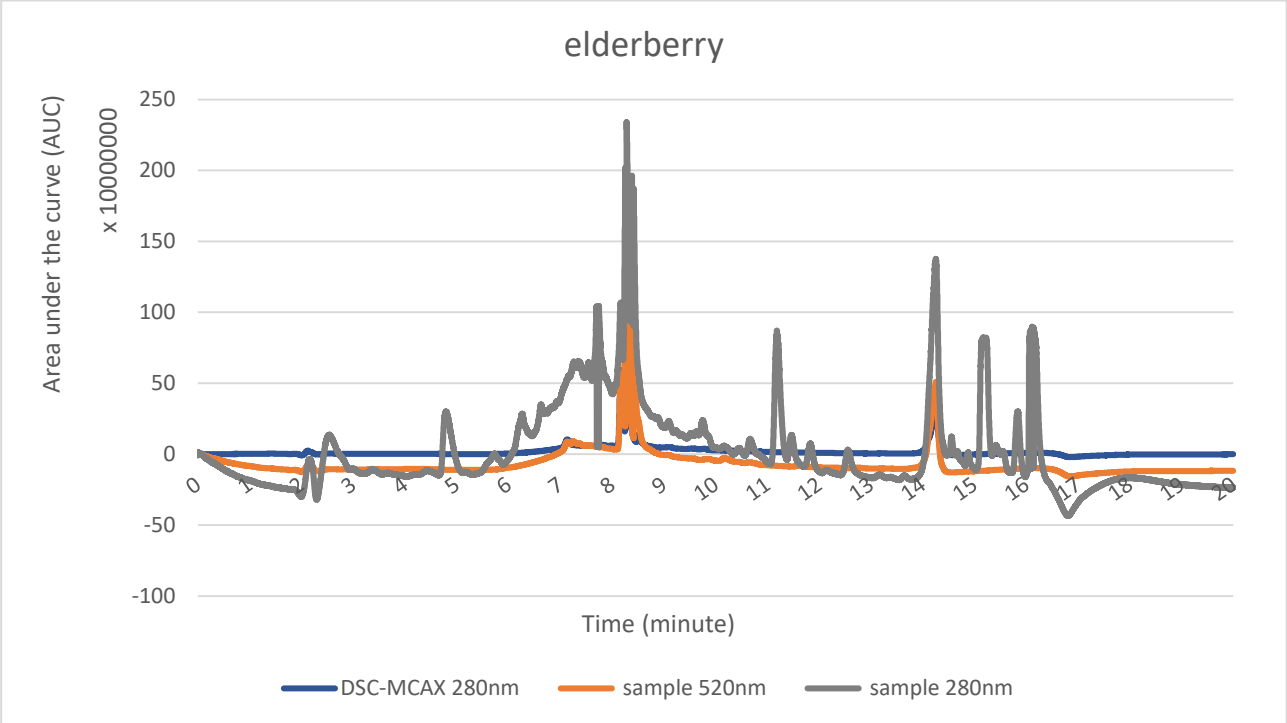

Figure 41. Elderberry purified with Discovery® DSC-MCAX SPE 52788-U

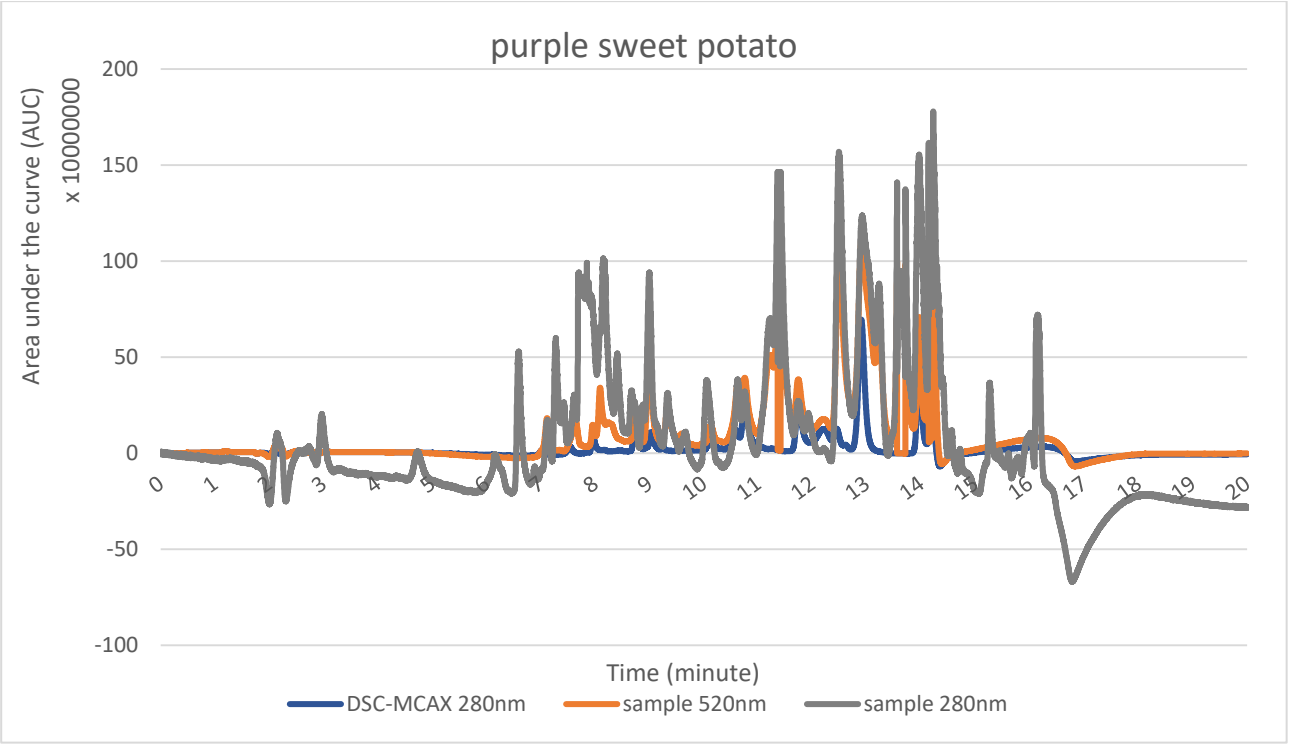

Figure 42. purple sweet potato purified with Discovery® DSC-MCAX SPE 52788-U

