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Laboratory Experiment

Resin Assisted Purification of Anthocyanins and Their Encapsulation

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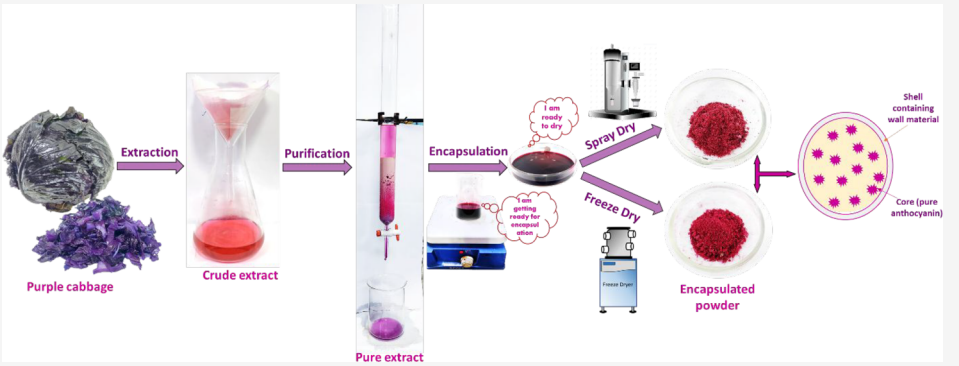

ABSTRACT: A procedure to purify anthocyanin, a natural pigment, following a green approach by using macroporous resin and its encapsulation for the maintenance of its stability is described. This laboratory experiment, which describes the benefits of using resin for the purification, is a learning tool for students working in the field on nutraceuticals. The resin can be used several times by the students as it follows an adsorption−desorption technique. The whole experiment was divided into two sections: the first is the purification, and the second part deals with encapsulation. Students successfully purified the anthocyanins using XAD-16 resin from the solvent extract of purple cabbage. Afterward, encapsulation of purified anthocyanin in maltodextrin was done using a freeze-drying and spray-drying technique. Feedback from students clearly indicates that the laboratory practical session indeed helped them to learn the greener approach of purification and encapsulation using industrial techniques.

KEYWORDS: *Graduate and Postgraduate Students, Anthocyanins, Adsorbent Resin, Freeze-Drying, Spray-Drying, Encapsulation, Laboratory Demonstration*

INTRODUCTION

Purification for students working in the field of nutraceuticals and natural products chemistry is of prime importance. A greener chemistry approach is in general more attractive as it takes care of sustainability and the environment. The disposal of used silica gel (commonly used) and the use of organic solvents and their disposal are of concern for any laboratory, and it is very important for students, who will be researchers in the future. Learning greener techniques is an added advantage for any student. Further hands-on training on the subject strengthens the concept and their belief.

Anthocyanins, a natural pigment, are a source of natural colorant for the food industry as well as for the nutraceutical industry. The unique property of this group of plant pigments, i.e., the pH-dependent color profile, has been successfully demonstrated as a simple and impressive experiment for high school and undergraduate students to teach the fundamentals of chemistry of pigments.[1,2] It is of more interest to the graduate and postgraduate students to learn the purification techniques of anthocyanins from the crude plant extracts. The interest in purified anthocyanins is not only their use as a natural food colorant, but also their excellent antioxidant properties.[3,4] With the increasing demand for replacement of artificial food colorants, the future market potential of natural plant pigments like anthocyanins in their purified form is very high. Furthermore, in the post-COVID situation, the additional benefit of immunity boosting antioxidant properties of anthocyanins is also captivating. Conventional extraction methods of anthocyanins provide coextractives *viz.* free sugars, phenolics, sugar alcohols, organic acids, copigments, proteins, etc. from the plant parts which reduce the yield significantly.[5] Some of these impurities affect the stability of extracted anthocyanins remarkably.[6] Apart from that, the stability of extracted anthocyanins is severely affected by several external factors like pH, temperature, light, oxygen, etc.[7] Therefore, priority should be given to purification of the crude extracts of anthocyanins to remove a majority of the coextractives and to

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protect the purified anthocyanins by encapsulation for further use. This paper demonstrates a simple yet effective learning method to purify a crude anthocyanin extract followed by encapsulation techniques of the purified anthocyanins.

