Improved Method for the Stabilization of Anthocyanidins

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Anthocyanidins, prepared by the acid hydrolysis of black bean and blackberry anthocyanins, were deposited in C_{18} solid-phase extraction (SPE) cartridges from four different manufacturers. The amount of the black bean anthocyanidins, delphinidin, petunidin, and malvidin (monitored at 520 nm) was unchanged for the first 7 days of storage in SPE cartridges under nitrogen atmosphere at 2 °C. After 45 days of storage the level of delphinidin, petunidin, and malvidin was reduced to 82, 63, and 49% of the original content, respectively. In contrast, the level of delphinidin, petunidin, and malvidin decreased to 17, 10, and 3%, respectively, of the original amount when these anthocyanidins were stored for 3 days in acidic methanol (0.01% HCl) at 2 °C. Similarly, after 46 days of storage in SPE cartridges under nitrogen atmosphere at 2 °C the amount of cyanidin (obtained from evergreen blackberry puree) decreased to 75% of the original content. Cyanidin almost completely disappeared (only 0.2% remained) when it was stored for 4 days in acidic methanol (0.01% HCl) at 2 °C. Anthocyanidin stability was markedly different depending on the brand of the SPE cartridge on which it was stored.

Keywords: *Solid-phase extraction; anthocyanidin stabilization; delphinidin; petunidin; malvidin; cyanidin*

INTRODUCTION

Anthocyanins, one of the major groups of natural pigments, are responsible for many of the colors of fruits, vegetables, flowers, and other plant tissues (Harborne, 1967). There is considerable interest in the utilization of food colorants from the natural sources to replace synthetic colorants, and therefore anthocyanins are of special interest. A problem with the use of anthocyanins for coloring foods and beverages is their instability in solution to light and pH changes and especially their bleaching by sulfur dioxide which is often used as a preservative (Harborne, 1988). Thus, improved methods of anthocyanin stabilization are a major concern in the food industry. This instability can be avoided by reacting anthocyanins with other flavonoids such as flavan-3-ols which stabilize the quinonoidal base and improve the resistance to sulfur dioxide (Timberlake, 1980). Alternatively, it is possible to employ acylated anthocyanins which are more stable than their nonacylated analogues (Brouillard, 1981; Francis, 1989). Anthocyanins are difficult to purify, and their tinctorial power is nearly 100 times lower than coal tar dyes (Riboh, 1977). The structural transformations of anthocyanins in aqueous solution have been summarized by Brouillard (1988) as follows (Figure 1): At pHs below 2, anthocyanins exist primarily in the form of red (glycosylated at C-3) flavylium cations (AH^+) [**1**]. Solvation of a flavylium salt in a slightly acidic or neutral aqueous solution results in the immediate formation of the neutral [**2**−**4**] and/or ionized quinonoidal bases [**5**−**7**]. However, the common 3-glycosides and 3,5-diglycosides change more or less rapidly to the more stable carbinol [**8**] and chalcone pseudobases [**9**]. Rapid and almost complete hydration of the flavylium cation (AH^+) occurs almost exclusively at the 2 position to give the colorless carbinol pseudobase at pH values ranging from 3 to 6. This in turn can equilibrate, at a slower rate, to an open form, the chalcone pseudobase, which is also colorless.

