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Solubilization of Water Soluble Anthocyanins in Apolar Medium Using Reverse Micelle

SEOK JOONG KIM,† YOUNG-HEE CHO,‡ WHANSOO PARK,‡ DAESEOK HAN,‡ CHANG-HOON CHAI,§ AND JEE-YOUNG IMM*,§

Department of Food Science and Technology, Catholic University of Daegu, Hayang 712-702, Korea, Korea Food Research Institute, Songnam 463-746, Korea, and Department of Food and Nutrition, Kookmin University, Seoul 136-702, Korea

Crude anthocyanins extracted from grape skin were solubilized in hexane containing 100 mM bis-(2-ethylhexyl)sodium sulfosuccinate (AOT) by forming stable reverse micelles (RMs). Anthocyanins solubilized in RMs showed about four times greater color intensity than that in aqueous medium. The color intensity of anthocyanins in RMs was primarily affected by the interaction between sulfonate head of AOT and flavylium cation of anthocyanins. The molar ratio of water to AOT (Wo) also influenced the color properties. As the Wo increased from five to 20, the color intensity increased and resulted in a bathochromic shift. This result suggests that increased micelle size facilitates complexation between AOT and flavylium cation. The color stability of anthocyanins in RM was higher than that of buffered anthocyanins during the storage at 30 °C. The current study might be utilized as a model system to predict color properties of anthocyanins in apolar medium.

KEYWORDS: Anthocyanins; reverse micelle; AOT; complexation; color

INTRODUCTION

Anthocyanin is one of the most widely distributed water soluble pigments responsible for pink, red, and purple color in fruit and vegetables (*1*). Anthocyanins are usually present as glycosides of polyhydroxy and polymethoxy derivatives and the number of sugar, hydroxyl, and methoxyl groups and the location of glycosylation affect color expression of anthocyanins (*2*). Anthocyanins are frequently used as a natural colorant in various food products such as juice, wine, candy, jelly, sauce, and cake (*3*). Recently, anthocyanins are regarded as biologically active substances because of their strong antioxidant activities, which are able to reduce the risk of cancer and heart disease (*4*, *5*).

Despite attractive color and pharmaceutical potential, low stability of anthocyanins limited their application as a food colorant (*6*). The stability of anthocyanins is affected by various factors such as pH, temperature, oxygen, ascorbic acid, light, metals, and their combinations (*7*). In addition, copigmentation, a molecular interaction between anthocyanins and variety of other compounds including polyphenols, amino acid induced changes in color expression and stability (*8*).

Up to the present, most studies done on anthocyanins were mainly focused on stability and processing aspects of anthocyanins in aqueous media (*9*, *10*) and no reports are available for the color expression of anthocyanins in apolar environments.

In this paper, RM system was used to characterize color expression of anthocyanin in an apolar medium since RM allows incorporation of water soluble anthocyanin into organic solvent. In the RM system, anthocyanin was successfully solubilized into hexane in the form of microemulsion.

Considering synthetic colorants become replaced by natural colorants, the demand for natural colorants in food products will continuously increase. A better understanding of the behaviors of anthocyanins in an apolar medium provides insight to control its stability in a lipid soluble environment and possibly allow us to use water soluble pigment in the lipid media such as oil and salad dressing. Therefore, the objectives of this study were to examine changes in color characteristics of anthocyanin in an apolar medium and propose factors affecting color properties.

MATERIALS AND METHODS

Chemicals. Surfactants such as AOT, CDAB, Tween 80, and SDS were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were high-performance liquid chromatography (HPLC) or analytical grade and purchased from Fischer Scientific (Springfield, NJ) otherwise stated.

Extraction of Crude Anthocyanins. Fresh grape (*Muscat bailey* A) was obtained from a local supermarket (Seoul, Korea), and grape skins were collected and frozen at −70 °C until anthocyanin extraction. Anthocyanins were extracted from grape skins with methanol containing 3% formic acids (methanol:formic acid:water = 70:3:27, v/v/v). The extract was filtered through Toyo no. 2 filter paper (Toyo Inc., Japan) and was concentrated by a rotary evaporator (Büchi Rotavapor R-124, Germany). The concentrated anthocyanin stock solution was distributed

* To whom correspondence should be addressed. Tel: +82-2-910-4772. Fax: +82-2-911-4771. E-mail: jyimm@kookmin.ac.kr.
† Catholic University of Daegu.
‡ Korea Food Research Institute.
§ Kookmin University.