Resin assisted purification is one of the industrially scaled techniques to purify natural products.[8] Some of these impurities affect the stability of extracted anthocyanins remarkably.[6] Apart from that, stability of extracted anthocyanins is severely affected by several external factors like pH, temperature, light, oxygen, etc.[9] Encapsulation is a common process to entrap active substances within a carrier material. It is a useful method to improve delivery of active ingredients and also to protect them from the external environment by creating a physical barrier, thus improving shelf life.[10,11] A number of encapsulation techniques are reported in the literature, e.g., spray-drying, spray chilling, freeze-drying, extrusion coating, inclusion complexation, coacervation, liposomal entrapment, cocrystallization, fluidized bed coating, nanoencapsulation, etc. Each of these techniques has its own advantages and drawbacks, and selection of the encapsulation technique should be based on the purpose of its use. Inherent instability of isolated anthocyanins and their susceptibility to degradation have been addressed by various encapsulation techniques.[12] As anthocyanins are intended to be used as natural food colorants and nutraceuticals, one or more food grade biopolymers have been employed as their encapsulants in most of the studies. Encapsulation of anthocyanins mainly within polysaccharides, *viz*. starch, cellulose, gums, maltodextrin, cyclodextrin, pectin, inulin, chitosan, alginate, etc., and protein matrices have been reported by various techniques as mentioned earlier.[12−14] Among these, freeze-drying and spray-drying are the most explored techniques for encapsulation of thermosensitive compounds like anthocyanins.[15,16] In both the techniques, anthocyanins are solubilized in an aqueous dispersion of biopolymeric encapsulants. Anthocyanins get entrapped within the biopolymer matrix by quick sublimation of water molecules from a frozen sample in the freeze-drying technique, whereas, in the case of spray-drying, rapid evaporation of water enables atomization of liquid samples into solid powder by maintaining a low temperature. The second part of the present experiment describes both these archetypal techniques of anthocyanin encapsulation.

Purification is an important step for any chemist especially for a natural product chemist or food technologists, where it is being performed by resins. It is an environmentally benign technique as well as a quick regeneration for a high output of target compound. A major advantage of using resins is that, apart from fewer environmental hazards, these materials can be regenerated with a minor treatment and can be used as many times as possible. Senior graduate students pursuing their degree in the field of organic chemistry, food technology, or material chemistry can learn this technique and use it for their future projects.

The aim of this publication is to generate interest among students through a learning experience about the purification and encapsulation of natural compounds/nutraceuticals with the example of anthocyanin. Students can adopt these techniques to facilitate their own research work. Our target is also to introduce the strength and opportunities of anthocyanin purification and encapsulation techniques in students' curious minds which they can avail in well-equipped chemistry laboratory.

PEDAGOGICAL GOALS

This laboratory experiment was designed and formulated through a collaborative effort from faculty of Agricultural Chemicals, ICAR-Indian Agricultural Research Institute, New Delhi, India, with input from students of the discipline. The experiment was designed to keep the skill and knowledge level of the students as a background, and it was formulated to improve skills and academic engagement.

Students of organic chemistry/medicinal chemistry, who are involved in courses where purification of natural products is important, food technology, where encapsulation of bioactive molecule(s) is important, and material chemistry, where resin assisted purification is being taught, are the major stakeholders of these techniques.

To help students in their understanding, courses/lectures involving chemistry of anthocyanin, encapsulation techniques, and resin assisted purification are prerequisite before attending the practical sessions. For better understanding, related lecture notes need to be visited.

Aims and Learning Outcomes

Students who attended this laboratory practical are well-versed in the purification of synthetic as well as natural products. During their coursework in M.Sc./Ph.D. programs in Agricultural Chemicals, it was taught to them. The basic concept and theoretical part of the purification technique were generally taught in the advanced course. However, students were not capable of handling the experiment in the laboratory. With this background, the experiment was started and demonstrated. Students from organic chemistry, food technology, as well as material chemistry also learn these techniques for purification, and the same will be useful in their future research endevors.

The pedagogical aim of the present experiment can be enlisted as follows:

- Hands-on experience with handling the macroporous resins starting from its activation, loading, and elution
- Exposing them to sorption mechanism, where time and ratio of adsorbent and adsorbate
- Recovery of resins after purification by proper cleaning
- Familiarizing students with the procedure of the encapsulation technique

After successful completion of the practical class, students must able to

- Use microporous resins for purification of different group of compounds especially natural compounds
- Calculate the ratio and time required for the complete adsorption or equilibrium concentration
- Recover the resin material after proper cleaning
- Encapsulate any compounds which are having stability/any other issue(s)

MATERIALS AND METHODS

Materials for the experiment follow: purple cabbage (*Brassica oleracea* var. *capitata* f. *rubra*), ethanol (Millipore Sigma, CAS: 64-17-5), hydrochloric acid (Merck Life Science Pvt. Ltd., CAS: 7647-01-0), XAD-16 (Millipore Sigma, CAS: 104219-63-8), maltodextrin (Merck Life Science Pvt. Ltd., CAS: 9050-36-6).

