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Finnish berry juice as a DNA stain: the search for active DNA binding compounds capable of staining nucleic acids

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Many berries grown in Finland contain anthocyanins in high amounts, providing the berry juice a bright colour and capability to stain a variety of materials. On the basis of earlier studies on Finnish berry juice, it was established that the berry juice contains a substance (or substances) that has a capability to stain nucleic acids, and suggested anthocyanins as a candidate molecule(s). Historically, DNA visualization during electrophoresis has been done by the use of synthetic dyes such as ethidium bromide but considering their toxicity in the light of environmental perception, there is a need to search for safer, preferably natural, alternatives. This study addresses berry juice as a natural dye for the staining of nucleic acids, particularly DNA, in the context of gel electrophoresis. Thus, the aim of this study is to evaluate the feasibility of berry-derived substances as sustainable alternative DNA stain that can be used during gel electrophoresis in a non-toxic way. Further, the aim is to characterize the molecules that are responsible for the binding of DNA and thus are able to stain it.
Frozen berries of Finnish origin were purchased from a grocery store and melted at room temperature. A filtered juice was obtained from unmashed, partially mashed, and totally mashed berries. Each juice preparation was used as a DNA dye in agarose gel electrophoresis, plasmid DNA acting as a probe. The above three berry juice preparations were also analyzed by the use of HPLC-DAD-ESI-MS/MS.
Gel electrophoresis revealed substantial DNA staining only with the juice preparation from partially mashed berries. The stained plasmid DNA band in gel appeared purple under UV-light, similar to staining obtained with ethidium bromide. Based on the HPLC-DAD-ESI-MS/MS analysis, processed through MassLynx software, anthocyanins and proanthocyanidin appeared more stable in the partially mashed preparations and presumably were responsible for the clear and repeatable staining observed by gel electrophoresis.
The DNA-staining molecule(s) from partially mashed berries most probably are anthocyanins, and their mode of staining presumably is intercalation. HPLC-DAD-ESI-MS/MS analysis of the three berry samples (unmashed, partially mashed, totally mashed) revealed a distinct variation in the amount of their compounds. These findings suggest that the extent of mechanical processing significantly influences the composition of DNA-binding compounds in the berry juice.

Keyword: Finnish berry juice, anthocyanins, proanthocyanidins, DNA staining, mechanical processing, HPLC-ESI-MS/MS, gel electrophoresi

Contents

Abbreviation

Glc	glucose
Gal	galactose
Arab	arabinose
Rham	rhamnose
Xyl	xylose
Cy	cyanidin
Dp	delphinidin
Pt	petunidin
Pn	peonidin
Pg	pelargonidin
Mv	malvidin
HES	hesperetin
NAR	naringin

A	adenine
G	guanine
C	cytosine
T	thymine
AGE	agarose gel electrophoresis
EhBr	3, 8-diamino-5-ethyl-6-phenylphenanthridinium bromide

1 Introduction

1.1 Bilberry

Bilberry (*Vaccinium myrtillus L.*) is a low-growing shrub that originates from northern Europe, but today it also grows in parts of North America and Asia. Often referred to as European blueberry, whortleberry, huckleberry, or blaeberry, bilberry is closely related to other members of the Vaccinium genus, such as the American blueberry (*Vaccinium corymbosum)* and cranberry (*Vaccinium macrocarpon*).[1] In Finland alone, wild bilberries are a cornerstone of the forest berry harvest, with annual yields averaging about 168.4×10^6 kg,[2] though yearly output can fluctuate substantially. Recognized by their deep blue–black berries (5–9 mm in diameter) and potent staining capacity, bilberries thrive in heaths, meadow clearings, and shaded, moist woodland understory.[3]

Bilberries naturally thrive in forest meadows and moist coniferous woodlands, preferring areas with moderate shade and humidity. The fruit itself is small, typically 5 to 9 millimeters in diameter, with a dark bluish-black color and contains numerous seeds. The American Herbal Products Association classifies bilberry as a Class 1 herb, indicating it is safe for consumption when used correctly. There have been no reports of mutagenic activity, nor are there any well-documented reasons not to use it.

Bilberries are eaten in various forms, including fresh, frozen, and dried berries, preserves, jams, juices, and more recently, as dietary supplements in liquid or powdered extract forms. Its health-promoting potential is due in great measure to its high content of phenolic compounds. These include flavonols such as quercetin and catechins, tannins, and phenolic acids, but the reigning constituents are anthocyanins, which are predominant in its phytochemical composition.[1,4] Anthocyanins have antioxidant and metal-binding properties and are also responsible for the red, blue, and purple hues of many fruits and vegetables.

Typical daily consumption of anthocyanin is around 200 mg. [5,6] Bilberry has some of the highest anthocyanin contents among berries. Compared with strawberry, cranberries, elderberry, sour cherry, and raspberry fruits, bilberries have much greater amounts of anthocyanin, typically between 300 and 700 mg per 100 grams of fresh fruit. The amount varies depending on the cultivar, environmental growing conditions, and ripeness at harvest.[7] Besides anthocyanins, 100 grams of fresh bilberries also provide small amounts of vitamin C (3 mg), quercetin (3 mg), and catechin (20 mg).[8]

Although the antioxidant activity of anthocyanins is most widely studied, their therapeutic potential is not confined to free radical scavenging. There is evidence to suggest that anthocyanins can influence cellular signaling pathways, gene expression, DNA repair, and even possess cancer chemo-preventive and antimicrobial properties.[9,10,11] Commercial bilberry extracts are frequently standardized to a 25% content of anthocyanidins (equivalent to a concentration of around 36% anthocyanins), but the concentration is widespread among products.[1,12]

In 2001 Upton recommended daily dosage also ranges widely, with some dosages from 20-60 grams of dried berries to 160-480 milligrams of powdered extract.[1]

As one of the richest natural anthocyanin sources, bilberry's strong color and potent antioxidant activity are both directly related to these polyphenols. Traditionally used to support eye health, bilberry has also been associated with reduced blood sugar, anti-inflammatory and cholesterol-lowering effects, as well as enhanced antioxidant protection. These properties suggest potential roles in the prevention or treatment of health disorders related to oxidative stress, inflammation, and metabolic derangement e.g., cardiovascular disease, cancer, diabetes, dementia, and other age-related diseases. Antimicrobial properties have also been documented for bilberry, further adding to its medicinal applications. Traditionally, blueberries have been used as natural dyes in Finland, Estonia, and Livonia for dyeing textiles in shades of black and blue. Blueberry anthocyanins are also well documented to provide health benefits to humans, and thus, these berries are expected to provide similar advantages to the herbivores feeding on them. [3,13,14]

1.1.1 Anthocyanins

Anthocyanin's name comes from the Greek anthos (flower) and kyanos (blue). [15,16,17] These pigments belong to a larger family of naturally occurring (poly)phenolic substances called flavonoids which are water-soluble flavonoid pigments found throughout the plant kingdom. Flavonoids are a group of plant-based compounds known for their wide variety of phenolic structures, and they play an important role in giving color to plant parts like flower petals and leaves.[18] Scientists have discovered over 4,000 different types of flavonoids, many of which are responsible for the rich and diverse colors we see in fruits, vegetables, and flowers.[19] flavonoids can be found in a wide range of everyday foods, including citrus fruits, apples, grapes, olive oil, tea, red wine, and many other plant-derived products. These polyphenolic

compounds give flowers, fruits, and leaves their vivid reds, purples, and blues, with the exact hue shifting markedly with pH: at very acidic pH (<2) they appear red, transition through purple and blue as pH rises, and eventually lose color at alkaline pH. Structurally, an anthocyanin consists of an anthocyanidin "core" (the aglycone) bound to one or more sugar units, most commonly glucose, galactose, xylose, arabinose, or rhamnose, attached at various positions on the core flavilium backbone.[14,20,21]

Indeed, anthocyanins are glycosylated derivatives of flavonoids, specifically polyhydroxy and polymethoxy compounds based on the flavylium cation, also known as 2-phenylbenzopyrylium (Figure 1). These molecules are aromatic and often carry a positive charge, which allows them to absorb visible light, giving each anthocyanin its characteristic color. In general, anthocyanins contain a sugar molecule (commonly glucose) attached at the 3-position (Table 1). Anthocyanins consist of two aromatic rings (A and B) connected by a three-carbon C-ring. Variations in the B-ring structure led to the formation of six distinct anthocyanidins. Due to their ionic nature, anthocyanins undergo molecular transformations based on pH levels, resulting in a spectrum of colors and shades. Typically, glycoside groups attach to the 3-position of the C-ring (3-monoglycosides) or the 5-position of the A-ring (3,5-diglycosides).

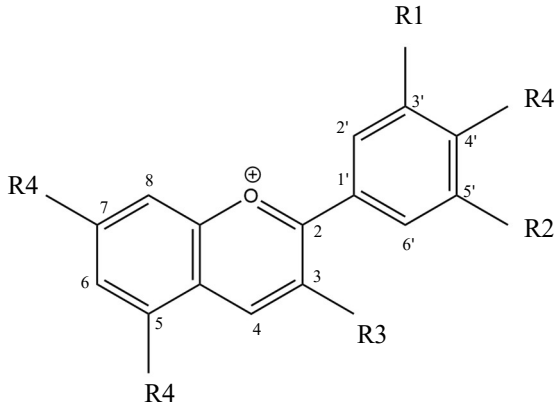


Figure 1 structure of anthoyanidins: R1 and R2 are H, OH, or OCH3, R3 is a glycosyl or H and R4 is OH or glycosyl

Table 1 Anthocyanins

	R	R1	R2	R3
Cyanidin 3-O-glycoside	OH	OH	H	Sugar
Delphinidin 3-O-glycoside	OH	OH	OH	Sugar
Malvidin 3-O-glycoside	OHC3	OH	OCH3	Sugar
Peonidin 3-O-glycoside	OHC3	OH	H	Sugar
Petunidin 3-O-glycoside	OH	OH	OCH3	Sugar

When the sugar is removed, the compound is referred to as an anthocyanidin or aglycone. The most common sugars found in anthocyanins include glucose (glc), galactose (gal), arabinose (arab), rhamnose (rham), and xylose (xyl), which can attach to the anthocyanidin as mono-, di-, or trisaccharides. Aglycones are rarely found in fresh plant materials except in the case of 3-deoxyanthocyanidins like luteolinidin and apigeninidin, which are present in sorghum. [22]

Pentose sugars such as arabinose and xylose can be found in some anthocyanins. In certain cases, sugars undergo acylation with acetic acid or one of the four cinnamic acids (p-coumaric, caffeic, ferulic, or sinapic), which enhances the stability of anthocyanin molecules under extreme pH and temperature conditions.

Although there are fewer than 20 naturally occurring anthocyanidins (Figure 1 and Table 2), differences in sugar type, sugar position, and the number and arrangement of hydroxyl and methoxyl groups yield hundreds of distinct anthocyanin molecules.[21,16,17]

There are only six anthocyanins widely distributed in plant foods: cyanidin (Cy), delphinidin (Dp), petunidin (Pt), peonidin (Pn), pelargonidin (Pg), and malvidin (Mv). Among them, cyanidin is the most abundant, accounting for about 50% of anthocyanidins found in edible plant parts, followed by pelargonidin (12%), peonidin (12%), delphinidin (12%), petunidin (7%), and malvidin (7%).