Anthocyanidins, the chromophic aglycons of anthocyanins, are red polyhydroxylated flavylium salts. They have limited solubility in water, are rapidly destroyed by alkali, and are very unstable compared to their glycosides (anthocyanins). The half-life of a typical anthocyanin, i.e., cyanidin 3-rutinoside, is about 65 days at room temperature in 0.01 M citric acid, pH 2.8. In contrast, the corresponding aglycon, cyanidin, has a half-life of only 12 h in the same solution (Iacobucci and Sweeny, 1983). Peonidin and malvidin are also much less stable than their corresponding 3-glucosides at pH values of 2.5 and 4.5 (Ohta et al., 1980). Due to their instability, the aglycons (anthocyanidins) are seldom found in plant tissues (Harborne, 1967). Anthocyanidins can be separated successfully by thin-layer chromatography (TLC) using silica gel plates. However, the disadvantage is that their colors fade rather rapidly on plates, and the results have to be analyzed under subdued light immediately after development (Harborne, 1967). High-performance liquid chromatography (HPLC) has become the preferred method for the separation of anthocyanidins (Wilkinson et al., 1977; Wulf and Nagel, 1978; Casteele et al., 1983; Andersen, 1985, 1987; Hong and Wrolstad, 1986, 1990; Takeoka et al., 1997). Due to their instability, anthocyanidins have to be analyzed immediately after samples are prepared. For instance, Price and Wrolstad (1995) stored anthocyanidins at −15 °C and analyzed them within 12 h after preparation. One method of preserving anthocyanidin color involves increasing the concentration of flavylium cations in acid solution. Self-

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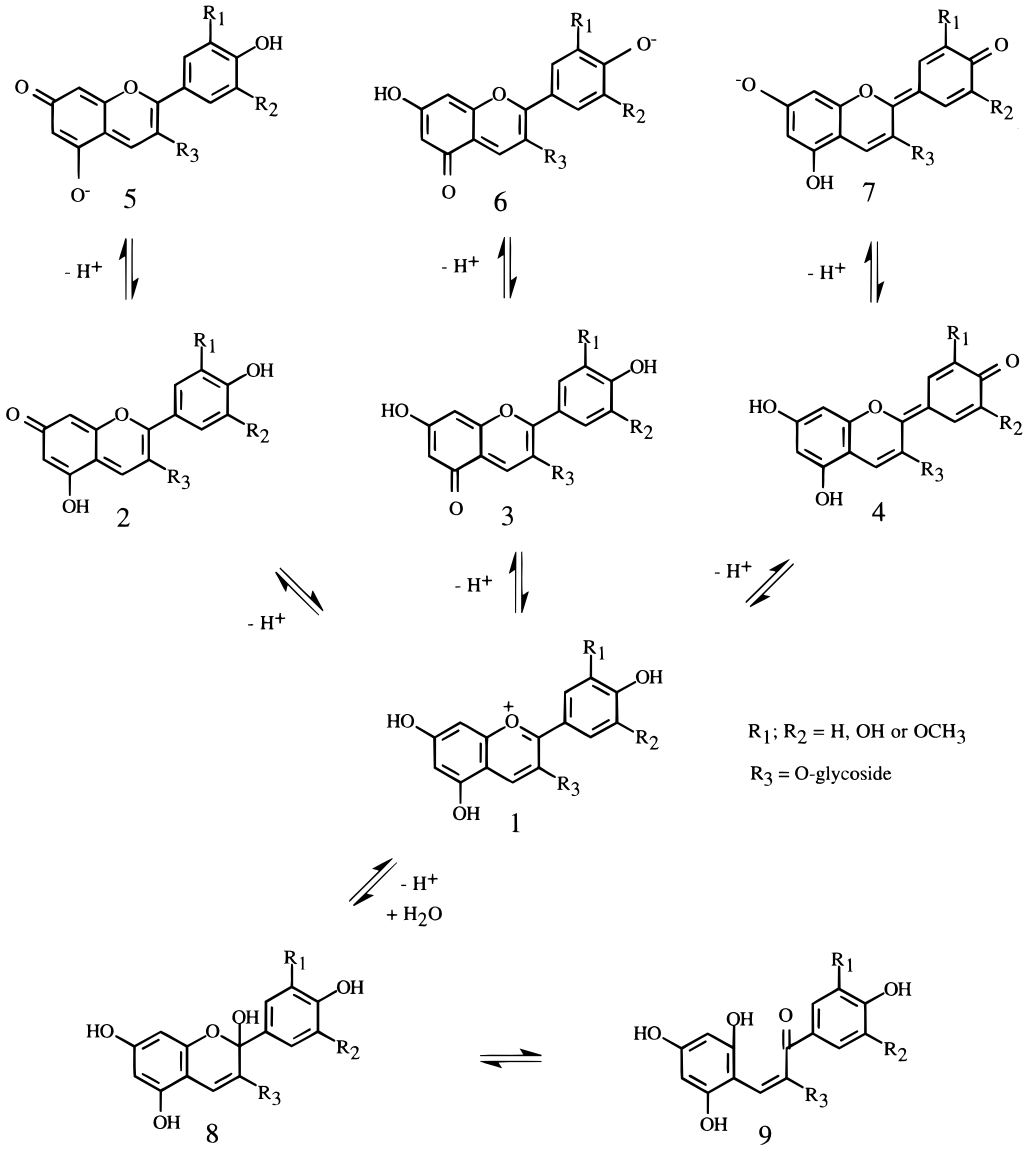