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in eppendorf tubes and was frozen until use. Prior to the experiment, the stock solution was thawed in a refrigerator and then was filtered through a 0.45 μm poly(vinylidene difluoride) (PVDF) syringe filter (Whatman International Ltd., England) to exclude any particulate matter and risk of microbial growth.

Preparation of RM. RMs were prepared by the injection method (*11*). Surfactant (AOT, 100 mM) was dissolved in hexane, and the anthocyanin stock solution was dropped into hexane using a syringe and vigorously stirred until it became a transparent single phase.

Visible Spectroscopy. The anthocyanin stock solution was dissolved in McIlvaine buffer (50 mM citric acid−Na_2HPO_4, pH 3.0) and incorporated into RM. The amount of stock solution incorporated in RM was adjusted to give an initial absorbance reading at 534 nm (λ_{max}) of 1.0−1.2. The absorption spectra of samples at various conditions were monitored in the visible range from 400 to 700 nm with a Jasco SSE-343 spectrophotometer (Jasco International Ltd. Co., Japan). All sample preparations and measurements were done in triplicate.

Color Intensity of Anthocyanins Solubilized in RM. The color intensities of anthocyanins in the McIlvaine buffer (pH 3.0) and RMs were measured as absorbance values at λ_{max}. For the comparison of color intensity, the equal volume of anthocyanin stock solution was added to the buffer or hexane containing 100 mM AOT, respectively. To examine the effect of water content in RM on color intensity, RMs with different molar Wo ratios from 5 to 20 were prepared. The desired Wo was obtained by injection of McIlvaine buffer (pH 3.0). Thus, all RM samples contained a fixed amount of anthocyanin stock solution and 100 mM AOT.

The effect of micelle number on color intensity was also examined at a constant Wo of 10. To make RMs with different micelle numbers, the concentrations of AOT (50−200 mM) and water (500−2000 mM) were increased with equal portions (the hydration degree was constantly kept).

Interaction between Anthocyanins and AOT. To examine the interaction between AOT and anthocyanins, surplus AOT (1 M) was added to 10 mL of biphasic hexane−water solution (1:1, v/v) where anthocyanin was solubilized in the water phase. After the mixture was vortexed, the movement of anthocyanin from water to hexane phase was observed at room temperature for 24 h.

The effect of AOT concentration (0−200 mM) on color intensity was examined in ethanol solution (20, 30, and 50%) containing anthocyanins. Effects of other surfactants such as CDAB, Tween 80, and SDS on color intensity were also monitored in 50% ethanol solution.

Storage Stability of Anthocyanins Solubilized in RM. The stability of anthocyanins solubilized in the buffer or RM was determined in a 30 °C incubator (Vision Scientific, Korea) for 14 days. The color stability was expressed as the residual percentage of absorbance at λ_{max} after the designated time interval. For the stability measurements, the samples in screw-capped amber vials (10 mL) were sealed with Parafilm (Whatman International Ltd.) and then placed in the incubator. There were no changes in the volume of samples after the storage.

Statistical Analyses. Data were analyzed by analysis of variance (ANOVA) using SigmaStat Version 2.01 (SPSS Inc., Chicago, IL). The Student−Newman−Keuls *t*-test was used for multiple comparison of the treatment means at the significance level of $P < 0.05$.

RESULTS AND DISCUSSION

Solubilization of Anthocyanins in RM. A transparent red-colored solution was obtained by the addition of anthocyanin stock solution into hexane containing AOT (**Figure 1c**). It suggests that water soluble pigment can be uniformly solubilized in apolar medium using the RM system. On the basis of the structure of RMs, anthocyanins were incorporated into polar cores formed by surfactant dissolved in the solvent. When the same amount of anthocyanin stock solution was added either to hexane or to McIlvaine buffer (pH 3.0), the color intensity of the RM solution (**Figure 1c**) was much greater than that of the buffer (**Figure 1a**). On the other hand, the anthocyanin stock solution was directly precipitated to the bottom of the vial when surfactant was not present in the hexane phase (**Figure 1b**).