Table 1 Naturally occurring anthocyanidins

Name	Abbreviation	Substitution pattern							Color
		3	5	6	7	3'	4'	5'	
Apigeninidin	Ap	H	OH	H	OH	H	OH	H	Orange
Aurantinidin	Au	OH	OH	OH	OH	H	OH	H	Orange
Capensinidin	Cp	OH	OMe	H	OH	OMe	OH	OMe	Bluish-red
Cyanidin	Cy	OH	OH	H	OH	OH	OH	H	Orange-red
Delphinidin	Dp	OH	OH	H	OH	OH	OH	OH	Bluish-red
Europinidin	Eu	OH	OMe	H	OH	OMe	OH	OH	Bluish-red
Hirsutidin	Hs	OH	OH	H	OMe	OMe	OH	OMe	Bluish-red
6-Hydroxycyanidin	60HCy	OH	OH	OH	OH	OH	OH	H	Red
Luteolinidin	Lt	H	OH	H	OH	OH	OH	H	Orange
Malvidin	Mv	OH	OH	H	OH	OMe	OH	OMe	Bluish-red
5-Methylcyanidin	5-Mcy	OH	OMe	H	OH	OH	OH	H	Orange-red
Pelargonidin	Pg	OH	OH	H	OH	H	OH	H	Orange
Peonidin	Pn	OH	OH	H	OH	OMe	OH	H	Orange-red
Petunidin	Pt	OH	OH	H	OH	OMe	OH	OH	Bluish-red
Pulchellidin	PI	OH	OMe	H	OH	OH	OH	OH	Bluish-red
Rosinidine	Rs	OH	OH	H	OMe	OMe	OH	H	Red
Tricetinidine	Tr	H	OH	H	OH	OH	OH	OH	Red

The anthocyanins sugar parts play a fundamental role in their water solubility. When sugars are removed or hydrolyzed, the solubility decreases, and the anthocyanin structure becomes unstable. The functional differences between various anthocyanins are influenced by several factors, including the number of hydroxyl groups, the type and number of sugar units, their attachment points, and the nature and number of aromatic or aliphatic acids attached to the sugars.

Anthocyanins are typically soluble in polar solvents and can be extracted from plant materials using methanol or acetone, often in combination with small amounts of

hydrochloric acid (HCl) or formic acid. The acid helps to lower the pH and stabilize the non-acylated anthocyanins during extraction. By the way, it has been observed that using solvents with up to 0.12 M HCl can cause partial hydrolysis of acylated anthocyanins, such as those found in red grapes.[23]

In bilberry, which ranks among the highest anthocyanin-producing fruits, the specific profile and concentration of these pigments can vary with genetic origin and environmental conditions. Light, particularly in the UV, visible, and far-red spectrum, strongly induces anthocyanin biosynthesis, whereas darkness suppresses it.[24] Northern-latitude growing conditions, long summer days and high proportions of red light favor especially high anthocyanin accumulation in *V. myrtillus*, and studies have shown that berries from Scandinavian clones contain more anthocyanins on a dry-weight basis than those of cultivated blueberries.[10]

Functionally, while anthocyanins are best known to attract pollinators and dispersers, also their protective roles in the plant, including frost tolerance, antimicrobial protection, and alleviation of photooxidative stress.[9] Fruit harvesting that damages the berry skin can decrease anthocyanin levels, and unripe fruits typically contain more total phenolics but less anthocyanin than fully mature berries, which accumulate these pigments as they mature.

Aside from their ecological importance, anthocyanins are renowned for their health-promoting properties. Their high antioxidant activity is directly proportional to food plant anthocyanin content,[25] and recent evidence indicates they can stabilize DNA, affect gene expression in adipocytes, enhance insulin release and sensitivity, and be anti-apoptotic, anti-inflammatory, and antibacterial in action.[26,4] Such pluriform bioactivities constitute the source of interest of anthocyanin-rich food like bilberry as “functional foods” and phytotherapeutic medicines.[27]

1.2 DNA

A fundamental molecule in all living cells is DNA (deoxyribonucleic acid), carrying the genetic information necessary for life. An early study identified the major chemical components of DNA: purine and pyrimidine bases, a sugar, and phosphate groups.[28]

By the 1930s, further research refined this understanding, identifying the four specific bases adenine (A), guanine (G), cytosine(C) and thymine (T) along with the sugar deoxyribose. In his landmark work, Erwin Chargaff demonstrated that in any given species,

the amount of adenine always equals thymine, and the amount of guanine equals cytosine. For example, the human genome typically consists of about 20% cytosine, 20% guanine, 30% adenine, and 30% thymine.[28, 29, 30]

DNA is structurally composed of repeating nucleotides units. Each nucleotide includes a phosphate group attached to the 5′ carbon of a deoxyribose sugar, and a nitrogenous base linked to the 1′ carbon via an N-glycosidic bond. The phosphate groups, being acidic, give DNA its name. In the DNA polymer, nucleotides are connected by phosphodiester bonds, linking the 3′ hydroxyl group of one sugar to the 5′ hydroxyl group of the next. This creates a chain with a defined directionality: one strand runs from 5′ to 3′ and the complementary strand runs 3′ to 5′. [31]

Through X-ray diffraction studies the helical structure of DNA was revealed. The data confirmed that DNA forms a right-handed and two-stranded helix. The two strands are held together by hydrogen bonds between specific base pairs, with adenine pairing with thymine, and guanine pairing with cytosine.[32]

Watson and Crick published their groundbreaking model of DNA over 60 years ago in 1953, Building upon these findings. They emphasized two key features: the double-helix structure and the complementary nature of base pairing, which explained how genetic information is preserved and replicated. Their work also confirmed Chargaff's earlier observation that the quantities of adenine and thymine, as well as guanine and cytosine, are always equal in double-stranded DNA. this molecular explanation strongly supported previous discoveries that identified DNA as the "transforming principle" of heredity.[31,33,34,35]

The regular spacing between the deoxyribose sugars in base pairs ensures a uniform helical diameter of 2 nm. The helix repeats its structure every 10 base pairs, with adjacent bases separated by 0.34 nm and rotated by 36°.[2]

An estimate showed the molecular weight of DNA at between 5×10^6 and 10^7, or roughly 30,000 to 40,000 nucleotides. Surprisingly enough, these measures imply that DNA is a pretty rigid molecule, a finding that would seem to be quite counterintuitive, considering that every nucleotide in the phosphate-sugar backbone has about five single bonds, which theoretically ought to confer a good deal of flexibility. This unexpected stiffness has been verified by the assistance of electron microscopy. With the assistance of high-resolution

imaging techniques, Williams (1952)[36] and Kahler et al (1953)[37] observed extremely thin, elongated fibers of DNA with the same width of approximately 15 to 20 Å.

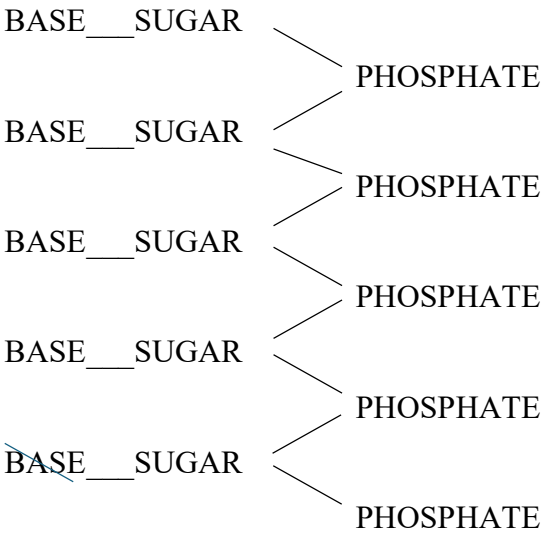


Figure 2 Chemical formula of a single chain of desoxyribonucleic acid.

DNA strands are long, and the sequence of bases along the chain can vary, encoding the genetic information necessary for life. Stability of double stranded DNA (dsDNA) at physiological temperatures is primarily maintained by hydrogen bonds between complementary base pairs and stacking interactions between neighbors. Nevertheless, brief and localized unzipping of the double helix can occur due to even some minor thermal fluctuations under these conditions. [38] DNA is a vital molecule that carries the instructions for life itself.

As science continues to evolve, researchers have developed powerful tools to study it, one of which is DNA staining. This technique is widely used in biological research, helping scientists explore the complexities of genetics.[39]

For DNA quantification assays, achieving fluorescence enhancements of approximately 1000-fold upon DNA binding, recent advances have also led to the development of highly sensitive fluorescent dyes.[40]

1.3 Gel Electrophoresis

With advancements in biotechnology, standard agarose gel electrophoresis (AGE) has rapidly improved in its ability to separate different DNA forms.[41,42] particularly in consistently and successfully staining linear DNA and single-stranded circular DNA.[43,44]

AGE has long been a fundamental technique for analyzing nucleic acids in laboratories worldwide. The practice of using EtBr (3, 8-diamino-5-ethyl-6-phenylphenanthridinium bromide) as a staining dye for visualizing nucleic acids emerged following Waring's study,[45] which highlighted its interaction with DNA. Due to its high sensitivity, EtBr quickly became a widely used reagent for nucleic acid staining. This dye is heat-sensitive, carries a positive charge in aqueous environments, and can alter the electrophoretic mobility of linear double-stranded DNA when present at saturating concentrations. As a result, Sigmon and Larcom recommended its use for post-electrophoresis staining of gels.[46,47]

1.3.1 visualization of biomolecules

The study of biomolecules, including proteins and nucleic acids, plays a crucial role in life sciences research and is essential for diagnostic purposes in both clinical and industrial settings.[48]

Fluorescent dyes have been widely employed to assess DNA fragment size, quantity, and quality, beginning with the use of EtBr in 1972 [49] and 1973. [50]

EtBr is a dark red organic fluorescent dye, highly soluble in water, and widely exploited for rapid visualization of DNA in gel electrophoresis experiments in biomedical laboratories.[51]

Typically, EtBr is discharged in dilute aqueous solutions having concentrations in the lower to middle micromolar range. [52] Toxicity and mutagenicity studies[53] showed that EtBr inhibits cell growth in tissue culture, effective frameshift.

Mutagenic action causes the development of chromosomal abnormalities and can intercalate with DNA and prevent transcription. Thus, the treatment of contaminated water with EtBr is recommended. Fortunately, other alternatives can be used. These include SYBR Gold, SYBR Green I (Molecular Probes, Eugene, OR, USA), and GoldView (SBS Genetech, Beijing, China), which show even greater (SYBR Gold, SYBR Green I) or comparable (GoldView) sensitivity compared to EtBr in agarose gel electrophoresis. More crucially, studies and manufacturing reports indicate that these alternatives may be far less mutagenic than EtBr.[40,54,55,56]

Nucleic acid stains are typically categorized into two classes: intercalating dyes and minor groove binders. Among these, Intercalating dyes, especially those based on cyanine, are commonly preferred because they produce intense fluorescence once they attach to DNA. Common examples of intercalating dyes include EtBr, propidium iodide, and SYBR® Green I (Life Technologies), while DAPI and Hoechst dyes are representative minor groove binders.[57]

EtBr intercalates between the base pairs of double-stranded DNA (dsDNA), a mechanism that underlies its well-known mutagenic properties.[40] Similarly, cyanine dyes like SYBR® Green I have been extensively applied in nucleic acid labeling and detection across various techniques, including flow cytometry, real-time PCR, and both capillary and gel electrophoresis. Research has shown that EtBr exhibits genotoxic effects at standard staining concentrations (0.5 μg/mL) and demonstrates cytotoxicity at higher doses, it classified as a potent mutagen, whereas SYBR® Green I has been characterized as a weak mutagen.[58] In response to safety concerns, newer dyes such as SYBR® Safe (Life Technologies) have been developed, claiming reduced mutagenicity compared to both EhBr and SYBR® Green I. These newer agents generally interact with the minor grooves of DNA rather than intercalating, thereby lowering their mutagenic potential. Examples include Diamond Nucleic Acid Dye (Promega, NSW, Australia), which has been reported to offer a lower limit of detection (LOD) compared to SYBR® Safe. [58]Additionally, dyes such as GelRed and GelGreen (Biotium) have been engineered to minimize mutagenicity by preventing cell membrane penetration. Examples of such assays include the QuantiFluor® dsDNA System (Promega), the AccuClear® Ultra High Sensitivity dsDNA Quantitation Kit (Biotium), and the Qubit™ system (Life Technologies).

