International Journal of Pharmacognosy and Chemistry
Content available at www.saapjournals.org ISSN (O): 2582-7723

Open Access Research Article

ANTHOCYANIN EXTRACTION AND PURIFICATION

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Article History

Received on: 04-08-2025
Revised on: 18-09-2025
Accepted on: 10-10-2025

***Keywords*:** Red Onion, Herbal Extraction, Anthocyanins, absorbance curve, food, pharmaceutics

Abstract

Anthocyanins, found naturally in numerous plants like fruits and vegetables, anthocyanins offer important pharmacological advantages, making their extraction for medical purposes crucial endeavor. While conventional methods ordinarilyuse methanol for extraction, this often yields extracts contaminated with other components. Consequently, there is a essential for extraction techniques that provide greater anthocyanin purity. Our current study addresses this via employing a modified extraction techniqueusing an acetone-chloroform system designed to produce an additional or more purified anthocyanin extract. This extraction approach achieved a high recovery percentage of 82.73 ± 1.96%. The research focused on two major anthocyanins-Peonidin-3-glucoside and Pelargonidin-3-glucoside-extracted from red onions and analyzed their spectral behavior. UV-Vis measurements revealed that the first compound peaked at the wavelength typical for peonidin derivatives, while the second matched the pelargonidin profile. When we introduced aluminum chloride, both pigments exhibited the expected bathochromic shifts, confirming our assignments. To isolate these molecules, we optimized an acetone–chloroform extraction procedure that consistently delivered fractions of high purity. Conclusion, this streamlined approach not only proves that specific anthocyanin can be efficiently recovered from red onion but also highlights their promise for applications in food science, nutraceuticals, and pharmaceutical formulations.

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DOI: https://doi.org/10.46796/ijpc.v6i4.754

Introduction

Anthocyanins are naturally occurring flavonoid pigments that easily dissolve in water, contributing vibrant reds, purples, and blues to various plants. Found within the vacuoles of fruit skins, flower petals, leaves, and stems, these compounds possess a polyphenolic structure along with delicate aromatic characteristics that suggest their significant biological benefits [1].

Research over the years has established that anthocyanins serve as potent antioxidants, effectively neutralizing free radicals before they can inflict cellular damage. Laboratory studies have also shown their antibacterial and anticancer properties, alongside evidence suggesting they contribute to maintaining healthy vision. Importantly, findings from cell culture and animal studies consistently indicate that anthocyanins are safe and non-toxic, even when administered in higher doses [2].

Because of their vivid coloration and health-supporting traits, anthocyanins have become prized ingredients across multiple industries. They're used as natural food colorants and functional food additives, explored for nutraceutical and pharmaceutical uses, and even incorporated into skincare and cosmetic products. As scientific interest grows, so does the range of ways we tap these plant-derived pigments for flavor, color, and wellness.(3-8) These compounds appear to exhibit antioxidant characteristics, contributing to the prevention of neuronal disorders. Consequently, they have a range of biological activities, including anti-inflammatory, chemotherapeutic, cardioprotective, hepatoprotective, and neuroprotective effects [9-11].

Onions represent a crucial and commonly grown vegetable, not only in India but globally. The bulb's coloration, attributed to flavonoid compounds, is an economically significant characteristic. Bulbs of red and white hues find their respective uses in culinary preparations and salads.

The bulbs and skins of onions are abundant in various bioactive compounds, including fructo-oligosaccharides (FOSs), organo-sulfur compounds (OSCs), thiosulfinates, polyphenols, and flavonoids. Onions come in a range of colors-white, yellow, red, pink, orange, and gold—largely due to two main types of flavonoids: anthocyanins and flavonols. Anthocyanins contribute to a wide spectrum of colors, from red and orange to blue and violet. In contrast, flavonols, such as quercetin and its derivatives, are responsible for producing yellow and brown shades in the outer layers of the onion's scale leaves [12-14].

Natural dyes like anthocyanins and flavonoids are more readily soluble in water and absorbed by our bodies compared to synthetic pigments. The combination of anthocyanin pigments from various sources can enhance the quality of functional foods, making them not only visually appealing but also beneficial for health [15]. Flavonoids were extracted from may herbalsources [16-19, with unique chemical structures, are present in onions. Studies have led to the discovery of approximately ten distinct types of anthocyanins in onions. The primary anthocyanins include 3-(3-glucosyl-6-malonylglucoside), 3-(6”-malonylglucoside), 3-(3”-glucosylglucoside), and the 3-glucoside of cyanidin. These compounds play a significant role in the coloration of various plants and contribute to their nutritional value. White onions contain a lower concentration of anthocyanins compared to red onions, possibly due to the varying anthocyanin content in each onion layer. This evidence suggests that red onions serve as a substantial source of anthocyanins [20].The objective was referred to modified extraction of onion anthocyanins as acetone - chloroform (extraction and partition of anthocyanins) and aimed merge for purifications manners.