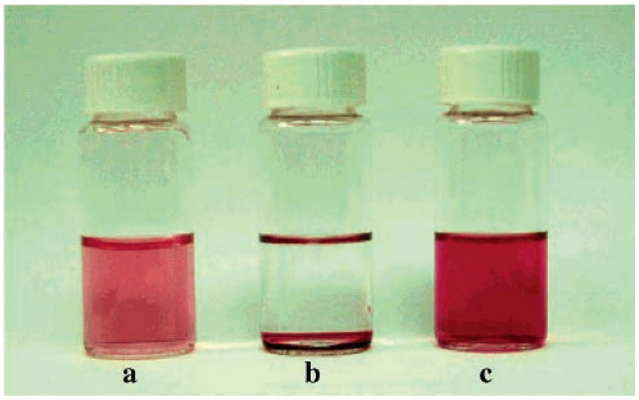

Figure 1. Anthocayanins solubilized in different medium. (**a**) McIlvaine buffer (pH 3.0), (**b**) hexane, (**c**) hexane containing 100 mM AOT.

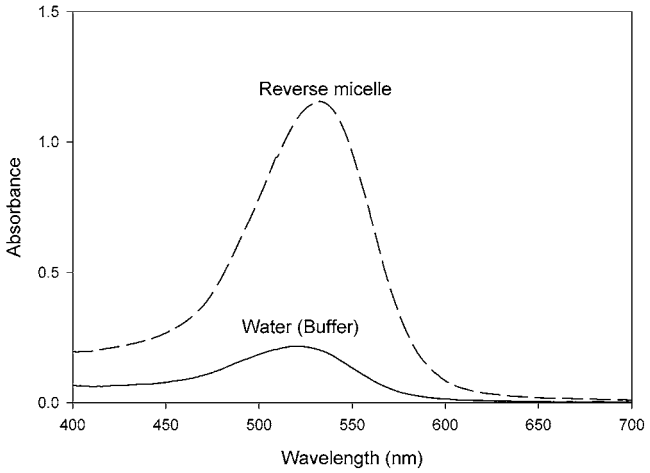

Figure 2. Absorption spectrum of anthocyanins solubilized in RM and McIlvaine buffer (pH 3.0). The equal volume of anthocyanin stock solution was added to the buffer and hexane containing 100 mM AOT, respectively. The molar water to AOT ratio (Wo) of RM was 10.

Aggregation behaviors of various surfactants in organic solvent are a quite complex phenomenon. Shape and size of micellar aggregates were highly dependent upon not only type and concentration of surfactant but also nature of the solvent (*12*). Among the various surfactants, AOT was chosen for this study since AOT readily forms uniform and spherical micellar aggregates in a variety of solvents (*13*). Hexane was selected as a solvent phase because it is a popular solvent used for RM formation and food processing such as edible oil extraction from soybean.

The color properties of anthocyanins in the buffer and RM solution (100 mM AOT, Wo = 10) were compared using spectral analysis. The color intensity of the RM solution was about four times greater than that of the buffer solution when the equal volume of the anthocyanin stock solution was solubilized (**Figure 2**). There was a shift of absorption maximum (λ_{max}) from 522 (buffer) to 534 nm (RM). This bathochromic shift might reflect the structural changes of anthocyanins in the presence of AOT. Broulillard (*14*) reported that the structural unit of anthocyanins related to the typical red color expression is the 2-phenylbenzopyrylium (flavylium), and modification of structure affects color properties. Intramolecular (hydroxylation, methoxylation, glycosylation, etc.) and/or intermolecular (flavone, organic acid, metal, etc.) interactions induced color changes (*2*).

It is well-known that the color properties of anthocyanins significantly change with pH (*10*). Thus, a pH change during

Figure 3. Migration of anthocyanin from the aqueous to hexane phase by the addition of AOT. (**a**) Before the addition of AOT. (**b**) After the addition of AOT. AOT (1 M) was added to 10 mL of biphasic hexane–water solution (1:1, v/v) where anthocyanin was solubilized in the water phase. The mixture was vortexed, and color transfer was observed.

RM formation was examined since the pH of the water pool in RM may be significantly different from the pH of the buffer in which anthocyanins have been solubilized. Walde et al. (*14*) reported that anionic surfactant, AOT, led to a pH decrease in the water pool of the RM system even when the water pool was buffered. In accordance with the report of Walde et al. (*15*), the pH in the water pool further dropped from pH 3 and the estimated pH after RM formation was below 2.0 (data not shown). Therefore, the pH change during RM formation was not the major contributor for increased color intensity since color expression of anthocyanins is not critically affected below pH 3.