In an effort to find Nontoxic Stains for DNA Detection safer alternatives to EtBr, researchers developed a new, less toxic staining method for detecting DNA in agarose gels.[57,59] Although this method is about two times less sensitive than EtBr, it offers a significant advantage in terms of safety. The approach uses berberine, a natural fluorescent compound, followed by a counterstain with an anionic dye called mordant yellow 3R1. This counterstain effectively reduces the background fluorescence from berberine, making the DNA bands stand out more clearly. Berberine itself has an interesting chemical structure. It

is formally known as 7,8,13,13 α -tetrahydro-9,10-dimethoxy-2,3-(methylenedioxy)-berberinium.

Its structure features a quaternary nitrogen atom and a flat, aromatic framework, which enables it to slide between the base pairs of the DNA helix, a binding mode similar to that of EtBr. When exposed to long-wave UV light, berberine-bound DNA fluoresces brightly, producing clear yellow bands that are easy to detect. While other non-toxic stains like methylene blue and Nile blue have been used for DNA visualization, they are generally much less sensitive and require longer staining times, making the berberine method a more practical and efficient alternative for many applications.

For different nucleic acid staining dyes, the average relative intensity signals across a range of DNA concentrations are obtained (0.5–50 ng). Among the dyes tested, only four, GelRed™, GelGreen™, SYBR® Green I, and Diamond™ Nucleic Acid Dye, were sensitive enough to detect DNA at as little as 0.5 ng. RedSafe™ was able to detect DNA down to 1 ng, but the signal was noticeably weaker. Findings recommended GelRed™ is the most sensitive option compared to RedSafe™ and EtBr, when using UV transillumination (302 or 312 nm excitation), Additionally, GelRed™ is reported by the manufacturer to be less toxic than EtBr. For blue light transillumination (460 nm excitation), Diamond™ Nucleic Acid Dye is recommended first, followed by GelGreen™, since both successfully detected DNA at 0.5 ng and are marketed as being less toxic and mutagenic than SYBR® Green I. However, the study also found that Diamond™ Nucleic Acid Dye can penetrate cell membranes, suggesting it might be less safe than GelGreen™, although it does offer stronger signal intensity.[58]

1.3.2 Basic Principles of Electrophoresis nucleic acid staining

The technique used to move charged particles through a solution by applying an electric field Electrophoresis.[60] The force that runs this movement comes from the voltage applied across the system. Two important electrical concepts in electrophoresis are Ohm's Law (V = IR, where V is voltage, I is current, and R is resistance) and the Power equation (P = VI, where P is power in watts). Particles in the solution move depending on their size, shape, and electrical charge.

Electrophoresis is commonly used to separate nucleic acids (DNA and RNA) and proteins in molecular biology. When separating nucleic acids, a constant voltage is usually

applied. To visualize these molecules after separation, scientists use fluorescent dyes that bind to nucleic acids and glow under specific lighting conditions.[61] EtBr binds to nucleic acids by inserting itself between their strands (intercalation), and it does so without a strong preference for specific sequences. Its chemical formula is $C_{21}H_{20}BrN_3$ and its molecular weight is 394.3 g/mol. When bound to nucleic acids, the fluorescence of EtBr increases around 30 times, making it easy to detect. It emits an orange-red fluorescence and can stain double-strand DNA (dsDNA), single-stranded DNA (ssDNA), and RNA. It is sensitive enough to detect as little as 10-50 nanograms of double-stranded DNA. However, its binding also slightly alters the mobility of DNA fragments by inserting itself into the double helix.

After molecules have been separated by electrophoresis, for staining nucleic acids the next step is to detect them. Both RNA and DNA are usually visualized using fluorescent dyes that glow much brighter when attached to nucleic acids compared to when they are free in the solution. The advantage of using these dyes is that the bands of nucleic acids become clearly visible against the less bright background. Despite being a potent mutagen, EtBr must be handled with great care. To safely dispose of it, it can be filtered through activated charcoal and then incinerated or chemically degraded in a solution containing sodium nitrite and hypophosphorous acid. EtBr can be added directly into the agarose gel at a concentration of about 1 μg/mL, although doing this slightly slows down the migration of nucleic acids. Gels containing EtBr must also be treated as hazardous waste. Alternatively, the dye can be added to the running buffer or used after the electrophoresis run by soaking the gel in a 1 μg/mL EtBr solution for a few minutes. After staining, the DNA bands are visualized using ultraviolet (UV) light at 300 nm and captured with photography.[47]

1.4 Interactions of Anthocyanins with Biomolecules

DNA plays a fundamental role in vital biological processes such as replication and transcription, making it a prime target for drug development. Small molecules can bind to DNA through various mechanisms, including intercalation between base pairs, insertion into the minor or major grooves, and electrostatic interactions with the DNA's phosphate backbone. Among these, intercalation and minor groove binding are the most common binding modes for small ligands.[62] Electrostatic interactions typically occur on the DNA helix's exterior, where positively charged, molecules are attracted to the negatively charged phosphate groups.

Many studies on ligand DNA interactions focus on changes in the fluorescence of flavonoid compounds when they interact with nucleic acids.[63, 64,65] Flavonoids may bind to DNA either through groove binding or by intercalating between the base pairs. In cases where the ligand itself is non-fluorescent, researchers often use chemical probes like EtBr, positively charged and planarly, whose fluorescence greatly increases upon intercalation with double-stranded DNA.[45, 66]

One study investigated how hesperetin (HES) and naringenin (NAR) (Figure 3), interact with DNA by combining spectrophotometric, circular dichroism, and voltametric measurements. By characterizing the ways in which these flavonoids bind to DNA, can be gained a valuable model for the development of DNA-targeting drugs.

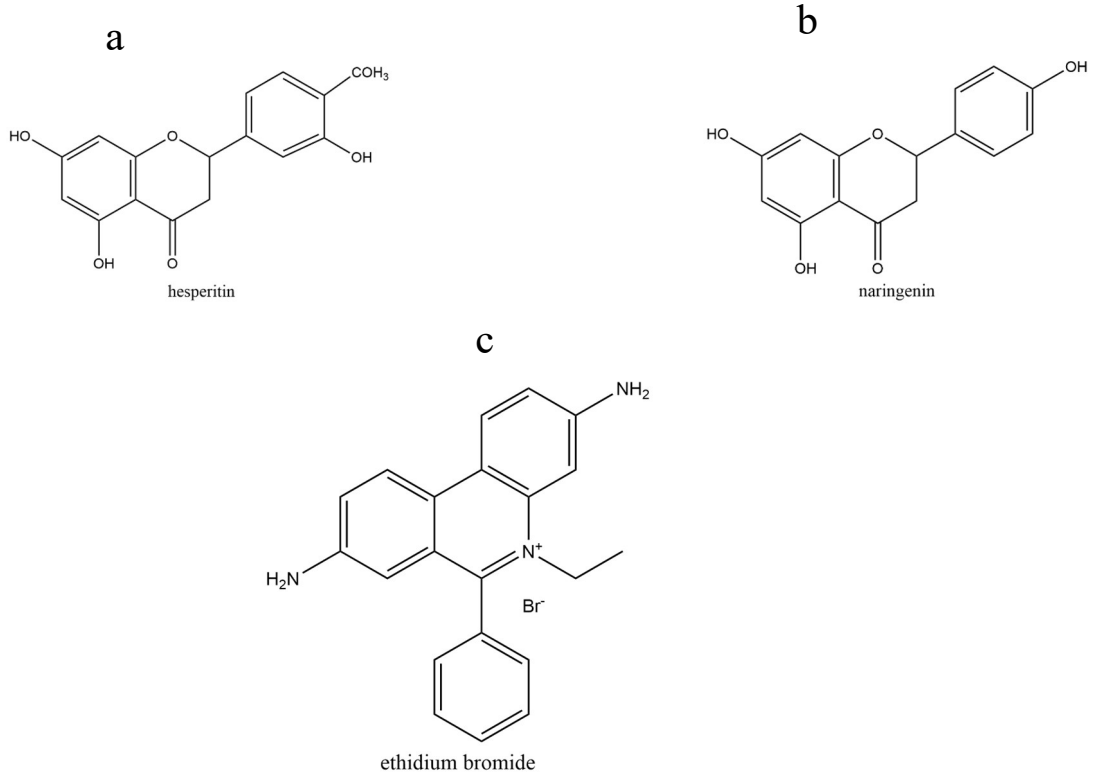


Figure 3 Structures of hesperitin (a), naringenin (b) and ethidium bromide (c)

To study how flavonoids interact with DNA, absorption spectroscopy is widely used. The absorption of DNA at 260 nm increases upon the addition of flavonoids, suggesting that these compounds may intercalate between the DNA strands. This interaction unwinds the double

helix slightly, exposing aromatic bases to more UV light and enhancing absorption intensity.[67]

The fluorescence of EtBr is naturally low when free in solution (excitation at 525 nm), but when it binds to DNA, increases significantly. This enhancement results from strong stacking interactions between EtBr and the DNA base pairs, stabilizing the intercalated complex.[51]

To further explore how flavonoids interact with DNA, fluorescence quenching experiments were conducted using a DNA-EtBr complex. It is known that the fluorescence of EtBr bound to DNA can be partially quenched by a second molecule.[68] The degree of quenching showed that the interaction between flavonoids and DNA can be induced. When flavonoids such as HES or NAR were gradually added to the DNA-EtBr complex, the fluorescence intensity of EtBr decreased consistently. This suggests that the flavonoids interact with DNA in a way that competes with EtBr, and binding constants were calculated from the fluorescence data accordingly.

1.4.1 Mechanism of Interaction of Anthocyanins with DNA

Human DNA is made up of long chains of nucleotides connected through phosphodiester bonds linking the 5′ and 3′ carbons of deoxyribose sugars. These nucleotide strands run in opposite directions and coil together to form the well-known double helix structure. [33] The bases in each strand pair through hydrogen bonding-adenine pairs with thymine (A = T) via two hydrogen bonds, and cytosine pairs with guanine (C ≡ G) via three hydrogen bonds. These base pairs are located in the core of the helix, while the sugar-phosphate backbone lies on the exterior.

In living organisms DNA features two grooves running along its length: a wider and deeper major groove and a narrower minor groove. The major groove allows easier access for molecules to interact with DNA due to its larger size, making it a common target for binding.

