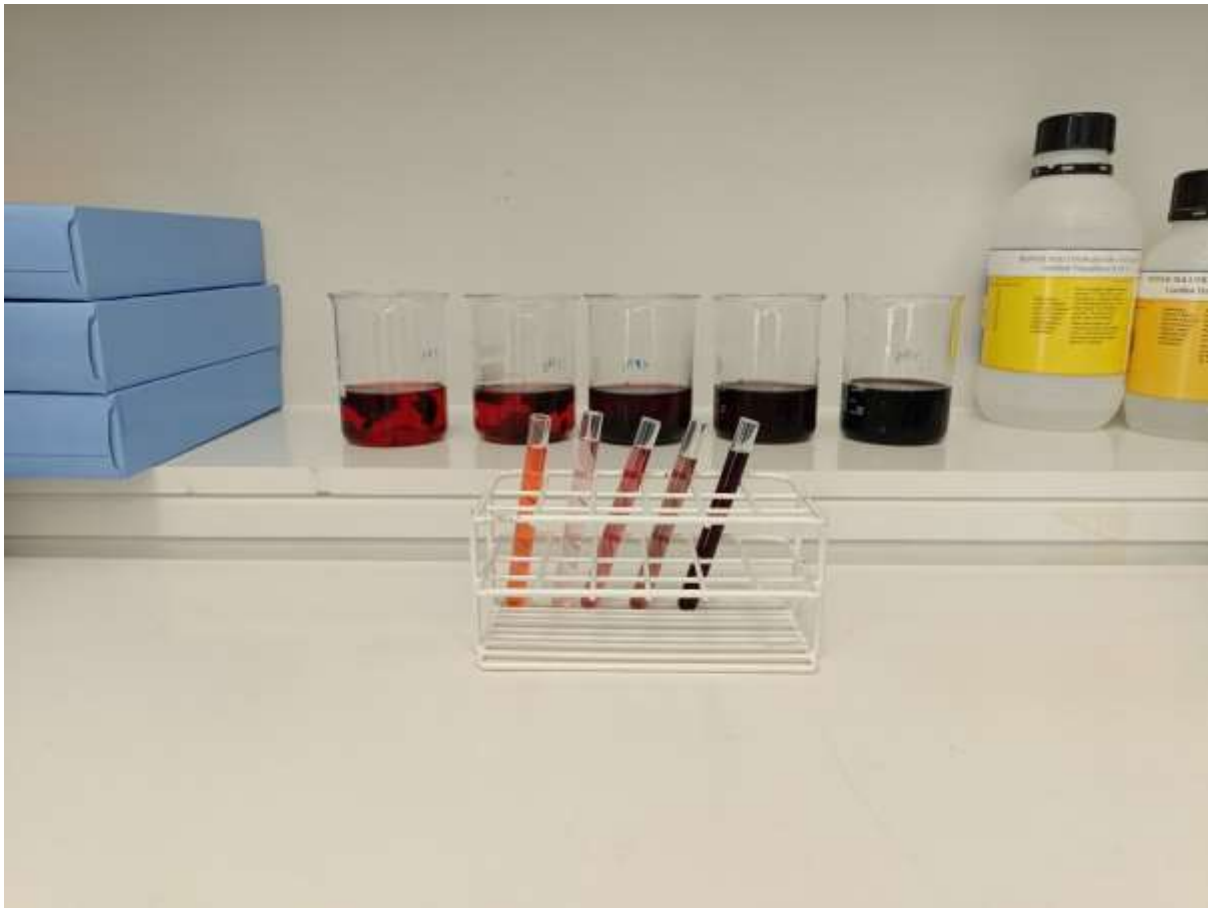
Chemical characterization and extraction optimization of *Aronia melanocarpa* polyphenols



AALBORG UNIVERSITY
STUDENT REPORT

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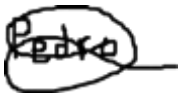
Pedro Martínez Alfaro: 

Abstract

The extraction, chemical characterization, and isolation of polyphenolic compounds from *Aronia melanocarpa* are the main objectives of this thesis. Anthocyanins are interesting because of their significant antioxidant activity and potential for health benefits. Acidified water (pH = 3) and water was chosen as a solvent solution after a variety of extraction methods, such as Soxhlet, maceration, and ultrasound-assisted extraction (UAE), were investigated. In order to enhance anthocyanin yield while avoiding degradation, process factors like temperature and extraction time were optimized. Maceration at 80°C for two hours was the most successful condition for macerations, yielding 5.64 mg of cyanidin-3-O-galactoside/g DM. And the best condition for ultrasound extraction was using acidified water, 50 % amplitude after 2 hours, yielding 9.50 mg of cyanidin-3-O-galactoside/g DM.

Extensive maceration and UAE tests were performed to evaluate compound stability and recovery, and the results showed that anthocyanins are comparatively stable for 6–8 hours at mid temperatures. Using a two-dilution technique with ethyl acetate and 70% ethanol, Amberlite XAD-7 resin was used in a dynamic column arrangement for isolation, preferentially recovering anthocyanins and hydroxycinnamic acids, in two different desorbents. Up to 95% fractionation efficiency was achieved for hydroxycinnamic acids and anthocyanins.

For detecting phenolic acids and anthocyanins at 325 nm and 520 nm, a unique HPLC-DAD approach was studied and verified. This technique made it possible to identify important substances like cyanidin-3-O-galactoside and chlorogenic acid. Total phenolic content and total anthocyanin were assessed using colorimetric assays (TPC, TA, TFC, and DPPH).

Overall, the study offers a viable and sustainable process that combines resin-based purification with improved thermal extraction to maximize the value of *Aronia melanocarpa* as a source of bioactive polyphenols.

Preface

This master's thesis was carried out at Aalborg University during the academic year 2024–2025. I would like to express my sincere gratitude to everyone who contributed to the successful completion of this work.

I am especially thankful to Mette Hedegaard Thomsen for their guidance, support, and constructive feedback throughout the entire process. I also wish to thank the members of the AAU energy for their helpful discussions, collaboration, and shared expertise, which greatly enriched this project.

Special thanks go to Lilian Melo Bondig for their assistance with laboratory work, particularly in the extraction and chromatographic analysis. Finally, I would like to thank my family and friends for their continued encouragement and support during this journey.

Aalborg Univeristy,

June 2, 2025

Abbreviations

- **BV** – Bed volumes
- **S** – Soxhlet Extraction
- **TPC** – Total Phenolic Compounds
- **TA** – Total Anthocyanins
- **TFC** – Total Flavonoid Compounds
- **TCT** – Total Condensed Tannins
- **A. melanocarpa** – *Aronia melanocarpa*
- **MPRs** – Macroporous Polymerized Resins
- **UAE** – Ultrasound-assisted Extraction
- **MAE** – Microwave-assisted Extraction
- **HWE** – Hot Water Extraction
- **DES** – Deep Eutectic Solvents
- **NADES** – Natural Deep Eutectic Solvents
- **MA** – Maceration
- **MeOH** – Methanol
- **EtOH** – Ethanol
- **EtOAc** – Ethyl acetate
- **Dem. water** – Demineralized water
- **GAE** – Gallic Acid Equivalent
- **QE** – Quercetin Equivalent
- **CE** – Catechin Equivalent
- **C3G** – Cyanidin 3-O-glucoside

Content

1 Introduction

To prevent further environmental harm, the circular economy (CE) concept minimizes waste and recycles resources in a circular manner. A shift to a CE can cut GHG emissions by as much as 70% [1]. Several nations have worked to achieve a CE in the following ways: In 2008, China enacted the first CE law; Germany and Japan followed suit. EU nations have lately suggested CE monitoring systems, and national CE regulations [2]. The three pillars of reduction, reuse, and recycling form the foundation of a CE's approach to food waste management. Additionally, in order to safeguard the environment and public health, the European Union Waste Framework Directive places a strong emphasis on the recycling and reuse of biowastes like FW [3]. By creating value-added compounds from biomass, biorefining is a process that can recycle and utilize FW [4].

Originally from North America, *Aronia melanocarpa*, also called black chokeberry, is now grown all over the world. It is a member of the Rosaceae family, and because it contains a lot of anthocyanins and other phenolic chemicals, its berries are deep purple.

Compared to other berries, aronia has the highest anthocyanin concentration, the highest overall polyphenol content, and one of the strongest DPPH radical scavenging activities [5], [6]. The primary class of phenolic chemicals has been determined to be procyanidins [7]. While epicatechin is the primary monomer, about 80% of procyanidins are made up of polymers with a polymerisation degree greater than 10 [8]. The phenolic chemicals called anthocyanins are what give the fruit its deep purple hue. They make up between 25 and 50 per cent of all phenolic compounds [7], [9]. With over 90% of the total anthocyanin content, cyanidin 3-O-galactoside and cyanidin 3-O-arabinoside are the most abundant anthocyanins; cyanidin 3-O-glucoside and cyanidin 3-O-xyloside are found in trace amounts [5], [6], [7].

Antioxidant, antimutagenic, antidiabetic, cardioprotective, gastroprotective, hepatoprotective, anti-inflammatory, anti-carcinogenic, and geroprotective properties are among the biological activities of aronia polyphenols [10], [11], [12]. There was a moderate correlation between the total anthocyanin content and the strong radical scavenging activity of A. melanocarpa and the total polyphenol content [6], [13].

Since juicing by-products account for 3% of all food waste in Europe, a significant portion of food waste is caused by the juice industry. Though many other species, including aronia, require more research, the majority of studies have concentrated on the use of the juicing by-products of the major horticulture crops, such as grapes, raspberries, citrus fruits, pomegranates, and apples [14]. Berries like aronia are being grown more frequently as a result of customer desire for functional meals. For instance, between 2004 and 2013, Poland, the greatest producer in the world, increased its output from 38,000 to 58,000 metric tons [10]. As a result, more fresh fruit or industrial processing byproducts ought to be taken into consideration for potential use. Aronia pomace is further distinguished by its wide range of morphologies. Fruit skin makes up the majority of it, however, minor amounts of seeds, flesh, and agglomerates of the aforementioned are also found [15]. There is no scientific literature on the polyphenolic composition of fruit fractions, despite a great deal of research on the

polyphenols in aronia fruit, extracts, and juice. When considering aronia pomace as a possible source of formulations rich in polyphenols, these facts are thought to be quite helpful.

This study uses high-performance liquid chromatography with diode-array detection (HPLC-DAD) to chemically characterize the polyphenols of *Aronia melanocarpa*, with a focus on anthocyanins. Evaluating how temperature and extraction time affect anthocyanin yield and stability is a key component of the investigation, since it offers insights into the best extraction conditions. Furthermore, XAD-7 resin solid-phase extraction is investigated as a method for anthocyanin isolation and partial purification. This study helps to effectively value *Aronia melanocarpa* as a rich natural source of polyphenols by combining extraction optimization with focused analytical characterisation.

Therefore, based on all the things mentioned above, the following research question was formulated:

How do extraction parameters such as temperature and time influence the yield and stability of anthocyanins from *Aronia melanocarpa*, and how can the use of XAD-7 resin be optimized for their effective isolation and concentration?

2 Literature review

This section reviews state-of-the-art technologies related to the extraction, separation, and isolation of polyphenols from *Aronia melanocarpa*. First, the chemical composition of A. melanocarpa is described, with emphasis on its rich content of anthocyanins, phenolic acids, and flavonoids. Then, both conventional and emerging extraction techniques are discussed, focusing on their application to polyphenol recovery from plant matrices. Particular attention is given to the effects of process parameters such as temperature, solvent composition, and extraction time. Finally, two subsections explore separation and isolation techniques, including the use of macroporous resins (for example, XAD-type resins), highlighting their role in purifying and concentrating anthocyanins from complex plant extracts.

2.1 Polyphenol Compounds

Black chokeberries' strong bioactivity is determined by biocompounds called polyphenols. Polyphenols, which comprise anthocyanins, flavonols, proanthocyanidins, and phenolic acids, are abundant in chokeberry fruits. The following polyphenol content was found by several authors in chokeberry fruit: 7849 mg/100 g DM [16]; 6351,38 mg/100 g DM [17]; 37,600 mg/kg DM [18]; 1079–2996 mg Gallic Acid Equivalents/100 g fresh mass [19]; 819–1330 mg GAE/100 g FW (fresh weight); 778–1285 g GAE/kg FW [20]. Polyphenols are also abundant in chokeberry waste and products. The amount of anthocyanins, such as cyanidin 3-glucoside, 3-galactoside, 3-xyloside, and 3-arabinoside, gives chokeberry fruit its dark blue colour. Myricetin and kaempherol 3-galactoside and 3-glucoside, as well as isorhamnetin 3-galactoside, 3-glucoside, 3-neohesperidoside, and 3-rutinoside, are additional flavonols found in chokeberry fruit [21], [22], [23]. One of the distinctive characteristics of chokeberries is the extent of proanthocyanidin polymerization. Epicatechin and trace amounts of catechin, which may only be found at the end of a molecular chain of proanthocyanidins, make up proanthocyanidins. C4, C6, and C4 C8 links bind individual subunits together [24], [25], [26].

Chokeberries mostly contain chlorogenic and neochlorogenic acids. Other phenolic acids are cryptochlorogenic acid, p-coumaric acid and its derivatives, caffeic acid and its derivatives, protocatechuic, vanillic, ferulic, salicylic, syringic, 4-hydroxybenzoic and ellagic acids. Dried juice also contains methyl esters of chlorogenic and neochlorogenic acids, 2,4,6-trihydroxybenzaldehyde, 3-hydroxybenzoic acid and phenylacetic acid derivatives [27]. Gentizinic and synapic acids were identified in chokeberry honey [28]. There were also reports on the content of eriodictyol 7-glucuronide; 3,7-diglycuronide, 7-xylose and naringenin-7-O-glucopyranoside and 7-O--d-glucopyranoside5,7,3,5-tetrahydroxyflavanone, as flavanones found in chokeberry fruit and products [27].

Polyphenol	Compound
Hydroxycinnamic Acids	neochlorogenic acid
	Chlorogenic acid
	Cryptochlorogenic acid
	Caffeic acid
	Protocatechuic acid
	p-Hydroxycinnamic acid

	3-O-p-Coumaroylquinic acid
	Dicaffeoylquinic acid
Flavonoids	(−)-Epicatechin
	(+)-Catechin
	Procyanidin B2
	Total procyanidins
	Quercetin-3-O-rutinoside
	Quercetin-3-O-galactoside
	Quercetin-3-O-glucoside
	Quercetin-3-O-vicianoside
	Quercetin-3-O-robinobioside
	Quercetin-3-O-arabinoside
	Quercetin-arabinoglucoside
	Quercetin 3-O-(6”-malonyl)-glucoside
	Quercetin-3-O-deoxyhexoside
	Quercetin-3-O-dihexoside
	Kaempferol-3-O-glucoside
	Eriodictyol-glucuronide
	Isorhamnetin-neohesperidoside
	Isorhamnetin-pentosylhexoside
	Isorhamnetin-rhamnosylhexosideisomer
	Isorhamnetin-3-O-rutinoside
Anthocyanin	Cyanidin-3-O-galactoside
	Cyanidin-3-O-glucoside
	Cyanidin-3-O-arabinoside
	Cyanidin-3-O-xyloside
	Cyanidin-3-O-rutinoside
	Cyanidin-3,5-hexoside-(epi)catechin
	Cyanidin-3-pentoside-(epi)catechin
	Cyanidin-3-hexoside-(epi)cat-(epi)cat
	Pelargonidin-3-O-arabinoside
	Delphinidin-3-O-rutinoside

Table 1: Content of polyphenol compounds in black chokeberry. Adopted from [29]

2.2 Type of anthocyanins

One of the subclasses of phenolic phytochemicals is anthocyanins. Glycoside is the form of anthocyanins, whereas aglycone is the form of anthocyanidin. Anthocyanins come in the forms of acylated anthocyanins and anthocyanidin glycosides, while anthocyanidins are classified as 3-hydroxy, 3-deoxy, and 0-methylated anthocyanidins. Cyanidin, delphinidin, pelargonidin, peonidin, petunidin, and malvidin are the most prevalent forms of anthocyanidins. In addition to the usual anthocyanins, plants can also contain acylated anthocyanins. The subtypes of acrylated anthocyanin are malonylated anthocyanin, caffeoylated anthocyanin, coumaroylated anthocyanin, and acrylated anthocyanin [30].

Because it lacks a ketone oxygen at the 4-position, anthocyanin, which is generated from flavonol, has the fundamental structure of a flavylium ion (Figure 1). The flavylium ion of anthocyanin has the empirical formula $C_{15}H_{11}O^{+}$ and a molecular weight of 207.24724 g/mol.

Conversely, anthocyanins are anthocyanidins that have been glycosylated. Anthocyanins' conjugated bonds give plants their red, blue, and purple hues.

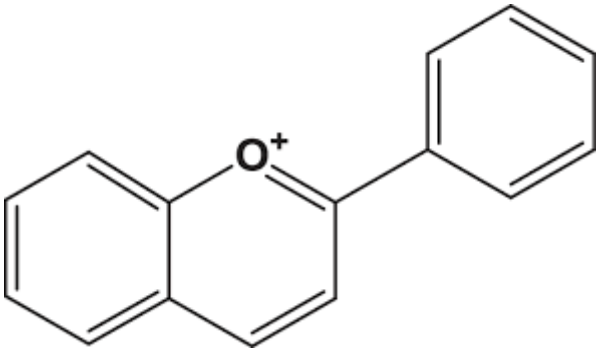


Figure 1: Flavylium ion structure in two dimensions. [30]

The most prevalent anthocyanidins found in plants include cyanidin, delphinidin, pelargonidin, peonidin, malvidin, and petunidin. These anthocyanidins are found in fruits and vegetables in the following proportions: 50%, 12%, 12%, 12%, 7%, and 7%, respectively [31]. Figure 2 displays their molecular structures. Cyanidine is a reddish-purple (magenta) pigment found in nature. It is the primary pigment found in berries [32] and other reddish foods like purple corn and red sweet potatoes[33]. Delphinidin shares a chemical property with the majority of anthocyanidins. In plants, it manifests as a purple or blue-reddish colour. The delphinidin pigment is what gives flowers their blue colour [34] The majority of the anthocyanidins are not like pelargonidin. It manifests as a red pigment in nature [35]. Pelargonidin gives some fruits and berries their red colour and flowers their orange colour [36], [37].

Another form of anthocyanidin that is widely present in plants is methylated anthocyanidin, which includes peonidin. Magenta is its visible colour [35] Red wines, berries, and grapes are rich sources of peonidin. Another O-methylated anthocyanidin is malvidin. Its obvious hue is purple, and it features a lot of blue blossoms, particularly Summer Wave Blue.[38] The primary red pigment in red wine is malvidin [39]. In mature red wine, it manifests as a dustier red [40]. Methylated anthocyanidins include petunidin. It is a water-soluble pigment that is either dark red or purple [35]. Purple flower petals and blackcurrants have been found to contain petunidin [41], [42].

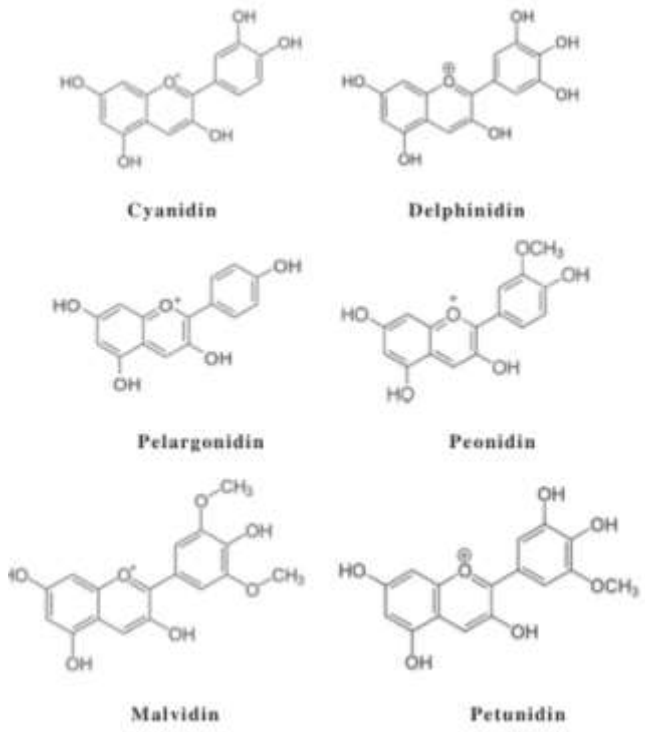


Figure 2: Major plant anthocyanidins. [30]

2.3 Anthocyanin Stability

Common anthocyanins have poor overall stability after purification, and their colour will specifically change or fade when the pH changes [43]. As a result, its direct use as a natural food pigment in the food sector has long been impacted. Nonetheless, practically all anthocyanins are quite stable in plant tissues under normal biochemical conditions, particularly in the absence of temperature and sunlight. This phenomenon implies that there may be a way to stabilize anthocyanins. In the food business, anthocyanins can be employed extensively as alternatives to artificial colours and antioxidants [44]. However, the main elements influencing the usage of anthocyanins are their colour and stability.

Anthocyanins' structure, including the quantity, kind, and mechanism of ligand attachment, has a direct impact on their stability. Despite their inherent instability, anthocyanins are more stable in acidic environments. Different processes can break them down into colourless, tan-soluble, or insoluble compounds. This deterioration happens when anthocyanins are extracted or purified, or when food is processed and stored. In order to employ anthocyanins as additives and to improve their purity, it is crucial to comprehend the variables that influence their stability and the mechanism of degradation. Research has shown that co-colour stabilization, which mimics the natural biochemical conditions of anthocyanins in plant tissues, is a successful technique for stabilizing these pigments. By avoiding hydration conversion, cochromatics can clearly increase the stability of anthocyanins and intensify their colour. In the early days, nature had a number of anthocyanin derivatives that are highly stable in neutral or slightly acidic aqueous solutions. At anthocyanin sites C3, C5, and C7, these compounds are joined by more

than two acylated glycoside groups. These groups prevent the colourful anthocyanins from hydrating into colourless pseudobases by raising the activation energy of the anthocyanins that are overshadowed by hydration equilibrium.

In general, as the degree of hydroxylation of glycoside ligands increases, anthocyanin stability declines. Nonetheless, anthocyanin stability is enhanced by a higher degree of methoxidation and further enhanced by the glycosylation of free hydroxyl groups [45]. Complex polyacylated anthocyanins also provide a "sandwich" structure that improves intramolecular chromophores by preventing nucleophile attack in water and preventing pigment discolouration. The substituent bonded to the benzene ring of the glycoside ligand plays a major role in the colour of non-acylated and monoacylated anthocyanins in acidic environments [46],[47]. While methoxidation results in a red shift, the colours become more purple the more hydroxyl groups there are. Anthocyanin glycosylation and acylation typically result in a purple shift. Apart from cochromatics, a number of external elements also affect anthocyanins' stability, including pH, oxygen, temperature, light, anthocyanin structure and concentration, enzymes, metal ions, and other auxiliary factors. The plant kingdom's colour diversity is unaffected by any of these variables. Co-coloration, or the formation of complexes between distinct colours, is the cause. Co-staining is thought to be an important component that can enhance anthocyanins' structural stability. The stability of anthocyanins in fruits, vegetables, and cereals has been the subject of numerous studies recently.

But generally speaking, anthocyanins are a naturally occurring pigment that is not very stable. The number of anthocyanin species present determines how stable plant anthocyanin extracts are; the greater the variety present, the more stable the extract.

2.3.1 pH

The most significant instability factor for plant anthocyanins is pH, and the medium's pH significantly impacts the colour of anthocyanins.

The pH of the solution affects the anthocyanins' colour. In very acidic solutions, non-acylated and monoacylated anthocyanins turn red, but in mildly acidic to neutral solutions, they turn reddish purple. In alkaline solutions, anthocyanins turn blue and colourless as pH rises. Anthocyanin extracts that primarily contain cyanin and geranium glycosides will appear orange to red, extracts that contain paeoniflorin glycosides will appear dark red, curetin, petunia, and mallow glycosides will appear red, and acylation of the glucoside group may appear blue [48]. However, over the whole pH range, anthocyanins containing two or more acyl groups show a respectable level of colour stability. Furthermore, the stability of anthocyanins is also influenced by the characteristics of acylated components [49].