EXPERIMENTAL PROCEDURE

The whole experiment was divided into two separate experiments. Two experiments were done in two different practical classes. Students were divided into four different groups to

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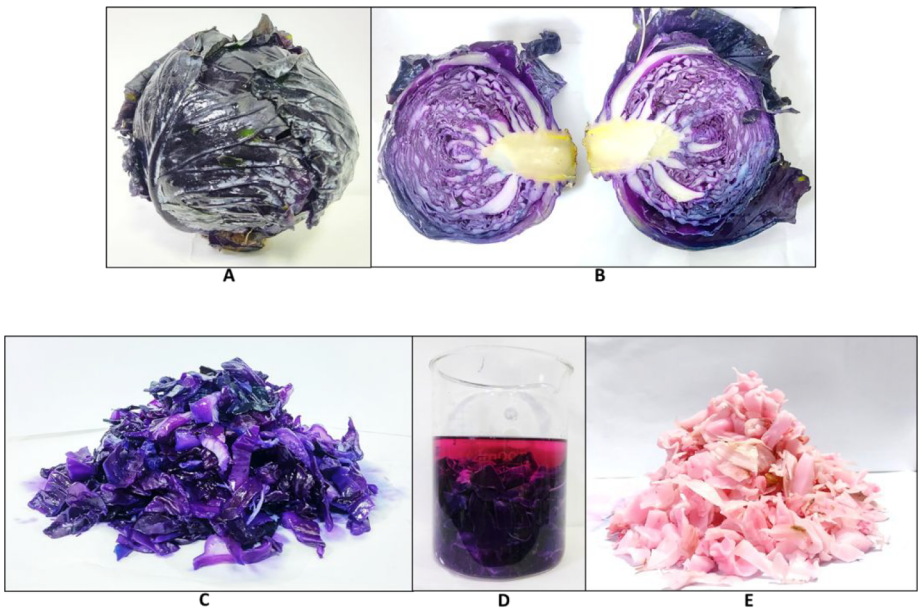

Figure 1. Purple cabbage (A, B). Chopped purple cabbage (C). Addition of acidified ethanol (D). Colorless residue material after complete extraction (E).

accommodate all the students as well as to expose students to hands on training for better understanding and academic engagement.

General Procedure

Experiment 1. *Extraction of Anthocyanins.* Chopped purple cabbage (196.2 g) was taken in a beaker (1 L), with acidified ethanol containing 0.1% HCl (500 mL) added to it as the extracting solvent, and placed into a bath sonicator for half an hour in the dark (Figure 1). After that, the extract was collected through filtration, and fresh solvent (500 mL) was again poured into the beaker; the process was continued until the material became colorless. Extracted fractions were combined, filtered, and concentrated by a rotary evaporator (at 35 °C temperature and 60 mbar pressure) to get a viscous liquid (7.84 g).

Purification of Anthocyanins. A macroporous polymeric adsorbent resin XAD-16 was selected for the demonstration. The resin was activated by thorough washing with distilled water at least two to three times, followed by ethanol. Finally, a thorough washing with distilled water was executed, and the resin was stored by submerging it in distilled water. The activated resin was packed in a glass column (45 cm length × 3.5 cm diameter) up to around 1/3rd of the column height. A sufficient amount of the crude anthocyanin rich extract was loaded onto the column by dissolving it in a minimum quantity of distilled water so that it could distribute uniformly into the resin. Sufficient time was provided for binding of anthocyanins to the polymer adsorbent resin by keeping the column undisturbed for 2 h without exposure to direct light. Then the column was washed thoroughly with an excess amount of distilled water to remove the unbound coextractives like sugar and other impurities. Finally, the adsorbed anthocyanins were eluted from the column by a thorough passing of acidified ethanol (0.1% HCl) until the elute becomes colorless (Figure 2). The eluted ethanolic fraction was concentrated by a rotary evaporator until complete removal of ethanol, and purified extract (0.323 g) was obtained. The obtained residue was then lyophilized to get purified anthocyanin crystals.

Detection of Anthocyanin. The color of anthocyanins is pH-dependent, and thus it is very easy to detect/identify whether a natural color is due to anthocyanin or something else. The color-changing property is mainly due to the ionic nature of anthocyanin.[17] Mostly, in acidic conditions anthocyanins are reddish in color, pink in neutral conditions, and blue in basic conditions. The pH-dependent structural change of anthocyanin is attributed to its structural changes at different pH values. At pH 1−3, it forms flavylium cation, whereas at pH 4−5, anthocyanins exist as pseudobases, and these have quinonoidal structures at pH 6−7, which are then transformed into a chalcone structure at pH 8−9.[18] So, upon changing the pH from 3 to 8, the color changes from reddish to bluish, which is the indicator of an anthocyanin structure, and that is why it is being used as the pH indicator.