DNA-binding molecules, also known as ligands, can interact with DNA in several ways. Among the three major components of DNA, deoxyribose sugars, phosphate groups, and nitrogenous bases, the sugars and phosphates serve as sites for general electrostatic and hydrophobic interactions, while the bases (purines and pyrimidines) enable more specific

interactions through hydrogen bonding. Some biological molecules, such as cytokines, can bind DNA through a combination of these interaction types, allowing highly selective binding to certain sequences.

When a ligand contains large, flat aromatic structures, it can insert itself between two adjacent base pairs, forming what's known as an intercalation complex. This intercalation process was first described by Lerman while studying amino acridines and their interactions with DNA.[62, 69,70 ,71] It occurs through π–π stacking interactions between the ligand's aromatic rings and the base pairs of DNAs. This insertion widens the space between base pairs and distorts the DNA helix, which is typically counterbalanced by slight unwinding of the DNA structure. A classic example of such an intercalating agent is EtBr.[71, 55]

DNA and RNA may both act as effective co-pigments for the native anthocyanins.[69] Such interaction is triggered primarily by intercalation between the adjacent base pairs and facilitates stable π–π stacking between bases of DNA and the anthocyanin molecules. Sarma and Sharma reported that adding DNA to a cyanidin solution caused a noticeable bathochromic shift (15-20 nm) in its absorption maximum (λmax), suggesting the formation of a charge-transfer co-pigmentation complex between cyanidin and DNA (ctDNA). [72](Figure 4).

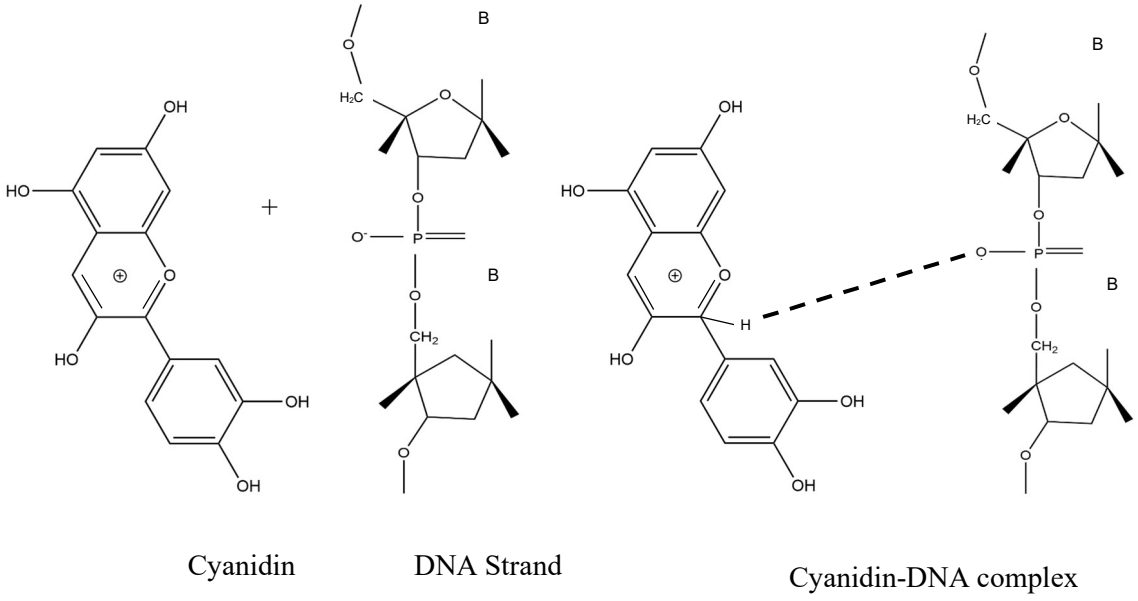


Figure 4 Formation of cyanidin- DNA complex

In addition, FTIR and UV-visible difference spectroscopy experiments have examined the interaction between delphinidin (Dp) and calf thymus DNA (ctDNA). At low Dp concentrations, its positively charged oxygen groups were found to interact primarily with the negatively charged phosphate backbone of DNA. It neutralized phosphate charges and was involved in helix stabilization. Spectral data also showed that Dp is capable of indirectly interacting with certain bases of DNA, guanine, adenine, and thymine via its hydroxyl and oxonium (O^+) groups.[73]

1.5 Aim of the study

The main aim of this study is to investigate the potential of Finnish berry juices, rich in anthocyanins, as natural, non-toxic alternatives to synthetic DNA stains commonly used in electrophoresis, such as ethidium bromide. The research is motivated by increasing environmental and safety issues over synthetic intercalating dyes, most of which have been proven to be mutagenic or carcinogenic. Considering that berries grown in Finland have high levels of anthocyanins, pigmented flavonoids with established bioactivity, the current research aims to identify what type of compound or compounds are capable of binding to DNA for purposes of gel-based visualization.

2 Materials and methods

Frozen Finnish bilberries (V. myrtillus L.) were purchased from a local grocery store in Turku, Finland and melted at room temperature. Frozen bilberries were weighed, and 2 grams of fruit were allowed to melt at room temperature. After melting, the samples were mixed with 1 mL of distilled water and subjected to three different levels of mechanical processing:

Unmashed: The berries were left intact, and only the juice released during melting was used for analysis.

Partially mashed: The berries were partially mashed using a mortar to slightly release the content.

Totally mashed: The berries were thoroughly mashed using a mortar to ensure complete and maximum juice release.

These three sample types were subsequently filtered using paper filters to separate the juice, which was then collected for further analysis.

2.1 Analytical materials and methods

Analytical grade reagents (methanol, acetone and formic acid) were used throughout the SPE and Sephadex LH20. Ultrapure water was produced using a Milli-Q purification system. Partially mashed juice was used in this process.

2.1.1 SPE (solid phase extraction)

SPE was performed using QAM cartridges. 1 ml of partially berry juice was injected into the QMA cartridge. For sample loading, the filtered juice from the sample preparation step was passed through the conditioned cartridge slowly. Juice sample in the cartridge were eluted with water, 5% aq. MeOH, 30% aq. MeOH, 50% aq. MeOH, 70% aq. MeOH and pure MeOH as shown in Table 3. 1 mL of the eluted mobile phase was used for subsequent AGE and quantitative analysis.

Table 2 Different fractions on SPE

	Eluent	Fraction	Volume/ml
1	Water	1A	1
2	5% MeOH	2A	1
		2B	1
3	30% MeOH	3A	1
		3B	1
4	50% MeOH	4A	1
		4B	1
		4C	1
		4D	1
5	70% MeOH	5A	1
		5B	1
6	100% MeOH	6A	1
		6B	1

2.1.2 Sephadex LH 20

For complete separation by Sephadex LH 20, 100 ml berry juice was added in the column, then different eluents have been used and operated at a flow rate of 5 mL/min with 100% of the solvent delivered through channel A. Of these, pure MeOH and MeOH mixtures, particularly the mixtures mixed with 0.1% formic acid, have been useful in flavonoid compound isolation and purification (Table 4).

Table 3 Fractionation scheme used in Sephadex LH-20

	Eluent	Fraction	Volume/ml
1	0.1% HCOOH	1A	500
2	10% MeOH	2A	500
		2B	500
3	20% MeOH	3A	500
4	30% MeOH	4A	500
5	40% MeOH	5A	500
		5B	500
6	50% MeOH	6A	500
		6B	500
		6C	500
7	80% Acetone	7A	500
		7B	500

1-6 prepared with 0.1% HCOOH, 7 prepared with MQ water

2.1.3 UHPLC-DAD-ESI-QqQ-MS/MS Analysis

Fractions of berry juice obtained from Sephadex were filtered using 0.22 μm polytetrafluoroethylene (PTFE) to remove particulates prior to analysis. Filtered samples were then pipetted into inserts in LC-MS vials using precision pipettes to ensure accuracy and prevent contamination.

Analytical separation and detection were carried out on an ACQUITY UPLC system (Waters Corporation, Milford, MA, USA) alongside a Xevo TQ (triple quadrupole) mass spectrometer (Waters Corporation, Milford, MA, USA). The two systems were connected via an electrospray ionization (ESI) source, which provided efficient ionization of analytes for detection by mass spectrometry.

The UPLC system included a binary solvent manager, sample manager, diode array detector (DAD), and ACQUITY UPLC BEH Phenyl column (100 mm × 2.1 mm i.d., 1.7 μm; Waters Corporation, Wexford, Ireland). The two solvents were mixed to create the mobile phase: acetonitrile (solvent A) and 0.1% aqueous formic acid (solvent B). Chromatographic

separation was achieved with a gradient elution program, which is shown in Table 5. The flow rate was maintained at 0.5 mL/min.

Mass spectrometric detection was done in multiple reaction monitoring mode, essentially in negative ionization. Parameters of ESI source were optimized as follows: capillary voltage, 1.8 kV; desolvation temperature, 650 °C; source temperature, 150 °C; nitrogen was used as desolvation gas (flow rate, 1000 L/h) and cone gas (flow rate, 100 L/h). Argon was used as collision gas. (TICswere also recorded under both positive (*m/z* 100–800, ESI+, 4 eV) and negative (*m/z* 15-800, ESI−, 4 eV) ionization conditions using MRM and full-scan MS modes. The UHPLC-DAD-ESI-QqQ-MS/MS system was operated using MassLynx 4.2 software.

UV and MS responses were monitored within a retention time window of 0 to 7 minutes. UV spectra were recorded on the diode array detector from 190–500 nm. Full-scan mass spectrometry data were collected in positive ionization mode to detect and identify anthocyanins.

The group specific method was utilized by Engström 2014 .[74]

2.1.3.1 Chromatographic techniques

UV chromatograms were recorded at two specific wavelengths using a diode array detector (DAD): 280 nm for the detection of general phenolic compounds, and 500 nm for the selective detection of anthocyanins. The identification of phenolic compounds was carried out by comparing the retention times, UV spectra, and mass spectra of the sample analytes with those of authenticated reference standards. To confirm the retention times, both methanolic solutions of the reference compounds and sample solutions spiked with the same standards were analyzed under identical chromatographic conditions.

2.1.3.2 Compound groups detected

The detected compounds in UHPLC-DAD-ESI-QqQ-MS/MS included a wide range of groups such as galloyl derivatives, flavonol derivatives (including kaempferol, quercetin, myricetin, isorhamnetin), flavone derivatives (including apigenin, luteolin, and acacetin), extension and terminal units of procyanidin (PC) and prodelphinidin (PD) oligomers and polymers, quinic acid derivatives, caffeic acid derivatives, ferulic acid derivatives, HHDP derivatives, as well as monomeric flavan-3-ols such as catechin, epicatechin, gallocatechin, and epigallocatechin.

Table 4 Gradient elution program used in UHPLC-DAD-ESI-QqQ-MS/MS .

	time (min)	Flow (ml/min)	Acetonitrile (A) %	0.1 % HCOOH (aq), (B) %	curve
1	0.0	0.5	0.1	99.9	0
2	0.5	0.5	0.1	99.9	6
3	5.0	0.5	30.0	70.0	6
4	6.0	0.5	35.0	65.0	6
5	6.1	0.5	90.0	10.0	6
6	8.1	0.5	90.0	10.0	6
7	8.2	0.5	0.1	99.9	6
8	9.5	0.5	0.1	99.9	6

2.2 Gel preparation for gel electrophoresis

To prepare the gel, the 0.4 g of agarose (Seakem® LE Agarose) powder is dissolved in electrophoresis buffer of 50 ml and microwaved until the powder is melted completely. When the solution has cooled down to around 50°C, 15 µl of berry juice is included to stain the DNA after the electrophoresis run. The warm mixture is then placed in a casting tray with a comb in it to make sample wells and left to set at room temperature. the open ends of the tray are taped in order to enclose the melted agarose; this tape is removed after gel solidification, and the gel is ready for use.