Red yellow salt-positive ions predominated in the extremely acidic medium (pH=0.5), giving the solution a red appearance. The concentration and colour intensity of the yellow salt's positive ions drop as the pH steadily rises because anthocyanin loses its cation on C-epoxy and turns into a blue quinone alkali. This is because the nucleophilic water attacks the red-yellow salt's positive ions, hydrating them to form a colourless methanol pseudobase. In an acidic solution, quinone base is inversely transformed into yellow salt ions. The methanol pseudobase loses the conjugated double bond between the A-ring and the B-ring as the pH rises, making it

unable to absorb visible light [50] The blue quinone base rose as the pH rose and the yellow salt's positive ions lost protons. Colourless methanol pseudobase is produced as the pH rises more, and ringopening regenerates chalcone. At this point, the methanol pseudobase and chalcone underwent reversible transformations, and the anthocyanin solution was colourless. Each equilibrium type's relative number is correlated with particular anthocyanin structures in addition to pH [51].

The acylated form of 2,5-diglucoside is very considerable, while the cation quantity is very little under the same conditions and at any pH. The phenomenon is significantly more noticeable when pH is higher than 5. Because quinone bases predominate at pH 6.0 to 7.0 and deoxyanthocyanins have minimal propensity to create colourless methanol pseudobases, they exhibit a greater colour at higher pH values. The equilibrium shifts in favour of quinone base even at pH=7 and higher. Certain anthocyanins undergo conversion to a base of the quinone type. Because they are acylated by glucoside at alkaline pH values (quinone base and more anions than cations), some anthocyanins, particularly those with only one hydroxyl group in the B-ring, are more colorful at these pH levels.

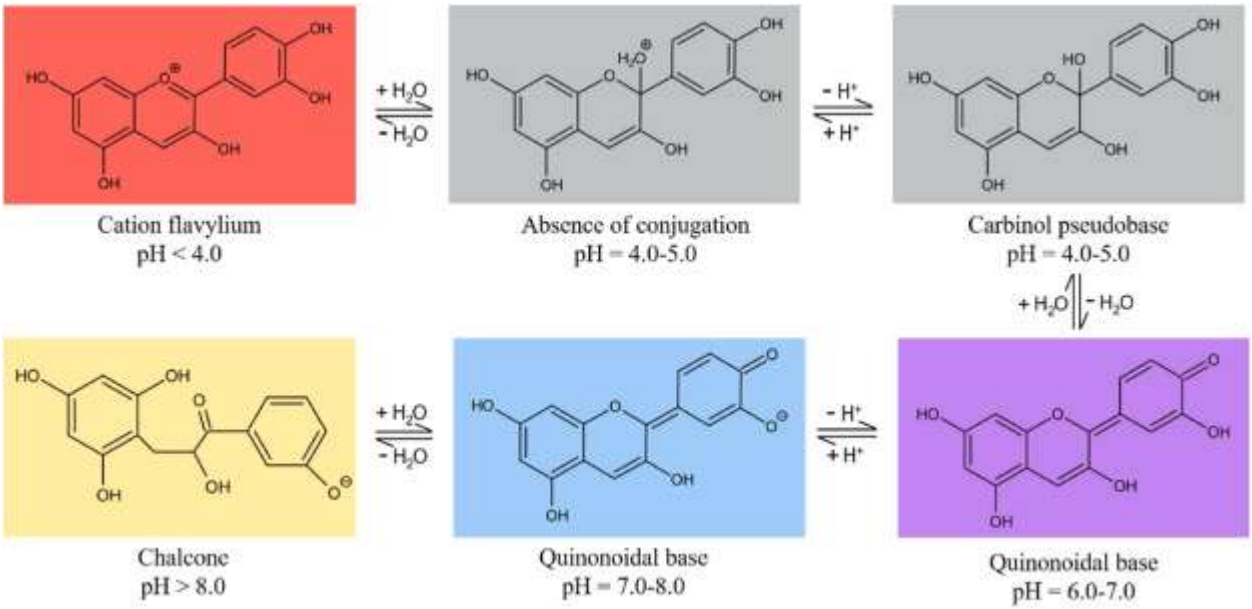


Figure 3: Anthocyanin chemical structures at various pH levels.[52]

2.3.2 Temperature

One important factor in the food industry is temperature. One of the most popular techniques for maintaining food safety during manufacturing and increasing its shelf life is heating treatment. According to earlier research, the anthocyanin degradation process was endothermic and readily affected by temperature [53]. As the temperature increased, the anthocyanins' degradation rate constant improved, but their half-life decreased [54]. According to earlier research, anthocyanins' thermal breakdown kinetics followed a first-order reaction kinetics model [53].

Currently, anthocyanins mostly undergo the following two heat breakdown pathways: (1) The strong aggression of water molecules causes anthocyanins to be deglycosylated into anthocyanidin glycosides at high temperatures, which are subsequently converted into methanol, chalcone, diketone, and even derivatives of benzoic acid and aldehyde [55]; (2) High temperatures encourage ring opening and the conversion of anthocyanins into chalcones, which are then further transformed into coumarin glycoside derivatives [55]. Low temperatures are better for preserving the structural stability of anthocyanins than high temperatures. A growing body of research has shown that anthocyanins are highly stable between 2 and 4 ◦C [54], [56].

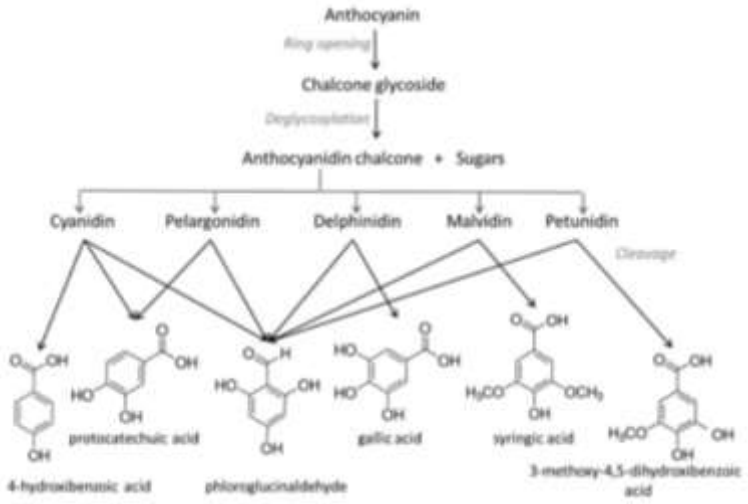


Figure 4: Possible mechanism of thermal degradation of the most common non-acylated anthocyanins [57]

2.4 Solubility of anthocyanins

In alkaline solutions, the anthocyanin pigment appears blue to purple, while in acidic solutions, it appears red. These pigments are frequently derived from purple fruits and vegetables, berries, flowers, and blackcurrants. Additionally, the most common extraction medium for isolating anthocyanin pigments is water. Additionally, certain food processing facilities extract anthocyanin pigments using alcoholic solutions. The reason for this is that anthocyanins dissolve in water and the majority of organic solvents. Conversely, the apolar organic solvent does not dissolve anthocyanin. Additionally, it is unstable in neutral or alkaline solutions [30].

Anthocyanidins, like cyanidin, are particularly soluble in water at lower pH values or in acidic conditions because they create the red flavylium cation [58]. Acidic environments strengthen the anthocyanin pigment's red colour and preserve the stability of the flavylium ion. Given that the red anthocyanin pigment is extremely persistent in acidic aqueous solutions, this property makes it a strong contender for use as a colorant. It works well as a colouring agent for red drinks. At higher pH values, where quinoidal bases prefer alcoholic solution, the flavylium ion deprotonates [59].

While glycosylated anthocyanin is very soluble in water, anthocyanin aglycone is more soluble in alcohol than its glucoside. [60] Anthocyanins' polyphenolic structure gives them a hydrophobic quality and renders them soluble in organic solvents like methanol and ethanol. In addition to the flavylium cation, the 3-hydroxyl group in the anthocyanidin's C-ring, which

is invariably connected to a sugar or sugars, may be the cause of the anthocyanidin's water solubility. The most soluble anthocyanidin in methanol is delphinidin, which is followed by acetone, ethanol, and water [61]. Cyandin is less soluble in methanol than delphidin is. The reason for this is that methanol has been used to extract cyanidin from grape skins with poor yield [62].

Due to malvidin's static dipole moment in water is larger than that of methanol and ethanol, it is more soluble in water than in these substances [63]. As the degree of acylation increases, its water solubility decreases. The degradation of acylated malvidin releases syringic acid at high pH levels (pH > 7). Even at higher temperatures, the diffusion coefficients of peonidin-3-glucoside and malvidin-3-glucoside in water at room temperature (25°C) are comparable [64]. A decreasing polarity of anthocyanidins has been reported in the order of delphidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin despite the fact that no study examines the solubility of these six anthocyanins in water [65]. Actually, cyanidin, delphinidin, and pelargonidin are more soluble in water than malvidin, peonidin, and petunidin. It might be because these anthocyanidins have one or more hydrophobic methoxy groups at positions 3′,5′, and 3′ and 5′ of the B-ring.

At lower pH levels, when intense protonation takes place, anthocyanins become more soluble in water [59]. Anthocyanins become more soluble in alcohol when HCl is added [63] Additionally, malonylation of anthocyanidin stabilizes its structure and increases its water solubility [60]. Consequently, anthocyanin aglycones' pigment hue is preserved for use as a food coloring when malonylation occurs. However, the quantity of hydroxyl groups that are affixed to the B-ring has a significant impact on the colour of anthocyanin.

2.5 Health benefits

The black chokeberry, *Aronia melanocarpa*, has drawn a lot of interest lately because of its remarkably high concentration of bioactive polyphenols, especially phenolic acids, flavonoids, and anthocyanins. These substances are widely known for having strong antioxidant properties, which are essential for scavenging reactive oxygen species and lowering oxidative stress, which is a major contributing factor to the onset of chronic illnesses. Because of this, *Aronia melanocarpa* is regarded as a potentially useful functional ingredient in the management and prevention of diseases linked to a certain lifestyle, underscoring the need of creating efficient techniques for the extraction and stabilization of its polyphenolic constituents.

2.5.1 Antioxidant effects

Among the anthocyanins found in *Aronia melanocarpa*, cyanidin-3-O-arabinoside has the best ability to scavenge radicals. It has also been demonstrated to be a potent inhibitor of pro-oxidative enzymes such as xanthine oxidase and 15-lipooxygenase [66] Due to the low amount of flavonols in fresh chokeberries, quercetin demonstrated the highest antioxidant activity in terms of both oxygen radical absorbance capacity (ORAC) and total radical-trapping antioxidant parameter (TRAP) [67]. However, flavonols' contribution to antioxidant activity was found to be less than 10 %. Procyanidins were found to be responsible for 40 % of the in vitro antioxidant activity of chokeberries, with anthocyanins (24 %), hydroxycinnamic acids (18 %), and epicatechin (11 %), following closely behind. Procyanidins are thought to be more

potent antioxidants than their monomers [68]. Despite lacking anthocyanins, green, unripe chokeberries have the highest antioxidant activity because of their high procyanidin and flavonoid concentration [69] The findings of [70] demonstrated that Aronia leaves have significant antioxidant activity and may have medicinal and nutritional value. X found that after a one-month training camp, rowers who consumed 150 mL of juice daily reduced the oxidative damage to their red blood cells caused by exercise [71].

SOURCE	$ORAC_H$ (µMOL TE/G)	$ORAC_L$ (µMOL TE/G)	DPPH	REFERENCE
BERRIES	158	2.4		[72]
BERRIES	160.2	-		[73]
BERRIES	-	-	181 (µmol TE/g)	[74]
JUICE	-	-	60 mg TE/ mL	[75]
EXTRACT	5165	-	-	[76]
EXTRACT	-	-	36 IC_{50} (mg/L)	[77]

Table 2: Antioxidant activity of Aronia and Aronia products determined by different in vitro assays

2.5.2 Anti-inflammatory activity

The fruit of the *Aronia melanocarpa* plant has anti-inflammatory qualities that help prevent the onset of long-term conditions like diabetes, heart disease, and immune system issues [78]. Inducible nitric oxide synthase (iNOS) and cyclooxygenases (COXs) are the two main pro-inflammatory enzymes that produce lipid mediators and nitric oxide, which are linked to the development of numerous inflammatory illnesses [79] In rats with endotoxin-induced uveitis, the study by [80] demonstrated the anti-inflammatory properties of *Aronia melanocarpa* extract. Because iNOS and COX-2 enzyme expression is decreased, the in vitro experiment suggests that the anti-ocular inflammatory action may entail reduction of nitric oxide, prostaglandin, and tumor necrosis factor (TNF) production. The study by [81] provided fresh proof that the extract can stop human aortic endothelial cells from responding proinflammatorily [82]. offer the first empirical evidence in favor of using the bioactive fraction therapeutically to treat a range of inflammatory airway conditions. Their investigation clearly demonstrated the anti-inflammatory efficacy through reduction of ROS secretion and triggered cell cycle arrest, in addition to the lowered production of iNOS and COX-2.

SOURCE	CELL OR ANIMAL TYPE	WORKING CONCENTRATION	MODE OF ACTION	REFERENCE
ARONIA CRUDE EXTRACT	RAW 264.7	10 µg/mL	↓ iNOS ↓ COX-2	[83]
ARONIA MELANOCARPA FRUIT EXTRACT	HAECs	10 µg/mL	↓ICAM-1 ↓VCAM-1 ↓NF-κB ↓ROS	[84]
ARONIA BIOACTIVE FRACTION (ABF)	BEAS-2B	1 µg/mL	↓TNF-α ↓IL-6,-8,-1β ↓CCL5 ↓COX-2, iNOS ↓ROS	[85]
ARONIA MELANOCARPA (MICHX.) ELLIOT CONCENTRATE (CYANIDIN-3-O-GALACTOSIDE, CAFFEOYLQUINIC ACIDS)	human peripheral monocytes RAW 264.7	10 ng/mL	↓TNF-α ↓IL-6, IL-8 ↓NF-kB ↓PGE_2	[86]
ARONIA MELANOCARPA (PROCYANIDINS C1, B5, B2)	RAW 264.7	500 ng/mL	↓NO	[87]

Table 3: Anti-inflammatory activity of Aronia and Aronia products

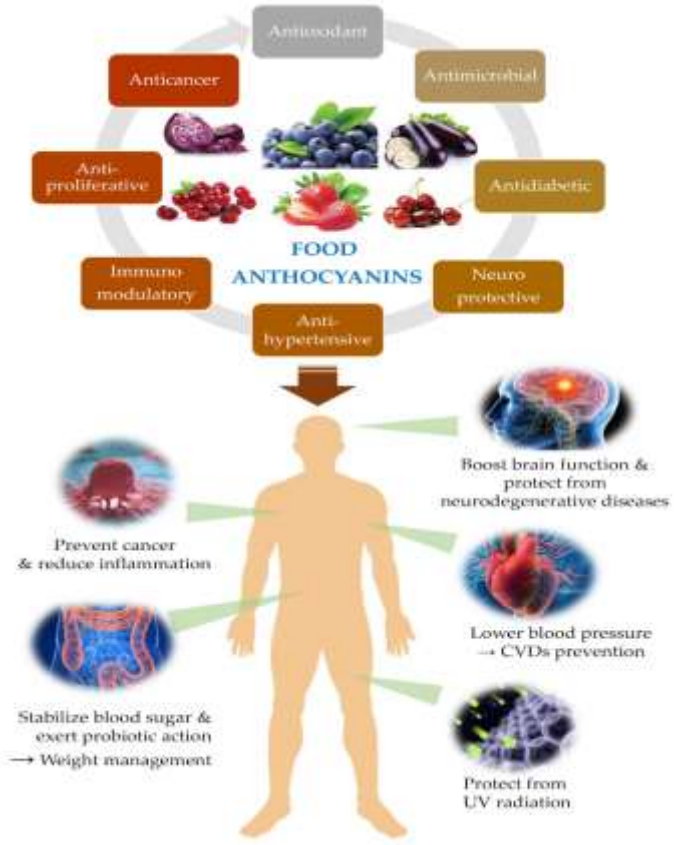


Figure 5: Overview of major human protective effects associated with ACNs consumption. [88]

2.6 Extraction methods

2.6.1 Conventional solvent extractions

The traditional methods for extracting anthocyanins are identical to those for extracting other phenolics. For instance, solvent extraction, sometimes referred to as solid-liquid extraction (SLE), is the most widely used technique for the extraction of plant components. It may effectively recover a high yield of anthocyanins from a range of sources. Generally speaking, soaking, leaching, separation, and purification are the fundamental processes for SLE. First, the pre-treatment samples are immersed for a predetermined amount of time in particular solvents. The anthocyanins are then transported to the particle surface by the solvents after being leached into them.

A number of factors, including solvent type. Temperature, extraction time, acid addition, particle size, and solvent/mass ratio affect the anthocyanin yield by the SLE method. Of these solvent types, acid addition and temperature are the main factors examined for SLE optimization.

Solvent extraction, filtration, concentration by decompression, and vacuum drying are often used for anthocyanins that are soluble in water or alcohol. Depending on their characteristics and raw materials, several solvents are employed to extract pigments. Water, acid base solutions, and organic solutions (such as ethanol, methanol, acetone, alkene, benzene, etc.) are the most often utilized ones. As anthocyanins are unstable in neutral or alkaline solutions and soluble in water and alcohol solutions, the extract should be acidic. The most effective

extraction solvent is methanol, which has an extraction efficiency that is 73% higher than water and 20% higher than ethanol. Acidic solvents, such as hydrochloric acid, acetic acid, and formic acid, break down plant cell membranes and dissolve water-soluble pigments ([89][90], [91]. Given the toxicity of methanol, an acidic solution of water or ethanol should be chosen for evaluating the pigment extraction solution's food safety as a food additive. Because they can break the bond between anthocyanins and metal ions and co-pigments, acidifying solvents of inorganic acids (such as HCl) have the potential to alter the natural form of complex pigments. The structure of anthocyanins is not significantly affected by weak organic acids [92].

Biomass	**Solvent**	**T^a(°C)**	**Time (min)**	**L/S Ratio (g/mL)**	**Recovery**	**Ref**
Aronia melanocarpa	Ethanol	46	252	1:23.8	6.12 mg/g	[93]
Black rice	Water	34.7	80	1:30	16.58 mg/100 g	[94]
Blueberry bagasse	Acidulant (1 % w/v) in water	50	5	-	17.17 ± 0.70 mg/100 g	[95]
Dried blackcurrant skins	Methanol/water	50	120	-	1712.3 ± 56.1 mg/100 g	[96]
Purple passion fruit peel	Potable water pH=2	52.03	180	29.79	577.59 mg/100 g	[97]

Table 4: Conventional Extraction Techniques

2.6.2 Soxhlet extraction

The Soxhlet method extracts the desired bioactive components by using a solvent at low pressure and boiling temperature. A small portion of the sample is placed in a thimble using this extraction technique, and the solvent is continuously circulated through the material until it becomes colorless, indicating that the extraction process is complete. Although this bioactive extraction method is straightforward and easy to use, it takes a long time and uses a lot of solvent, which eventually raises the process's cost. The breakdown of thermolabile components and a lower quality of the final product are the results of high-temperature processing at prolonged extraction times [98].

2.6.3 Supercritical fluid extraction

Since SFE is regarded as one of the most environmentally friendly and sustainable technologies, it has been used extensively in recent decades. Because supercritical CO_2 (critical temperature (CT) = 31.3 C; critical pressure (CP) = 72.9 atm) is nontoxic, inexpensive, and protects the extracts from air oxidation, it is frequently used as the solvent in this procedure. The non-polar nature of this solvent is a drawback. For this reason, polar molecules, such as anthocyanins, can be extracted more easily by adding a co-solvent [99]. Ethanol, methanol, and aqueous solutions of these alcohols in concentrations between 1% and 15% are the most often utilized cosolvents [100].

SFE and PLE are typically exposed to medium-to-high pressures. PLE is predicated on the use of liquids at temperatures higher than their typical boiling points, whereas SFE uses solvents at pressures and temperatures higher than their critical points. SFE extracts the target compound by utilizing the supercritical fluid's characteristics. In essence, there are two basic steps in the process: first, the supercritical fluid extracts the substance, and then a change in temperature or pressure quickly removes the fluid. The extraction procedure is frequently performed by alternating between a dynamic phase, in which the solvent continuously passes through the solid, and a static phase, in which the solvent remains in constant contact with the solid [101].

Biomass	Pressure (bar)	Tª(ºC)	Cosolvent	Sample	Flow rate	Recovery	Ref
Indian blackberry (*Syzygium Cumini*) fruit pulp	162	50	Ethanol	10 g of sample	2g/min	231.28 mg C3G/100 g of fruit	[102]
Haskap (*Lonicera caerulea* L.) berry pulp paste	450	65	Ethanol 42.39 %	L/S ratio 5.4/3.2 (w/w)	15 min static time 20 min dynamic time at 10 mL/min	25 mg of C3G/g of paste DW	[103]
Colombian blueberry (*Vaccinium meridionale*) fresh and mature fruit	300	40	No cosolvent	160 g and 800 g	32 g/min	26.7 mg extract/g of sample	[104]
Roselle (*Hibiscus sabdariffa* L.) dry calyces	89	70	Ethanol 75 %	1.5 g	6 mL/min	26.7 g of dried extract/100 g of sample	[105]
Merlot red grape (*Vitis vinifera*) pomace	100	95	Ethanol	30 g	32 g/min	700 mg of M3G/kg of grape DW	[106]

Table 5: Supercritical fluid extraction

2.6.4 Ultrasound-assisted extraction

Cavitation bubbles created by ultrasound frequencies have the ability to compress and expand before collapsing, giving samples shear force. Cell wall pores increase as a result of the mechanical effect, which also occasionally results in cell wall rupture. As a result, mass transfer and anthocyanin extraction yields will be enhanced by ultrasound-assisted extraction (UAE) [107], [108]. UAE is distinguished by its low energy consumption and uses fewer solvents and no CO2 [109], [110]. UAE is regarded as a promising alternative approach for extracting anthocyanins because it is safe for human consumption, reasonably easy to scale up [107], [111], and particularly intriguing for the food business [107], [109], [110]. When compared to alternative methods with comparable anthocyanin yields, the UAE method's primary drawback at the moment is its high cost [111]. Based on ultrasound frequencies, the UAE can be separated in two groups: high-power ultrasound with low frequency and high amplitude between 20 and 100 kHz, and low-power ultrasound with low frequency and low amplitude between 100 and 1000 kHz. Because it can cause vibrations from surrounding cells and particles, which generate fluid currents and disruptive forces, the latter category is more frequently used in food processing and can increase the yield of anthocyanins from samples. However, it is well established that the asymmetrical collapse of air bubbles close to the cell wall surface and fluid microprojects, when combined with ultrasound administration, can result in a sharp rise in temperature, which can exceed 70 ºC in cooled reactors. This effect clearly affects anthocyanin stability: 10 minutes of ultrasonic exposure at 20 kHz drastically decreased strawberry juice's anthocyanin concentration by 3.2 % [112]. The study's conclusion is that the extraction time affects UAE's efficiency because vibrational energy is released as heat. However, under 40 kHz ultrasonic, the total monomeric anthocyanin content from jussara pulp grew gradually over 180 minutes [110]. This could suggest that the efficacy of UAE depends on the right protocol being chosen for a certain sample.