Materials and Methods

The main outlined method of isolations and purification involves the following steps:

First step: Extraction of Anthocyanins: Acetone is used to extract anthocyanins from the plant material.

Second steps: Isolation and Partial Purification: The process of chloroform partitioning extra isolates as well as partially purifies anthocyanin pigments.

Third steps: Phase Separation: The introduction of chloroform leads to the separation of phases. In the aqueous layer, you'll find anthocyanin, phenolic compounds, sugars, organic acids, and other water-soluble substances. Meanwhile, the bulk phase consists of immiscible organic solvents, lipids, carotenoids, chlorophyll pigments, and various nonpolar compounds.

The Advantages of this method yields an extract devoid of lipophilic contaminants.

As well as Prevention of Pigment Degradation: This method does not involve a concentration step, thereby minimizing the risk of acid-dependent pigment degradation.

Preparation of Onion: Combine approximately 100 g of powdered Onion material with an equal volume of acetone byblender equipped with a stainless steel container for homogenizing also at 500 rpm.

a. **Ratio of mixing Considerations**: An onion powder to solvent (1:1 ratio) for materials rich in pectic substances, a developedpartition proportion of acetone may necessary, set as 1:1.4.

b. **Handling Dried - High-Sugar of Samples**: Dried materials with rich sugar content, the sample was dispersed in water prior to extraction with acetone. Theoverall guideline was to suspend the dried sample with amount of deionized water equivalent to present in the fresh tissue. If a blender is utilized, it should be explosion-proof.

c. **Alternative Mixing Method**: As an alternative, the plant material and acetone can be mixed using a chemical-resistant stir-bar. This method ensures a thorough and safe mixing process.

2. **Extraction Separation**: Toward isolate the anthocyanin extract from the insoluble plant material, begin by filtering the mixture through Whatman no. 1 filter paper. This is done using vacuum suction with a Buchner funnel, which facilitates a clear separation of the anthocyanin extract from the leftover plant debris. This method ensures that the filtrate is free from any solid residues, allowing for a purer anthocyanin solution.

a. **Re-extraction**: Towards re-extract the anthocyanins from the plant material, perform a re-extraction using a 70% (v/v) aqueous acetone solution until you obtain a clear or lightly tinted liquid. If the pH of the plant material is 4 or above, it is advisable to use an acidified aqueous acetone instead. Once the extraction is complete, combine the resulting filtrates and discard the remaining plant material.

b. **Extraction Repetition**: Typically, three consecutive extractions should suffice.

c. **Acidified Acetone**: To ensure the stability of anthocyanins, using acidified acetone is crucial as it keeps the aqueous fraction at a low pH. This acidic environment not only enhances the stability of the anthocyanins but also maintains the chloroform and acetone solvents in an acidic state.

d. **Pigment Stability**: Based on the authors’ experience, the pigments derived from a wide array of plant materials have demonstrated stability in the aqueous acetone extract. Certain potato cultivars that exhibit elevated levels of polyphenol oxidase activity have been found to experience problems with pigment degradation.

d. **Enzyme Inactivation:** This issue can be mitigated by placing the uncapped aqueous acetone extract in a boiling water bath for duration of 5 minutes. Following the heating process, any acetone volume lost due to evaporation should be replenished. This enzyme inactivation step has been deemed unnecessary for the majority of materials.

4. **Filtrate Transfer**: Transfer the filtrate into a separatory funnel and add twice the volume of chloroform. Gently mix the contents by inverting the funnel several times. Allow the mixture to sit overnight at 4°C or until you can clearly see the separation of the two phases. **Small Sample Sizes**: Transfer the filtrate into a separatory funnel and add twice the volume of chloroform. Gently mix the contents by inverting the funnel several times. Allow the mixture to sit overnight at 4°C or until you can clearly see the separation of the two phases.
5. **Aqueous Phase Transfer**: Relocate aqueous phase; represented as upper portionto a 0.5 liter boiling flask. Rotary evaporator was used at 40°C under vacuum to eliminate ruminants acetone/chloroform.
   a. **Anthocyanin Presence**: The presence of anthocyanin pigments in the aqueous phase is reflected in the solution's coloration, which can range from pink to red, purple, or blue.
   b. **Extraction, Isolation, and Purification of Anthocyanins**: For efficient solvent removal, the evaporating flask should be filled to less than half its capacity. Typically, the flask volume should be four to five times the volume of the solvent.
   c. **Evaporation Time**: To minimize pigment degradation, extended evaporation times should be avoided. Ideally, evaporation should be completed within 5 to 10 minutes.