To create wells for loading DNA samples, special combs are inserted into the liquid gel before poured it. As the gel hardens, the combs are removed carefully, creating small wells. The gel is then covered with an electrophoresis buffer, typically either Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE), which assists in passing on the electric current and maintaining a stable pH along the run.

The gel, still in its plastic tray, is placed horizontally into the electrophoresis box and completely covered with running buffer. Different dilution of DNA plasmid (PAPH6) was obtained by adding TE buffer and these samples prepared in 1:12 with gel loading dye Purple (6X) buffer (NEW ENGLAND Biolabs) are then gently micro pipetted into the wells. 1 Kb Single strand DNA ladder (Invitrogen) was used as a molecular size marker. The box is

closed with its lid, the power leads are connected, and the electric current is switched on. You can confirm the current is flowing by spotting bubbles forming at the electrodes.

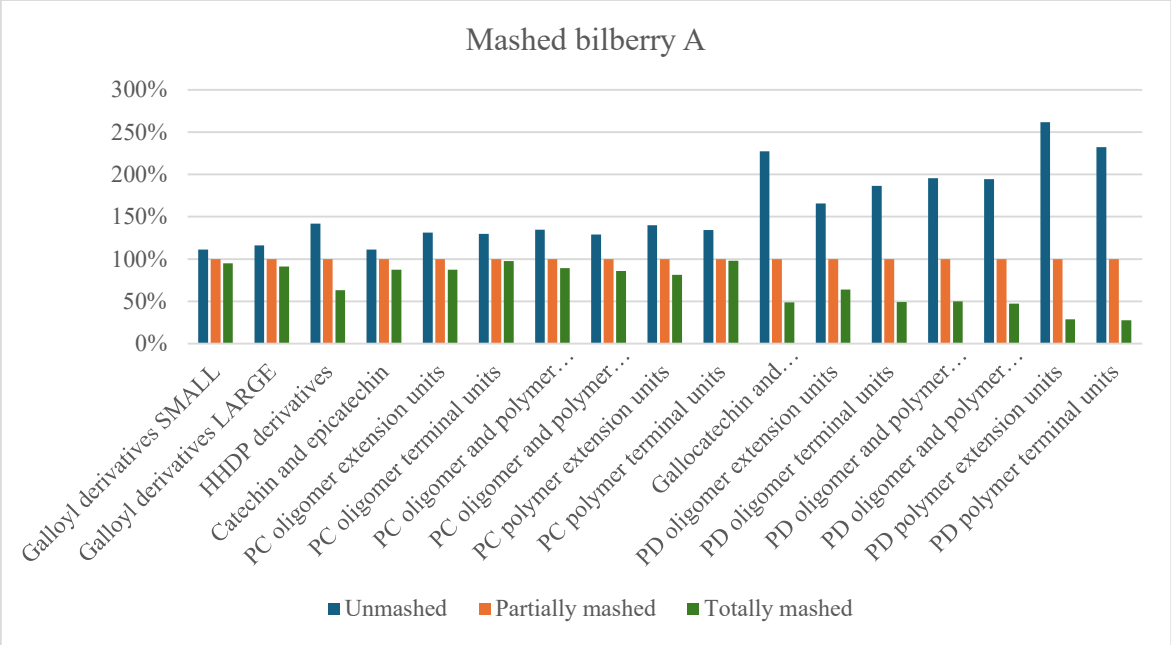
Due to its negative charge, DNA migrates toward the positive electrode, which is usually marked in red. The movement of DNA through the gel can be tracked visually by monitoring colored dyes which are added to the samples for this purpose. DNA samples are mixed with a fluorescent dye for visualization, that is bound by the DNA. The stained DNA fragments after electrophoresis can be seen clearly under ultraviolet (UV) light.

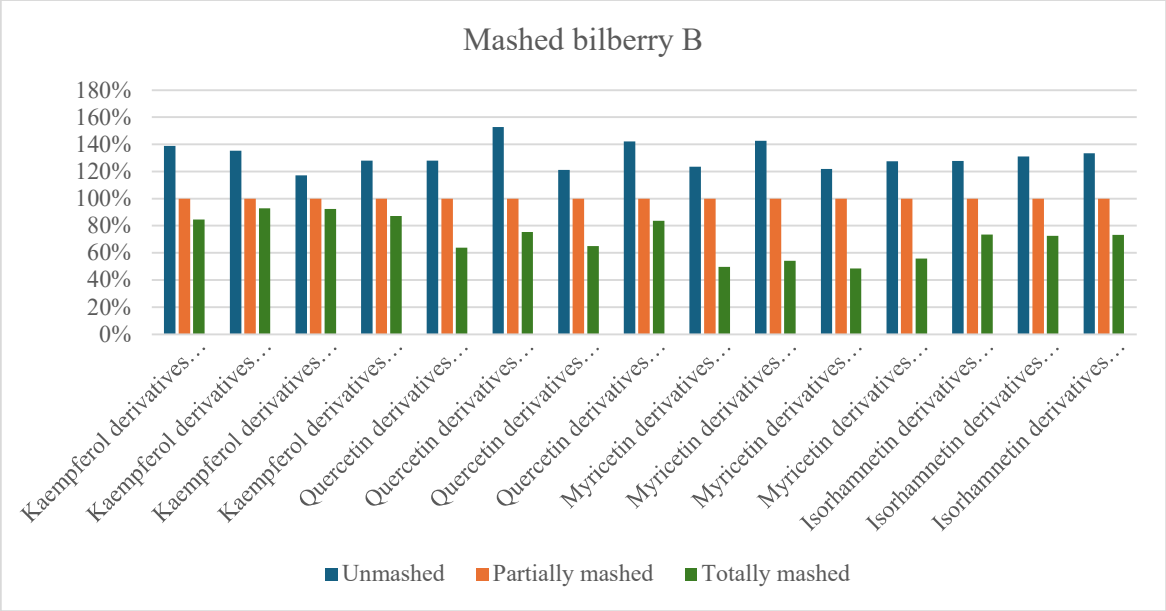
3 Results and Discussion

3.1 Mashed treatment

For quantitative analysis of phenolic compounds, the chromatographic data (Figure 5) shows that un-mashed berries consistently yielded the highest peak integration values across a wide range of anthocyanin and polyphenolic compounds. The partly mashed samples, normalized to 100%, provided a balance between anthocyanin retention and improved DNA stainings. Totally mashed samples exhibited the lowest integration values, which may reflect the combined effects of enzymatic hydrolysis and oxidation occurring during complete cellular breakdown.[75,76]

However, not all compound classes followed this trend. Caffeic acid derivatives, ferulic acid derivatives, and apigenin derivatives maintained relatively high levels even under full mashing conditions. This suggests that these compounds are either more chemically stable or more effectively released through cell wall degradation. Vice versa, PC polymers were significantly reduced, by more than 70%, in fully mashed berries, likely due to oxidative cleavage or polymer fragmentation catalyzed by polyphenol oxidases.





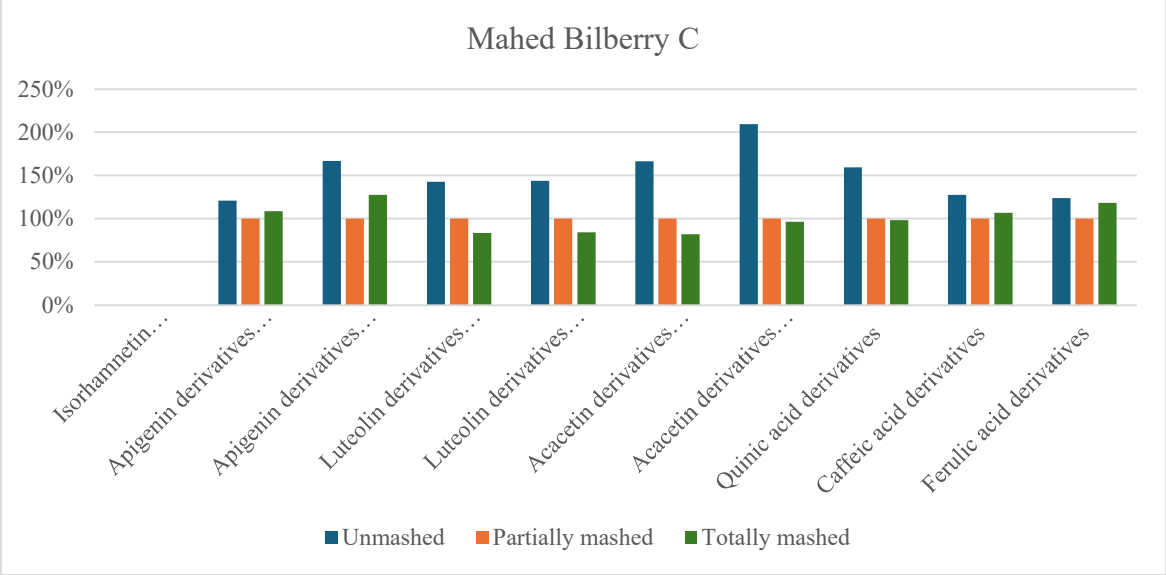


Figure 5 Three mashing types of bilberry phenolic compounds categorized under three derivative types: A (tannins and proanthocyanidins), B (flavonol derivatives) and C (other flavonoids and phenolic acids)

The anthocyanin profiles obtained at 500 nm clearly highlight the influence of berry processing on the integrity and availability of these pigments (Figure 6). The unmashed berry extract displayed the broadest and most intense absorbance peaks, indicating a richer

anthocyanin content compared to the partially and totally mashed samples. This is consistent with previous reports showing that anthocyanins are susceptible to oxidative degradation when exposed to air and endogenous enzymes released during cell disruption.[76]

To evaluate the functional impact of mashing on extract performance, the samples were applied in DNA staining using AGE. While UPLC-DAD-ESI-MS/MS analysis (Table 6) confirmed that unmashed berries had the highest integrated peak areas for anthocyanins, the partially mashed samples exhibited superior staining characteristics, achieving 100% staining efficacy. This observation aligns with findings that moderate mechanical processing can enhance pigment extraction without triggering extensive degradation.[77]