Biomass	Time (min)	T[a](°C)	Solvent (%)	L/S Ratio (mL/g)	Power (W)	Frequency (kHz)	Recovery	Ref
Purple sweet potato	60	60	Ethanol 90 % (0.1 % HCl)	100:5	200	-	214.92 mg of C3GE/100 g of potato DW	[113]
Red cabbage	75	40	Ethanol 42.39 %	3:1	-	37	58.67 mg of C3G/L of extract	[114]
Red cabbage	15	30	Water	100:2	100	30	20.9 mg of P3G/L of extract	[115]
Jambolan (*Syzygium cuminin* L.) fruit	7.5	30	Ethanol 79.6 %	15:1	Power density: 112.5 W/L/40/4	-	54.2 mg C3GE/g of fruit DW	[116]
Fig (*Ficus carica* L.) peel	21	30-35	Ethanol 100 %	100:15	310	-	4.32 mg C3R/g of fig peel DW	[117]
Black carrot pomace	20	50	Water	3:1	102	24	12.4 mg of C3XGG/L 69.7 mg of C3XG/L 16.0 mg of C3XGGS/L 73.4 mg of C3XGGC/L 34.2 mg of C3XGGC/L	[118]

Mulberry (*Morus nigra*) pulps	10	48	Methanol 76 % pH=3	12:1.5	200	24	149.95 µg of C3G + C3R + C3MG + C3DG/g of mulberry FW	[119]
Blueberries (V. *Angustifolium* Aiton)	11.5	65	Ethanol 60 % acidified	50:1	100	40	13.22 mg C3G3/g if blueberries DW	[120]
Blackthorn (*Prunus spinosa* L.) Fruit Epicarp	5	Room T[a]	Ethanol 47.98 % acidified (citric acid, pH=3)	100:5	400	40	11.76 mg of C3R + P3R/g of fruit epicarp DW	[121]
Purple Majesty potato	5	33	Ethanol 70 %	200:5	35	20	346.3 mg C3/kg of potato FW	[122]

Table 6: Ultrasound-assisted extraction

2.6.5 Pressurized Liquid Extraction

High pressure (3.8-10.3 MPa) is used in pressurized liquid extraction (PLE), also referred to as accelerated solvent extraction (ASE). High pressure has the ability to keep solvents liquid over a wide temperature range (40 to 200 ºC) [123] and improve solvent-phytochemical contact in samples, enabling the quick extraction of anthocyanins with minimal solvent [124], [125], [126], [127]. Since the technique relies on high temperatures, the methodology is always used to extract less heat-sensitive chemicals. Red onion anthocyanins began to degrade early in the PLE extraction process [126]. Therefore, it is important to carefully consider the balance between degradation and extraction kinetics when using PLE to extract anthocyanins. The anthocyanin profile, temperature, and duration time all have an impact on degradation kinetics, thus, it is necessary to look into the PLE extraction settings to make sure the benefits of PLE are realized. Pretreatments such as pH modification have an impact on the extraction effectiveness of the PLE method in addition to temperature and extraction time. Furthermore, solvents that are easy to remove and non-toxic are favoured for PLE [128]. For instance, hot compressed water is used as a solvent for PLE, which is both a cost-effective and environmentally favourable substitute. Water is a poor solvent for extracting phytochemicals at room temperature. On the other hand, water's dielectric constant approaches that of methanol when heated to 200 ºC [129]. Nowadays, PLE is frequently used to extract anthocyanins from a variety of foods, such as sweet potatoes, jabuticabas, açai berries, blueberries, and onions [124], [126], [130], [131]. For instance, when compared to low-pressure solvent extraction, PLE enhanced the anthocyanin production from jabuticabas by 2.15 times. Simultaneously, the manufacturing cost was reduced by 40 times [127].

Biomass	T (ºC)	P (bar)	Time (min)	Solvent/Flow Rate (mL/min) (Amount of Sample)	Recovery	Ref
Jambolan (*Syzygium cumini* L.) fruit	90	117.2	5 rinsing time; 10 extraction time/cycle (2 cycles)	Ethanol 80 % (acidified 0.1 % TFA)/- (4g)	47.05 mg C3GE/g of fruit DW	[132]
Purple sweet potatoes	90	-	15 (2 cycles)	Ethanol 80 % (acidified 0.1 % HCl)/- (10g)	252.34 mg C3GE/ 100 g of potatoes DW	[133]
Broken black bean (*Phaseolus vulgaris* L.) hulls.	60	100	26	Ethanol: citric acid 30:70 (pH=3.4)/5 (5 gDW).	3.96 mg C3GE/g of sample DW	[134]

Table 7: Pressurized Liquid Extraction

2.6.6 Microwave-Assisted Extraction

Since 1986, microwave-assisted extraction (MAE), a relatively recent technology, has been used to prepare samples [135]. The method promotes cell disintegration by rapidly, effectively, and uniformly heating the solvent and target tissues using non-ionizing electromagnetic radiation (300-300000 MHz) [136]. MAE makes reduced solvent usage, shorter extraction periods, and selective and effective extraction possible [136]. On the other hand, improved compound diffusion to solvents facilitates the extraction of non-targeted compounds while simultaneously increasing the extraction yield [136]. Furthermore, as was already indicated, using heat to extract anthocyanins has several drawbacks, including the potential to cause deterioration. Therefore, it is crucial to prevent overheating during MAE. For instance, when it came to extracting anthocyanins from powdered blueberries, MAE at 53 ºC was more effective than hot reflux extraction . It is commonly acknowledged that for MAE, heat-induced anthocyanin degradation predominates at temperatures higher than 60 ºC [137]. Low temperatures, however, may not necessarily stop anthocyanin deterioration. According to the study by Garofulic et al., the production of anthocyanins dramatically dropped with increasing temperature and/or irradiation period. Liazid et al.'s work, however, demonstrated that MAE at 100 ºC considerably raised the quantity of anthocyanins extracted from grape skin peels; the amount only decreased at temperatures higher than 100 ºC [136]. Based on the tissues that make up the samples, these findings suggest that the anthocyanin extraction temperature for MAE should be optimized. MAE is currently a commonly used technique in the food sector due to its affordability, environmental friendliness, efficiency, and ease of assembly on both large and small scales.

Biomass	Time (s)	Tª(ºC)	Solvent (%)	L/S Ratio (mL/g)	Irradiation Power (W)	Recovery	Ref
Fig (*Ficus carica* L.) peel	300	62.4	Ethanol 100% (pH=3)	100:5	400	411 mg of C3R/100 g of fig peel DW	[138]
Red cabbage	300	100	Water pH=3	30:1	200	110.0 mg of C3G/L	[139]
Red cabbage	600	90	Ethanol 50% (pH=3)	20:1	600	220.2 mg of C3G/L	[139]
Purple sweet potato	500	-	Ethanol 30% (citric acid pH=2)	3:1	320	31 mg of C3GE/100 g of potato	[140]
Lonicera edulis	90	-	85 % Ethanol	25:1	280	292.16 mg/100 g	[141]
Cranberry	8	50	52 % Ethanol	28:1	-	3.06 mg/g	[142]
Red rice	100	-	Ethanol 85 % acidified	22:1	400	3.82 mg of C3G/100 g of rice	[143]
Rosa pimpinellifolia L. fruits	1037.4	60	Ethanol 26.85 % $(NH_4)_2SO_4$ 19.15 %	40:1	400	1373.04 mg C3GE/g of fruit DW	[144]
Grape juice waste	138.6	55	Double-distilled water	19.2:1	35	132 mg M3/100 g of grape juice waste DW	[145]

Table 8: Microwave-Assisted Extraction

2.6.7 Enzyme-Assisted Aqueous Extraction (EAE)

Enzymes can be employed by EAE as stand-alone extraction techniques or as supplementary instruments in other extraction procedures to boost extraction yields by degrading the vegetal cell walls. The enzyme's effect on the cell wall serves as the foundation for the enzymatic extraction process. Extracellular cell walls, cell walls, and oleosomes are some of the barriers that must be overcome in order to access the bioactive compounds stored in vegetal cells. These barriers are each made up of different components. They are produced naturally and hydrolyzed by certain enzymes. Proteases, hemicelluloses, pectinases, and cellulases are the four groups of enzymes that are more frequently used to hydrolyze portions of vegetal cell walls. Each of the first three groups has a distinct effect on the cell wall and can be used separately or in combination. Therefore, depending on the enzyme used, a selective extraction of various bioactive compounds can be accomplished [146]. Since they may extract pigments more quickly and efficiently than traditional ethanol extraction, cellulases, hemicelluloses, pectinases, and proteases have been used to improve and speed up the pigment extraction of a variety of plant materials [147]. However, due to their ability to hydrolyze enzymatic proteins, which reduces or eliminates the specific activity of the enzyme combination, proteases must be utilized independently of the other families of enzymes. A variety of natural sources, including bacteria, fungi, fruit and vegetable extracts, and animal organs, can provide the enzymes used in EAE [148]. The characteristics and settings under which an enzyme acts vary depending on its source. Therefore, the denaturing temperature of a mammal enzyme is typically 40–45 °C, but the denaturing temperature of microorganism-produced enzymes is higher than 60–65 C. However, some enzymes can withstand temperatures above 100 °C for a number of minutes because they are thermoresistant. Various commercial enzymes are currently available for purchase.

When compared to traditional methods, EAE has been used to increase the yield of pigment, phenols, and anthocyanins extracted from various natural matrices [146], [147], [149], [150].

Biomass	Enzyme mixture	pH	Tª (ºC)	L:S Ratio/Enzymes: Mixture Ratio/ Hydrolysis Time (min)	Recovery	Reference
Blueberry	Pectinase	4.5	45	8:1/-/60	2.346 mg of TA/ mL of extract	[151]
Raspberry (*Rubus idaeus* L.) pomace	Ultrazym AFP-L	-	45	100:15/1:100/60	0.32 mg of C3S + C3G + C3R/ g of sample FW	[152]
Raspberry wine residues	Pectinase	3	40	30:1/0.16:100/30	0.853 mg of C3G/g of sample	[153]
Raspberry wine residue	Pectic enzyme	-	52	100:1/0.2:100/66	0.75 mg of C3G/g of sample	[153]
Mulberry wine residue	Pectinase	5.9	45	20:1/-/58	6.04 mg of C3G/g of sample	[154]

Table 9: Enzyme-Assisted Aqueous Extraction

2.7 Resin Isolation

In comparison to other types of resins, microporous polymer resins, often referred to as microporous adsorption resins, have a higher porosity and surface area when dry and are strongly cross-linked. Their adsorption ability is better than that of other types of resins, including gel structure resin, because they usually have a surface are between 300 and 800 m^2/g and particle sizes between 0.2 and 1.25 mm [155]. MPRs are often made via suspension polymerization, which combines monomers with organic solvents that are poor solvents for the polymer and totally miscible with the monomers. For microporous resin to have its unique porous structure, the solvent must be removed after polymerization [156] During synthesis, a number of physical and chemical factors affect the qualities of the resin. Physical elements like time, temperature, and stirring speed are important. For example, smaller polymer particle sizes result from greater polymerization temperatures. Furthermore, non-uniform polymer distribution and particle surfaces can be caused by very low or high stirring speeds [157]. The type of monomers, the amount of initiator, the amount of crosslinker, and the diluent are among the chemical characteristics that are critical in determining the properties of microporous resin. For example, the degree of swelling and solvent retention depend on the kind of monomers (hydrophobic or hydrophilic). Moreover, surface roughness and particle size are influenced by monomer concentration. Furthermore, particles with more crosslinkers have larger surface areas and tougher surfaces. Phase separation between the aqueous and organic phases during synthesis is also strongly influenced by the type and amount of diluent used [158]. For microporous resins to have the appropriate qualities and functionality, these factors must be carefully controlled and optimized.

Ion exchange, catalysis, adsorption, separation, and purification are just a few of the many uses for microporous polymer resins. Styrene-divinylbenzene resins are perfect for applications that need an effective adsorption process because of their high specific surface area, porosity, and adsorption capacity. Additionally, the business manufactured the popular Amberlite XAD family of polymer resins, which vary from one another in terms of adsorption capacity, pore width, specific area, particle size, and porosity. The Amberlite XAD-2, XAD-4, XAD-7, and XAD-16 series are a few examples. These are frequently used to separate and adsorb metabolites from crude plant extracts [159]. Since their creation, inexpensive polymer resin brands and models that share the same physical and chemical properties as conventional Amberlite resins have become available on the market and are frequently employed in the purification and separation of bioactive substances. NKA, ADS, EXA, and D100 are a few examples of these inexpensive microporous resins [160]. Given the large range of resins on the market, it is crucial to choose the best resin for the separation and purification process, depending on the particular adsorption characteristics needed for the target chemicals. As a result, when choosing the best resin, some factors such as its polarity, surface area, pore size, and adsorption and desorption capabilities need to be considered. When choosing the right type of resin, one crucial factor that distinguishes its adsorption ability is its polarity, which should be taken into account. Macroporous resins are categorized as nonpolar, moderately polar, weakly polar, and polar based on their polarity. The solute material can only dissolve in a

solvent with a similar solubility according to the theory of resemblance and intermiscibility [161]. As a result, polar resins may adsorb polar chemicals, whereas nonpolar resins are appropriate for adsorbing nonpolar compounds. To a certain degree, weakly polar resins can adsorb both polar and nonpolar molecules, while moderately polar resins can adsorb intermediately polar compounds. When choosing for separation and purification, surface area and pore volume are also crucial considerations. The pore structure limits the size of molecules that may be adsorbed [162], whereas the surface area controls the quantity of material that can be adsorbed [162]. Additionally, choosing the best resin depends heavily on the resin's adsorption and desorption capabilities. These capabilities show how well the target compounds may be adsorbed by the resin and released during the desorption process [163]. Therefore, when selecting the best resin for separation and purification, it is crucial to establish a correlation between these aspects.

The use of microporous polymer resins (MPRs) to purify bioactive substances from medicinal plants, including phenolics[164], saponin, alkaloids[165], and flavonoids[166], has increased recently. This is mostly explained by their capacity to use adsorption and desorption processes to amass a sizable amount of chemicals on their surface [167]. Furthermore, MPRs have a number of advantages over other purification methods, including preparative high-performance liquid chromatography, high-speed counter-current chromatography, and gel chromatography. These benefits include ease of regeneration, simplicity, great capacity, low cost, and excellent selectivity. As a result, MPRs are now the go-to option for purifying bioactive substances. The benefits and drawbacks of the most popular techniques for purifying bioactive chemicals are presented in Table 10.

Purification technqiue	Advantages	Disadvantages
Ion exchange column chromatography	High purity and simple operation	High operating expenses, time-consuming, and low selectivity
High-performance liquid chromatography	Simple, rapid, and high purity	Only applicable for small-scale separation and high cost
Macroporous resin column chromatography	Simple operation, ease of regeneration low cost and high selectivity	Low purity

Table 10: The advantages and disadvantages of various flavonoid purifying methods. Adopted from [168]

2.7.1 Modes of operation in Macroporous resin

The resin adsorption process can be carried out in three different ways: chromatographic, dynamic, and static. The dynamic mode is used in large-scale operations as opposed to static or batch resin adsorption, which is frequently used in laboratories. The primary distinction between the techniques is flow. Chromatographic adsorption requires controlled flow to separate analytes, whereas static adsorption does not. Chromatography, on the other hand, seeks to separate specific components, whilst static and dynamic adsorption are mostly used for purification. Different systems are made to reduce expenses while improving yield, purity, and specificity, depending on the goals of the separation. These systems perform better than

other separation techniques such as liquid-liquid extraction, chemical precipitation, membrane filtering, and distillation [169], [170].

The breakthrough curve, which establishes the operation and dynamics of an adsorption column, is essential for assessing the performance of a packed column in dynamic adsorption. Plotting the proportionate instant concentration to the beginning concentration against time or effluent volume produces breakthrough curves. When the column capacity is achieved, the breakthrough curve, which resembles a step function, shows an instantaneous increase in the adsorbate concentration [171].

2.7.2 Resin XADs

MPRs are frequently used to isolate bioactive components and purify plant extracts because of their low cost and strong adsorption capability. This study focuses on MPR XADs, which are heavily crosslinked with acrylic ester, aliphatic ester, or styrene-divnylbenzene. Except for two that are somewhat polar, they are entirely nonpolar. In previous investigations, they were found to be effective in extracting polyphenols.

The resins of the Amberlite XAD series are polymer spheres that vary from one another in terms of their chemical and physical properties, including degree of polarity, porosity, specific area, particle size, volume, pore diameter, and chemical composition. Since the speed of the adsorption processes is controlled by diffusion through the polymer matrix, these characteristics have a direct impact on it. Metals and organic chemicals found in environmental, biological, and plant samples have often been separated and/or concentrated using these resins. XAD-7 has medium polarity, whereas XAD-2, XAD-4, and XAD-16 resins are non-polar [172].

Amberlite	Matrix	Surface area (m^2/g)	Particle size (mesh)	Pore size (A)
XAD-2	Sytrene-divinylbenzene	300	20-60	90
XAD-4	Sytrene-divinylbenzene	750	20-60	100
XAD-7	Aliphatic ester	500	20-60	450
XAD-8	Acrylic ester	140	40-60	250
XAD-16	Sytrene-divinylbenzene	800	20-60	200
XAD-1180	Sytrene-divinylbenzene	500	20-60	400
XAD-2000	Sytrene-divinylbenzene	600	20-60	45
XAD-2010	Sytrene-divinylbenzene	660	20-60	280

Table 11: Polymer resins and their physical and chemical characteristics.[173]

SOURCE	EXTRACTION	PURIFICATION	COMPOUNDS	REFERENCES
CHOCKEBERRIES	Water with citric acid (1 %) (SWE)	XAD-7	Malvidin-3-galactoside, cyanidin-3-galactoside	[174]
BLACK CURRENT	600 mL of acidified water with 0.01 % HCl (v/v) (SPE)	XAD-7HP	Delphinidin-3-O-rutinoside, cyanidin-3-O-rutinoside	[175]
RED CABBAGE	mixture of methanol, acetone, HCl, and water (SLE)	XAD-7HP	Cyanidin-3-glycoside	[176]
MUSCADINE (VITIS ROTUNDIFOLIA) JUICE POMACE	-	XAD-16	Anthocyanin	[177]
PURPLE SWEET POTATO	7 % acetic acid in 80 % methanol was purged with nitrogen gas (SLE)	XAD-7HP	Cyanidin and peonidin	[178]
MUSCADINE (VITIS ROTUNDIFOLIA) JUICE POMACE	-	XAD1180	Anthocyanin	[177]

Table 12: Overview of Macroporous resins (MPR) XADs and studies on adsorption of anthocyanins

Reviewing recent studies (Table 12), XAD 7 and XAD 16, which are somewhat polar, reveal high adsorption and desorption capacity in extracting some polar phenolic compounds, such as epigallocatechin-3gallate. XAD7 is well-performed in the recovery of glycosylated phenolic compounds that can be developed by strong binding of these molecules due to hydrogen bonds. Performs better in the adsorption of glycosylated substances than XAD4 and XAD16; it is more polar. [179]

3 Project Objectives

To answer to the section 1 hypothesis. The primary goals are founded on current research in the Bioenergy and Bioproducts group at Aalborg University Esbjerg, international research, and the most recent literature review. The three primary goals are:

1. Screening of extraction methods
2. Isolation of phenolics using XAD-7
3. Establish a system of analytical techniques to assess each process step's effectiveness

The following tasks will be used in order to accomplish these primary goals:

1. **Screening of extraction methods**

- Screening of solvent in Soxhlet extraction, working with the normalization of the data to study the stability of anthocyanins
- Screening of temperature using Maceration extraction, working with the absorbance to study the anthocyanin efficiency
- Screening of time using Maceration extraction using the total anthocyanin pH differential method, and total phenolics to study the efficiency
- Study of the Ultrasound extraction to see if it improves the extraction of anthocyanins and phenols

2. **Isolation of phenolics using XAD-7**

- Total anthocyanins are measured in order to assess the adsorption. The 5% breakthrough point is found using the analytical method.
- Study the desorption curve for total phenolics and total anthocyanins using two different solvents to separate these two phenolic groups.
- Confirm the separation efficiency via HPLC-DAD results

3. **Establish a system of analytical techniques to assess each process step's effectiveness**

- HPLC-DAD analysis to evaluate:
    - Neochlorogenic and chlorogenic acid
    - Cyanidin 3-O-galactoside and Cyanidin 3-O-glucoside

- Spectrophotometric determination
    - Total anthocyanins
    - Total phenolic compounds
    - Total condensed tannins
    - Total antioxidant capacity using DPPH

4 Materials and methods

4.1 Biomass

After juice extraction, the aronia biomass residue was obtained from Elkaerholm (Denmark). The biomass was dried in a 30 – UF1060 oven (Memmert, Schwabach, Germany) at 60 °C for 48 hours. Once dried, the biomass was carefully weighed, packed into plastic bags, and stored at room temperature until further use.

Figure 6: Aronia melanocarpa biomass

4.2 Chemicals

The chemicals used in this study were EtOH, hydrogen peroxide, acetonitrile, MeOH, sulfuric acid, hydrochloric acid, anhydrous sodium carbonate, Folin-Ciocalteu phenol reagent, quercetin, gallic acid, aluminum chloride, catechin, DPPH, DMACA. Ultrapure water is produced from the Synergy Water Purification System (Mercl KgaA, Darmstadt, Germany) for all analyses.

4.3 Equipment

4.3.1 The colorimetric analysis

The microwell plate coulometric test, which includes Total Phenolic Compounds(TPC), Total Flavonoid Compounds (TFC), Total Condensed Tannins (TCT), and the antioxidant capacity utilizing DPPH, was analyzed using the spectrophotometer Infinite M1000Pro (Tacan, Salzburg, Austria). The range of $R^2 > 0.99$ was identified as the calibration range. With the exception of Total anthocyanin (TA), the colorimetric analyses were measured in cuvettes and measured at Cary 60 UV-Vis Spectrophotometer (Agilent, Santa Clara, USA).

4.3.2 Resin Adsorption

Resin	Peristaltic pump	Tubes	Peristaltic Tube	Column
Amberlite XAD-7 HP (Thermo scientific, Cat.No: 202245000, Lot: A0431603)	Masterflex Easy Load II 77200-60 (ColeParmer, Vernon Hills, USA)	Silicone tubing, inner diameter 6 mm	Masterflex L/S Precision Pump Tubin, Platinum-Cures Silicone, L/S 25	Chromatographic columns with frit and bedded rim A chromatographic column with sintered frit (porosity: 0), with PTFE stopcock, made of DURAN tubing. 600 mm, 30 mm in diameter. Capacity 430 mL. Art nr 5 4457 06

Table 13: Equipment used in the dynamic adsorption setup.

4.4 Extraction methods

Aronia melanocarpa's polyphenolic potential for nutraceutical and functional food uses depends on their effective extraction from which point they can be fully utilised. Maximising yield while keeping stability depends on choosing the suitable extraction technique and circumstances, including solvent type, temperature, and time, considering the structural sensitivity of anthocyanins to environmental variables. With an eye on optimising conditions for anthocyanin recovery utilising aqueous solvents under controlled heat procedures, this section investigates extraction methods.

4.4.1 Soxhlet extraction (Screening of solvent)

In this part of the study, it was decided to study the influence of the solvent used on the stability of anthocyanins. Water and acidified water were the solvents used to see which contributes more to anthocyanins' stability.

Run	Solvent	Number of cycles	Biomass (g)	Volume Solvent (ml)
1	Acidified water	9	10	200
2	Acidified water	9	10	200
3	Water	9	10	200

Table 14: Run of experiments made in the Soxhlet and the different parameters used in the different runs.

4.4.2 Maceration (Screening Temperature)

Macerations were run in a water bath, which allowed us to control the temperature. The conditions for the maceration were:

- **Biomass:** 20 g
- **Solvent:** 200 ml (acidified water, pH 3)
- **Time:** 2 hours
- **Liquid to Solvent Ratio:** 10 ml/g

- **Temperature Variations:** 20ºC, 40ºC, 60ºC, 80ºC

The main objective of the different macerations was to study the degradation of anthocyanins and to achieve the maximum amount of anthocyanins possible. Apart from the parameters mentioned before, time was also crucial to determine and study the anthocyanin yield.

Figure 7: Maceration set up experiments

4.4.3 Maceration (Screening time)

After screening the temperature, it was decided to study the effect of time in the degradation of anthocyanins. The conditions of the experiments were:

- **Biomass:** 20 g
- **Solvent:** 200 ml (acidified water, pH 3)
- **Time:** 24 hours
- **Liquid to Solvent Ratio:** 10 ml/g
- **Temperature Variations:** 40ºC, 60ºC, 80ºC

Samples to study the stability of anthocyanins were taken at 2, 4, 6, 8, and 24 hours for 40, 60, and 80 ºC.

4.4.4 Ultrasound extraction

Ultrasound-assisted extraction was employed to investigate the potential of cavitation-driven mass transfer in enhancing anthocyanin recovery from *Aronia melanocarpa* biomass. In this study, extractions were performed using an ultrasonic bath at a constant amplitude. Acidified water (pH = 3) and water were used as the solvent. The biomass-to-solvent ratio was fixed, while the ultrasound power was varied to assess its influence on anthocyanin yield.

Run	Biomass (g)	Solvent	Solvent Volume (mL)	Time (min)	Amplitude (%)
1	20	Acidified water (pH 3)	200	120	100
2	20	Water	200	120	100
3	20	Acidified water (pH 3)	200	120	75
4	20	Water	200	120	75
5	20	Acidified water (pH 3)	200	120	50
6	20	Water	200	120	50

Table 15: Run of experiments for the ultrasound extraction

Samples were taken every 20 minutes to evaluate the extraction dynamics and identify the time point at which anthocyanin yield plateaued or degradation began.

4.5 Resin Isolation XAD-7

To perform the resin adsorption, first, a maceration extraction was performed to prepare the extract. The conditions of the maceration were:

- Temperature: 80 ºC
- Time: 2 hours
- Solvent: acidified water (pH=3)
- Solvent to mass ratio: 10 mL/g

The extract was filtered through DWK Life Sciences DURAN™ Filter Disc Sintered Glass, Porosity 1, and then through DWK Life Sciences DURAN™ Filter Disc Sintered Glass, Porosity 3.