Experiment 2. *Encapsulation of Anthocyanins.* Removal of solvent from any liquid formulation can be done by different techniques and freeze-drying or lyophilization is one such technique. Sublimation of frozen solvent at reduced pressure followed by desorption of the unfrozen solvent is the basic principle of the technique. First, the liquid solvent is frozen to a solid, and then the solid solvent is sublimed at reduced pressure. This technique is mainly applicable for those materials where degradation of compounds/formulation at a higher temperature or under other conditions is possible. Similarly, spray-drying is another drying technique for liquid formulations. The spray-drying technique involves transformation of the liquid formulation into powder material by passing it through a hot, dry medium. The output of spray-dried material depends on the nature of the feed materials, and it can be a powder, or an agglomerate with a granular structure. This technique is very commonly used in industrial purposes, and it is applicable for all kinds of materials except those which are sensitive to high temperature.

Purified anthocyanin crystals (0.323 g) and maltodextrin (2.584 g) were mixed (at 1:8 ratio) with 10 mL of distilled water. The mixture was stirred with the help of a magnetic stirrer for 30 min to get a slurry (Figure 3A,B). Half of the slurry was frozen at −20 °C followed by freeze-drying at −80 °C and 0.08 mbar

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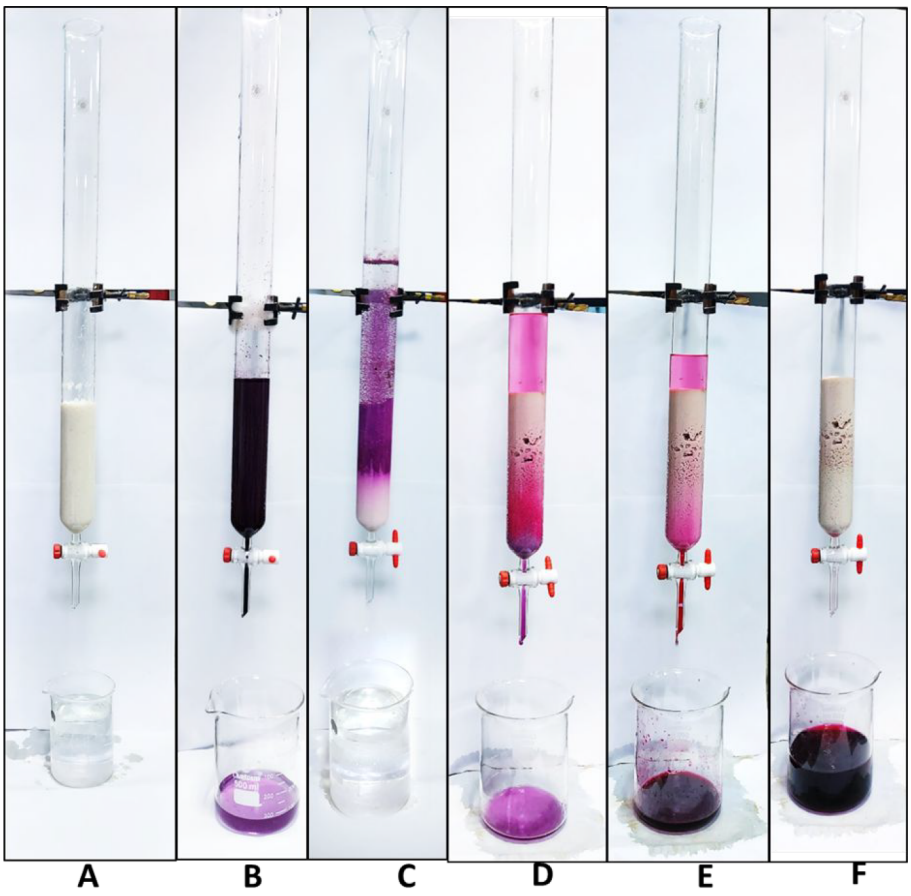

Figure 2. Column loaded with activated XAD-16 resin (A). Loading of crude anthocyanin extract (B). Washing with distilled water for removal of unbound compounds (C). Elution of anthocyanins by acidified ethanol (D, E). Complete elution of bound anthocyanins (F).