Figure 1. Structural transformations of anthocyanins in aqueous solution at varying pH. 1 = flavylium cation (red to orange color), 2−4 = neutral quinonoidal bases (purple to violet color), 5−7 = ionized quinonoidal bases (blue color), 8 = carbinol pseudobase (colorless), 9 = chalcone pseudobase (colorless) (adapted from Brouillard, 1988).

association of anthocyanins occurs at higher concentrations (Asen et al., 1972) which results in greater stability. The resulting vertical, hydrophobic stacking of aromatic rings between anthocyanins (self-association) prevents the nucleophilic attack of water at C-2 of the anthocyanidin nucleus (Goto and Kondo, 1991). At low concentration of flavylium cations (achieved by dissolution in weak acid (10^{-3} N), the quinonoidal bases of pelargonidin and cyanidin are formed to only small extents and fade to the carbinol bases (colorless) almost immediately (Timberlake and Bridle, 1966). Attempts to improve the stability of anthocyanidins have been reported. However, those works emphasized chemical modification of the anthocyanidins such as the synthesis of 3-deoxyanthocyanidins (Sweeny and Iacobucci, 1983) or attachment of a methyl or phenyl group at C-4 (Timberlake and Bridle, 1968) which enhances resistance to fading in the presence of SO_2 or ascorbic acid. However, all of these colorants would be considered new compounds. Harper (1968) dissolved pelargonidin chloride in aqueous methanol solutions and reported that the flavylium ion was stable at pHs below 3 though its half-life was not described. When dissolving a flavylium salt in slightly acidic or slightly alkaline solution, the neutral and/or the ionized quinonoidal bases are formed immediately and, in general, slowly evolve to the highly stable colorless carbinol pseudobase or its ionized form (Brouillard, 1983). The color retention of anthocyanidin solutions measured at constant pH over an interval of time gave results similar to those obtained by varying the pH (Timberlake and Bridle, 1966). Due to the limited commercial availability of anthocyanidin standards, naturally existing anthocyanins have been used as the main source in most studies to verify the presence of anthocyanidins *in vitro.* For instance, acid hydrolysis of Concord grape anthocyanins gives five of the most common anthocyanidins: delphinidin, cyanidin, peonidin, petunidin, and malvidin (Wilkinson et al., 1977; Hong and Wrolstad, 1986; Takeoka et al., 1997). Additional sources include the following: cyanidin as the sole anthocyanidin in blackberry (Barritt and Torre, 1973), delphinidin in eggplant (Markakis, 1974), and radish containing only pelargonidin (Giusti and Wrolstad, 1996). In practice, authentic anthocyanidins are needed for all anthocyanin related studies. Anthocyanidins are best obtained from acid hydrolysis of pure