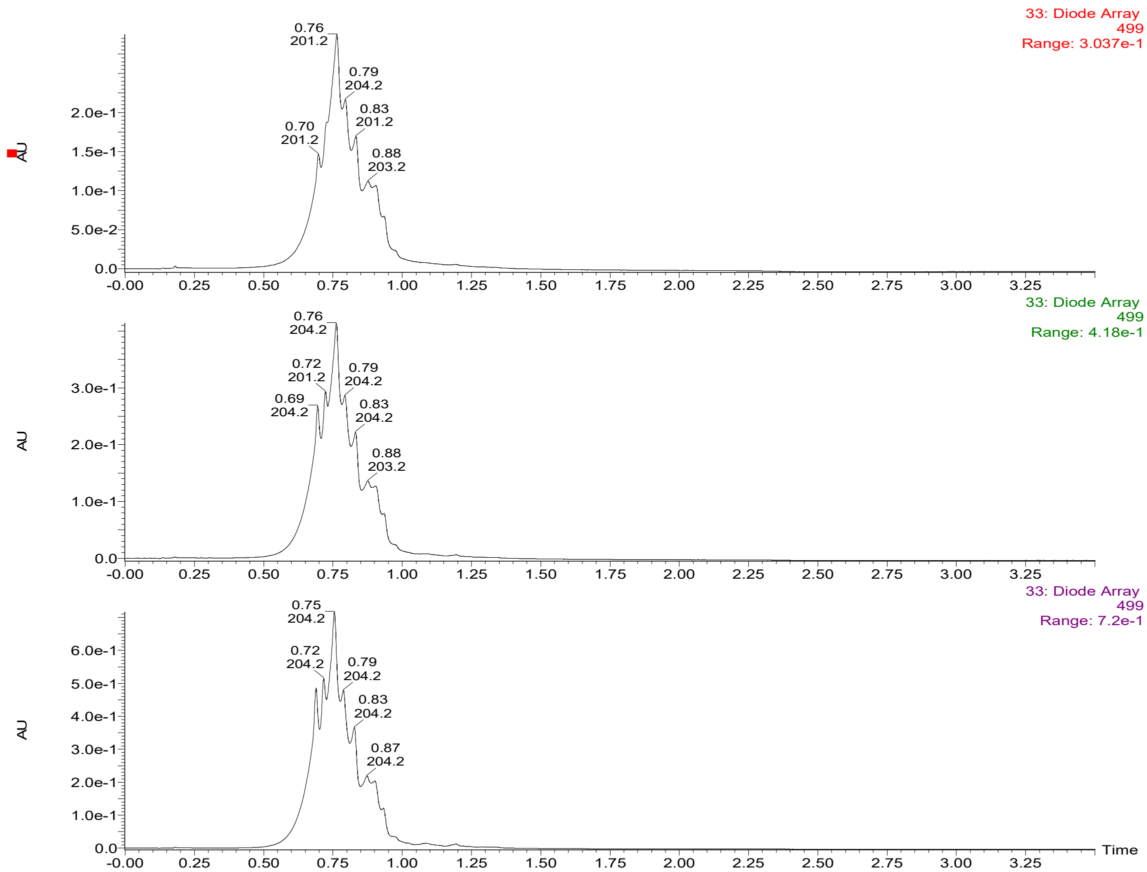


Figure. 1 UV spectra of Anthocyanins in 500 nm for three mashed types of bilberry

The anthocyanin profiles at 500 nm for the three mashing treatments are clearly delineated, with the unmashed sample exhibiting and higher absorbance range than both the partially mashed and totally mashed preparations.

The UPLC-DAD-ESI-MS/MS analysis revealed nine distinct anthocyanins in blueberry extracts across different sample preparations: un mashed, partially mashed, and totally mashed (Figure 7). The anthocyanins identified corresponded to the following (*m/z*) values: 419 (cyanidin-3-arabinoside), 433 (peonidin-3-arabinoside), 435 (delphinidin-3-arabinoside), 449 (cyanidin-3-glucoside), 463 (peonidin-3-galactoside), 465 (delphinidin-3-glucoside), 479 (petunidin-3-glucoside), 493 (malvidin-3-galactoside),[78] and 519 (pelargonidin-malonyl-dihexoside)[79], integration profile of this ion shown in figure 8. These compounds are among the most abundant anthocyanins commonly reported in blueberries.[21,80]

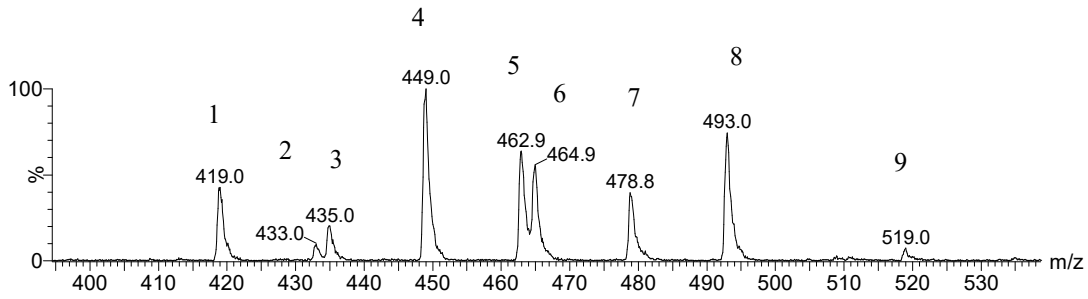


Figure 2 Nine ion masses of Anthocyanins in 500 nm for three mashed types of bilberries

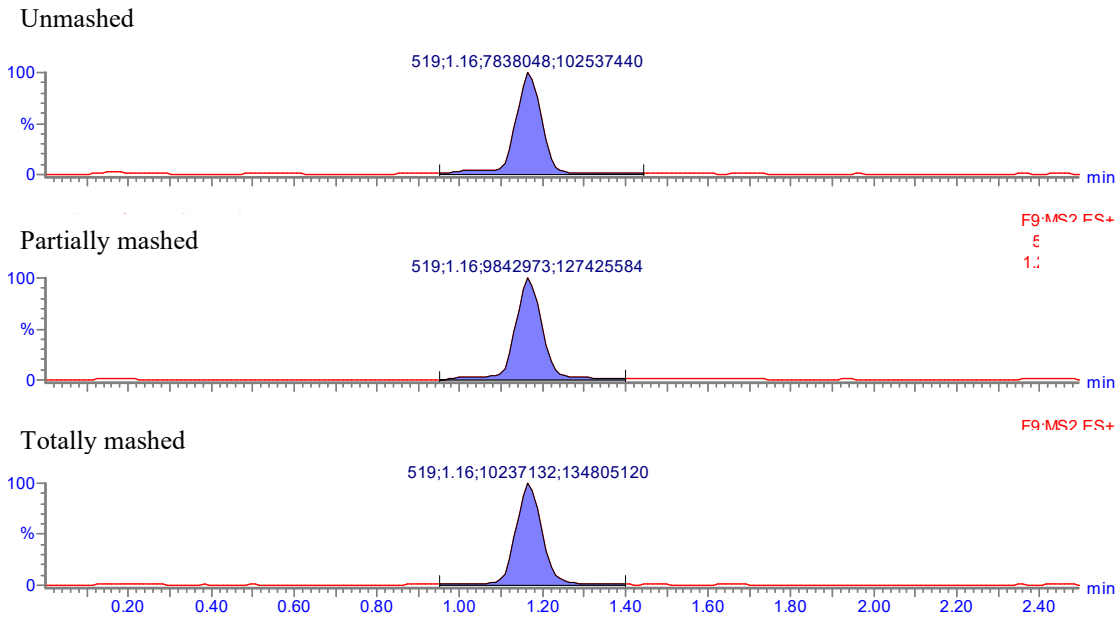


Figure 8 pelargonidin-malonyl-dihexoside (519 *m/z*) ion integration profile in three mashed types of bilberries at 500 nm

The total peak areas for all detected anthocyanins were highest in the unmashed sample, followed by the partially mashed, and lowest in the totally mashed preparation, as shown in Table 6. The major anthocyanins contributing to the peak intensity were cyanidin-3-glucoside (*m/z* 449), delphinidin-3-glucoside (*m/z* 465), malvidin-3-galactoside (*m/z* 493) and pelargonidin-malonyl-dihexoside (519).

Table 6 individual peak integration of ion molecular mass in different mashed type at 500 nm

Peak (*m/z*)	419	433	435	449	463	465	479	493	519
Name	Cyanidin-3-arabinoside	Peonidin-3-arabinoside	Delphinidin-3-arabinoside	Cyanidin-3-glucoside	peonidin-3-galactoside	Delphinidin-3-glucoside	Petunidin-3-glucoside	Malvinidin-3-galactoside	pelargonidin-malonyl-dihexoside
Unmshed	66613596	13212734	37759772	131347720	87401648	117781464	68256456	89329112	8335952
Partly mashed	53667924	10279676	30143166	105805136	71876736	88773312	55340724	78314336	10429713
Fully mashed	50336424	9781654	19076796	95273296	64456900	55846416	41681556	70171560	10911620

The pelargonidin-malonyl-dihexoside concentration increased slightly during mashing which could indicate its formation as a breakdown product or transformation intermediate under mechanical stress.

A consistent decline of approximately 20–40% in peak integration values was observed as the degree of mechanical processing increased, indicating progressive anthocyanin degradation (figure 8). For example, cyanidin-3-glucoside showed a reduction from 100% (unmashed) to 68% (totally mashed), representing a about 32% loss. Similarly, delphinidin-3-glucoside content decreased by more than 50%. These losses can be attributed to enzymatic oxidation initiated during mechanical tissue disruption, which activates polyphenol oxidase and peroxidase leading to anthocyanin polymerization and subsequent degradation.[75]

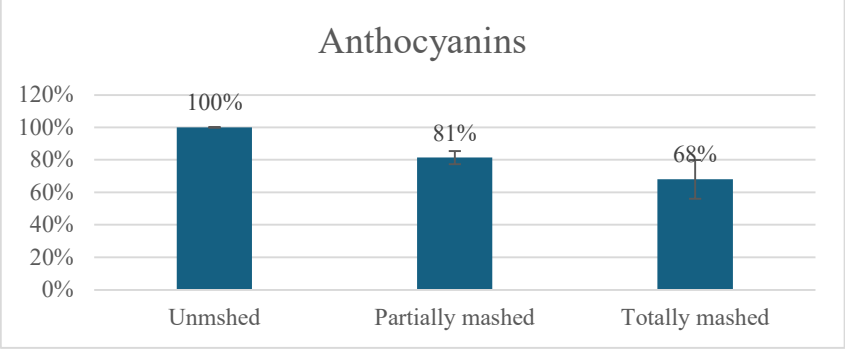


Figure8 Relative concentrations of anthocyanins in three different treatments measured at 500 nm

AGE of DNA extracted from PAPH6, inoculated bilberry tissue (Figure 9) reveals that the degree of mechanical disruption strongly affects both the amount and the integrity of recovered genomic DNA. In the unmashed samples, only a very faint smear and indistinct bands are visible beneath the DNA-standard ladder (200 ng/µl through 12.5 ng/µl). Partially mashed produces clear, discrete bands in the 25 ng and 50 ng standards, demonstrating substantially improved yield without excessive fragmentation. totally mashed samples yield the greatest total DNA signal, evident as a broad smear extending below 1 kb.

The observed pattern highlights a trade-off between DNA yield and molecular integrity during sample preparation. When bilberries are left intact, rigid cell walls and unbroken plant structures hinder efficient cell lysis, trapping bacterial genomes and producing inadequate template for downstream assays. Partially mashed emerges as the optimal compromise, because it sufficiently disrupts the plant matrix to release bacterial cells and their DNA while preserving fragment lengths in the several-kilobase range.