d. **Aqueous Extract Volume Adjustment**: To prepare the remaining aqueous extract, adjust it to a known volume, typically 100 ml, using acidified deionized distilled water. If you plan to analyze the sample within two days, store it at 4°C. For longer storage, which can extend up to a year or more, keep it at -18°C. It's important to avoid repeated freeze-thaw cycles to maintain the integrity of the extract.

Anthocyanin Purification

The purification process is crucial for anthocyanin-containing extracts. This is due to the solvents typically used for extraction done not specifically target and purified anthocyanins as well as **presence of other materials** A significant quantity of other materials was also extracted with anthocyanin and re-concentratedto colored extracts. These materials could potentially affect the stability and analysis of the anthocyanin pigments. There were two maneuvers for purification:

Solid-Phase Extraction: The purification of anthocyanin can be achieved using solid-phase extraction via mini-column (C_{18}); chains bonded on silica, were used in the process for recollecthydrophobic organic compounds; anthocyanin and phenolic, while allowing matrix interferences; like sugars or acid to be castoff as withdrow.

Further Purification: The pigments retained on the mini-columns can be further purified by washing with ethyl acetate. This step helps in removing phenolic compounds that are not anthocyanin.

Table 01: Materials equipped for purification summarized table

Material	Description
Methanol	Absolute
Acidified Water	0.01% HCl: deionized distilled water(v/v)
Aqueous Anthocyanin Extract	Refer to Basic Protocol 1 or Alternate Protocol
Ethyl Acetate	40 C° melting point
Acidified Methanol	0.01% (v/v) HCl in methanol
C18 Cartridge	C_{18} sorbent bonded on silic, Sep-Pak Cartidge (360 mg), ODS-4 Octadecyl Silane (500 mg sorbent), Whatman; or equivalent
Boiling Flask	50- 100-ml capacity and vacuum pump / water aspirator, 40°C
Rotary Evaporator	
Freeze-Resistant Container	Qualified 2 ml slandered tubes

- **Column Initiation process** conditioning a C18 cartridge was achieved via introducing 2 times column volumes of methanol into the sorbent bed.
- **Column acidification** by introducing 3 times column volumes of acidified deionized distilled water into the cartridge with ensures removed residual methanol.
- **Load of anthocyanin extract** into the cartridge via forcing an aqueous anthocyanin extract through the cartridge.
- **Wash cycle** on the cartridge using two column volumes of acidified water. This step aids in the removal of non-adsorbed compounds such as sugars and acids.
- **Rewashing cycle** on the cartridge using 2 times column volumes of ethyl acetate. This step targets the removal of polyphenolic compounds, including phenolic acids and flavonols.
- **Elute the anthocyanin pigments** using acidified methanol and collect the eluent in a 50- to 100-ml boiling flask.
- **Removed methanol** using a rotary evaporator set at 40°C and 50 rpm under vacuum conditions.
- **Redissolve the pigments** in acidified deionized distilled water

- **Purified anthocyanins extractstorage** at 4°C if analysis will be conducted within 24 hours. For longer storage periods, keep the sample at −15°C or lower; at −70°C) in a freeze-resistant container to minimize pigment degradation.

Spectroscopic Characterization of Anthocyanin

Anthocyanin characterization was based on UV-Vis spectra were recorded between 200-600 nm [21], before and after adding 3 drops of 5% (m/v) aluminum chloride in methanol. The sugar position in anthocyanins was determined based on spectral shifts by calculating the ratio of absorbance at 440 nm to the specific absorption maximum (λmax) for each isolated pigment [22].

Results and Discussion

The isolated yield of the compound was 4.74 ± 0.26 g, resulting in 1.08 ± 0.16 g of anthocyanin. The purification process involved 0.76 ± 0.041 partial purification steps, followed by a separation step. The overall recovery percentage was 82.73 ± 1.96%.