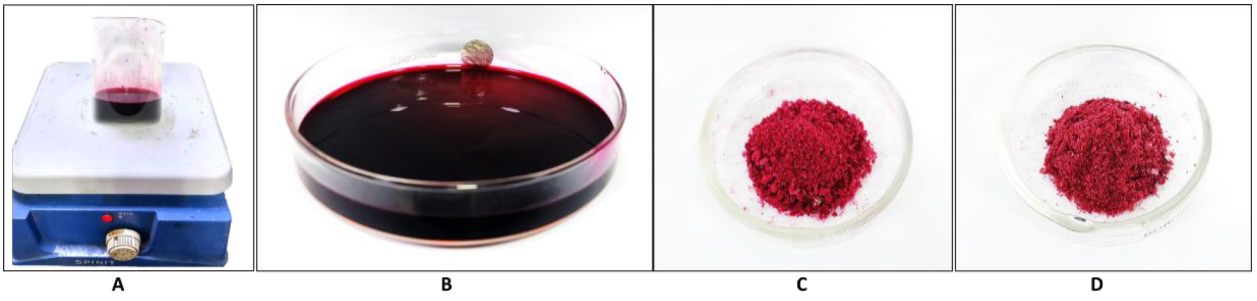

Figure 3. Stirring a mixture of anthocyanin and maltodextrin in aqueous media (A). Stable slurry (B). Freeze-dried encapsulated powder (C). Spray-dried encapsulated powder (D).

pressure. The freeze-dried encapsulated anthocyanin rich powder was preserved in an airtight container and stored in a desiccator at −20 °C (Figure 3C). The other half of the slurry was fed into the spray-drier where it was nebulized with pressure ranging from 1.97 to 2.38 kg cm^{-2} under vacuum (184 mm Hg). The inlet temperature was maintained at about 151.4−170 °C, and the outlet temperature was about 61.7 °C. From the cyclone chambers, the resultant powdered formulation was collected, combined, and stored in a sealed container at −20 °C (Figure 3D).

Hazards and Safety Precautions. All the experimental steps were carried out after wearing personal protective equipment (PPE), including safety gloves and goggles. All of the chemicals used for this study were noncarcinogenic, nonmutagenic, and nonreprotoxic. A fume hood should be used while using concentrated hydrochloric acid (HCl) though a very diluted percentage is used in the experiment. Proper handling and maintenance of instruments like a spray-drier and freeze-drier should be done under the supervision of an experienced and prudent person. With the photosensitive and antioxidant nature of anthocyanin, precautions such as the use of amber colored glassware, use of diffused light during the experiment, and quick execution of the experiment due to chance of losses due to oxidation were taken properly and explained to students in detail.

RESULTS AND DISCUSSION

Anthocyanin Extraction

The conventional method of anthocyanin extraction from plant parts is solid−liquid extraction using organic solvents.[19,20] In

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most of the studies, acidified aqueous ethanol or methanol was employed for extraction of anthocyanins.[6,8] It was observed that addition of a small amount of acid to the solvent increases the extraction efficiency of anthocyanins as they are more stable as flavylium cations at lower pH (<3). However, at too low a pH, acylated anthocyanins undergo hydrolysis.[21] In this study, an ultrasonication assisted method was employed for extraction of anthocyanins from purple cabbage using acidified (0.1% HCl) ethanol as a solvent. Ultrasonication helped in quick extraction of anthocyanins from the cells by the cavitation process. With successive batch addition of solvent, more and more anthocyanins from purple cabbage were extracted as observed from Figure 4, where the change of color of the extract was noted

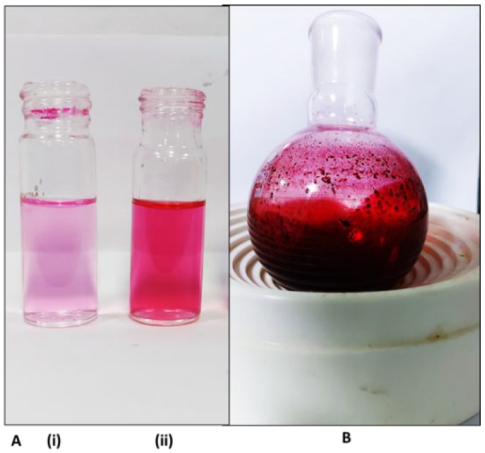

Figure 4. Initial extract (A, i). Final extract (A, ii). Concentrated crude extract (B).

starting from the initial batch of solvent addition to the final pulled extract. The physical yield of the concentrated crude extract was recorded as 39 g kg^{-1} fresh weight.