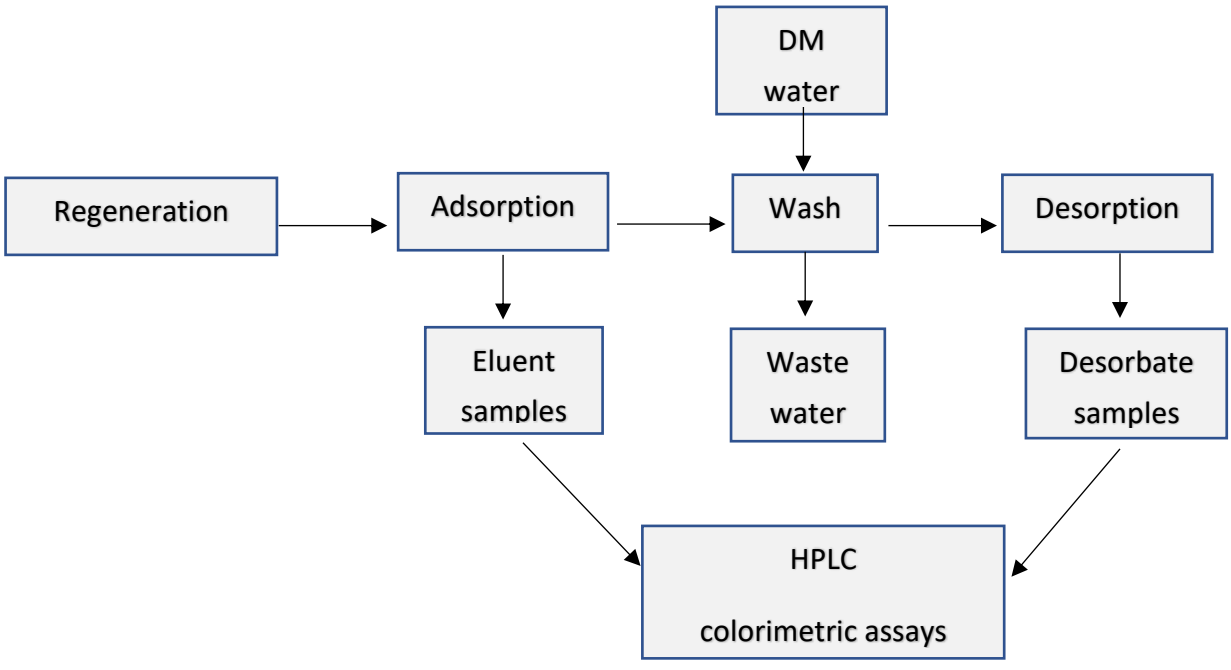


Figure 8: Schematic diagram of resin isolation step

The first step in the purification process was conditioning the column with 2 bed volumes (BV) of pH 3 water that had been adjusted with HCl and applied at a flow rate of 8 BV/h. After that the extract was added to the column at a rate of 2 BV/h for adsorption. Weakly bound chemicals were eliminated from the column by washing it with 2 BV of pH 3 water at a rate of 2 BV/h following adsorption. In order to desorbate phenolic compounds, 4 BV of ethyl acetate was used at a rate of 4 BV/h. Following that, anthocyanins were eluted at a rate of 4 BV/h using 4 BV of 70 % ethanol. A sequential wash using 2 BV of 1 M NaOH and 2 BV of 2 % hydrogen peroxide, both applied at 2 BV/h, was carried out to regenerate the column. Then, at the same flow rate, 2 BV of deionized water was used to rinse the column. To finish the cycle and have the column ready for use later, 1 BV of ethanol was finally run through it at a rate of 2 BV/h.

The decision to use two different solvents in the desorption phase was made to study and try to separate phenols from anthocyanins. Ethyl acetate is a semi-polar solvent, allowing the efficient desorption of compounds such as flavonoids and phenolic acids.

Ethanol is a polar solvent that can desorb hydrophilic polyphenols and anthocyanins that may still be retained on the resin. Allowing the separation of phenols from anthocyanins.

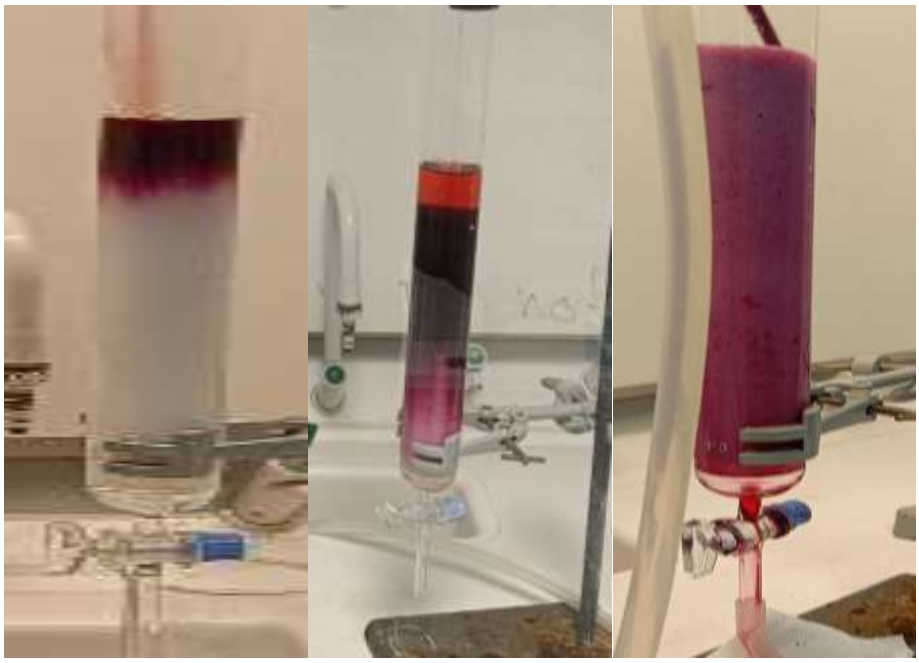


Figure 9: Visual representation of anthocyanin purification using XAD-7HP resin. The left image shows the beginning of adsorption, the middle image displays the resin fully saturated with anthocyanins, and the right image illustrates the desorption step.

4.5.1 Evaluation of adsorption and desorption

The focus of this study was to study the behaviour of the anthocyanins in the resin XAD-7HP. To study whether the total phenolics and total anthocyanins methods are suitable for studying the 5 % break point in the adsorption, and to study the desorption curve.

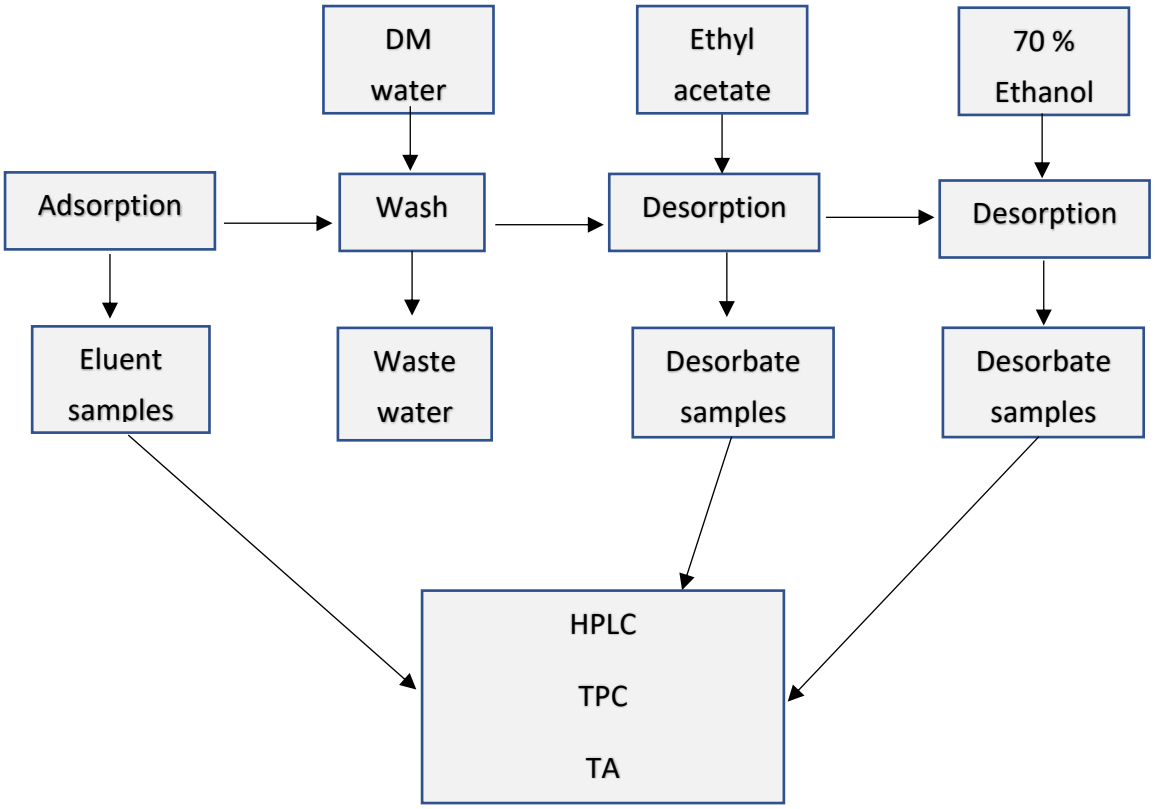


Figure 10: Flowchart of the adsorption and desorption process

Samples for the adsorption were taken every 0.5 BV and were prepared for TA, TPC, and HPLC analysis. In the desorption, samples were taken every 15 ml, and samples were also prepared for TA, TPC, and HPLC analysis.

4.6 Analytical methods

4.6.1 Total anthocyanin pH differential method

Prepare two buffer solutions first. While potassium chloride (KCl) and hydrochloric acid (HCl) are used to make the pH 4.5 buffer, sodium acetate and HCl are used to make the pH 1.0 buffer. These buffers are necessary to produce different pH environments that impact anthocyanin structure and absorbance characteristics.

After that, dilute your sample in every buffer. Dilute an aliquot of the sample using the pH 1.0 buffer. Take an equal quantity and dilute it in the pH 4.5 buffer in a different tube. Make sure both dilutions are performed precisely and reliably.

Vortex the samples completely after making the dilutions to guarantee adequate mixing and homogeneity. This step is essential for getting accurate absorbance readings.

Use a spectrophotometer to measure each sample's absorbance at 520 and 700 nm. These wavelengths are selected to measure the absorbance of anthocyanins while accounting for haze or turbidity.

Utilizing the following formula, determine the absorbance difference (A):

$$A = (A_{520\ nm} - A_{700\ nm}\ \text{at pH 1}) - (A_{520\ nm} - A_{700\ nm}\ \text{at pH 4.5})$$

The structural shift of anthocyanins between the two pH levels, which modifies their absorbance properties, is explained by this discrepancy.

Lastly, use the following formula to determine the sample's anthocyanin concentration:

$$\text{Anthocyanin (mg/L)} = \frac{\text{A} \times 1000 \times \text{MW} \times \text{DF} \times \text{DV}}{\varepsilon \times \text{l} \times \text{W}}$$

Every term in the equation has a distinct function in this calculation. The structural shift of anthocyanins under various pH settings is reflected in the variable A, which is the absorbance difference between pH 1.0 and pH 4.5. The standard anthocyanin molecule, cyanidin-3-glucoside, has a molecular weight of 655.2 g/mol, which is equivalent to the MW. While the DV represents the total dilution volume utilized in the test, stated in liters, the DF, or dilution factor, takes into consideration how much the original sample was diluted prior to measurement. The strength with which the chemical absorbs light at the specified wavelength is indicated by the ε (epsilon), which is the molar absorptivity of cyanidin-3-glucoside, which is 20,500 L/mol·cm. The l represents the cuvette's path length, which in conventional spectrophotometers is usually 1 cm. W is the weight, expressed in grams, of the original sample that was used in the analysis. The anthocyanin content is then expressed as milligrams (mg) per liter of solution by converting the final value from grams to milligrams using the factor 1000.

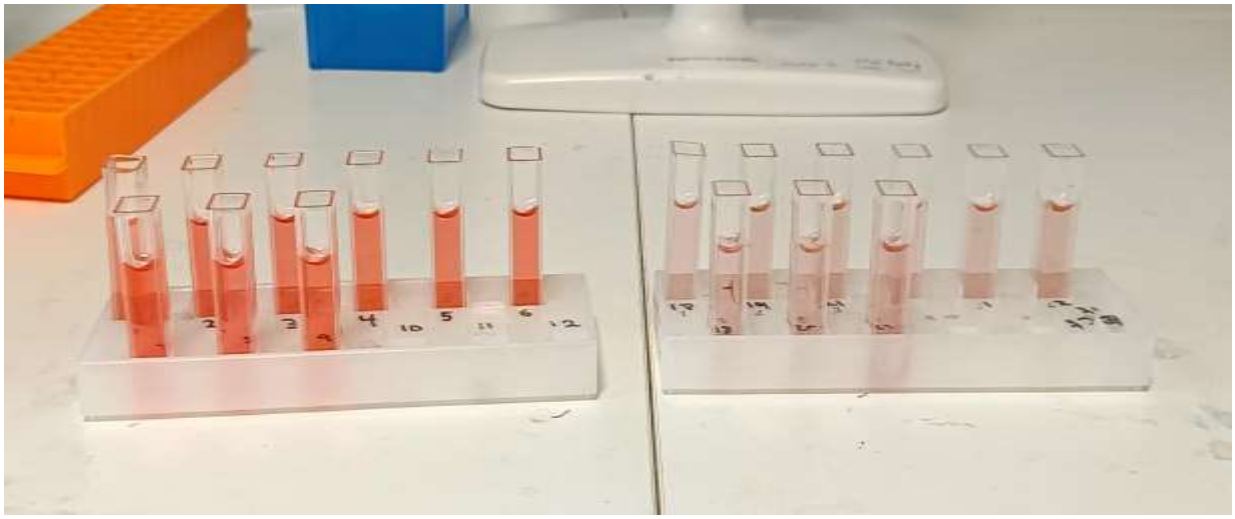


Figure 11: Cuvettes containing samples prepared for anthocyanin quantification using the pH differential method. The left rack contains samples in pH 1.0 buffer, and the right rack contains the same samples in pH 4.5 buffer

4.6.2 Colorimetric assays

In order to assess the outcomes of the optimization, separation, and isolation of polyphenols for analysis of groups of or total polyphenols, colorimetric assays, namely total phenolic compounds (TPC), total flavonoid compounds (TFC), and total condensed tannins (TCT), were used. Additionally, the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used to assess

Radical Scavenging Activity (RSA). Every microwell plate reading was taken at the plate reader. The calibration microwell plates from the four tests are displayed in Figure 12.

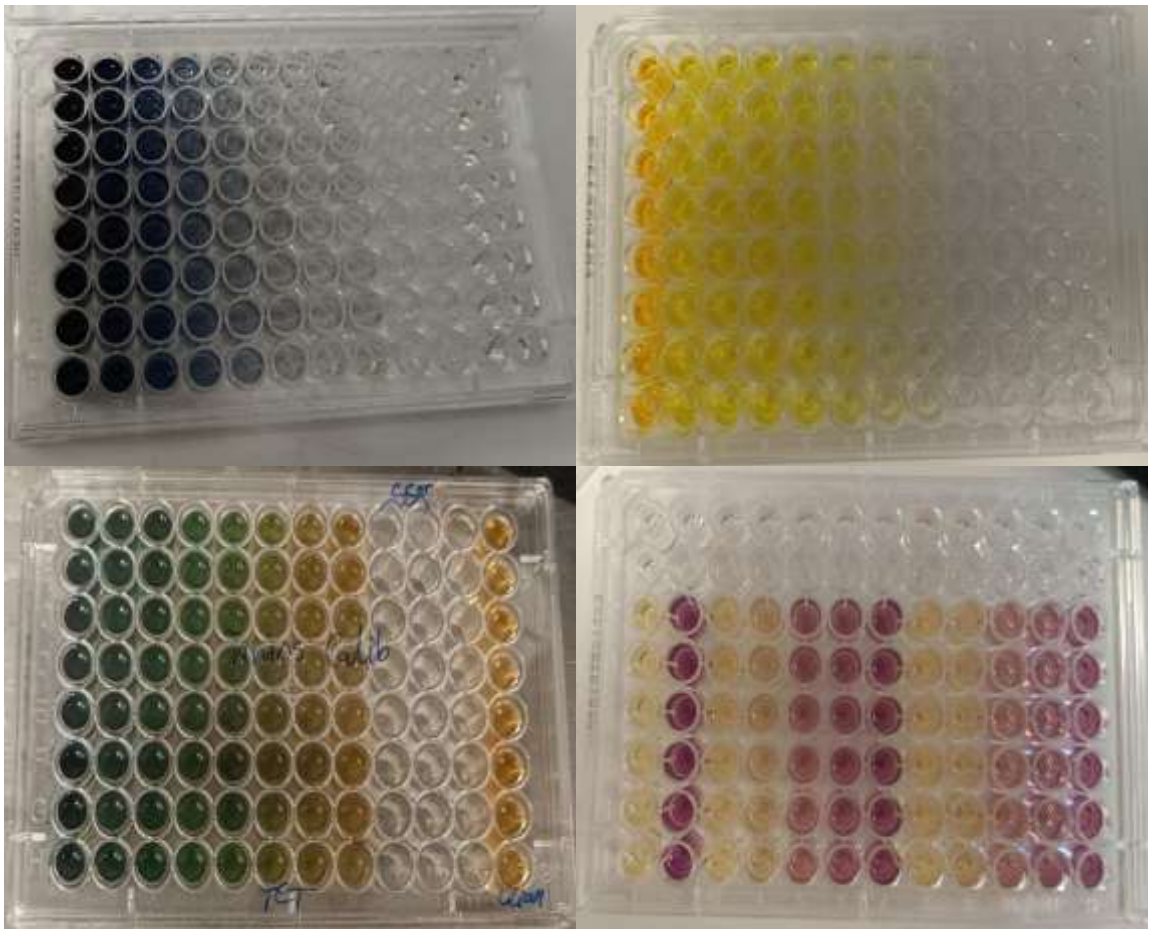


Figure 12: 96-microwell plates from the four colorimetric tests that were employed in the investigation. Radical Savaging Activity (RSA), Total Phenolic Compounds (TPC), Total Flavonoid Compounds (TFC), and Total Condensed Tannins (TCT) are listed from top left.

The TPC method was applied using the Folin-Ciocalteu TPC assay, using the microwell plates method.

The microwell plate used to create the TPC assay calibration curve is depicted in Figure 12. A multi-channel pipette was used to transfer 50 µL of the standard solution, which contained 1 mg/ml of gallic acid in Milli Q water. Dilutions were made from column to column. Next, 100 µL of a diluted Folin-Ciocalteu phenol reagent solution (x10 in MilliQ water) was applied to each well. Ten minutes were spent incubating the dish. All wells were then filled with 100 µL of 5 g/l sodium carbonate in Millipore water. To prevent irradiation and evaporation, the aluminized adhesive paper was adhered to the plate. After 90 minutes of dark incubation, the plate was read at 725 nm using a plate reader. The sample concentrations were computed using Equation 1 (Equation 2):

$$y = ax + b$$

Equation 1: Calibration curve equation

$$C_{GAE}\left[\frac{g}{L}\right] = \frac{\text{ABS @ 725 nm}}{a} - b$$

Equation 2: The calculation of Total Phenolic Compounds (TPC) in gallic acid equivalents.

In the TFC assay, the sample quantities were changed. Appendix 2-TFC contains the updated protocol.

The microwell plate used to create the TFC assay calibration curve is depicted in Figure 2. A multi-channel pipette was used to transfer 50 µL of the standard solution, which contained 1 mg/ml of quercetin in Milli Q water. Dilutions were made from column to column. Next, 50 µL of MeOH was applied to each well. Then 50 µL of 2% (w/v) aluminum chloride in MeOH was added. To prevent irradiation and evaporation, the aluminized adhesive paper was adhered to the plate. After 10 minutes of dark incubation, the plate was read in a plate reader set at 420 nm. Every absorbance must be less than 1. R^2>0.99 was used to create the calibration curve equation (Equation 8). Equation 9's sample concentrations were computed using the equation:

$$y = ax + b$$

Equation 3: Calibration curve equation

$$C_{QE}\left[\frac{g}{L}\right] = \frac{\text{ABS @ 420 nm}}{a} - b$$

Equation 4: The calculation of Total Phenolic Compounds (TFC) in quercetin equivalents.

Despite the protocol, it cannot be used to determined the total flavonoids, as the extract contains anthocyanins, which react with the aluminium chloride showing another colour different compared with the calibration curve.

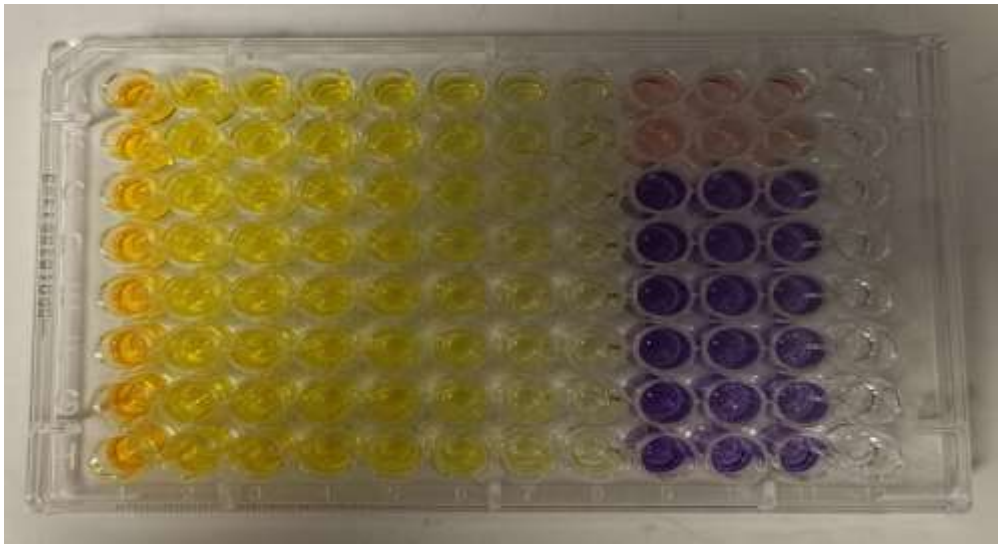


Figure 13: Calibration curve and three samples of TFC method.

The wells that look yellow are the ones using the standard for the calibration curve, but when looking into the columns with the three samples, the colour is clear purple due to the presence of anthocyanins. Making the method not reliable for total flavonoids.

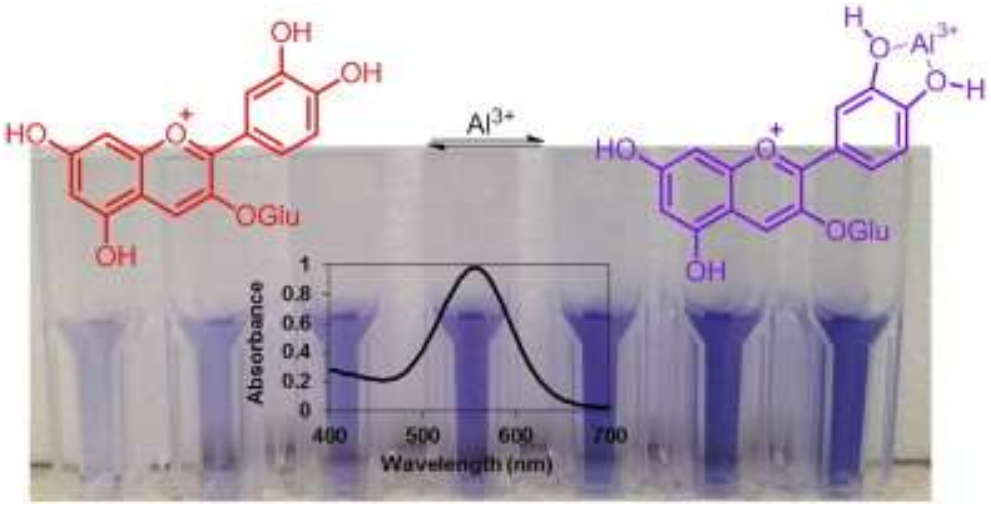


Figure 14: Reaction of anthocyanins with aluminium chloride. [180]

The microwell plate used to produce the TCT assay calibration curve is shown in Figure 12. A multi-channel pipette was used to transfer 50 µL of the standard solution, which contained 1 mg/ml catechin in MeOH. From one column to another, dilutions were created. All wells are treated with 100 µL of 1 % (w/v) DMACA in MeOH. Next, 50 µL of 32 % (w/v) HCl was added to each well. To prevent irradiation and evaporation, the aluminized adhesive paper was adhered to the plate. A plate reader was used to read the plate at 640 nm after it had been incubated for 15 minutes in the dark. Equation 11 was used to compute the sample concentrations based on Equation 10:

$$y = ax + b$$

Equation 5: Calibration curve equation

$$C_{CA}\left[\frac{g}{L}\right] = \frac{\text{ABS @ 640 nm}}{a} - b$$

Equation 6: The calculation of Total Condensed Tannins (TCT) in catequin equivalents.