Interaction between Anthocyanins and AOT. AOT, the anionic surfactant, has a polar sulfonate ($-SO^{3-}$) head and two nonpolar hydrocarbon tails. Yang and Russell (*16*) depicted RMs as microheterogeneous media where solubilized molecules are subject to partitioning between different phases (free water, bound water, and surfactant layer). In this context, anthocyanin (flavylium cation) located in the water core of the micelle might be able to contact negatively charged sulfonate, which protrudes into the water pool of RM. Rabie and Vera (*17*) reported that zwitterioic amino acids were able to electrostatically interact with AOT at the RM interface.

To verify complexation between anthocyanins and AOT, AOT (1 M) was added to biphasic hexane and the anthocyanin solution and transfer of anthocyanins from water to hexane phase was examined. As shown in **Figure 3**, anthocyanins spontaneously migrated to the hexane phase upon vortexing. The hexane phase containing AOT resulted in much greater solubilization of anthocyanin than bulk water phase. This result indicated a strong interaction of anthocyanin with AOT in the hexane phase.

This complexation was probably due to interaction between the sulfonate head of AOT and the anthocyanin molecules. The nonpolar hydrocarbon tail, the other part of AOT, cannot have substantial affinity for water soluble substances. Plucinski and Reitmeir (*18*) also reported a strong interaction between AOT and polyelectrolytes by showing movement of polyelectrolytes from the aqueous to apolar phase.

As a monophasic solvent, ethanol (20−50%) was used to solubilize both anthocyanin and AOT and the effect of complexation on the color intensity was examined. Regardless of ethanol concentrations, color intensity increased as the concentration of AOT increased (**Figure 4**). This result suggests that the anthocyanin intensity expressed in the RM system was closely related to the interaction between AOT and anthocyanins. Interactions of anthocyanin molecules with other surfactants including CDAB (cationic head), Tween 80 (neutral head), and SDS (anionic head) were tested using ethanol solution (50%).

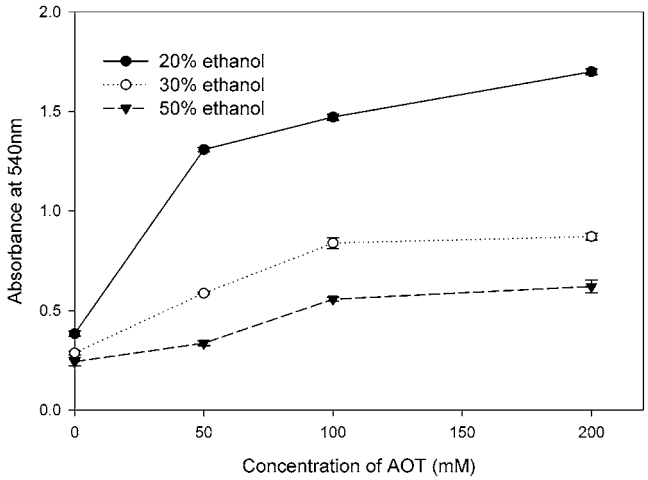

Figure 4. Effect of AOT concentration on the color intensity of anthocyanins in ethanol solution.

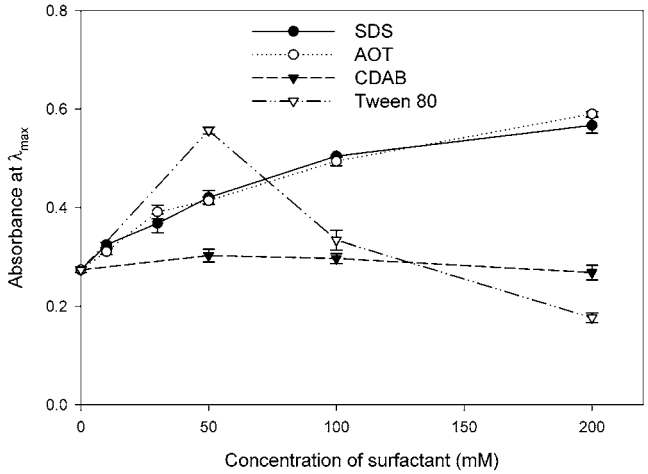

Figure 5. Effect of various surfactants on the color intensity of anthocyanin in ethanol solution (50%, v/v).