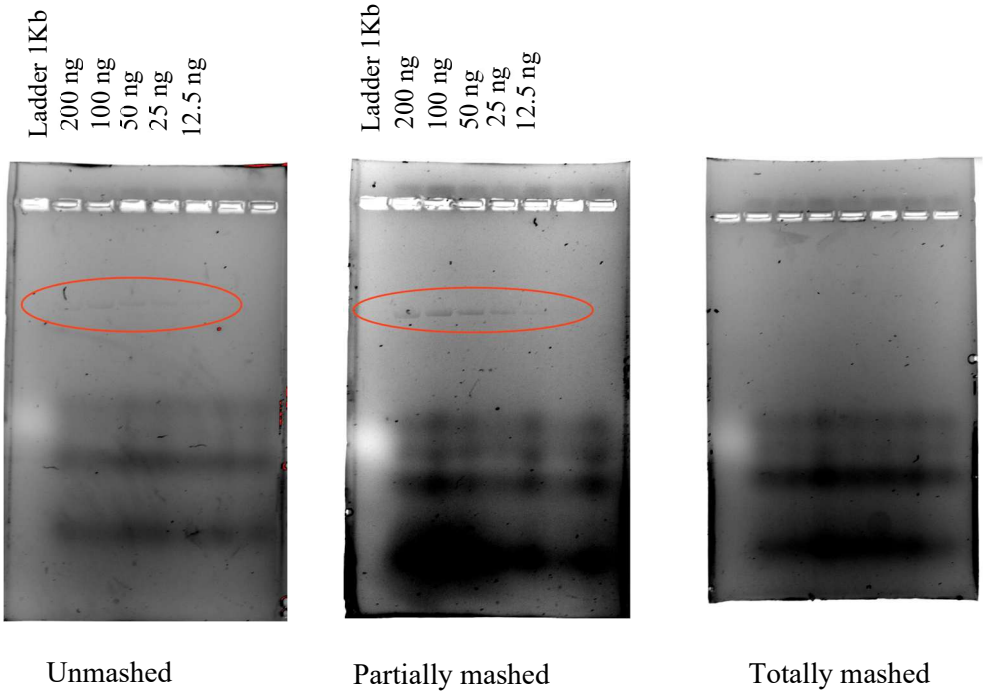


Figure 3 AGE results, Ladder (standard of size), DNA concentration in 200, 100, 50, 25, 12.5 ng/µl

3.2 SPE (solid phase extraction)

Solid-phase extraction (SPE) of bilberry anthocyanins yielded a characteristic elution profile when monitored by full-scan positive-ion LC–MS (Figure 10, Table 7). Across all seven major glycosides (*m/z* 419-493), the 50 % aqueous methanol fraction consistently produced the highest peak integrals. For example, cyanidin-3-arabinoside (*m/z* 419) increased from 573 621 counts in 30 % MeOH to 24 853 988 counts in 50 % MeOH, then fell sharply to 16 494 163 in 70 % MeOH. Similarly, delphinidin-3-glucoside (*m/z* 449) peaked at 90 797 032 counts in 50 % MeOH, versus only 3 980 143 in 30 % and 15 748 233 in 70 % MeOH.

Petunidin-3-glucoside (*m/z* 479) and malvidin-3-galactoside (*m/z* 493) followed the same pattern, each showing maximum recovery at 50 % eluent. Notably, the pelargonidin-n-malonyl-dihexoside ion (*m/z* 519) was undetectable in all SPE fractions, suggesting absence in the starting material after SPE cleanup.

Table 5 individual peak integration of anthocyanins from SPE method

Peak (*m/z*)	419	433	435	449	463	465	479	493	519
Eluent	Cyanidin-3-arabinoside	Peonidin-3-arabinoside	Delphinidin-3-arabinoside	Cyanidin-3-glucoside	peonidin-3-galactoside	Delphinidin-3-glucoside	Petunidin-3-glucoside	Malvinidin-3-galactoside	pelargonidin-malonyl-dihexoside
Water	35351	42996	77449	4221	26935	128834	73482	76609	0
5%MeOH	134473	59560	16372	901581	47623	18839	41152	34548	0
30%MeOH	573621	297052	580062	3980143	767078	5711664	3870817	2871720	0
50%MeOH	24853988	3976817	23743092	90797032	35030604	90550624	63075932	65811844	0
70%MeOH	16494163	2094293	5876241	15748233	14281839	5024141	3538032	6933878	0
100%MeOH	4504813	497067	1069587	3321088	2422028	1078416	523722	843343	0

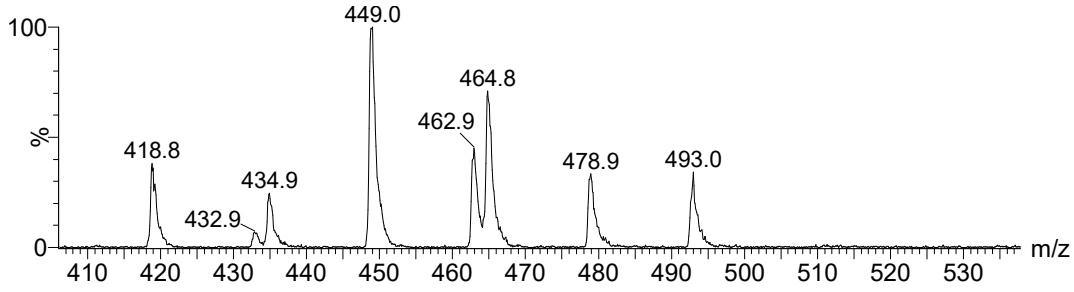


Figure 4 *Molecular ions* of a*nthocyanins detected from the SPE fraction at the positive full scan mode.*

The SPE elution pattern reflects the balance between anthocyanin polarity and sorbent affinity. At low MeOH concentrations (water to 30 % MeOH), polar compounds remain largely retained on the cartridge, resulting in low recovery. As the eluent strength increases, anthocyanidins become more efficient, peaking at 50 % MeOH, and anthocyanins are out of column. Beyond this point, proanthocyanidins are out of the column at 70 % and 100 % MeOH.[81]

The complete absence of the 519 *m/z* ion after SPE suggests that pelargonidin-n-malonyl-dihexoside was removed during cartridge washing. Malonylated anthocyanins are known to exhibit stronger ionic interactions and reduced desorption efficiency in aqueous methanol systems,[82] which could explain its disappearance.

Optimizing SPE for comprehensive anthocyanin profiling therefore requires careful selection of eluent strength. A 50 % aqueous methanol step maximizes total yield of the main glycosides, including cyanidin, delphinidin, petunidin, and malvidin derivatives.

The AGE analysis evaluated the distribution of bioactive compounds specifically nucleic acid in berry juice fractions extracted by SPE. The SPE procedure started with water as the first eluent followed by sequential MeOH elution at increasing concentrations from 0% to 100% MeOH.

The AGE profile demonstrated distinct banding patterns throughout the MeOH fractions because macromolecular compounds are separated according to solvent polarity. The water fraction (0% MeOH) displayed weak bands, that indicated the presence of highly polar and possibly low-molecular-weight compounds at low concentrations which results from HPLC-MS/MS. The 5% MeOH and 30% MeOH fractions displayed stronger band intensities which indicated the presence of moderately polar compounds including phenolics compounds ,analyzed in HPLC-MS/MS, that dissolve well in low to moderate MeOH concentrations.[82]

Figure 11 shows the results of DNA staining using different SFE fractions. The 50% MeOH fractions contained distinct bands which were more intense than the previous fractions. The elution of compounds with intermediate polarity including polymeric polyphenols reaches its optimal point at these specific concentrations. The bioactive flavonoids and tannins which exhibit moderate polarity tend to accumulate in these fractions because they elute optimally at mid-range MeOH concentrations.[83] The 100% MeOH

fraction displayed either faded or blurry bands which suggested that non-polar compounds or degraded or low-interacting substances were eluted.

The unclear bands may result from protein denaturation or the presence of interfering substances that affect the gel migration. The AGE results show that berry juice components separate effectively by polarity through gradient methanol elution with the 30-70% MeOH range containing the highest amount of bioactive compounds. The recovery of polyphenol-rich fractions peaks when SPE protocols use methanol concentrations in the 30–70% range.[80]

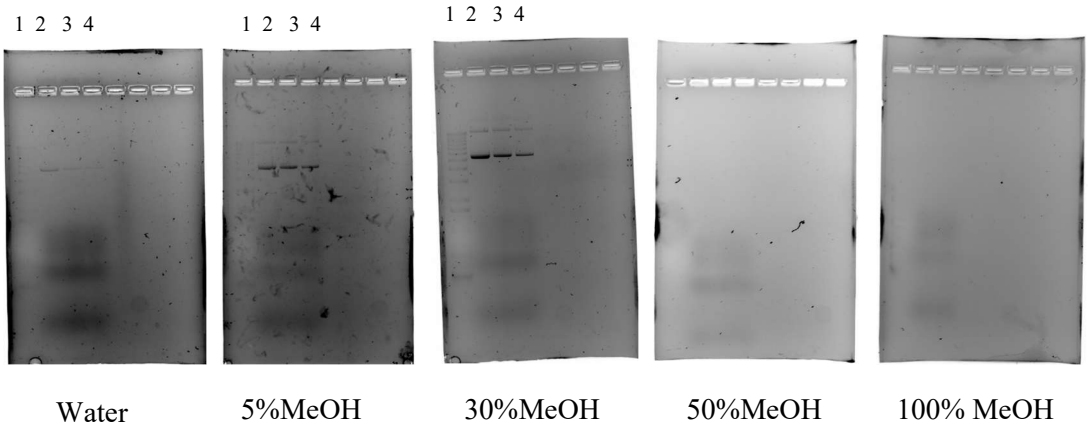


Figure 5 AGE results, DNA Ladder as a molecular size marker (1), DNA was used in 200(2), 100(3), 50(4) ng/µl concentration in AGE

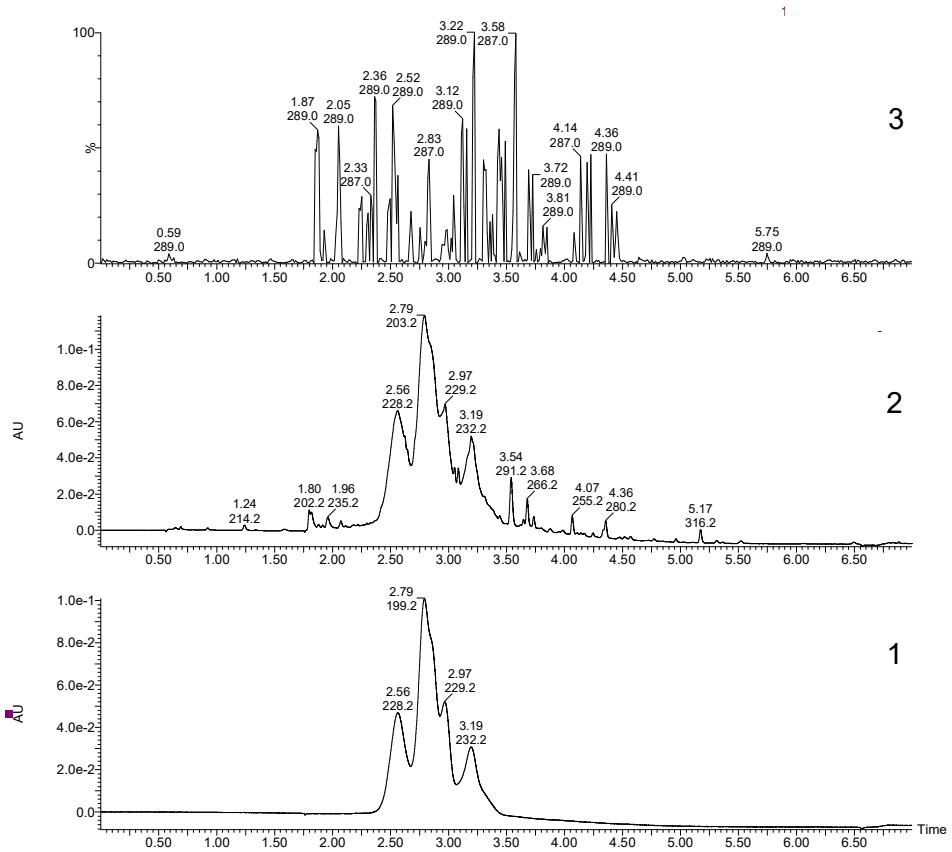
3.3 Sephadex LH20

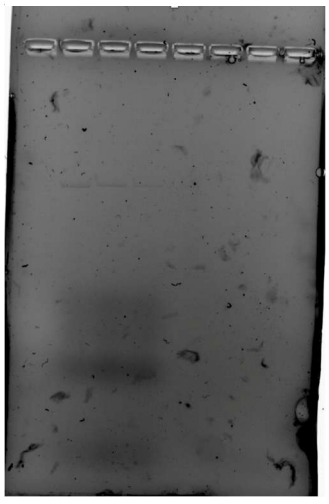
Fractions eluted with 10 % MeOH (polar), 1a, and 80 % acetone (non-polar), 1b, were analyzed by UHPLC–DAD at 280 nm (phenolic compounds) and 499 nm (anthocyanins) and by ESI+ mode. Representative chromatograms are shown in Figure12.