Anthocyanin Purification

To remove the coextractives like sugars, phenolics, proteins, etc. from the crude anthocyanin extract, an adsorptive purification technique has been exemplified in this study using XAD-16, a polymeric adsorbent resin. Activation of the resin is a prerequisite step before purification study. Washing of the resin with distilled water and ethanol was done to remove the preservatives used to store the resin and the previously adsorbed polar organic compounds, if any. This practice opens up the adsorption sites of the microporous resin, thus provides opportunity to the anthocyanin molecules to get adsorbed on those active sites. XAD-16 promises to be a good sorbent and offers high chemical stability as its surface is modified with polystyrene moiety. The polymeric functionalized surface of XAD-16 attributes hydrophobic interactions and hydrogen bonds with anthocyanins to occupy the active site more preferentially than other impurities like sugars. Moreover, the appreciable surface area of about 800 m^2 g^{-1} of this macroporous resin also contributed to favorable adsorption of anthocyanins. In Figure 2C, it was clearly observed that the selective binding of anthocyanins to the adsorbent resin was so strong that washing with water could not able to elute the resin-bound anthocyanins. However, coextractives like sugars can be easily removed by water due to their aqueous solubility and noninteraction with the resin. It can be clearly confirmed by the positive Fehling's test of the colorless elute. After complete removal of the coextractives by a continuous water wash, the adsorbed anthocyanins could be eluted from the resin by acidified ethanol. Figure 5 compares the color of the anthocyanin extract

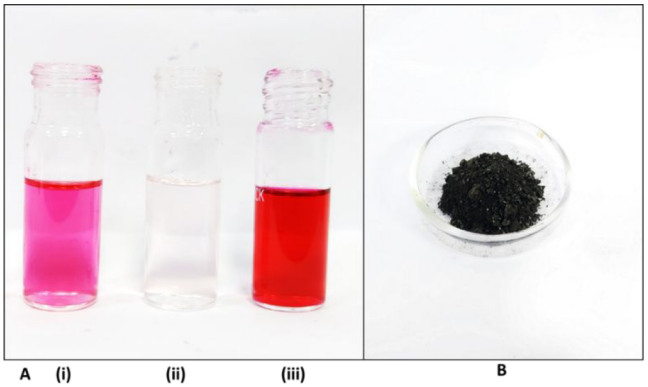

Figure 5. Crude extract (A, i). Water wash from anthocyanin loaded resin during purification (A, ii). Acidified ethanolic elute from anthocyanin loaded resin during purification (A, iii). Purified anthocyanin crystal after lyophilization (B).

before resin purification (Figure 5A,i), water wash (Figure 5A,ii), and resin purified anthocyanin extract (Figure 5A,iii). Further, the lyophilized anthocyanin extract after purification appeared to be a crystalline powder instead of a viscous semisolid (Figure 5B), emphasizing the removal of sugars from the crude extract. The yield of the purified anthocyanin crystals was recorded as 1.31 g kg^{-1} fresh weight, i.e., only 3.36% of the crude extract. It indicated the removal of impurities from the crude extract.

A confirmatory test was performed via HPLC by analyzing crude and purified extract samples of the same concentration with respect to anthocyanin content. In the case of crude extract, the area under the curve of four major anthocyanin peaks detected at 520 nm was 181604 AU, whereas for pure extract of the same concentration, the area under the curve of the same four peaks was 851558 AU (Figure 6), suggesting almost 4.7 times enrichment of the extract with pure anthocyanins. The difference in color of the same concentration of both the extracts and the prominence of the peaks of anthocyanins in the purified extract as well clearly indicated the efficiency of resin purification of crude anthocyanin extract.

Anthocyanin Encapsulation

Encapsulation helps to prolong the shelf life of purified anthocyanin rich extract. The coating materials play an important role in the stability of anthocyanin since they influence the physicochemical properties of the encapsulated material. The ability to form a film, resistance to gastrointestinal tract, biodegradability, low price, etc. are the qualities of good encapsulating agents.[22,23] Maltodextrin has been chosen as the encapsulating material of anthocyanins for this study because of its solubility in water, gel formation properties, and low viscosity.