As a reference, gallic acid (1 mg/mL) in Millipore water was utilized. DPPH was made in MeOH at a concentration of 120 µM, or 48 mg/L.

Calibration, samples, and a schematic illustration of the microwell plate are shown in Figure 12. This is how it was prepared: for color correction, rows A and B were used. Columns 1 and 2 contained samples from column 3, while column 3 contained one positive and one negative (blank). The negative control samples make use of the sample solvents. Using a multichannel pipette, 8 x 22 µL of gallic acid (1 mg/ml) was pipetted as the positive control, and 8 x 22 µL of sample solvent was pipetted as the negative control (blank). The same number of pips was applied to the samples.

4.7 HPLC-DAD method

The main phenolic components in Aronia melanocarpa extracts were identified and measured using High-Performance Liquid Chromatography with Diode Array Detection (HPLC-DAD), with an emphasis on anthocyanins and hydroxycinnamic acids. This analytical method enables

simultaneous monitoring of numerous wavelengths for more thorough compound profiling by fusing the spectrum analysis capabilities of a diode array detector with the separation efficiency of liquid chromatography.

HPLC settings	Value
HPLC	Agilent 1200 HPLC system
Column	Kinetex (2.6 µm, 100 Å, 100 x 2.1 mm)
Injection Volume	15 µl
Mobile phase flow rate	800 µl min^{-1}
Solvent A	100 % Acetonitrile
Solvent B	100 % Methanol
Solvent C	100 % Aqueous

Table 16: HPLC settings

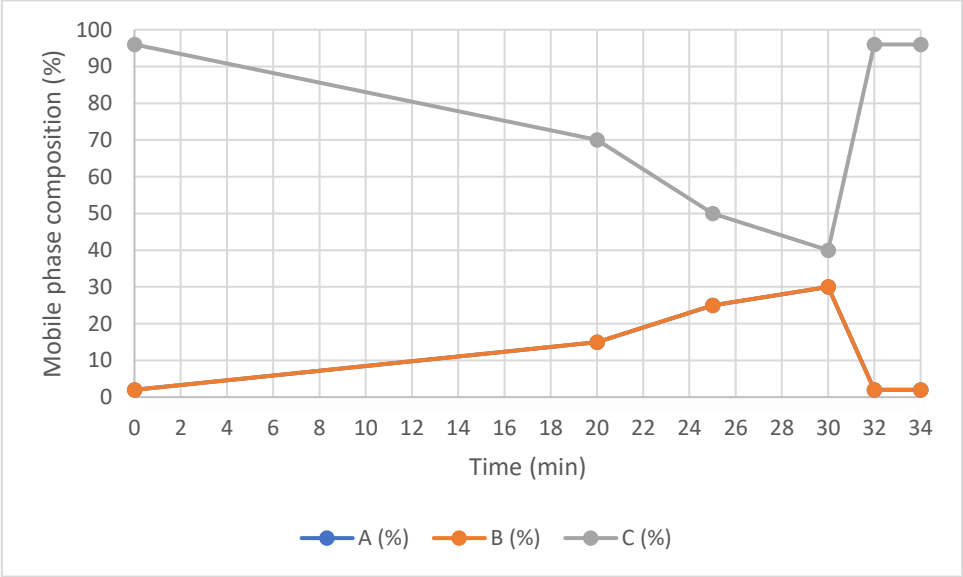


Figure 15: Mobile phase composition

Compound	Polyphenol group	Retention time (min)	Wavelength (nm)
Neochlorogenic acid	Hydroxycinnamic acid	11.7	325
Chlorogenic acid	Hydroxycinnamic acid	15.6	325
Cyanidin 3-O-galactoside	Anthocyanin	18.1	520
Cyanidin 3-O-glucoside	Anthocyanin	18.9	520

Table 17:Polyphenols analyzed with their retention time and wavelength

Chromatograms for anthocyanins and phenolic acids were investigated at 520 and 325 nm, respectively. Phenolic acids have considerable UV absorbance at 325 nm, while anthocyanins absorb strongly in the visible spectrum, especially at 520 nm, due to their flavylium cation structure. Selective identification of compound classes based on their distinctive absorbance profiles was made possible by the use of two wavelengths.

At a particular retention time (Rt), which represents how long it takes for a molecule to elute from the chromatographic column under the specified mobile phase conditions, each chemical in the chromatogram shows up as a peak. The process of identification involved comparing the UV-Vis spectra and retention periods of the samples with external standards. Quantification was performed using calibration curves prepared with known concentrations of reference compounds such as cyanidin-3-O-galactoside, cyanidin 3-O-glucoside, neochlorogenic acid, and chlorogenic acid.

5 Results and Discussion

This section describes the results from this study. The first part was to optimize the extraction of phenolics from *Aronia melanocarpa*. The second part consists of optimizing the resin isolation of the phenolics using the resin XAD-7.

5.1 HPLC methods

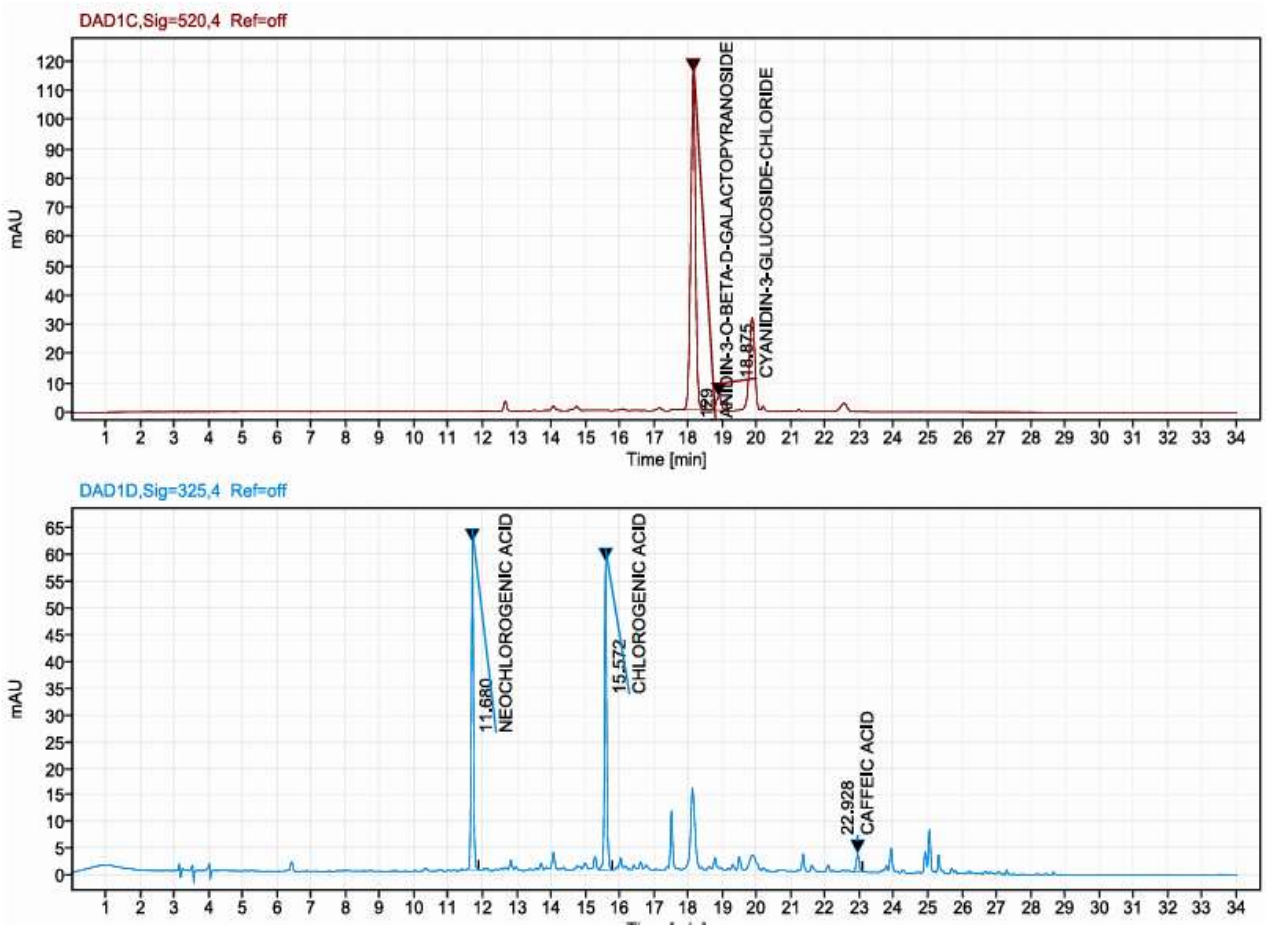


Figure 16:HPLC-DAD chromatograms of an Aronia melanocarpa extract obtained at 80°C for 2 hours. The upper chromatogram (520 nm) reveals major anthocyanins, predominantly cyanidin-3-O-galactoside, while the lower chromatogram (325 nm) shows prominent hydroxycinnamic acids, including chlorogenic and neochlorogenic acids.

Two chromatograms generated by High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD) of an *Aronia melanocarpa* extract are presented here. Two hours of at 80°C heating produced the extract.

Top Chromatogram—Red Trace Detection at 520 nm

Wavelength: 520 nm, which is typical of anthocyanins particularly in their coloured flavylium cation form at acidic pH.

Finding Compounds:

• Cyanidin-3-O-galactoside (Rt ≈ 18.1 min): Usually the most plentiful, this is the major anthocyanin in Aronia berries.

• Seen at 18.9 min are cyanidin-3-O-glucoside and other minor anthocyanins.

• The strong, high peak at about 18.1 min points to an anthocyanin dominating the profile in the extract.

Detection at 325 nm (blue trace) from a bottom chromatogram

Optimal for identifying phenolic acids, including hydroxycinnamic acids, wavelength is 325 nm.

Named Compounds:

• Rt ≈ 11.68 min, neochlorogenic acid

Usually, the most abundant hydroxycinnamic acid in Aronia, chlorogenic acid (Rt ≈ 15.57 min) is well known for its antioxidant action and adds to the general polyphenolic profile of the extract.

5.2 Extraction of anthocyanins

This section presents the results from the different extractions performed in this study. First, the effects of using acidified water or water as solvent in a Soxhlet extraction. Then, it the extraction was optimized, taking into account variables like temperature and time.

5.2.1 Soxhlet Extraction

First, a Soxhlet extraction was used as a standard extraction to determine the influence of acidified water and water in the anthocyanins stability. The amount of biomass used for Soxhlet extraction was 10 g and 200 ml of solvent. The main goal is to see an effect on the stability of anthocyanins depending on the solvent used.

Normalizing spectra allows for a more accurate comparison of anthocyanin degradation over cycles by minimizing variations due to differences in sample concentration or path length. This ensures that observed changes in absorbance are due to chemical transformations rather than experimental inconsistencies.

5.2.1.1 Acidified water

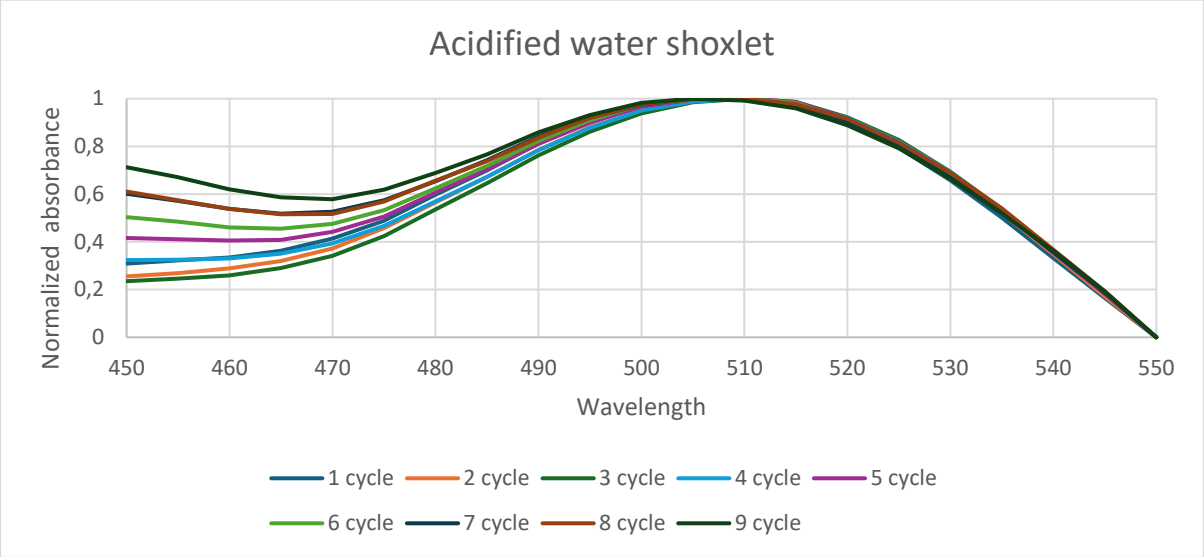


Figure 17: Normalized absorbance using acidified water as solvent in Soxhlet. The range selected to study the anthocyanin degradation was 450 and 550 nm.

As it can be seen from Figure 17, with acidified water after 9 cycles, anthocyanins do not have a clear tendency to degrade as they have the highest peak at around 510 nm after 9 cycles, meaning that it helps the stability of anthocyanins.

5.2.1.2 Water

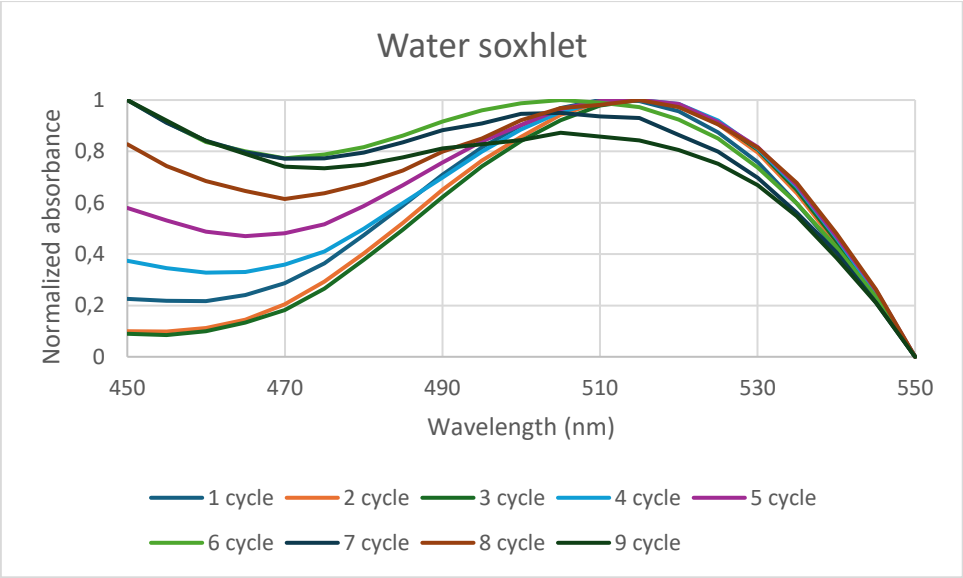


Figure 18 Normalized absorbance using water as solvent in Soxhlet. The range selected to study the anthocyanin degradation was 450 and 550 nm..

In contrast, when using water as the solvent, when normalizing the data, it can be seen clearly that anthocyanins tend to degrade with cycles, meaning that acidified water contributes more to the stability of anthocyanins.

As working with Soxhlet extraction, temperature cannot be controlled, it was decided to study batch extractions using acidified water as solvent, as it has been clear in Soxhlet results that it helps the stability of anthocyanins. In batch extraction, temperature and time can be parameters to study in order to study anthocyanin degradation.

5.2.2 Batch Extraction

In this section, the optimal temperature for anthocyanin extraction was studied. It was decided to study for 20, 40, 60, and 80ºC. These macerations lasted for 2 hours, and samples were taken every 20 minutes to determine if anthocyanins were degraded.

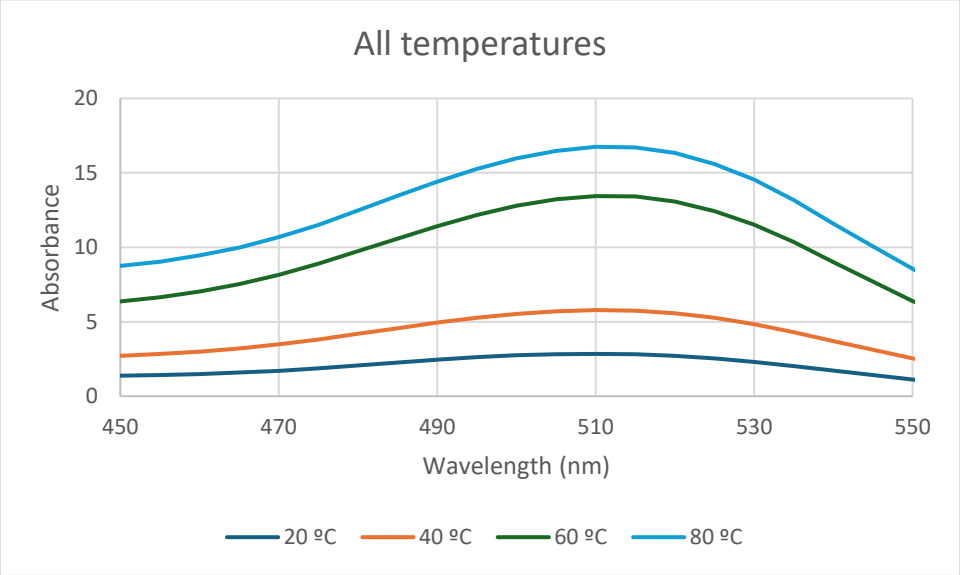


Figure 19: Absorbance of different temperatures in bath extractions.

From Figure 19, it can be seen how absorbance increases with the temperature, making a better curve in the range of anthocyanins wavelength. It is clear that temperature has a clear tendency to increase the anthocyanins yield. But to understand if there is degradation, it is important to normalize the data and see if there is a degradation. As degradation occurs at higher temperatures, it was only normalized the data for 80ºC.

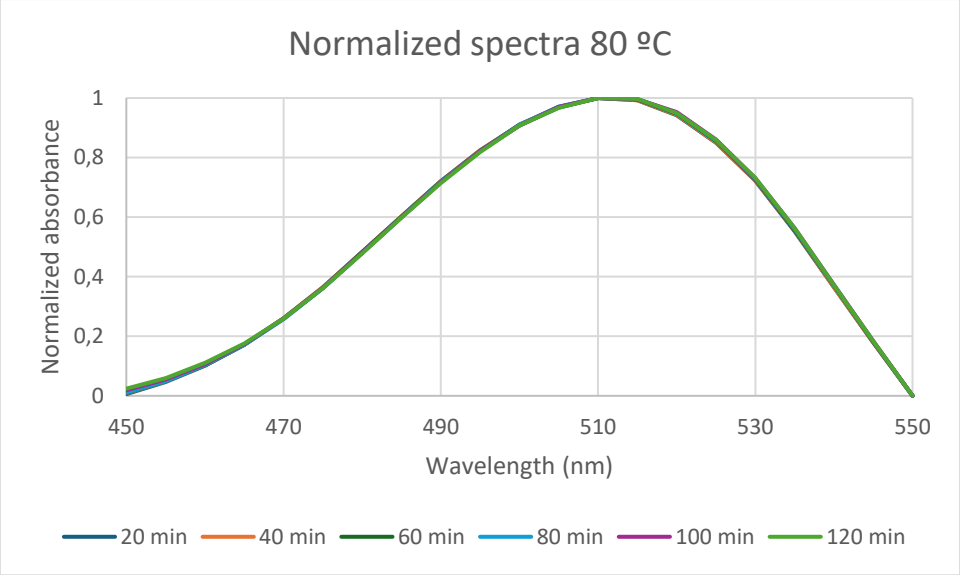


Figure 20: Normalized spectra for batch extraction at 80ºC.

Figure 20 shows that after 2 hours, there is no tendency for anthocyanins to be degraded. So, after these results, it was decided to study batch extraction for 40, 60, and 80ºC, as they show a better performance than 20ºC.

5.2.3 Maceration

Maceration was performed using 20 g of biomass in 200 ml of solvent, having a solvent-to-biomass ratio of 10 ml/g. The parameter taken into account was the time, performing the extraction at 40, 60, and 80 ºC.

The extraction was performed for 24 hours as we wanted to study the stability of anthocyanins with time and temperature. For that, samples were taken at 2, 4, 6, 8, and 24 hours. The solvent used was acidified water (pH=3), as based on the results from the Soxhlet, acidified water improved the anthocyanin stability. All experiments were done in triplicate.

The analysis of total anthocyanins has been made based on the pH differential method.

5.2.3.1 Results for anthocyanin

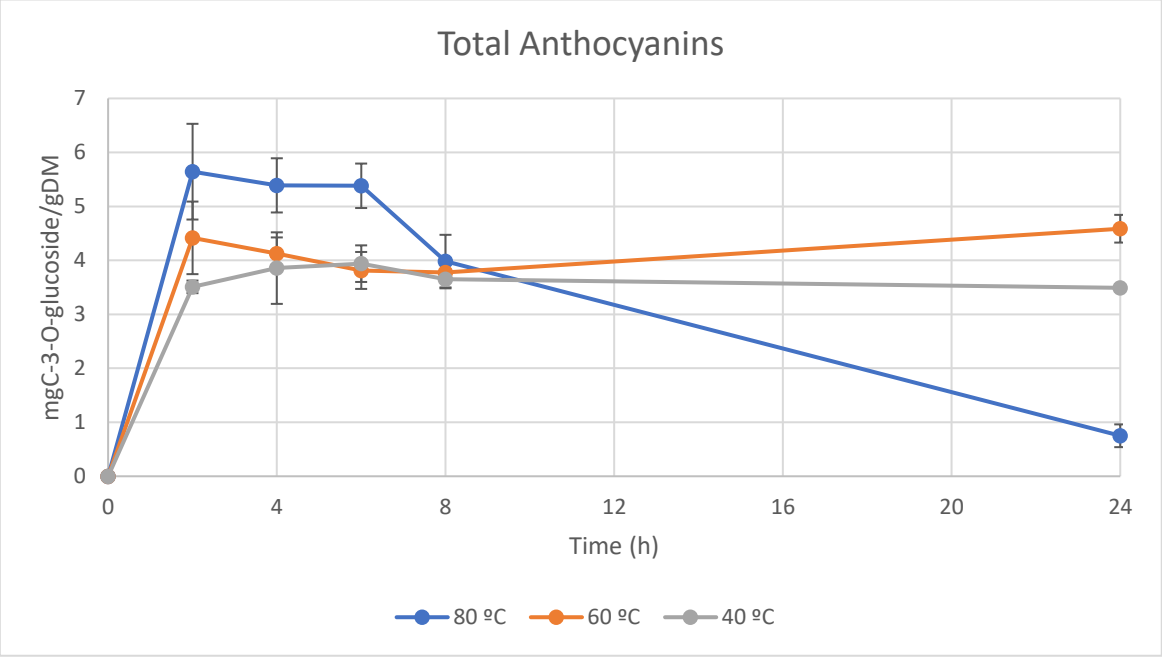


Figure 21: Total anthocyanin content in macerations at different temperatures.

This graph shows the total anthocyanin content (mg C-3-O-glucoside/g DM) over time (hours) at three different temperatures: 80°C, 60°C, and 40°C. Key observations are as follows:

1. **80°C**:
    - Peak total anthocyanin content at 80°C: 2 hours results in **5.64 mg C-3-O-glucoside/g DM**
    - Two hours later the anthocyanin concentration starts to drop drastically, suggesting major deterioration resulting from the high temperature, therefore compromising anthocyanin stability over time.
2. **60°C**:
    - Anthocyanin content stabilising around 4–5 mg C-3-O-glucoside/g DM progressively increases up to 6 hours.
3. **40°C**:
    - The total anthocyanin content increases gradually and stabilizes at around 6–7 mg C-3-O-glucoside/g DM
    - No significant degradation is observed, indicating that low temperatures preserve anthocyanins better but result in lower extraction efficiency.

Initially increasing anthocyanin concentration, higher temperatures (80°C) cause fast deterioration because of thermal instability. While low temperatures (40°C) give stability but produce reduced total anthocyanin content, moderate temperatures (60°C) balance extraction efficiency and stability.

These findings led to the decision to look at ultrasonic extraction for two hours, as anthocyanins typically break down at high temperatures over this length of time.