anthocyanins (Harborne, 1967). The pigments will normally crystallize out from the cooled solution and may be collected. If they fail to crystallize, the aglycons may be extracted into amyl alcohol (Du and Francis, 1973; Bobbio et al., 1983; Andersen, 1985, 1987), ethyl acetate (Baldi et al., 1995; Takeoka et al., 1997), or using C_{18} SPE cartridges for extraction and purification (Pettipher, 1986; Hong and Wrolstad, 1986, 1990; Wrolstad et al., 1990; Giusti and Wrolstad, 1996; Takeoka et al., 1997). Due to instability of anthocyanidins, all of the analytical steps should be carried out in subdued light with immediate analysis after sample preparation. The instability of anthocyanidins probably explains their poor availability from commercial sources. Since anthocyanidins are very labile in solution, it is possible that the red flavylium cations can be preserved if they are in a dry salt form. Thus, anthocyanidins can be entrapped, retained within a "solidlike" matrix, and eluted out as desired. The C_{18} SPE cartridge was selected for this purpose. Brouillard (1983) reported that physical adsorption on a suitable surface of any of the visible light-absorbing chromophores will provide a good means of preventing color loss, by taking the pigment out of the bulk solution. It was reported that the colorants extracted from a variety of plant sources can be adsorbed onto inorganic oxides such as silicic acid, titanium oxide, or alumina, which were coated with a styrene polymer. The coated resins were mixed with aqueous anthocyanin solutions and filtered off, and the pigments recovered with ethanol (Francis, 1989). A key feature of good color stabilization is that many layers of the pigment must be adsorbed (Brouillard, 1983). Another way to retain anthocyanin color is to remove as much water as possible thereby shifting the equilibrium from the carbinol pseudobase to the red flavylium cation. In this study, the anthocyanidins were obtained from the acid hydrolysis of black bean and blackberry anthocyanins.

Due to the limited commercial availability of anthocyanidin standards and their instability, methods to preserve these compounds are needed. The aim of this study was to develop an improved method of stabilizing anthocyanidins in vitro and to use it as a source of "in-house" anthocyanidin standards whenever needed. To the best of our knowledge, this is the first report on the stabilization of anthocyanidins without chemical modifications.

EXPERIMENTAL PROCEDURES

Materials. Black beans were obtained as previously described (Takeoka et al., 1997). Evergreen blackberry puree was supplied by the Sabroso Co., Sandy, OR. Solvents were of HPLC spectroquality grade unless otherwise stated. Solid-phase extraction cartridges containing 100 and 500 mg of C_{18} sorbent were obtained from four sources: Supelco, Bellefonte, PA; Varian Associates, Inc., Harbor City, CA; J&W Scientific, Inc., Folsom, CA; Waters Corporation, Milford, MA. The majority of experiments were carried out on the SPE cartridges from Varian Associates, Inc.

Extraction Methods. The anthocyanins in black beans were extracted according to a previously described procedure (Takeoka et al., 1997) with some modification. After evaporation of the acidified methanol extract (0.5% HCl), the residue was redissolved in ethanol:water (20:80; v/v) and extracted with ethyl acetate. The aqueous phase was collected. For blackberry puree, the original puree was centrifuged, and the supernatant was then deposited in Extrelut 20 cartridges (Merck, Darmstadt, Germany). Anthocyanins were eluted with acidified methanol (0.5% HCl), and the extract was subjected to the above procedures.

Acid Hydrolysis. The aqueous extracts were evaporated to near dryness, hydrolyzed with 2 N HCl for 60 min at 100 °C, cooled in an ice bath, and filtered through a 0.5 μm disposable filter membrane.

Sample Preparation. One milliliter of hydrolysate was pipetted into each C_{18} SPE cartridge (3 cm^3/500 mg sorbent) and washed two times with 2 mL aliquots of 0.01% HCl (to remove sugars) using a Visi-1 single SPE tube processor (Supelco) with moderate pressure. Using an empty processor, air pressure was used to expel any excess remaining liquid. The anthocyanidins were retained in the cartridges which were placed in a glass jar. A stream of nitrogen was applied to eliminate oxygen transfer into the jar which was immediately capped, sealed with Teflon tape, and stored in the dark at room temperature (ca. 25 °C) or in a refrigerator (2 °C). The anthocyanidins were eluted with 1 mL of 0.01% HCl in methanol just prior to HPLC analysis. The color retention was calculated using the peak areas from the HPLC chromatograms (color retention % = [peak area measured at 520 nm at time t/peak area measured at 520 nm at time 0] $\times$ 100). The stability of anthocyanidins was measured at selected time intervals (days or weeks) until the anthocyanidin peak(s) disappeared on HPLC chromatogram.