Among different encapsulation methods, spray-drying and freeze-drying have been commonly used due to their significant merits in terms of reductions in product volume, storage space and transportation cost, simplicity of process, ease of handling, wide availability of equipment facilities, and high stability of finished product due to the low moisture content. Between these two techniques, freeze-drying is being used more extensively where there are chances of degradation of the active ingredient and/or formulants.[24]

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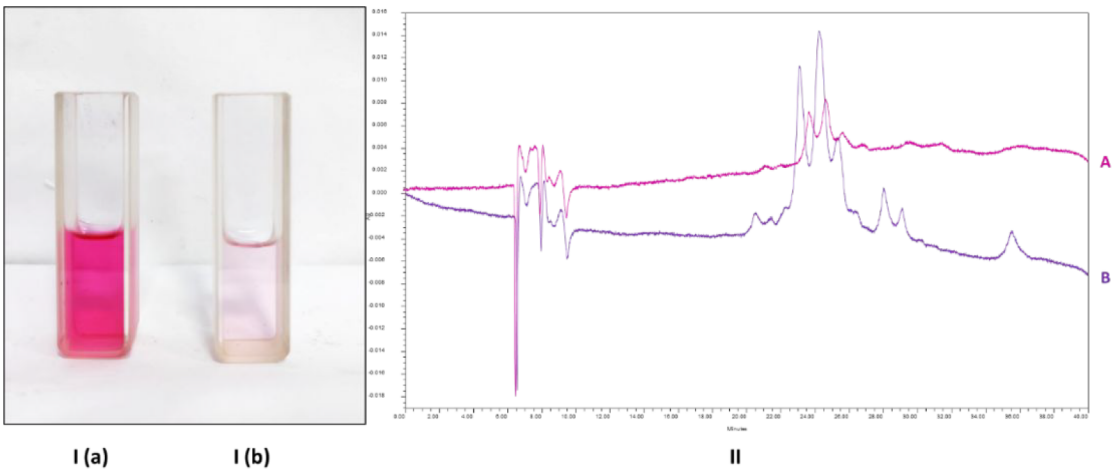

Figure 6. Color difference between purified extract (I a) and crude extract (I b), same concentration. HPLC chromatogram of crude extract (II a) and purified extract (II b).

Freeze-drying and spray dying are two relatively simpler and less cumbersome encapsulation techniques as compared to others.[25,26] Therefore, in the present study these two simple techniques were chosen for demonstration. Few studies reported the use of maltodextrin as a carrier in freeze-drying and spray-drying encapsulation techniques.[27,28] During the primary phase of freeze-drying, under reduced pressure and heat supplied through radiation or conduction, frozen water gets sublimed to water vapor leaving behind the solid encapsulated particles of the sample. During secondary drying, the residual bound water molecules are removed by isothermal desorption from the sample. On the other hand, in the spray-drying technique, a quick flush of air was used to evaporate the liquid during atomization, leaving behind the dried capsules. The mixture to be fed into the spray-drier is ensured to be stable enough to withstand the high energy process. In both the techniques, anthocyanin molecules were encapsulated within maltodextrin, which ultimately formed a dry layer as a shell or a coating over the core by quick evaporation of water molecules from the surface. The shell ultimately protected the core containing anthocyanins from the outer environment by forming a barrier. Purified anthocyanin crystals, maltodextrin (encapsulant) and encapsulated anthocyanins obtained by two encapsulation techniques has been illustrated in Figure 7. If a lyophilizer is not available in the laboratory, then the spray-drying technique needs to be adopted provided the drier is available.

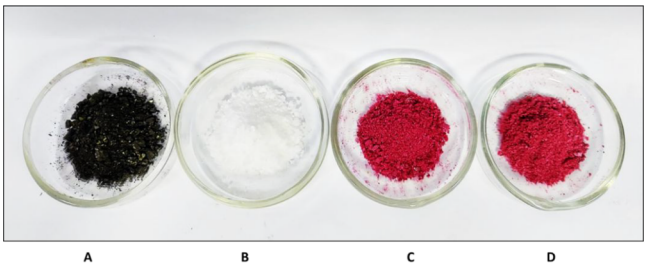

Figure 7. Purified anthocyanin crystals (A). Maltodextrin (B). Freeze-dried encapsulated anthocyanin powder (C). Spray-dried encapsulated anthocyanin powder (D).

Student Perceptions

Both the experiments were conducted in the laboratory, followed by feedback analysis which was done as a part of a pedagogical step. A total of 20 students were engaged in the study out of which 12 were pursuing an M.Sc. in Agricultural Chemicals and rest were pursuing a Ph.D. in Agricultural Chemicals. Although it was done with the students of Agricultural Chemicals, students from other related branches as well can learn, especially those studying disciplines like organic chemistry, food technology, as well as material chemistry. Questionnaires answered by the students of the Agricultural Chemicals division were compiled and analyzed for the better understanding of the students' point of view. The questionnaire items asked whether the experiment helped students to better understand the extraction, purification, and encapsulation of any bioactive compound. It further raised the point regarding whether the demonstration enabled them to replicate the work in their laboratory or not. The responses were evaluated using the ten-point Likert scale as shown in Figure 8. Among 20 students, 65%, 60%, and 55% strongly agreed that they could replicate the extraction, purification, and encapsulation techniques respectively in their laboratory. On the other hand, 65% students were confident they could use the same techniques for other plant sources. Above all, the demonstration improved the preexisting knowledge of 80% of the students. In a nutshell, this demonstration was helpful, interesting, and likely replicable, and it improved their concepts on the mentioned techniques.