Table 02: Yield and recovery of Onion anthocyanin

Isolated Anthocyanin g/kg	Partial Purification steps g/kg	Separation step g/kg	Recovery %
4.74 ± 0.26	1.08 ± 0.16	0.037 ± 0.0041	82.73 ± 1.96

Anthocyanin content depends strongly on both the solvent system and the plant matrix. Ultrasound-assisted methods have demonstrated that parameters such as solvent composition, pH, temperature, and sample-to-solvent ratio must be fine-tuned for each material to maximize yield (23). Using acidified acetone preferentially dissolves anthocyanins while minimizing co-extraction of lipophilic substances (e.g., lipids, chlorophylls), yielding a cleaner pigment fraction and simplifying downstream steps. Omitting evaporation or heating concentration step preserves pigment integrity, since exposure to elevated temperatures or prolonged acidic conditions can accelerate anthocyanin degradation [23-24].

Extracting anthocyanins from onion powder using acidified acetone offers clear benefits. Acetone efficiently dissolves these water-soluble pigments yet leaves behind most nonpolar substances-such as membrane lipids and chlorophyll-thanks to its moderate polarity. The outcome is an anthocyanin-rich extract with far fewer lipophilic impurities, which greatly eases any subsequent purification steps [25]. The decision to skip concentration steps was vital for preserving the stability of anthocyanins. Concentration methods, which often involve heating or evaporation, can lead to significant degradation of these pigments, especially under acidic conditions [1]. By minimizing the exposure of anthocyanins to high temperatures and acidic environments, this approach helps maintain the integrity and bioavailability of these important compounds [25].

This protocol offered strategies aimed at enhancing the extraction process, tailored to the specific characteristics of the active plant material. Consequently, the ratio of plant material to solvent was adjusted based on the abundance of substances known to potentially impede efficient extraction. Similarly, the inclusion of a water pretreatment step was recommended for dried samples or that exhibiting high sugar content, with the goal of facilitating the subsequent extraction with acetone. The chosen mixing method also significantly influenced the efficiency and safety of the extraction process; blending, for example, presented itself as a rapid and efficient technique [26].

The protocol included several extraction cycles, starting with an initial extraction followed by additional re-extractions, and utilized vacuum filtration to enhance the overall efficiency of the process. These methods were specifically chosen to maximize the yield of anthocyanins while reducing both time and resource consumption (27). Additionally, the use of acidified acetone significantly improved the stability of the extracted anthocyanins. Maintaining the integrity of these compounds is crucial for their later analysis or application [28, 29].

The choice of solvent for extracting anthocyanins is crucial as it directly impacts both the yield and purity of the extracts. Although methanol is a widely used solvent, acetone was selected for its effectiveness in producing a more refined extract. Following this, chloroform was applied for partitioning, which aids in removing unwanted impurities. This method is similar to the technique utilized by Solovchenko *et al*. (2010) (30) for recovering anthocyanins from apple peels [3]. The current study found a total anthocyanin content of 37 mg/kg in the red onion samples analyzed. This result is consistent with a range of values documented in previous research, although some studies have reported significantly higher concentrations, such as around 250 mg/kg for cyanidin-3-glucoside, which is the primary anthocyanin present in red onions [31]. The concentration of anthocyanins can vary significantly due to several factors, including the specific cultivar of onion, the part of the plant being analyzed, and the extraction methods used.Gonzalez-de-Peredo *et al*. (2021)[32] reported a notably low concentration of cyanidin at 0.056 mg/g when analyzing red onions using UHPLC [6]. In a similar vein, Park *et al*. (2018) [33] and Oancea *et al*. (2020) [34] found a wider range of anthocyanin yields from various red onion varieties, with values spanning from 0.02 to 0.12 mg/g and 748 to 840 mg/100 g, respectively, depending on the extraction methods employed [7, 8].

Efforts to isolate and characterize anthocyanin pigments resulted in the identification of two distinct compounds. The analysis of Compound 1 showed a peak absorbance at 537 nm, with a shift of -21 nm when aluminum chloride was introduced, which corresponds to the known properties of Peonidin-3-glucoside. In contrast, Compound 2 displayed a maximum absorbance at 519 nm and an AlCl3

shift of -22 nm, aligning with the characteristics of Pelargonidin-3-glucoside.