High-Performance Liquid Chromatography (HPLC). High-performance liquid chromatography was performed as previously described (Takeoka et al., 1997).

RESULTS AND DISCUSSION

The stability of black bean anthocyanidins under two different storage conditions is illustrated in Figure 2. At room temperature (25 °C), stored in the dark in acidic methanol, the amount of delphinidin, petunidin, and malvidin decreased to 14%, 8%, and 2% of the original content, respectively, after 24 h and completely disappeared after 48 h. The red flavylium cation was rapidly transformed into the colorless carbinol pseudobase and eventually into the chalcone pseudobase which is also colorless. A slight improvement of color retention was observed when the anthocyanidin solution was stored in the refrigerator (2 °C). After 3 days, the starting level of delphinidin, petunidin, and malvidin declined to 17, 10, and 3%, respectively. It is well-known that temperature is a major factor which affects the color stability of anthocyanins (Harborne, 1967; Markakis, 1974; Iacobucci and Sweeny, 1983; Francis, 1989). However, when anthocyanidins were stored in C_{18} SPE cartridges under a nitrogen atmosphere and in the refrigerator (2 °C), the color retention increased dramatically with better stability observed for a longer storage time. After 45 days, the three major anthocyanidins in black bean that included delphinidin, petunidin, and malvidin were still detectable by HPLC (Figure 3). The red color of the three anthocyanidins in black beans was unchanged during the first 7 days of storage and then slowly degraded with time. After 45 days of storage, the amount of delphinidin, petunidin, and malvidin still remained at 82, 63, and 49% of the original level, respectively. The exact reason is not known. The possibilities are that in the "solidlike" matrix of the C_{18} cartridge, the anthocyanidins formed salts in the presence of hydrochloric acid (left over from washing off the sugars with 0.01% HCl) and/or hydrogen bonding occurred between the hydroxyl groups of anthocyanidins and reactive surface silanol (SiOH) groups. The anthocyanidin flavylium salts were retained inside the cartridges and self-stabilized under a nitrogen atmosphere, subdued light, and low temperature and moisture. It is also possible that the increased stability was the result of self-association between the

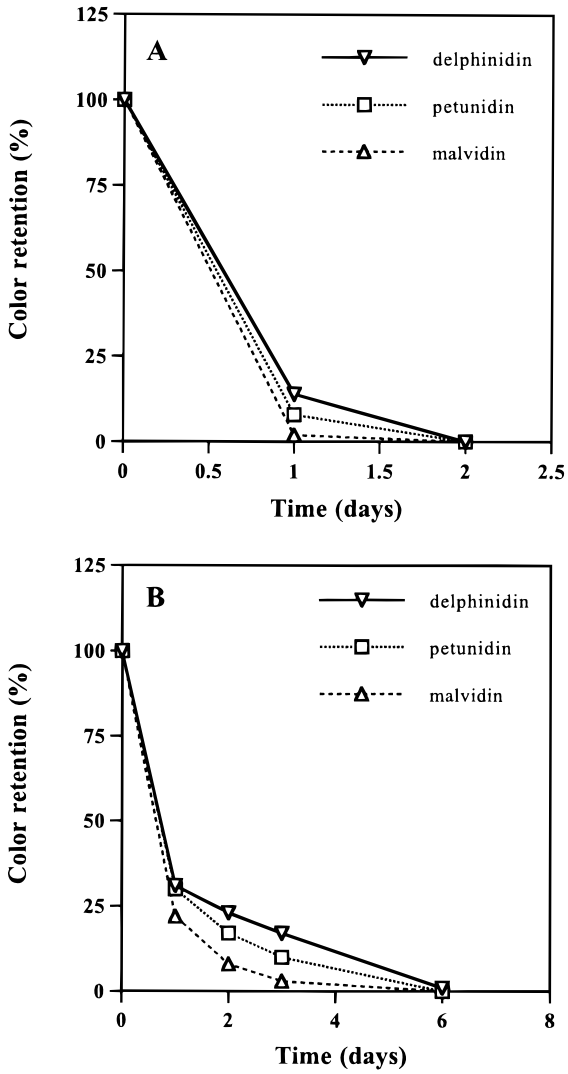

Figure 2. Effect of temperature on the stability of black bean anthocyanidins dissolved in acidic methanol (0.01% HCl). Storage temperature was (A) 25 °C and (B) 2 °C. Color retention % = (peak area measured at 520 nm at time *t*/peak area measured at 520 nm at time 0) × 100.