CONCLUSION

The present experiment offers a very good and a greener alternative to purification of anthocyanins, that is available in the literature. Students are able to use macroporous resins for purification including its activation, loading, and elution. Recovery of the resins after purification is the key learning step, which is otherwise not taught in the common laboratory experiments. Learning from the encapsulation experiment using industrial techniques is unique in the sense that these students are devoid of this exposure. Interaction with students and their feedback reveal the narrowing down of the knowledge gap among all the students irrespective of their knowledge level. The learning of masters and as well as Ph.D. students reveals the practical material as ideal for laboratory experimentation in the

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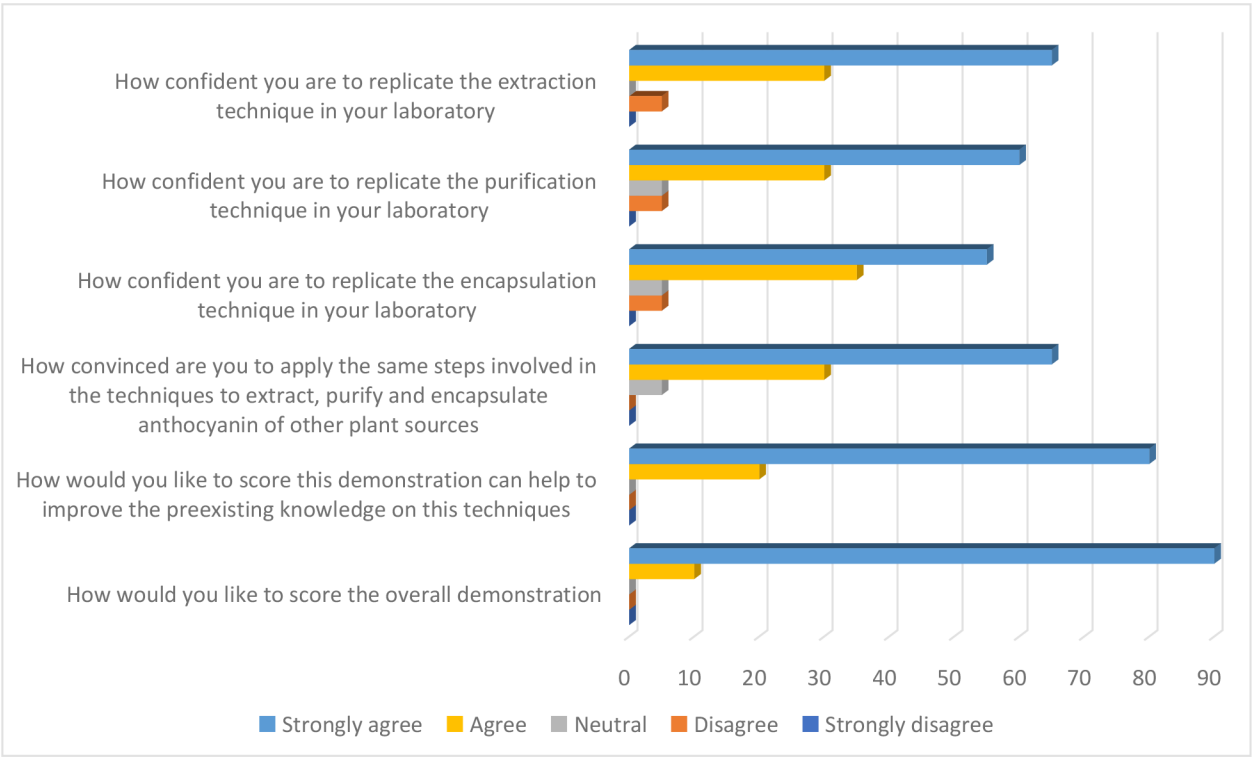

Figure 8. Students' evaluation of the demonstration based on the ten-point Likert scale.

advanced course on natural products/nutraceuticals. The described method will surely encourage students and teachers as well to adopt it in their chemistry/biochemistry lab while encountering the similar problems associated with natural products.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available at https://pubs.acs.org/doi/10.1021/acs.jchemed.2c00918.

Video showing the methodology (MP4)
Questions for students (PDF, DOCX)
Information for instructors (PDF, DOCX)
Information for students (PDF, DOCX)

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Notes

The authors declare no competing financial interest.

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