Table 3: Spectroscopic Characteristics and Identification of Anthocyanin Compounds

Compound No.	λmax (nm)	AlCl3 shift	Peak assignment
1	283-537	-21	Peonidin -3-glucoside
2	265-519	-22	Pelargonidin-3-glucoside

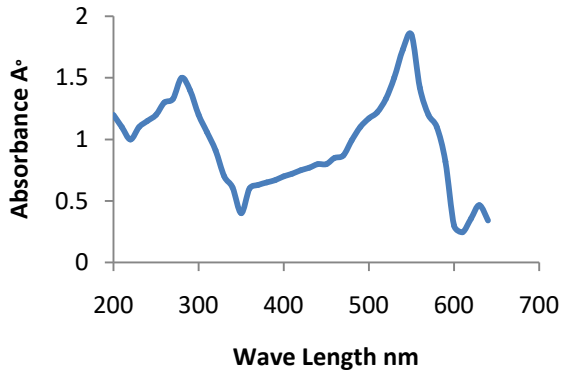

Fig 01: UV-Vis Absorption Spectrum of Anthocyanin Pigments in Anthocyanin Pigments in Red Onion Extract

The spectral analysis of the isolated compounds indicated distinct absorption peaks. For Compound 1, the observed maxima were at 283 nm and 537 nm, whereas Compound 2 displayed peaks at 263 nm and 520 nm. These spectral profiles corresponded with literature values reported for peonidin and pelargonidin, respectively [35]. Furthermore, the absence of a visible color change upon the addition of aluminum chloride, in conjunction with a lack of shift in either absorption band maximum, strongly indicated the presence of ortho-dihydroxy anthocyanins, specifically belonging to the peonidin and pelargonidin classes. This particular test effectively served to distinguish these compounds from derivatives of cyanidin, petunidin, and delphinidin, which were typically characterized by a positive color reaction under these conditions [27]. Analysis proceeded with the calculation of the Abs440/Absλmax ratio for each isolated band. A value of 22 was determined for Band 1 and 24 for Band 2. This specific ratio served as a key indicator of the glycosylation position. According to existing literature, ratios around 22 for peonidin and 24 for pelargonidin typically suggested glycosylation at the 3rd position. Established ratio ranges provided further context: values between 10 and 20 generally corresponded to 3,5-diglycosides, ratios from 20 to 30 indicated 3-glycosides, and values exceeding 30 were indicative of no glycosylation at either position. Based on these findings, the anthocyanin pigments in Band 1 and Band 2, exhibiting Abs440/Absλmax ratios of 22 and 24 respectively, were identified as peonidin-3-glucoside and pelargonidin-3-glucoside [27].

Conclusion

The research presented successfully extracting and purifying anthocyanin from red onions. The modified acetone-chloroform extraction technique proved highly effective, achieving a notable recovery rate of 82.73 ± 1.96%. This method not only yielded a substantial amount of isolated anthocyanin (1.08 ± 0.16 g from an initial 4.74 ± 0.26 g of compound) but also significantly enhanced the purity of the extract by minimizing co-extraction of undesirable lipophilic substances.A critical aspect of this protocol was the deliberate decision to forgo evaporation or heating concentration steps. This choice was instrumental in preserving the integrity and stability of the delicate anthocyanin pigments, which were highly susceptible to degradation under elevated temperatures or prolonged acidic conditions. The strategic use of acidified acetone, coupled with multiple extraction cycles and vacuum filtration, further optimized the process, ensuring both efficiency and the maintenance of pigment quality.Through meticulous UV-Vis spectroscopic analysis, the confidently identified and characterized the two primary anthocyanins. Compound 1, with its peak absorbance at 537 nm and a characteristic -21 nm shift upon aluminum chloride introduction, was confirmed as Peonidin-3-glucoside. Similarly, Compound 2, showing a maximum absorbance at 519 nm and a -22 nm AlCl3 shift, aligned perfectly with the profile of Pelargonidin-3-glucoside. The calculated Abs440/Absλmax ratios of 22 for Peonidin-3-glucoside and 24 for Pelargonidin-3-glucoside further corroborated their identification as 3-glycosides, consistent with established literature.While the total anthocyanin content found in our red onion samples (37 mg/kg) falls within the documented range, acknowledging the variability influenced by cultivar, plant part, and extraction methods, our study's primary contribution lies in the optimized purification methodology. This streamlined approach offers a robust and efficient pathway for obtaining high-purity anthocyanin fractions. Such advancements are pivotal, underscoring the considerable promise of these specific anthocyanins for diverse applications across food science, nutraceutical development, and pharmaceutical formulations, paving the way for their broader utilization in health and industry.

Conflict of Interest

The authors declare no conflict of interest.

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