5.2.3.2 Result for phenols

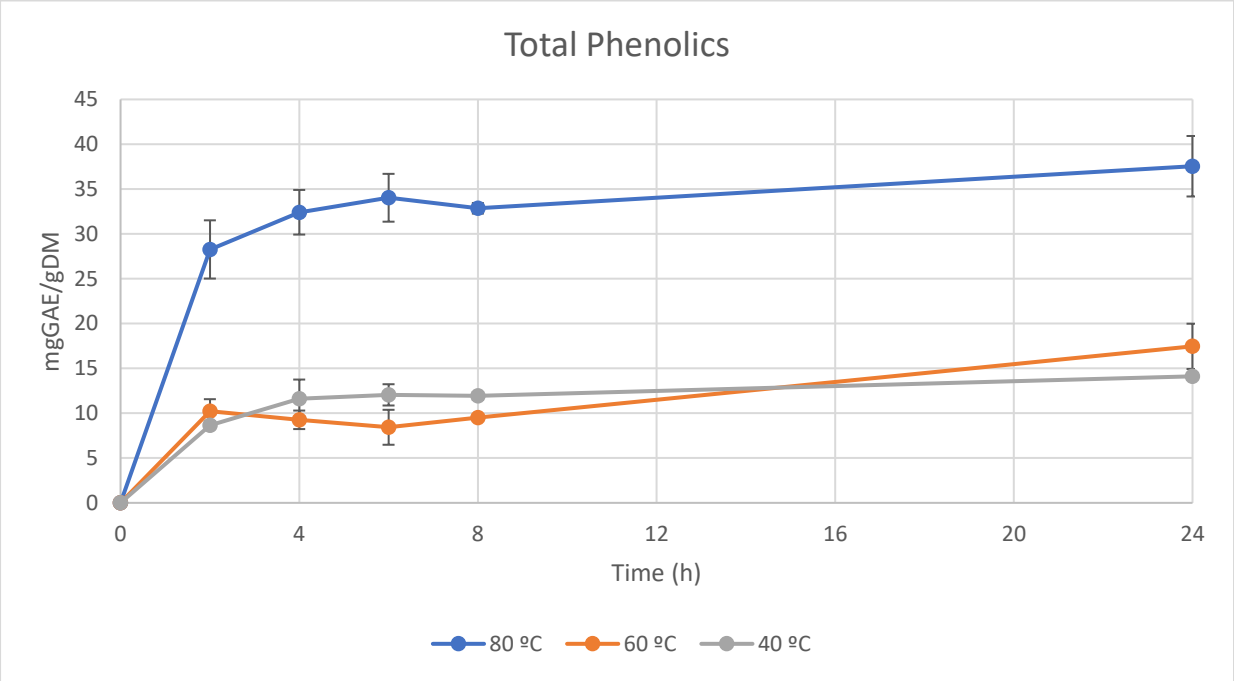


Figure 22: Total phenolics with time and temperature.

The results of total phenolic content extraction, expressed as mg GAE/g DM, are presented in Figure 22.

At all three temperatures, phenolic content rapidly increased in the first 2 hours. The highest yield was reached at 80ºC, where total phenols reached a value of around 35-40 mg GAE/g DM within the first 4-6 hours and then stabilized.

Most importantly, in contrast with anthocyanins, phenols do not degrade with high temperatures, as at 80ºC, phenols do not degrade like anthocyanins.

5.2.3.3 DPPH analysis

For the DPPH analysis, it was decided to study the samples at 80ºC, as 80ºC showed the best performance in terms of phenolic extraction and anthocyanin extraction. To understand the influence of phenols and anthocyanins, DPPH was studied for samples after 2, 6, and 24 hours.

Time(h)	mgGAE/gDM	mgC-3-O-glucoside/gDM	IC50
2	28.27	5.65	0.62
6	34.03	5.38	0.59
24	37.55	0.75	0.37

Table 18: Values for total phenols, total anthocyanins, and IC50 with time. The extract used was obtained with an 80ºC maceration.

Since lower IC50 values indicate higher antioxidant activity, this suggests that despite anthocyanin degradation, other polyphenols or degradation products contribute to maintaining or even enhancing antioxidant capacity.

5.2.3.4 HPLC results from macerations

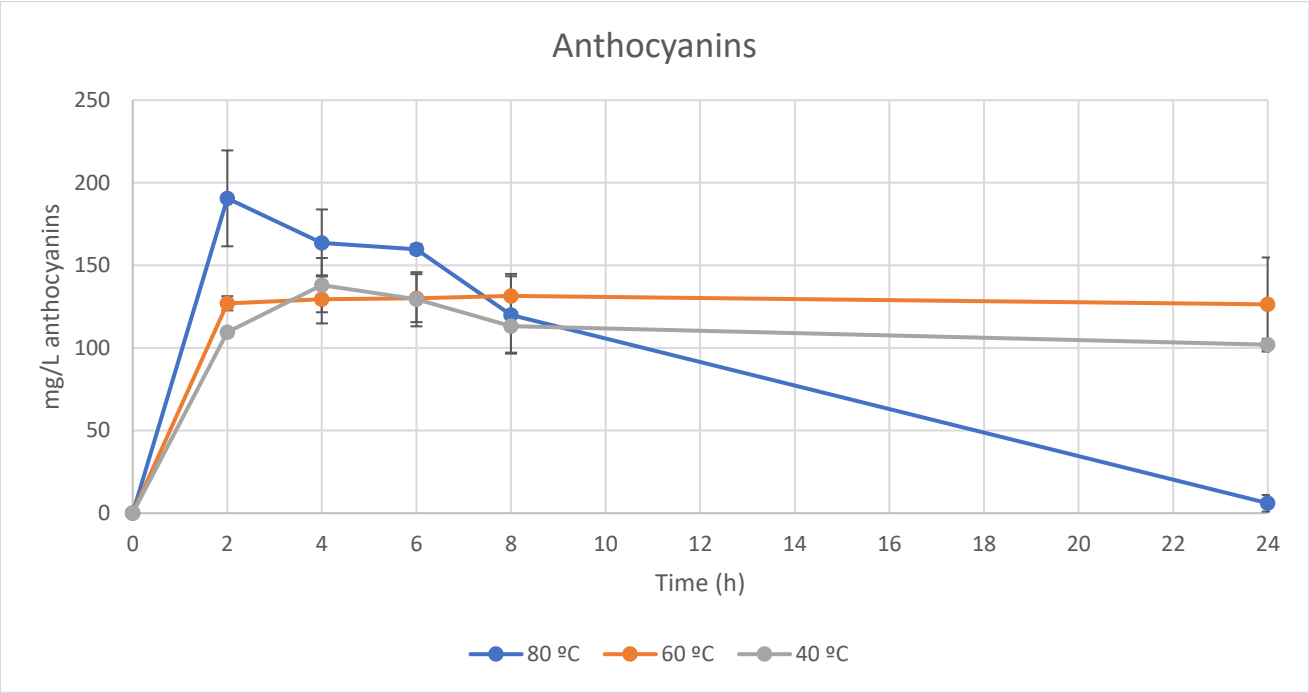


Figure 23: Concentration of anthocyanins with time. The concentration of anthocyanins is based on the sum of cyanidin 3-O-galactoside and cyanidin 3-O-glucoside.

The graph shows the same tendency as the total anthocyanins pH differential method. This result shows that although the pH differential method is not very accurate, it provides a great understanding of the extraction efficiency.

The HPLC results confirm the theory about the temperature's influence on the degradation of anthocyanins. Key observations are as follows:

- At 80 ºC, anthocyanin concentration peaks after 2 hours (**190.57 mg/L**) and then declines steadily, reaching almost zero after 24 hours, because of thermal degradation.
- At lower temperatures as 60 ºC and 40 ºC are more stable, but the extraction efficiency is lower, being the highest concentration being **138.06 mg/L.**

The HPLC results confirm the conclusion that the optimal extraction parameters for anthocyanins are **80 ºC** and **2 hours**.

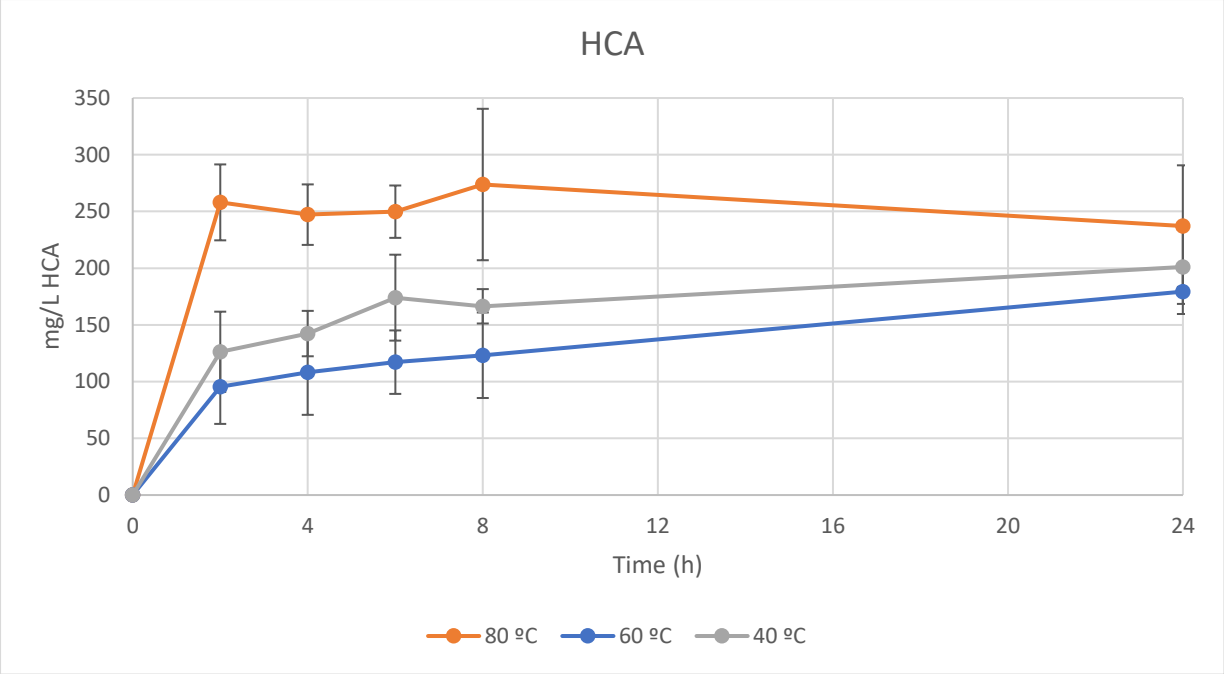


Figure 24: Concentration of HCA with time. The concentration of anthocyanins is based on the sum of chlorogenic acid and neochlorogenic acid.

5.2.4 Ultrasound extraction

The ultrasound extraction was performed using 20 g of biomass in 200 ml of solvent, having a solvent-to-biomass ratio of 10 ml/g. The parameters taken into account were:

- % Amplitude: 100, 75, and 50.
- Solvent: water and acidified water (pH=3).

It is important to take into consideration that the temperature was not kept constant, but it has been studied how temperature changes with time for the different ultrasound extractions, as we have seen that this is a key parameter.

The extraction was performed for 2 hours. Samples were taken every 20 minutes to see the total anthocyanin content and temperature.

The analysis of total anthocyanins has been made based on the pH differential method. Results will be presented first, the ones from water as a solvent, and then acidified water.

5.2.4.1 Water

Figure 25 presents the total anthocyanin content extracted using water as the solvent. The highest concentration was achieved with a 50 % amplitude after two hours, reaching **8.53 mg C-3-O-glucoside/g DM**. This was followed by extraction at 75 %, yielding **5.90 mg C-3-O-glucoside/g DM**, and at 100 %, yielding **4.86 mg C-3-O-glucoside/g DM.**

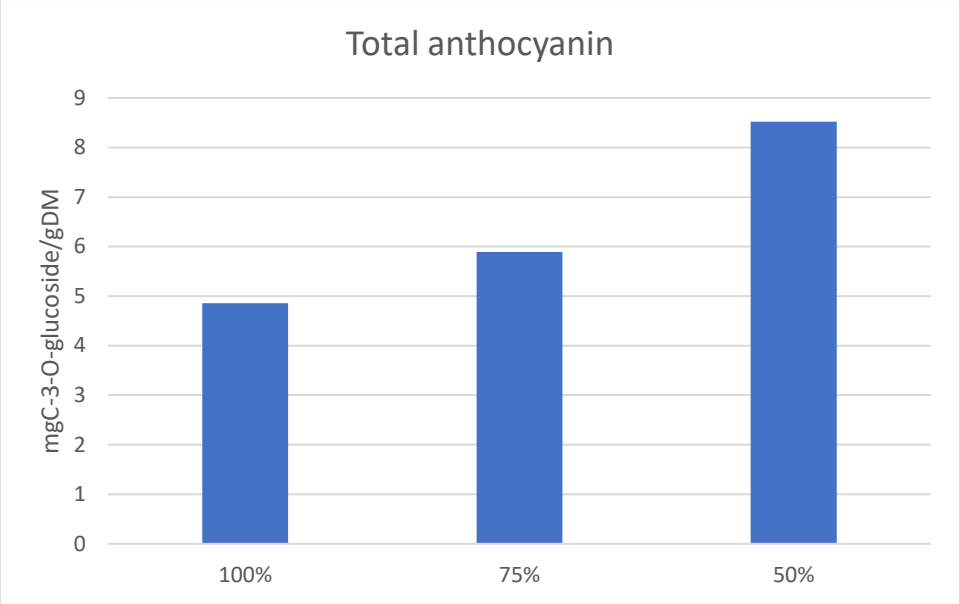


Figure 25: Total anthocyanin after 2 hours in ultrasound extraction using water as solvent at different amplitudes

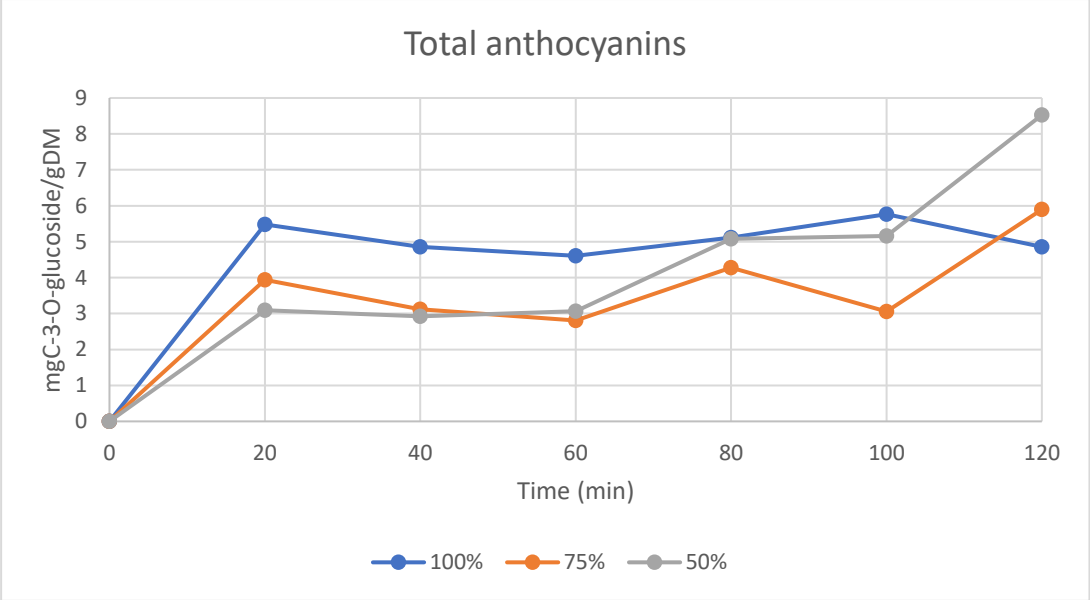


Figure 26: Total anthocyanins using water as solvent at different amplitudes with time.

Table 19 explains why the total anthocyanin content decreases as the amplitude increases. Higher amplitudes result in higher temperatures, which compromise the stability of anthocyanins. When the temperature exceeds 80 ºC—identified as the critical temperature for anthocyanin stability during maceration—the degradation of anthocyanins becomes significant.

	100 %	75 %	50 %
time(min)	Temperature (ºC)	Temperature (ºC)	Temperature (ºC)
0	20.0	20.0	20.0
20	84.9	75.6	63.9
40	88.7	**84.6**	77.7
60	**89.3**	81.3	78.9
80	75.5	68.4	**82.8**
100	63.4	68.3	71.6
120	53.0	71.2	65.4

Table 19:Temperature using water as solvent at different amplitudes.

5.2.4.2 Acidified water

Figure 27 presents the total anthocyanin content extracted using acidified water (pH = 3) as the solvent. The highest concentration was achieved with a 50 % amplitude after two hours, reaching **9.50 mg C-3-O-glucoside/g DM**. This was followed by extraction at 75 A, yielding **6.67 mg C-3-O-glucoside/g DM**, and at 100 A, yielding **5.90 mg C-3-O-glucoside/g DM**

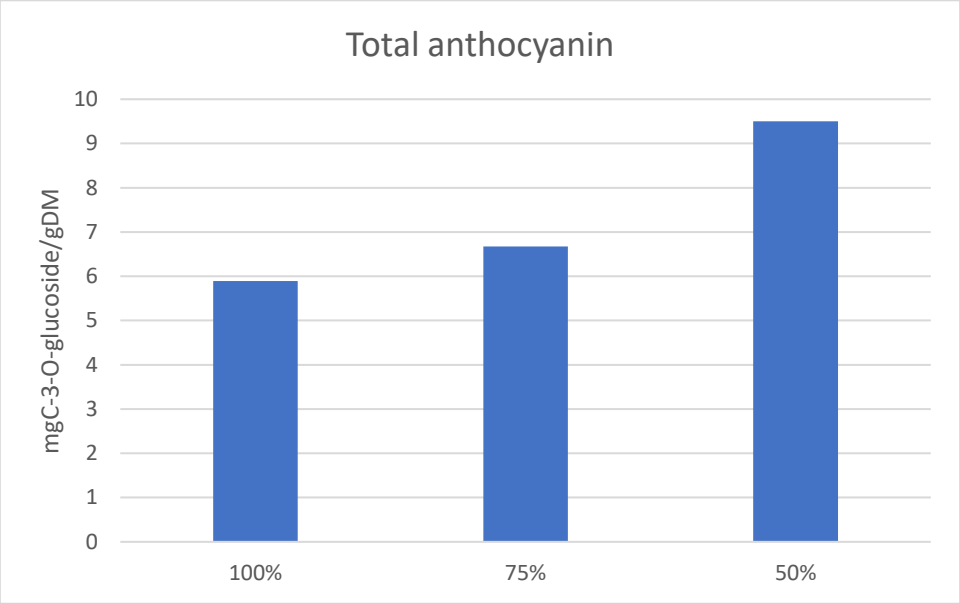


Figure 27: Total anthocyanin after 2 hours in ultrasound extraction using acidified water as solvent at different amplitudes

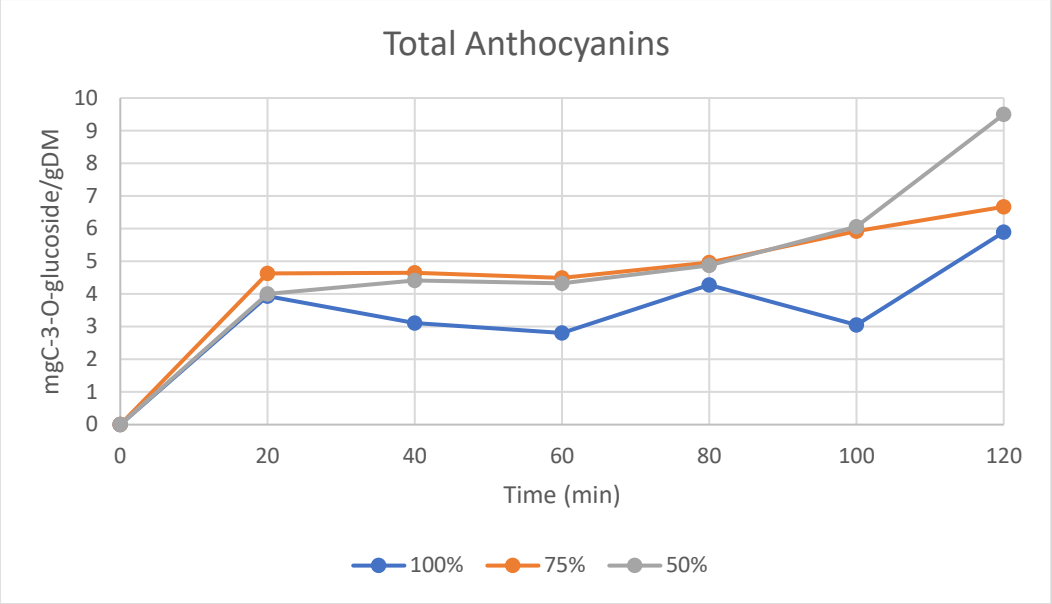


Figure 28: Total anthocyanins using acidified water as solvent at different amplitudes.

The explanation provided for water as a solvent also applies when acidified water (pH = 3) is used as the solvent. Higher amplitudes lead to increased temperatures. which negatively affects the stability of anthocyanins. When the temperature surpasses 80ºC—recognized as the critical temperature for anthocyanin stability during maceration—significant degradation occurs, resulting in lower total anthocyanin content.

	100 %	75 %	50 %
Time(min)	Temperature (ºC)	Temperature (ºC)	Temperature (ºC)
0	20	20	20
20	84.9	73.6	61.1
40	88.7	**83.1**	**77.1**
60	**89.3**	80.6	75.9
80	75.5	81.3	75.7
100	63.4	75.1	59.2
120	53	69.4	64.7

Table 20: Temperature using acidified water as solvent at different amplitudes.

It is important to highlight that the highest concentration of anthocyanins obtained through maceration was **5.64 mg C-3-O-glucoside/g DM** using acidified water as the solvent. In comparison, ultrasound extraction demonstrated better performance, achieving **8.53 mg C-3-O-glucoside/g DM** with water as the solvent with 50 % amplitude and **9.5 mg C-3-O-glucoside/g DM** with acidified water as the solvent with 50 % amplitude

5.2.4.3 HPLC results

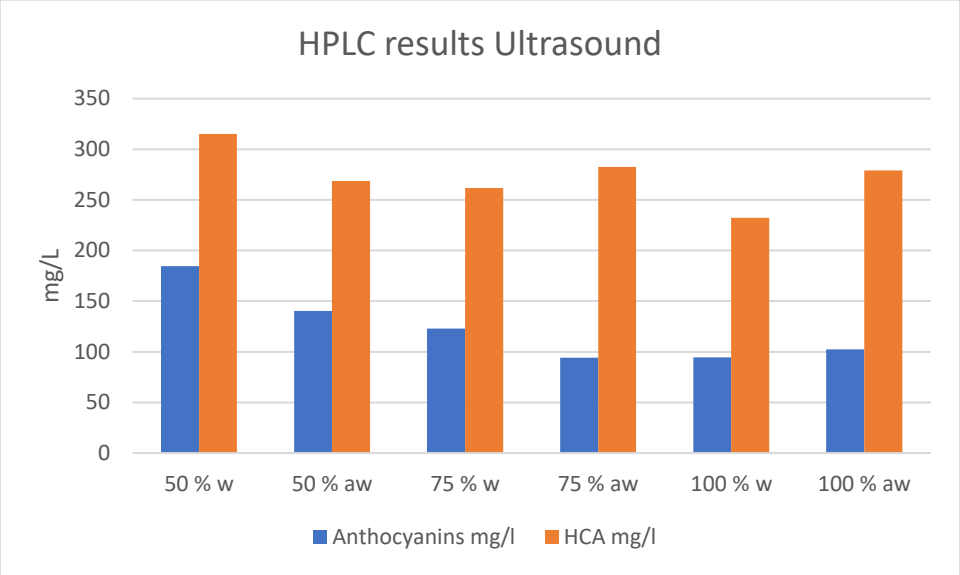


Figure 29: HPLC results from ultrasound extraction based on anthocyanin and HCA. w means water as the solvent, while aw means acidified water.

As expected from the total anthocyanins pH differential method, anthocyanins tend to degrade when increasing the amplitude, which is related to the higher temperatures at higher amplitudes. Highest anthocyanin extraction is at 50 % amplitude and using water as solvent (around **180-190 mg/L**)

On the other hand, HCA extraction is less sensitive to the amplitude, remaining fairly constant. Highest HCA yield at 50 % amplitude using water as the solvent **(~310 mg/L)**.