As shown in **Figure 5**, CDAB did not show significant effect on the color intensity. Tween 80 resulted in increased color intensity at 50 mM but it was probably due to turbidity. The color intensity significantly decreased when 200 mM Tween 80 was present in the medium ($P < 0.05$). Intensity of anthocyanins was significantly increased as the concentration of SDS increased in the medium ($P < 0.01$). The increase of color intensity with response to SDS concentration was almost identical to that of AOT, and there was no significant difference for color intensity between SDS and AOT. Even though SDS and AOT contain different numbers and shapes of nonpolar tails, the effect of nonpolar fraction of surfactants on anthocyanin intensity was not critical. Considering that AOT and SDS have the same sulfonate group as a head component, the increased anthocyanin intensity in the RM system could be ascribed to strong ionic interactions between the polar sulfonate group of AOT and the flavylium cation of anthocyanins solubilized in the water phase of RM.

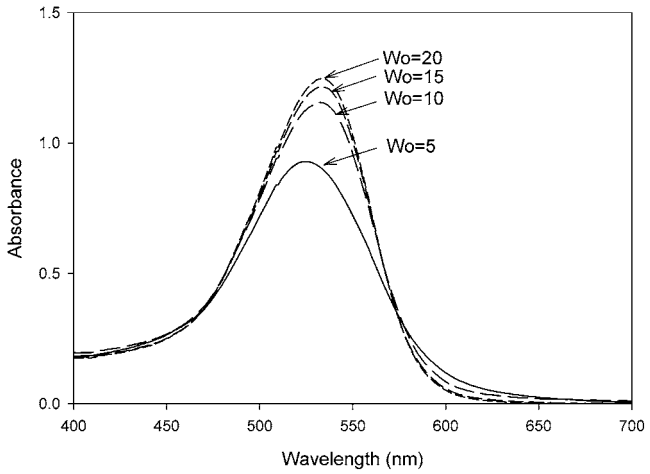

Figure 6. Effect of molar water to AOT ratio (Wo) on the absorption spectrum of anthocyanins in RM.

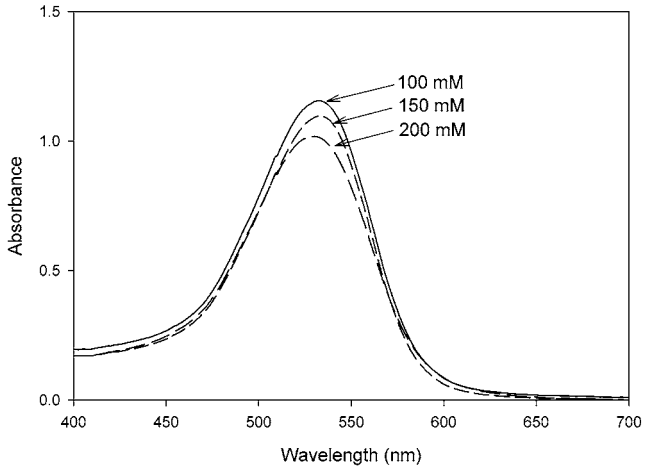

Figure 7. Effect of AOT concentration on absorption spectrum of anthocyanins in RM at a given Wo value (Wo = 10).

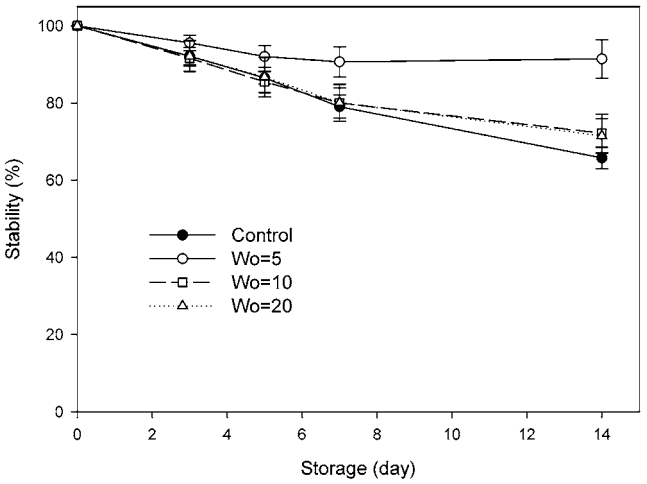

Figure 8. Color stability of anthocyanins in RM during storage at 30 °C.

Effect of Water Pool Size on Color Properties of Anthocyanins in RM. The Wo is an important parameter for color expression of anthocyanins solubilized in RMs since it determines the degree of hydration and subsequently micelle size. The higher the quantity of water in the system, the larger is the radius of micellar particles. On the contrary, as the surfactant concentration increases, the micelle becomes smaller (*19*).