flavylium cations. The almost planar structures and extended electronic delocalization of the anthocyanidins make them suitable to form molecular complexes (Brouillard and Dangles, 1994). A thin intensely red-colored layer was visible in all of the SPE cartridges.

Similar results were obtained for cyanidin which was isolated from acid hydrolyzed evergreen blackberry puree. Stored at room temperature in the dark, the red color of cyanidin in acidic methanol faded away (0% color retention) in less than 24 h. The color retention was increased to 4 days when the acidic cyanidin solution was stored in the refrigerator (2 °C) (Figure 4A). Stability increased dramatically when cyanidin was stored in C_{18} SPE cartridges. The color was unchanged after 3 days; only 5% was lost after 15 days, and 54% of the cyanidin still remained after 67 days of storage (Figure 4B). The stability of cyanidin stored in C_{18} SPE cartridges (100 mg sorbent mass) was studied at two different temperatures. After 35 days of storage, no cyanidin remained in the cartridges stored at 25 °C, while 70% of the cyanidin still remained in the cartridge stored at 2 °C (data not shown). As mentioned before, temperature is an important factor that affects the color stability of anthocyanidins. Additional study showed

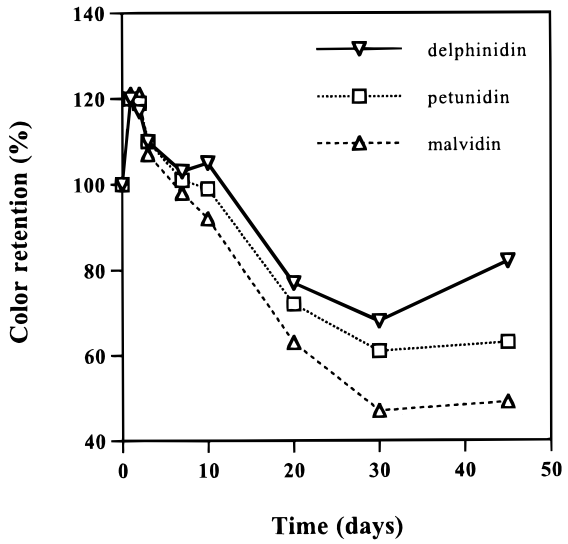

Figure 3. Stability of black bean anthocyanidins stored in C_{18} SPE cartridges (3 cm^3/500 mg sorbent) at 2 °C.