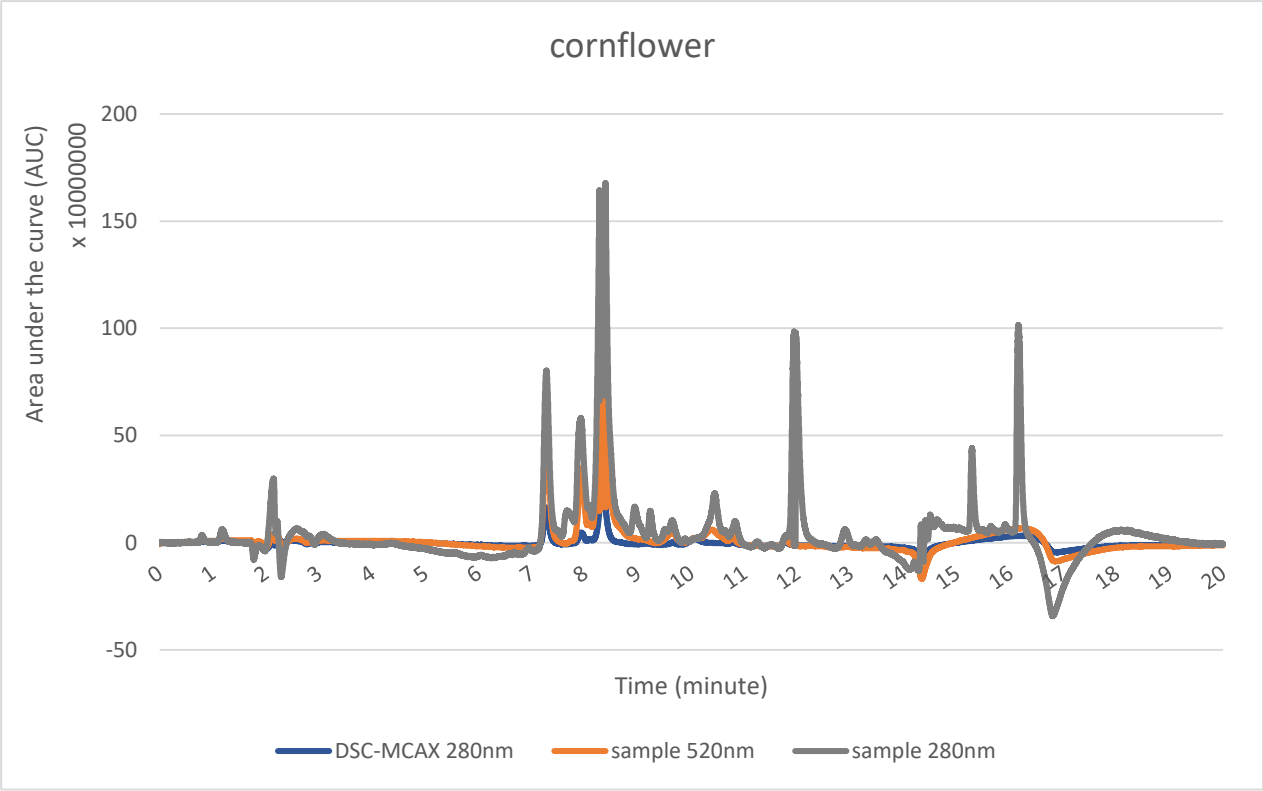

Figure 43. cornflower purified with Discovery® DSC-MCAX SPE 52788-U

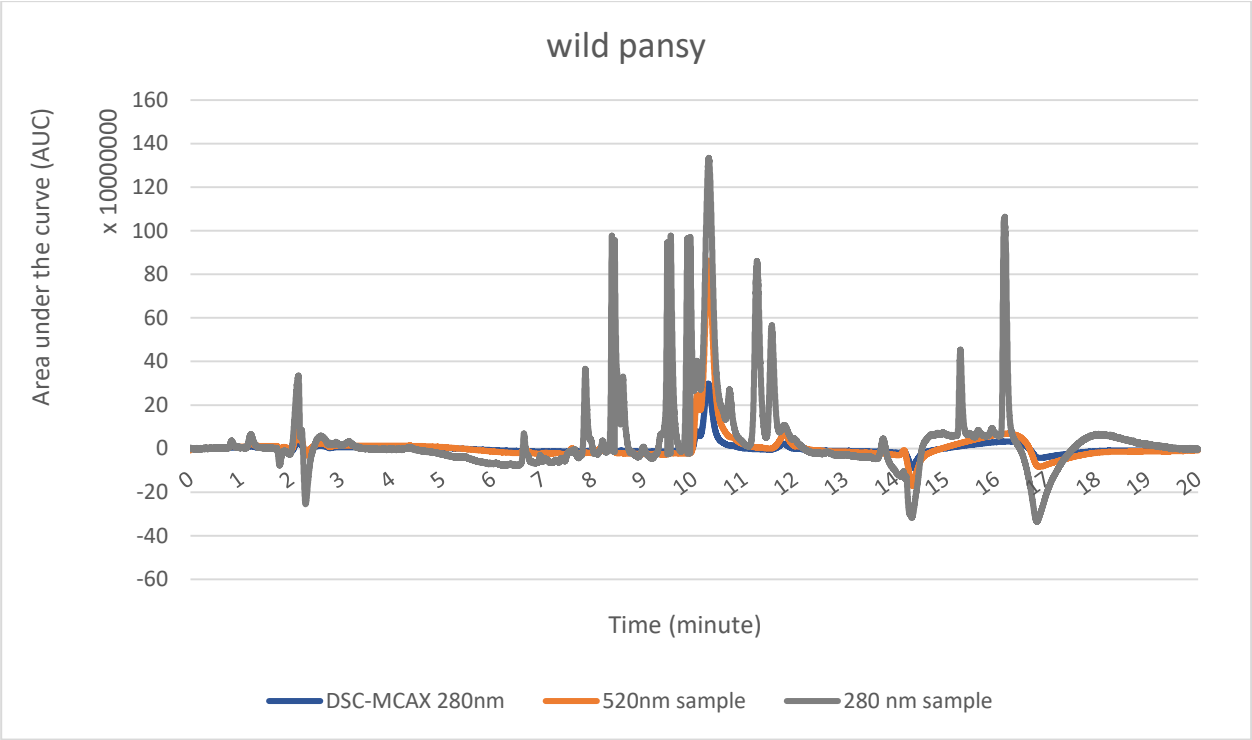

Figure 44. wild pansy purified with Discovery® DSC-MCAX SPE 52788-U

The objective of the research to find a method for purifying anthocyanins from different plant matrices has been achieved. Comparing the method proposed by the completed research, this one proves advantageous compared to the proposals already present in the literature [29, 31].