Different sizes of RMs were prepared using a given anthocyanin and AOT concentration (**Figure 6**). At Wo = 5, the lowest color intensity was observed and λ_{max} was close to that of buffered anthocyanin solution (523 nm). As the Wo increased from 5 to 20, the color intensity at λ_{max} significantly increased ($P < 0.05$) and λ_{max} was shifted to longer wavelength (534 nm at Wo = 20). This result could be explained by the interaction between AOT and water pool size containing anthocyanins. According to Silber et al. (*12*), water is highly structured up to approximately Wo = 10 and water is essentially frozen at Wo < 5. The presence of free water increases as Wo increases, and free water is the dominant species for Wo > 20. The intermediate situations hold true for 10 < Wo < 20 when a RM is prepared using AOT.

Hamada et al. (*20*) reported that the ionic headgroup of AOT was not entirely dissociated into sodium counterion and sulfonate at lower Wo because of high rigidity and ionic strength in water pool. On the basis of the previous result demonstrated in **Figure 5**, the intensity of anthocyanin was increased by the formation of a sulfonate−flavylium cation complex as follows:

$$[\text{sulfonate}^- - \text{Na}^+] + [\text{flavylium}^+] \rightleftarrows [\text{sulfonate} - \text{flavylium}] + \text{Na}^+$$

At low Wo, such as 5, the above complexation may not be facilitated because of the rigid characteristic of water and the high concentration of sodium counterion (theoretically 11.1 M in the water pool). As Wo increases, solvation capacity of ions such as sulfonate and sodium can be increased by the increased free water content. Subsequently, chances for the interaction between free sulfonate and flavylium cation will be increased. The complexation favored in higher Wo resulted in increased anthocyanin intensity with a bathochromic shift as shown in **Figure 6**.

To further investigate the effect of AOT−anthocyanin interaction on color intensity, RMs were prepared with fixed Wo (*10*) but with different AOT concentrations (100−200 mM). Thus, the prepared samples had equal micelle sizes and structures but varied in number. As shown in **Figure 7**, color intensity at λ_{max} significantly decreased ($P < 0.05$) as the concentration of AOT increased. The exact same pattern was found with other Wo values such as 5, 15, and 20 (data not shown).

This result might be due to competition between flavylium cation and sodium ion for sulfonate. Considering that color intensity was measured at the same Wo, the interaction between sodium and sulfonate should be close to equal regardless of AOT concentrations. However, the anthocyanin concentration in each micelle droplet would be decreased in the case of samples containing a greater number of micelles (e.g., RMs prepared with 200 mM AOT). This situation reduced formation of the sulfonate−flavylium complex and eventually resulted in decreased color intensity.

Stability of Anthocyanins Solubilized in RM. The stability of anthocyanins solubilized in RM was examined during the storage at 30 °C up to 14 days (**Figure 8**). The overall stability of anthocyanins solubilized in RMs was better than that of buffered aqueous anthocyanin (control) in the same storage condition. The highest storage stability was found at Wo = 5 and maintained 91% of initial color intensity after 14 days storage. The stability of anthocyanin at Wo = 5 was significantly higher than those of other Wo values at more than 5 days of storage ($P < 0.05$). The stability was not critically influenced by Wo in the range of 10 and 20.

This result indicates that complexation between AOT and flavylium cation affects not only color intensity but also storage

stability. Malien-Aubert et al. (*21*) and Eiro and Heinonen (*22*) reported that complexation of anthocyanin with other compounds such as flavonoids or phenol derivatives effectively contributed to color intensity and stability.

Although color intensity of RM at Wo = 5 was lower as compared to RMs with higher Wo values (**Figure 6**), it showed greater stability. Water at low Wo has more structured and viscous characteristics (i.e., more frozen) and might add more rigidity in complexation between anthocyanin and AOT. As Wo increases, free water in the water pool increases and behaves as bulk water.

In conclusion, water soluble anthocyanins were solubilized in organic solvent using the RM system. The changes in color intensity and stability of anthocyanins in RMs were explained by the interaction between surfactant (AOT) and flavylium cation. The amount of water incorporated in RMs also affected color properties. For the possible commercial application, the current study might be utilized as a model system to predict color properties of anthocyanins in apolar medium.

ABBREVIATIONS USED

RM, reverse micelle; AOT, bis(2-ethylhexyl)sodium sulfosuccinate; CDAB, cetyldimethylethylammonium; SDS, sodium dodecyl sulfate; Wo, molar water-to-surfactant ratio ($[H_2O]/[AOT]$); λ_{max}, wavelength of maximum absorbance in the visible range.

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