5.2.5 Comparative analysis of extraction methods

Developing and optimizing the extraction of polyphenols from *Aronia melanocarpa* presents a multifaceted challenge. This thesis integrates several tasks, including the evaluation of key extraction parameters such as temperature and time. Each stage requires careful optimization to ensure high yield, compound stability, and selective recovery of anthocyanins. The complexity lies not only in designing and executing these processes but also in systematically assessing their outcomes using analytical techniques such as HPLC-DAD and colorimetric assays.

EXTRACTION METHOD	CONDITION	TOTAL ANTHOCYANIN CONTENT	REFERENCE
MACERATION	80 ºC after 2 hours using acidified water as the solvent	5.64 mg C-3-O-glucoside/g DM	This study
MACERATION	60 ºC after 24 hours using acidified water as the solvent	4.42 mg C-3-O-glucoside/g DM	This study
ULTRASOUND	100 % Amplitude after 2 hours using acidified water as the solvent	9.50 mg C-3-O-glucoside/g DM	This study
ULTRASOUND	100 % Amplitude after 2 hours using water as the solvent	8.53 mg C-3-O-glucoside/g DM	This study
SOLVENT EXTRACTION	Solvent: Ethanol Tª: 46ºC Time: 252 min L/S Ratio (g/mL): 1:23.8	6.12 mg/g DM	[93]
ULTRASOUND EXTRACTION	Solvent: 1% HCOOH in 50% EtOH Amplitude (%): 80 Sonication time (min): 10	5.67 mg/g DM	[181]

Table 21: Comparison of results for total anthocyanins extraction

Depending on the conditions used, the total anthocyanin content (TAC) that was produced using various extraction techniques varied considerably. While solvent extraction using ethanol and formic acid-enhanced UAE was another study's findings, both maceration and ultrasound-assisted extraction (UAE) were carried out in this investigation.

The maximum anthocyanin concentration (9.50 mg C-3-O-glucoside/g DM) was obtained from ultrasound-assisted extraction using acidified water for two hours at 100% amplitude out of all investigated conditions. The outcome emphasizes the UAE's higher efficiency, which is probably caused by cavitation's mechanical impacts, which intensify cell disruption and encourage mass transfer. The anthocyanin yield dropped to 8.53 mg C-3-O-glucoside/g DM when the same ultrasonic settings were used with non-acidified water, demonstrating the beneficial effect of acidity in stabilizing anthocyanins and enhancing extraction efficiency. Based on the tendency of the graphs, it would be interesting to study the extraction yield for longer times, as it seems to be increasing the total anthocyanin content.

A moderate TAC of 5.64 mg C-3-O-glucoside/g DM was obtained after two hours of maceration at 80°C with acidified water. On the other hand, a longer maceration period of 24 hours at 60°C produced a lower yield of 4.42 mg C-3-O-glucoside/g DM.

These findings are also contextualized by the findings of other studies. 6.12 mg/g was obtained by solvent extraction with ethanol at 46°C for 252 minutes, whereas 5.67 mg/g was obtained by ultrasound-assisted extraction with a solution of 1% formic acid in 50% ethanol at 75% amplitude for just 10 minutes. These techniques did provide detectable anthocyanin content, but they were still less effective than the ultrasound extraction using acidified water used in this investigation. Interestingly, compared to the 2-hour sonication in this investigation, the external UAE procedure's short sonication time (10 minutes) might account for the decreased efficiency.

Maceration at 80°C for two hours was chosen as the optimal method to produce the extract used in the resin adsorption studies, even though ultrasound-assisted extraction (UAE) in this study produced a higher anthocyanin yield. Practical factors during processing led to this decision: the UAE extract was more viscous (Figure 30), most likely as a result of pectins and other polysaccharides being released from the plant matrix under sonication. The subsequent adsorption process on the resin may have been affected by the increased viscosity, which could present handling and filtration difficulties. Maceration, on the other hand, yielded a more controllable extract with a sufficient anthocyanin yield.

Figure 30: Ultrasound extract

5.3 Resin Isolation XAD-7 using 10 BV

After optimizing the extractions, it is now time to study the isolation of anthocyanins using the resin XAD-7. Section 3.4 presents the conditions of the resin adsorption used in this study.

5.3.1 Adsorption

Step 2 is the adsorption. To determine the breakpoint, samples were taken every 15 minutes and analyzed for total anthocyanin content. A key aspect to understand Figure 12 is knowing

both the initial anthocyanin concentration in the extract and the concentration of anthocyanins in the solution over time.

- The initial concentration of **5.19 mg C-3-O-glucoside/g D** represents the maximum anthocyanin content available for adsorption.
- The concentration in the outgoing solution allows us to track how much anthocyanin remains unadsorbed at each time point.

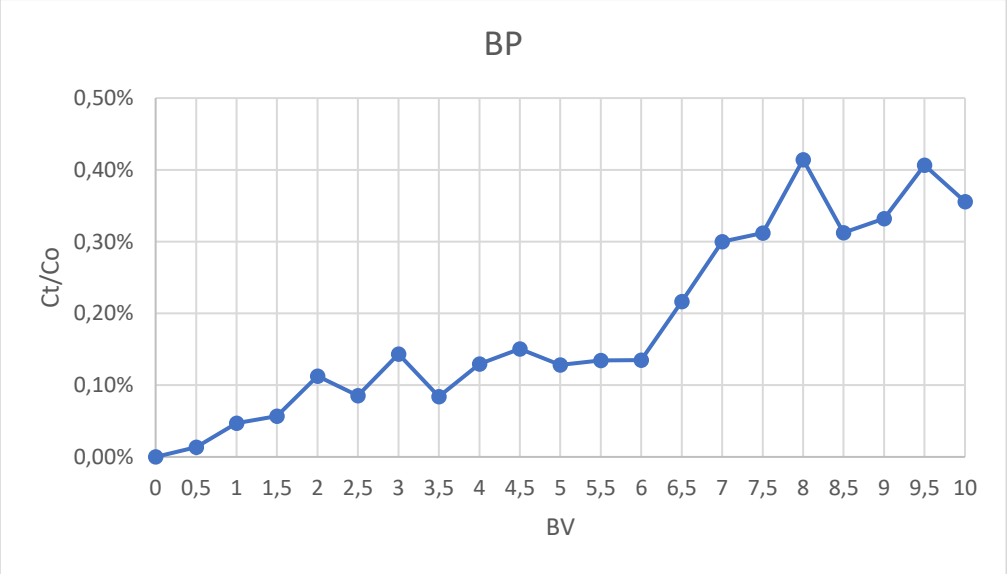


Figure 31: Breakpoint curve with respect to the bed volumes. Being Co the initial concentration and Ct the concentration of anthocyanins going out the resin.

As Figure 31 does not reach the 5 % after 10 BV that represents the break point, an estimation was made based on the results of Figure 31. This estimation has been made in Figure 32.

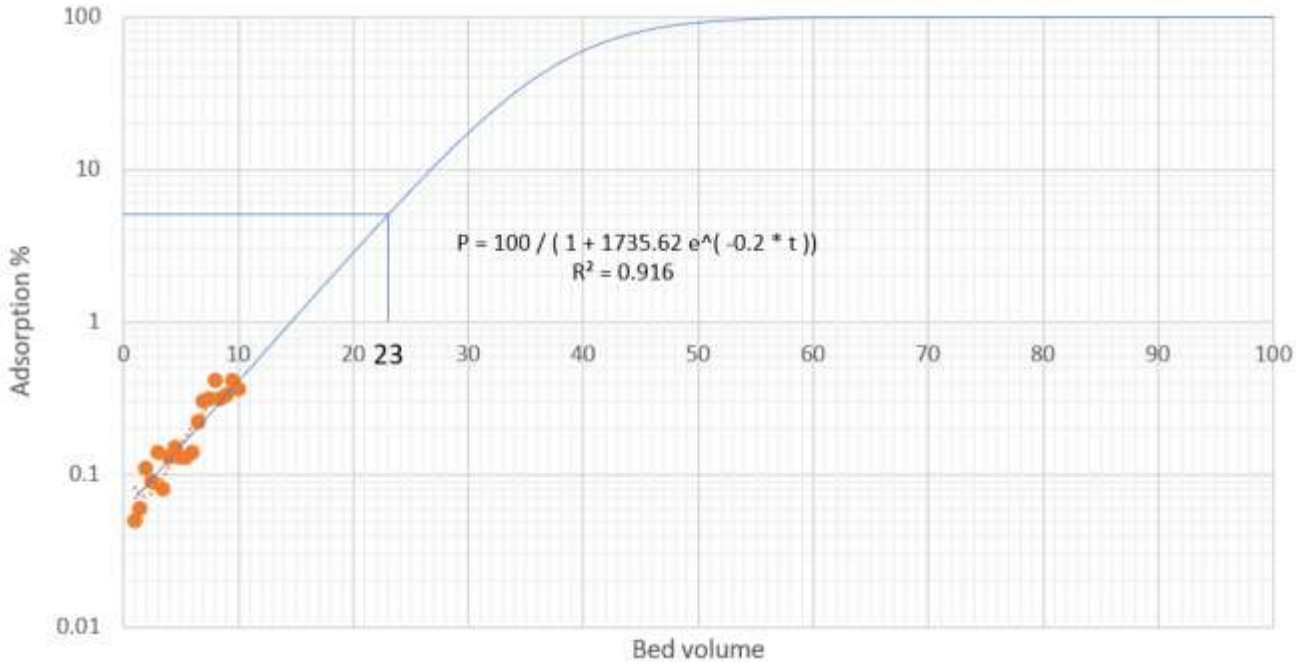


Figure 32: Estimation of bed volumes needed to reach break point.

Based on the estimation, the bed volumes needed to reach the breakpoint for anthocyanins are **23 bed volumes**.

This estimation may not be entirely accurate because, when determining the breakpoint, the total anthocyanin content was analyzed using samples that were only diluted by a factor of two. This limited dilution may not have allowed the buffer to fully interact with the sample, potentially affecting the accuracy of the calculation. As a result, the estimated breakpoint might not truly reflect the system's actual behavior, since the buffer may not have exerted its complete influence on the anthocyanin concentration.

5.3.2 Desorption

Steps 4 and 5 are the ones responsible for the desorption. To obtain non-anthocyanins phenols and anthocyanins separated the first eluent was ethyl acetate and then ethanol to take the anthocyanins. Samples were taken every 15 ml to study the desorption curve.

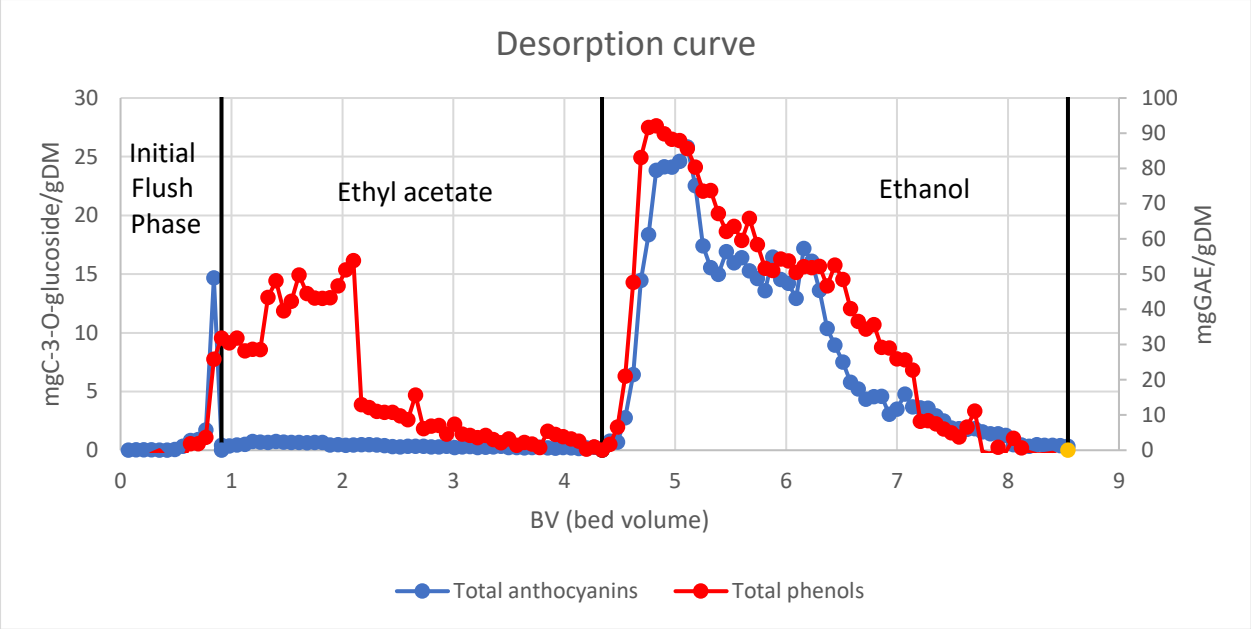


Figure 33: Desorption curve. The red line represents the total phenols and the blue one the total anthocyanins content.

Figure 33 shows the total anthocyanin and total phenol content with respect to the bed volumes. The first phase is the initial phase, where there is still acidified water when the ethyl acetate starts. When all the water is out, it starts the ethyl acetate phase, where it can be seen that it does not extract the anthocyanins from the resin but extracts the phenols, being the highest amount of phenols recovered after **2.1 BV** and a concentration of around **52 mgGAE/gDM**. After the ethyl acetate finishes, the ethanol starts, where it is clear how it increases the total anthocyanin content at the beginning, reaching a peak after **5.11 BV** and a concentration of around **26 mgC-3-O-glucoside/gDM**. After the peak, the concentration goes down as most of the anthocyanins are desorbed. It is also interesting to see how the total phenols analysis shows high concentrations that can be related to the anthocyanins content, as both curves tend to follow the same tendency, but this can only be affirmed by an HPLC analysis.

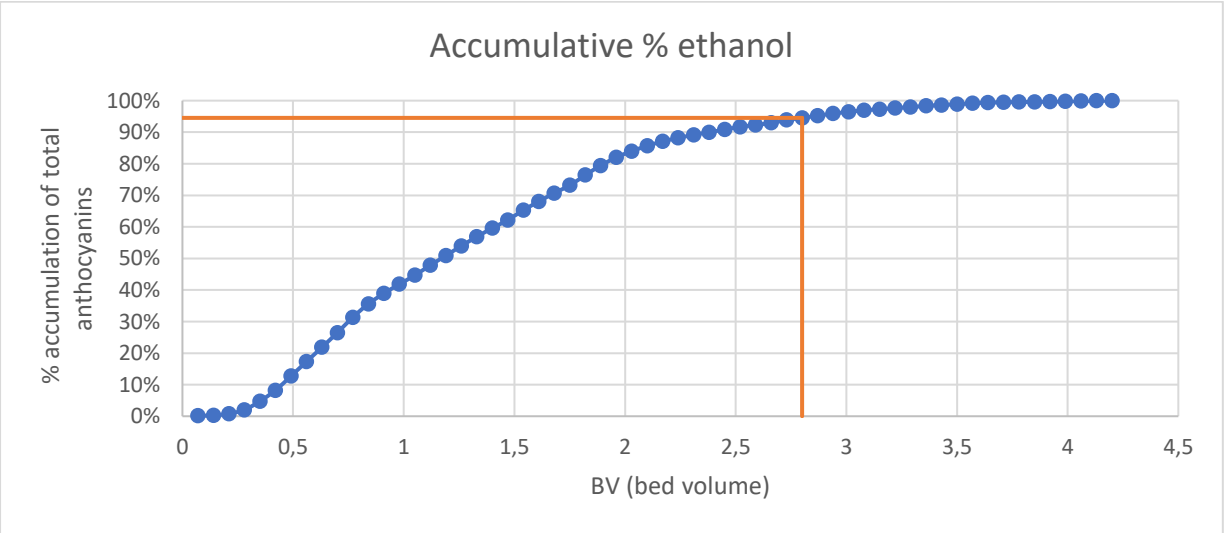


Figure 34: Accumulation of total anthocyanins with respect to the bed volume.

Figure 34 shows the accumulative % of anthocyanins desorbed with respect to the bed volumes in the ethanol phase. This shows the amount of bed volume needed to desorb 95 % of the anthocyanins that it is **2.8 BV.**

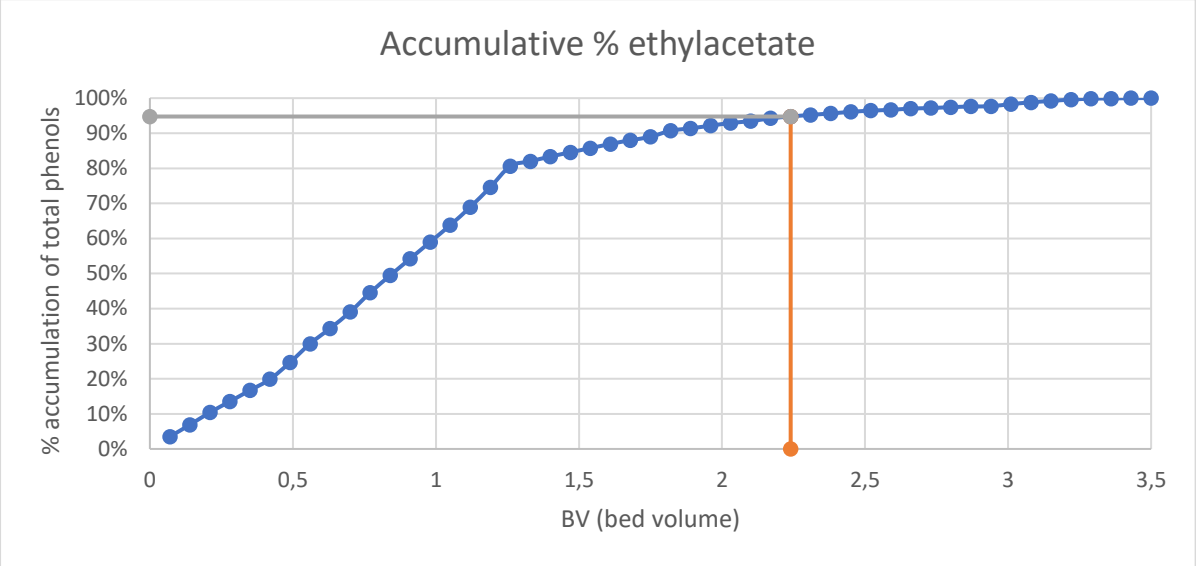


Figure 35: Accumulation of total phenols with respect to the bed volume.

Figure 35 shows the accumulative % of phenols desorbed with respect to the bed volumes in the ethanol phase. This shows the amount of bed volume needed to desorb 95 % of the phenols, which is **2.24 BV.**

5.3.3 Results from Celabor

After analyzing the results for the desorption curve, the samples have been rotovaporated and then freeze-dried all together in order to determine which phenols we have in the desorbed extract. The freeze-dried solid was sent to Celabor. The results from the laboratory of Celabor are:

Compounds	mg/g	%
Neochlorogenic Acid	16.31	1.631
Chlorogenic Acid	17.642	1.764
Epicatechin	0.773	0.077
Kaempferol		
Rhamnetin		
Isorhamnetin	LQ	LQ
Taxifolin	0.201	0.02
Hesperidin		
Quercetin-7-glucoside	LQ	LQ
Hyperoside	7.189	0.719
Quercetin	0.547	0.055
Caffeic Acid	0.403	0.04
Ferulic Acid	0.08	0.008
Vanillic Acid	0.15	0.015
Gallic Acid	0.132	0.013
Catechin	0.091	0.009
Procyanidin B1	0.188	0.019
Procyanidin B2	3.082	0.308
Protocatechuic Acid	11.525	1.152
Rutin	3.391	0.339
Kaempferol-3-rutinoside	0.282	0.028

Table 22: Results from Celabor

Based on the results, it can be seen in yellow the main compounds contained in the extract. As in our laboratory we have chlorogenic and neochlorogenic acid, the HPLC results are going to be based in:

- Neochlorogenic
- Chlorogenic
- Cyanidin 3-O-glucoside
- Cyanidin 3-O-glucoside

These four compounds will help us to understand the behaviour of anthocyanins using galactoside and glucoside as the reference, and using neochlorogenic and chlorogenic acid as the reference for hydroxycinnamic acids.

5.4 Resin Isolation XAD-7 using 31 BV

After optimizing the extractions, it is now time to study the isolation of anthocyanins using the resin XAD-7. Section 3.4 presents the conditions of the resin adsorption used in this study.

5.4.1 Adsorption

Step 2 is the adsorption. To determine the breakpoint, samples were taken every 15 minutes and analyzed for total anthocyanin content. A key aspect of understanding Figure 12 is knowing

both the initial anthocyanin concentration in the extract and the concentration of anthocyanins in the solution over time.

- The initial concentration (**5.17 mg C-3-O-glucoside/g DM**) represents the maximum anthocyanin content available for adsorption.
- The concentration in the outgoing solution allows us to track how much anthocyanin remains unadsorbed at each time point.

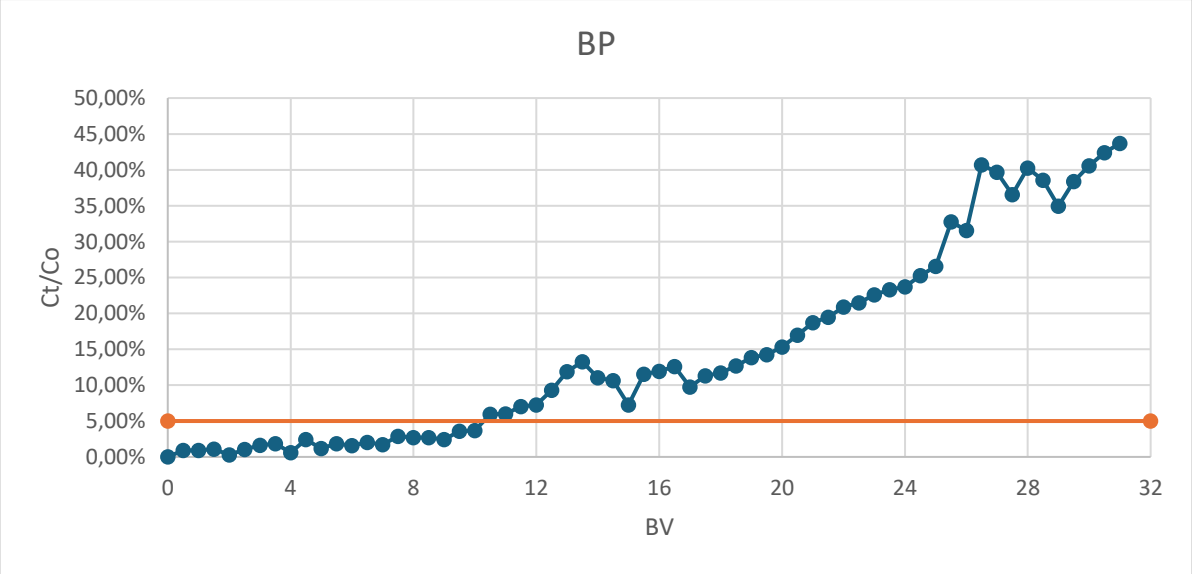


Figure 36:Breakpoint curve with respect to the bed volumes. Being Co the initial concentration and Ct the concentration of anthocyanins going out of the resin.

From Figure 36, some conclusions can be made:

- **Initial phase (0-6BV):**

  The ratio Ct/Co stays really low and almost 0% at the start of the adsorption process. This suggests that the resin is efficiently adsorbing anthocyanins, so preventing their presence in the effluent. The most accessible adsorption sites on the resin enable effective anthocyanin retention.

- **Gradual Increase (6–18 BV):**

  The resin's accessible adsorption sites begin to occupy themselves as additional solution runs through the column. This causes the Ct/Co ratio to gradually rise, so some anthocyanins are lost and start to show up in the effluent. The process of breakthroughs starts here.

- **Breakthrough Point (BP) Identification (~5% Ct/Co):**

  The horizontal orange line represents the commonly used threshold for the breakthrough point, which is often set at 5% of Ct/Co. At this point, the resin is no longer capable of efficiently adsorbing all anthocyanins, indicating that the column is

approaching saturation. From the graph, this occurs around **10.5 BV**, meaning that after 10 bed volumes, the resin starts allowing anthocyanins to pass through at a noticeable rate.

- **Saturation Phase (18+ BV):**

  Beyond this point, the adsorption capacity of the resin continues to decline as more sites become saturated. The Ct/Co ratio increases progressively, reaching **45% at around 30 BV**. This means that a significant portion of the anthocyanins is no longer being retained and is instead passing through the column.

5.4.2 Desorption

Steps 4 and 5 are the ones responsible for the desorption. To obtain non-anthocyanins phenols and anthocyanins separated the first eluent was ethyl acetate and then ethanol to take the anthocyanins. Samples were taken every 15 mL to study the desorption curve.