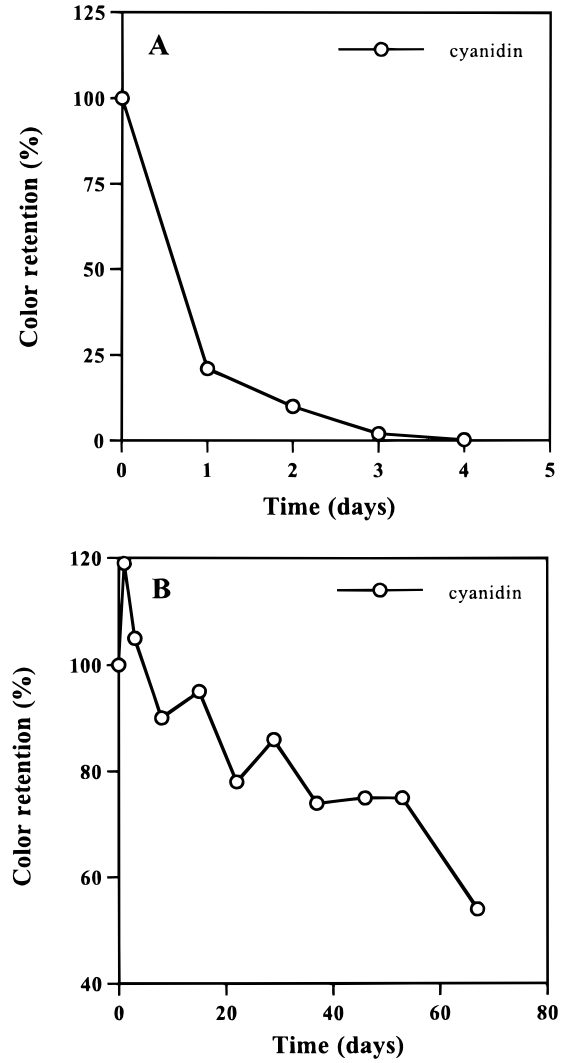

Figure 4. Stability of cyanidin (obtained from evergreen blackberry puree) stored at 2 °C in (A) acidic methanol (0.01% HCl) and (B) C_{18} SPE cartridges (3 cm^3/500 mg sorbent).

that the stability of cyanidin is slightly improved when the C_{18} cartridge samples are stored at lower temperatures (−8 °C) (data not shown).

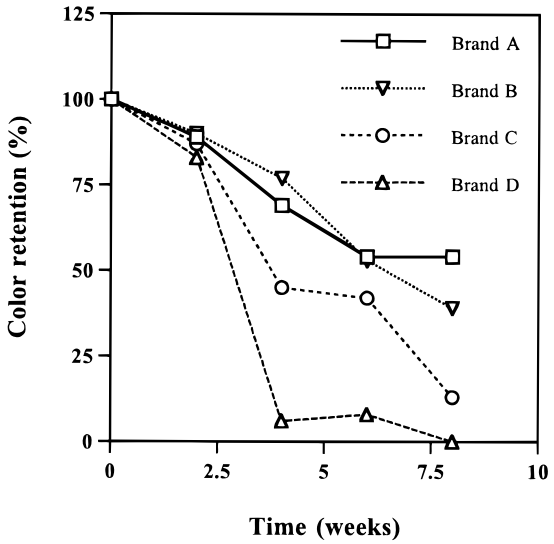

Figure 5. Stability of cyanidin (obtained from evergreen blackberry puree) stored at 2 °C in four different brands of C_{18} SPE cartridges (3 cm^3/500 mg sorbent).

It has been demonstrated that storing anthocyanidins in C_{18} SPE cartridges improves their color stability. Since these cartridges are available from various sources, we decided to test the stability of anthocyanidins on different brands of C_{18} cartridges. Figure 5 illustrates the behavior of cyanidin stored on different manufacturers' C_{18} cartridges. The color stability of cyanidin was similar on all four brands of C_{18} cartridges after 2 weeks of storage (83−90% remained). However, the behavior of cyanidin was completely different with longer storage times. After 8 weeks, the amount of cyanidin stored in brand A C_{18} cartridges remained at 54%, followed by the brand B at 39%, and 13% for the brand C, and none remained when brand D C_{18} cartridges were used. After nine months of storage at 2 °C red-colored bands were still visible in the brand A and B cartridges, while the bands were brown-colored in the brand C and D cartridges. It is possible that trace metal oxide impurities such as magnesium, iron, or aluminum present in the sorbent may have had an effect on the anthocyanidin stability. The metal oxides may indirectly influence adjacent silanol groups resulting in their enhanced acidity (Poole and Poole, 1991).

CONCLUSIONS

C_{18} SPE cartridges can be used to improve the stability of anthocyanidins. Temperature is an important factor that affects the stability of anthocyanidins both in solution and in adsorbed form. The color retention of anthocyanidins also depended on the chemical properties of individual C_{18} sorbents within the SPE cartridge. Different brands of C_{18} sorbents resulted in drastically different color retention. This novel method is convenient and can be utilized as an "in-house" source of anthocyanidin standards when commercial standards are not available.

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