The purification method obtained involves two individual steps. The first is extraction with H2O with HCl 1M from the plant matrix, while the second is the purification process with the Discovery® DSC-MCAX SPE 52788-U stationary phase. This method ensures a purity of over 90 % of anthocyanins.

The elution of anthocyanins occurs with the solution of phosphate buffer (pH 6) and methanol (1:1). The anthocyanins elute from the stationary phase after the purification process, as at this pH, anthocyanins do not carry a charge. Thus, they can detach themselves from the Discovery® DSC-MCAX SPE 52788-U stationary phase, an ion exchange stationary phase, and be collected. The method obtained from the research proves to be advantageous compared to the method previously proposed in the literature [29]. The method involves the use of stationary phase such as Sephadex-100. The results obtained with this process are interesting because from an initial purity of 7.5 %, a final purity of 32 % is achieved, so the method allows obtaining a purity 4 times greater than the initial concentration. Although this is a

positive result, the degree of purity obtained by this method is far from that obtained by the just completed research [29].

Furthermore, the method proposed by the just completed research proves to be more effective than the one involving the use of the XAD 7HP stationary phase for the purification of purple fleshed potato. In this case, a solution containing different concentrations of ethanol was used as the extraction solvent, where the solution with the higher concentration of ethanol represented the better solution in terms of recovery. The final extract post-purification with the XAD 7HP stationary phase is a solution rich in anthocyanins but not pure. Therefore, the method proposed by the just completed research is optimal both for the extraction solvent and for the degree of final purity. In fact, the final purity obtained with the newly discovered method is significantly higher than the method proposed by this article [30].

Instead, when evaluating the purification processes present in the literature, which have shown results of equal value to those obtained with the method proposed by the research, the method developed in this research remains advantageous compared to the others proposed in the following articles. In fact, the purification method to obtain a purified form of anthocyanins proposed by [15, 31], guarantees an extract of high purity but also requires several steps. Indeed, the method obtained from the research involves a process of extracting anthocyanins from the matrix and a single purification step. The purification protocol proposed by these two publications requires a first liquid-liquid extraction with ethyl acetate, then the solution enriched with anthocyanins is introduced into C18 Silica gel Spherical. The solution enriched with anthocyanins from this last stationary phase will need to be processed again on an ion exchange stationary phase. Thus, although these two proposed methods guarantee a high degree of purity, they require a significant expenditure of time as well as materials and solvents.

Furthermore, the purification method is more reliable even than those proposed by previously published studies [32, 33], even these methods use ion exchange stationary phases such as cation exchange resin 001X7. Ensuring a purity comparable to the method obtained with the Discovery® DSC-MCAX SPE 52788-U stationary phase. On the other hand, however, they do not refer to recovery values and are research based only on two matrices, red cabbage and mulberry. Instead, the method obtained from the research guarantees its reliability on a greater number of types of vegetables, having developed research on vegetables, tubers, and berries but also on flowers and leaves.

5. CONCLUSIONS

The goal set at the beginning of the research, aimed at extracting pure anthocyanins from purple cabbage, elderberry, purple basil, purple sweet potato, wild pansy, and cornflower, has been successfully achieved through meticulous experimentation and analysis. In all matrices subjected to the extraction process, the obtained anthocyanin extracts exhibit remarkable purity, underscoring the effectiveness of the developed methodology.

However, the aim of current studies must be to increase the recovery of anthocyanins in order to obtain a greater quantity of pure extract. This could make the cost more sustainable not only if the extract is intended for beneficial purposes, as anthocyanins are known for their antioxidant properties, which can help fight oxidative stress and protect cells from free radical damage. In addition, several scientific studies have highlighted the potential of anthocyanins in relation to cardiovascular homeostasis, improved cognitive function and even reducing the risk of certain chronic diseases.

However, a significant challenge concerns the use of another solvent instead of methanol as an extraction solvent, known for its toxicity, which in extreme cases can cause blindness and breathing difficulties. One solution could be a eutectic solvent that has the same polarity as methanol and is able to dissolve the anthocyanins trapped in the stationary phase. Perhaps it could also replace the use of buffers based on phosphate salts (HK_2O_4P), since the removal of the salts that enter solution and will be present in the pure extract requires a large volume of water. It is therefore necessary to invest in green chemistry that is as environmentally sustainable as possible.

One example would be the extraction of anthocyanins both from the starting matrix and in the purification process with the eutectic solvent NADES. Indeed, with this eutectic solvent, it is possible to achieve extraction with high recovery and completely without having any impact on the environment as is the case with organic solvents [34].

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