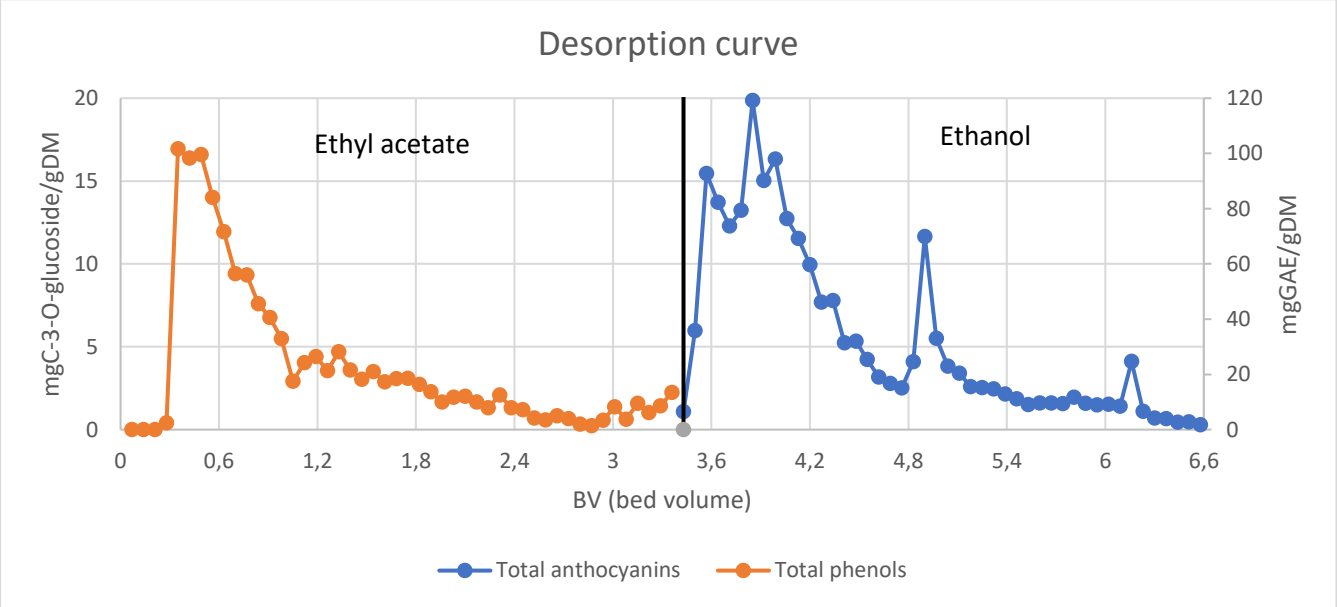


Figure 37: Desorption curve. The red line represents the total phenols and the blue one the total anthocyanins content.

Figure 37 shows the total anthocyanin and total phenol content with respect to the bed volumes. It starts the ethyl acetate phase, where it can be seen that it does not extract the anthocyanins from the resin but extracts the phenols, being the highest amount of phenols recovered after around 0.5 BV and a concentration of around 100 mgGAE/gDM. After the ethyl acetate finishes, the ethanol starts, where it is clear how it increases the total anthocyanin content at the beginning, reaching a peak after around 3.9 BV and a concentration of around 16 mgC-3-O-glucoside/gDM. After the peak, the concentration goes down as most of the anthocyanins are desorbed.

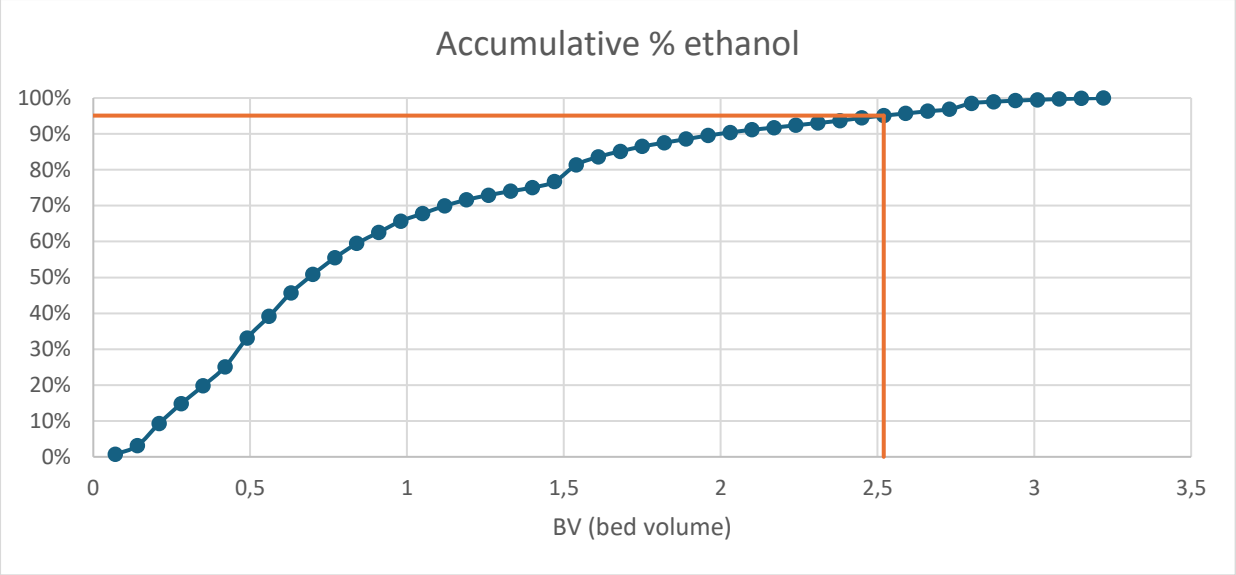


Figure 38: Accumulation of total anthocyanins with respect to the bed volume.

Figure 38 shows the accumulative % of anthocyanins desorbed with respect to the bed volumes in the ethanol phase. This shows that the amount of bed volume needed to desorb 95 % of the anthocyanins that it is **2.52 BV.**

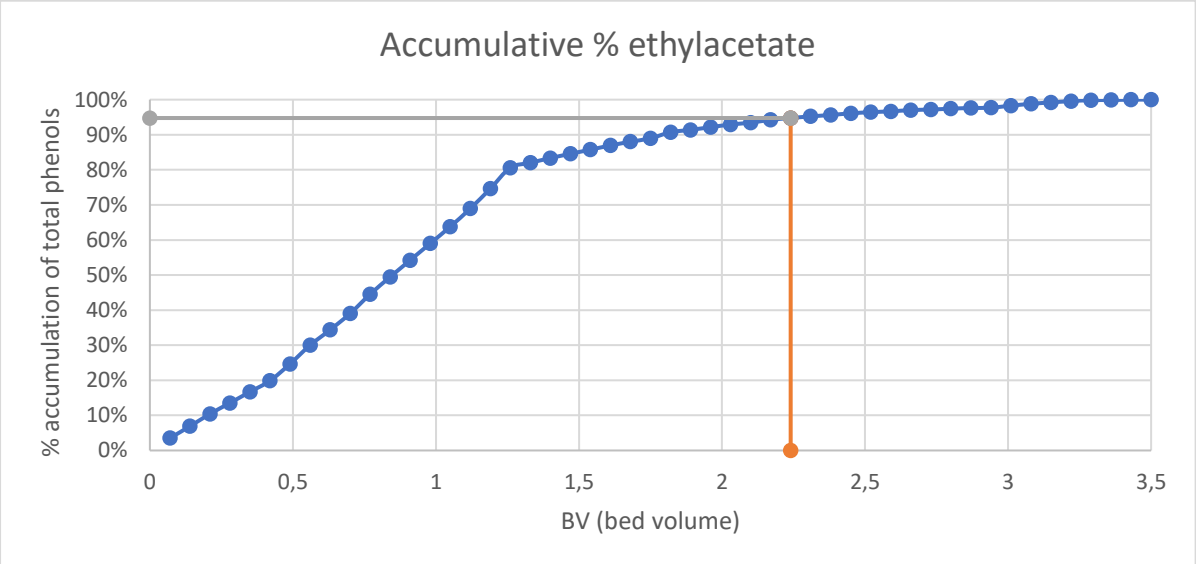


Figure 39: Accumulation of total phenols with respect to the bed volume.

Figure 39 shows the accumulative % of phenols desorbed with respect to the bed volumes in the ethanol phase. This shows the amount of bed volume needed to desorb 95 % of the phenols, which is **2.24 BV.**

5.4.3 HPLC results

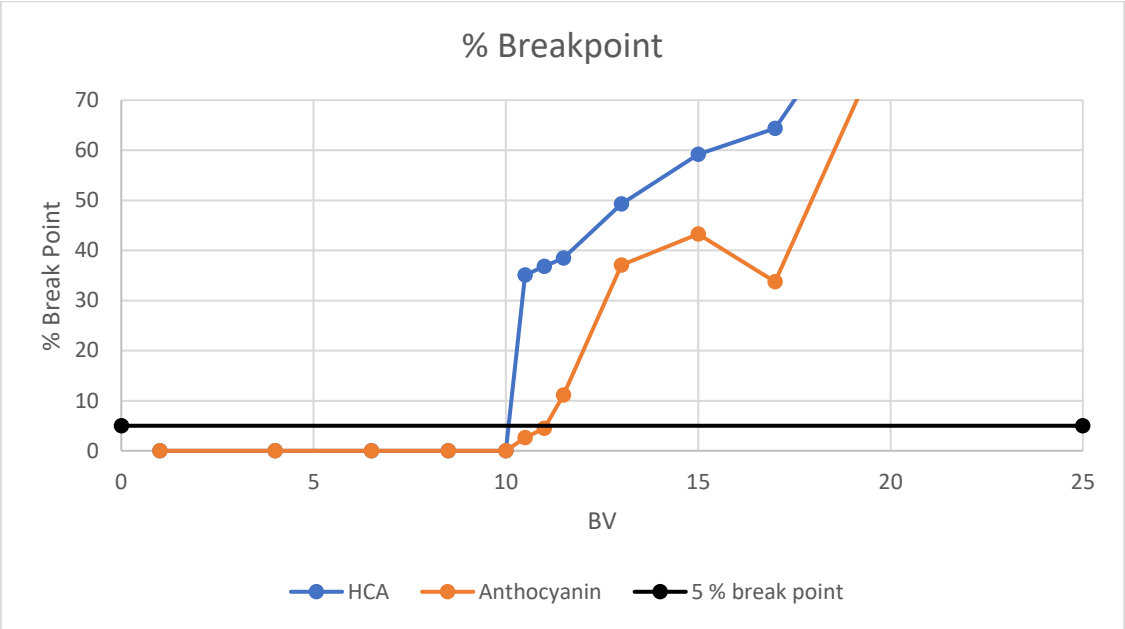


Figure 40: Breakpoint curve for HCA and anthocyanins.

Both HCA and anthocyanins remain below 5 % until around 10 BV. HCA adsorbs less strongly or saturates the resin earlier than anthocyanins. Anthocyanins have a higher affinity for the resin, delaying their breakthrough. The 5 % breakpoint is crossed by:

- HCA around 10 BV
- Anthocyanins around 10.5-11 BV

The optimal loading volume before significant loss would be < **10 BV**.

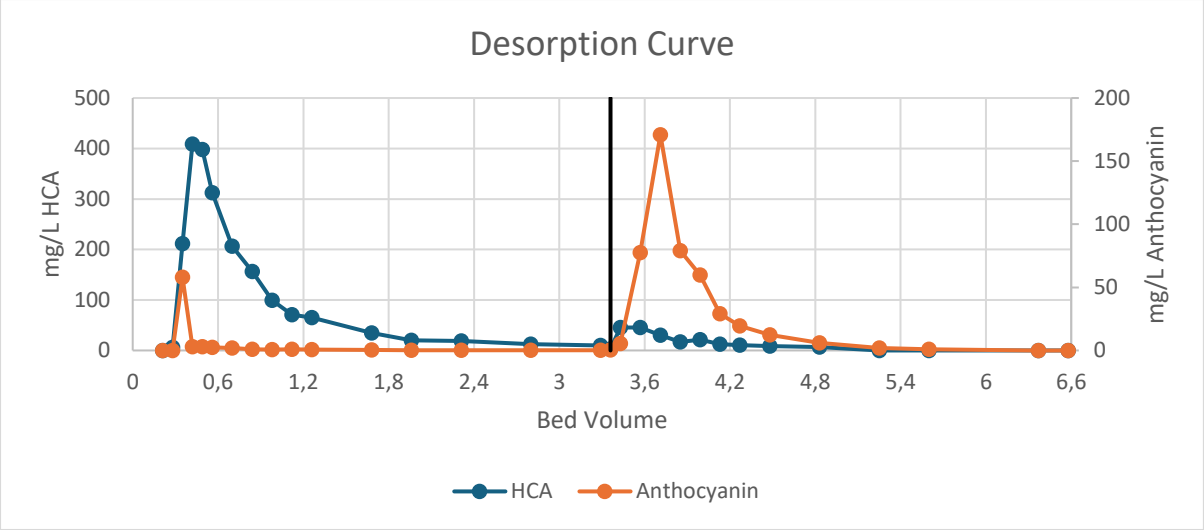


Figure 41: Desorption curve of HCA and anthocyanins. HCA are the result of the sum of chlorogenic acid and neochlorogenic acid. And anthocyanins are the sum of cyanidin 3-O-galactoside and cyanidin 3-O-glucoside.

HPLC results confirm how two-step elution with various solvents can selectively recover anthocyanins and hydroxycinnamic acids (HCA) from a resin column. Since the HCAs are comparatively less polar molecules, ethyl acetate was first used to desorb them. Ethyl acetate broke successfully their weaker connections with the resin and enabled their early elution, as evidenced by the graph's quick peak in HCA concentrations. A second elution was performed using 70 % ethanol, a more polar solvent, to target the desorption of anthocyanins after HCAs were removed. These polar pigments demonstrated a pronounced concentration peak at around 3.6 BV, indicating their higher polarity and stronger affinity for the resin. This two-solvent method effectively separated the compounds according to polarity and interaction intensity, as seen by the distinct separation between the two elution peaks and their low overlap. Phenolic acids and anthocyanins can be recovered effectively and sequentially using this method, which is very beneficial for purification and analysis later on.

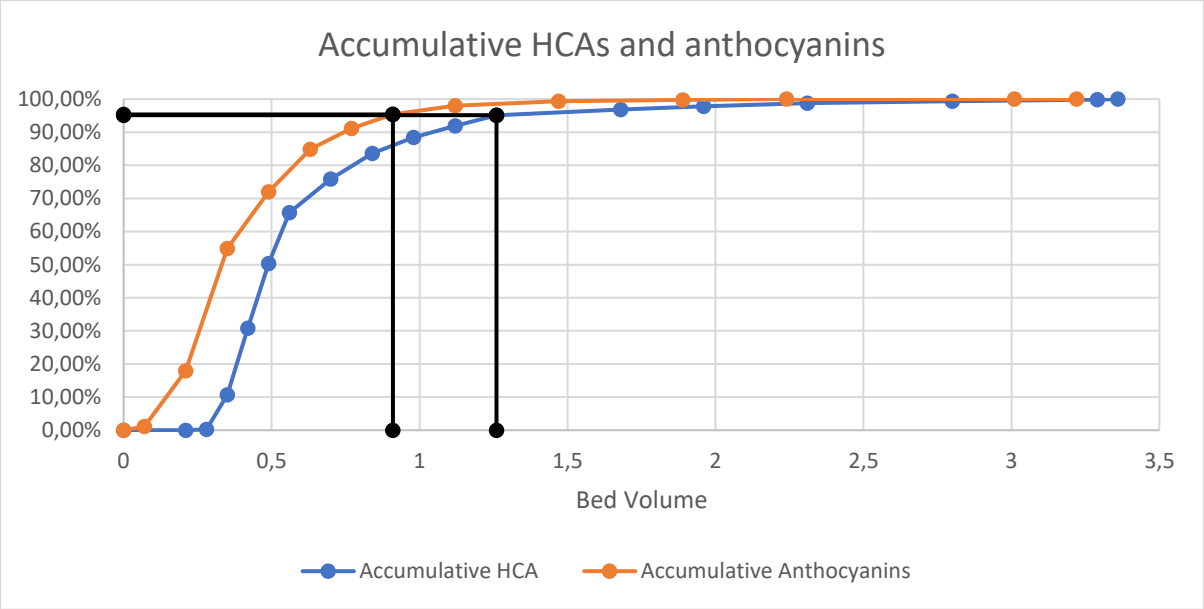


Figure 42: Accumulation of HCAs and anthocyanins with respect to the bed volumes.

The cumulative desorption profiles of anthocyanins and hydroxycinnamic acids (HCAs) are shown in this graph, which shows the efficiency of each constituent group's recovery from the resin column as a function of bed volume. Around 95 % of the total anthocyanins were eluted after 0.91 BV. HCAs, on the other hand, reach 95 % desorption at 1.26 BV. Ethyl acetate, a less polar solvent, efficiently desorbs the less polar HCAs in the early fractions, while 70 % ethanol, a more polar solvent, effectively recovers anthocyanins in the later fractions. This difference demonstrates the different interaction strengths of the compounds with the resin and is consistent with the selective elution strategy employed. The effectiveness of the desorption process is shown by the nearly 100 % recovery of both compound classes. This selective desorption method works effectively for applications that need phenolic acids and separated anthocyanins later.

5.5 Mass Balance

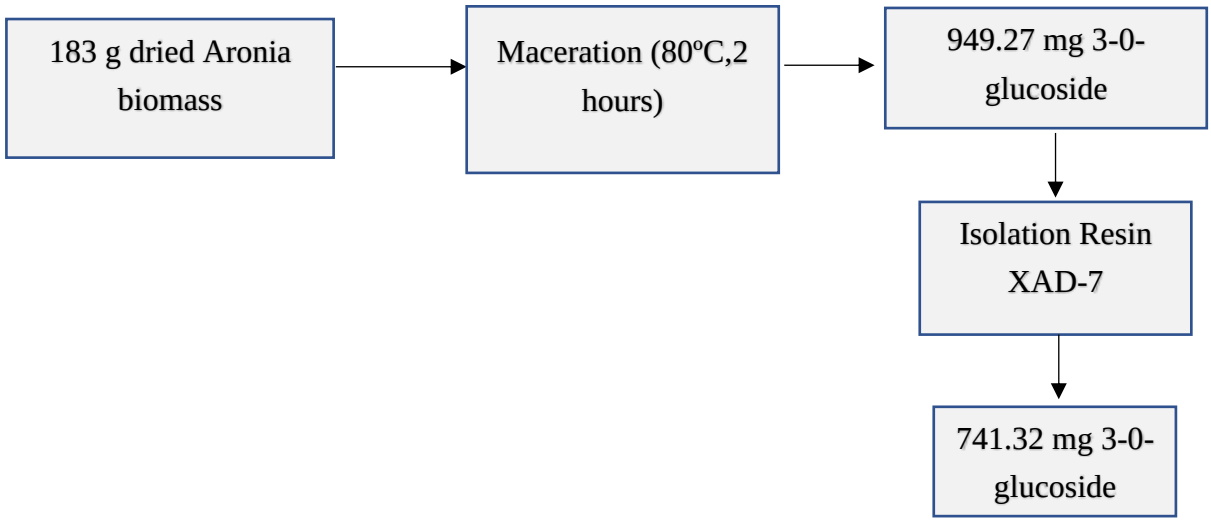


Figure 43: Approximate mass balance of the process when working with 10 BV in the resin

The basis for the development of this mass balance was the original resin adsorption experiment, which made use of a loading volume equivalent to 10 bed volumes (BV). 183 grammes of dried Aronia biomass were macerated for two hours at 80 °C using acidified water. This operation produced an extract with 3-O-glucoside type anthocyanin content of 949.27 mg. The extract was then isolated using an XAD-7 resin. Recovering 741.32 mg of anthocyanins after adsorption and desorption revealed a retention efficiency of practically 78.1%. This indicates that throughout the separation procedure, the resin effectively retains anthocyanins.

Anthocyanins are quite valuable; consequently, effective extraction and purification have a significant financial influence. Purified anthocyanins such as cyanidin-3-O-galactoside are currently on the market and can cost up to 4,000 DKK per 10 mg [182], which reflects their beneficial characteristics as well as their demand in the food, cosmetic, and nutraceutical industry.

6 Conclusion

This thesis studied the extraction and separation of polyphenolic compounds from *Aronia melanocarpa*, mostly anthocyanins, in order to maximise production, stability, and prospective scalability. As this work shown, utilising a mix of conventional and innovative approaches, the efficiency and quality of phenolic recovery from plant biomass may be much enhanced by carefully managing extraction conditions and strategically deploying solid-phase resins.

The results highlighted the significance of temperature and time for the anthocyanin extraction. Macerating at 80°C for two hours with acidified water (pH 3) without clearly generating any deterioration resulted in the highest anthocyanin content, up to 5.64 mg C3G/g DM. The results presented a sustainable and food-safe replacement by avoiding the use of organic solvents, while yet consistent with values reported in the literature using ethanol. Extended duration studies confirming that anthocyanins remain quite stable for 6–8 hours at moderate temperatures further underlined the need to optimise thermal treatment; longer exposure at 80°C can start to compromise stability.

Dynamic adsorption and desorption of Amberlite XAD-7 resin made effective fractionation and partial purification of polyphenols possible. The dual-solvent elution method employed ethyl acetate and 70% ethanol successfully separated hydroxycinnamic acids from anthocyanins with a recovery efficiency of up to 95%. Combining colorimetric and HPLC-DAD assays confirmed the performance and selectivity of the resin, therefore proving its feasibility as a cheap and extensible technique for polyphenolic isolation from natural sources.

Colourimetric tests validated the chemical characterisation; nonetheless, anthocyanin interference limited the total flavonoid content (TFC) method. Assays of total phenolic content (TPC), antioxidant activity (DPPH), and total anthocyanin (TA) indicated, however, the considerable bioactive potential of the Aronia extracts. Developed and refined to concurrently detect anthocyanins and phenolic acids was the HPLC-DAD method. Using dual-wavelength detection at 520 nm for anthocyanins and 325 nm for hydroxycinnamic acids, the technique let one profile individual chemicals by means of UV-Vis absorbance charts. To get enough resolution and repeatability, chromatographic parameters—gradient elution, flow rate, and detection parameters—were precisely changed. Important compounds such cyanidin-3-O-galactoside, cyanidin 3-O-glucoside, chlorogenic acid, and neochlorogenic acid were found by this approach.

Finally, this work offers a useful and scientifically verified technique for the polyphenolic extraction and separation from *Aronia melanocarpa*. Application of green solvents, enhanced thermal extraction, and resin-based purification marks a step towards the manufacturing of scalable, sustainable bioproducts.

7 Future perspectives

The extraction and isolation of *Aronia melanocarpa* polyphenols, especially anthocyanins, for industrial and functional uses can be further advanced by pursuing a number of prospective avenues based on the findings of this thesis. Although this work successfully established a dependable method for resin-based fractionation and showed how temperature and time affect extraction stability and efficiency, there is significant potential to improve yield and process integration through innovative techniques and system design.

The methodical investigation of ultrasound-assisted extraction (UAE) at regulated temperatures is one important area of growth. Also, to study the UAE for longer times, as the graphs seem to be increasing, without reaching stability. The potential of the UAE to speed up extraction and enhance anthocyanin release in brief periods of time was demonstrated by preliminary experiments carried out for this thesis. However, localized warmth produced by ultrasound may encourage the breakdown of anthocyanins if left unchecked. To precisely separate the mechanical consequences of cavitation from undesired thermal stress, future research should use temperature-monitored ultrasound devices, such as thermostatically regulated baths or ultrasonic probes with built-in cooling. To improve this environmentally friendly extraction method, a kinetic study that combines anthocyanin release, degradation behavior, and antioxidant capacity under various ultrasonic parameters (time, power, and temperature) will be very helpful.

Future studies should concentrate on creating a continuous or semi-continuous extraction–isolation system that efficiently combines extraction with resin-based purification. At the moment, extraction and isolation are carried out independently, which could restrict scalability and raise the amount of time and resources needed. It may be possible to separate and concentrate phenolic fractions in real time by using a flow-based system where the extract flows straight through a column filled with resin. To identify anthocyanin saturation and regulate elution steps, such a system could be customized with online monitoring tools (such as UV sensors). In addition to cutting down on processing, this strategy would make it easier to scale up for commercial uses in the food, nutraceutical, or cosmetic industries.

In conclusion, the next logical step in the value-adding of *Aronia melanocarpa* is to advance the research through controlled ultrasound extraction and combine extraction and isolation into a continuous, effective method. The creation of high-purity, stable, and bioactive polyphenol-rich extracts for sustainable bioproducts may be greatly aided by these advancements.

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