The chromatographic profiles obtained from UPLC-DAD-ESI analysis using 10% MeOH (1a) as the elution solvent showed a clear hump, with profile 1 is typical of anthocyanins and profile 2 low molecular weight polyphenols. This feature was especially noticeable in the UV spectrum at wavelengths around 280 nm and 500 nm. However, no distinct hump was observed for the corresponding PC polymers, at profile 3, under these elution conditions. This makes sense, as anthocyanins are more polar and water-soluble, making them more easily eluted in aqueous methanolic solutions.

The chromatographic profile using 80% acetone (1b) as the elution had an entirely different pattern. A well-defined and broad hump was observed corresponding to proanthocyanidins, while signals typical of anthocyanins was considerably absent. This alteration is attributed to the solvent-dependent elution efficiency of different polyphenolic subclasses. Acetone, especially in higher concentration, has been found to elute more hydrophobic and higher-molecular-weight polyphenolic compounds such as PC polymers more efficiently. [84]

High levels of PC polymer elution have been linked to noticeable nucleic acid staining, similar to the effects seen with anthocyanins. Like anthocyanins, proanthocyanidins are polyphenolic compounds known for their strong binding affinity to biological macromolecules such as DNA. This suggests that proanthocyanidins may also bind to or even intercalate with DNA, which likely contributes to the staining patterns observed in assays.





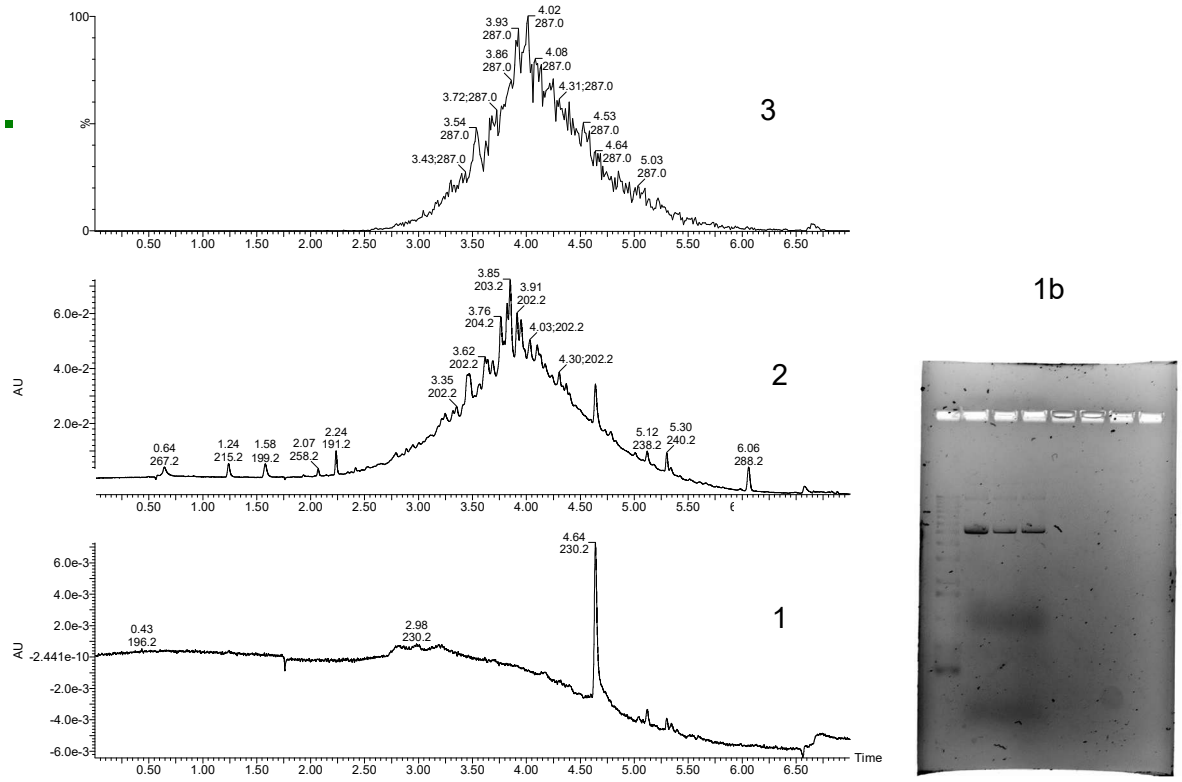


Figure 6 Anthocyanin profile (1), polyphenol profile (2), PC polymer profile (3) in 10% MeOH and 80% acetone

Moreover, a noticeable change in the chromatographic profile was observed after Sephadex LH-20 process. Specifically, the prominent peak previously detected and partially masked in the HPLC-MS/MS analysis, attributed to the anthocyanin molecular ion, was no longer present. The complete disappearance of the *m/z* 519 ion after SPE suggests that pelargonidin-N-malonyl-dihexoside was also removed during this process. Additionally, eight distinct peaks observed in the SPE chromatogram were also detected in the Sephadex LH-20 fraction.

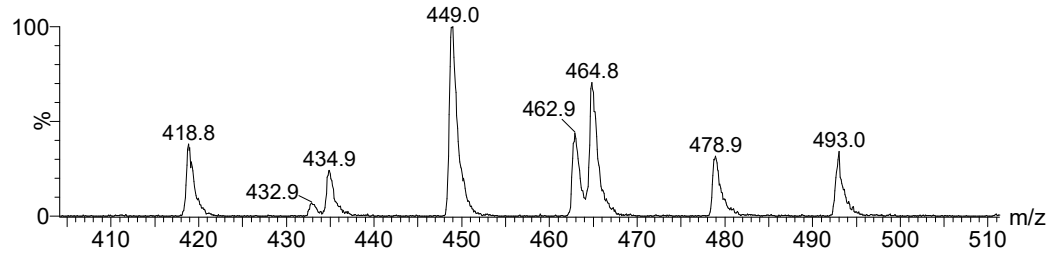


Figure 7 anthocyanins in full mass positive ionization mode- Sephadex LH 20 method

Table 8 presents the individual peak integration values of PC polymers analyzed by HPLC-MS/MS, following Sephadex LH-20 under various eluent. The values for both extension and terminal units varied noticeably depending on the solvent used. Among the tested solvents, 80% acetone produced the highest integration values, extension units and terminal units, representing 100% of the relative polymer content. In contrast, water, 10% methanol, and 40% methanol showed no detectable extension units, suggesting minimal or no polymer elution under these conditions. A gradual increase in polymer content was observed with increasing methanol concentration from 20% to 50%, with 50% methanol yielding a moderate amount of PC polymers, accounting for 4% of the total. These findings indicate that solvent polarity strongly influences PC polymer elution, with acetone-rich solvents being the most effective in enhancing polymer recovery.[94]

Table 6 individual peak integration of PC polymers from Sephadex LH-20

Name	PC polymer extension units	PC polymer terminal units	%
Water	0	5362	1 %
10% MeOH	0	1278	0 %
20% MeOH	3137	4906	1 %
30% MeOH	9621	6285	2 %
40% MeOH	0	254	1 %
50% MeOH	29767	10316	4 %
80% Acetone	824472	92087	100 %

4 Conclusion

The research demonstrates that mechanical processing techniques strongly impact the yield of anthocyanins in blueberry extracts. The process of cell wall rupture through partial mashing enables pigment extraction but full mashing possibly causes oxidative breakdown which results in decreased anthocyanin yield. The optimal processing conditions need to strike a balance between extraction efficiency and chemical stability because pigment performance remains essential for bio-staining applications. The current observations match previous research findings regarding anthocyanin reactions to processing and storage conditions.[85,75]

While non-mashed berries retain the highest concentration of intact anthocyanins, partially mashed berries offer a functional balance between stability and efficacy. These features enable stable and effective staining of nucleic acids. Full mashing leads to notable degradation of key compounds, especially polymeric flavonoids, while certain phenolic acids remain relatively stable. From a practical standpoint, partial mashing appears to be the most effective treatment for generating anthocyanin-rich extracts suitable for AGE-based DNA staining, owing to the enhanced extractability and bioactivity of the pigment matrix under moderate processing conditions.[76,77]

SPE with 50 % aqueous methanol provides the most efficient recovery of bilberry anthocyanins, particularly the major cyanidin, delphinidin, petunidin, and malvidin glycosides, while preserving their structural integrity. Lower methanol concentrations fail to elute sufficient quantities, and higher strengths reduce yield and may exclude more strongly retained, labile compounds such as malonylated derivatives. Accordingly, a 50 % MeOH elution step represents the optimal balance for routine anthocyanin profiling from bilberry, although targeted fractionation or alternative sorbent chemistries may be required to capture malonylated species fully.

Sephadex LH-20 chromatography separated polyphenolic compounds by separating anthocyanins and enhancing the proanthocyanidin content.

The extraction of phenolic subclasses including proanthocyanidins and anthocyanins strongly depends on the solvent polarity. The UHPLC–DAD and ESI+ results showed that 10% methanol and 80% acetone fractions contained phenolics and proanthocyanidins because of their strong absorbance at 280 and 500 nm. The chromatographic peaks in

UHPLC–DAD and ESI+ analyses became more pronounced when using 80% acetone as a less polar solvent which extracted higher-molecular-weight and more hydrophobic compounds such as proanthocyanidins. The results confirm earlier studies which showed that aqueous acetone solutions are optimal for eluting tannins and proanthocyanidins.[86, 87] Anthocyanins show better elution with methanol–water mixtures because they are highly polar and water-soluble compounds.[88] The method was employed to extract proanthocyanidins for studying their DNA staining properties .

The results demonstrate that high concentrations of eluted proanthocyanidins can bind to nucleic acids which results in DNA staining effects comparable to those of anthocyanins. The polyphenols exhibit DNA binding and intercalation properties which is supported by research on their bioactive binding behavior .[89]

Based on the analysis of UHPLC-DAD-ESI-QqQ-MS/MS data in the chemistry laboratory and the visualization of gel results under UV light in the biology laboratory, it was concluded that both anthocyanins and proanthocyanidins play significant roles in staining nucleic acids.

5 References

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