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Effect of Storage Temperature on the Stability of Anthocyanins of a Fermented Black Carrot (*Daucus carota* var. L.) Beverage: Shalgam

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The effect of temperature on the stability of shalgam anthocyanins stored at 4, 25, and 40 °C for 90 days was investigated. The effect of pasteurization and sorbate addition on the anthocyanin stability as compared to control was also studied. The monomeric anthocyanin content and color density decreased with increasing time as a function of storage temperature whereas the percent polymeric color and browning increased. The same trends were observed in control, pasteurized, and sorbate-added shalgam samples. Shalgam anthocyanins consisted of two nonacylated and three acylated cyanidin derivatives. Acylated anthocyanins were more stable when compared to nonacylated ones at all storage temperatures. The activation energies, 11.11−11.64 kcal/mol, were calculated from the reaction rate constants evaluated taking first-order reaction kinetics. The highest anthocyanin retention was observed at 4 °C storage temperature with a half-life between 231 and 239 days.

KEYWORDS: Shalgam; black carrot (*Daucus carota* var. L.); anthocyanin; storage stability

INTRODUCTION

Shalgam, a purplish red-colored, cloudy, sour soft drink is a local lactic acid-fermented beverage prepared mainly from black carrot (*Daucus carota* var. L.) and is consumed in Turkey. Black carrot is directly fermented by enriched lactic acid bacteria with the addition of salt, turnip, and/or red chili powder. Lactic acid bacteria are enriched by prefermentation of bulgur flour mixed with sourdough. A similar beverage known as Kanji in Asia is produced by spontaneous lactic acid fermentation of black carrots with the addition of salt, crushed mustard, and/or red chili powder (*1*, *2*). The purplish red color of these fermented beverages comes from the anthocyanins present in black carrot.

Anthocyanins are the most abundant group of pigments; they are responsible for the red, blue, and purple colors of many fruits and vegetables. Besides their color attributes, the interest in anthocyanins has intensified because of their possible health benefits. Anthocyanins contribute greatly to the antioxidant properties of certain colorful foods (*3*−*5*).

Anthocyanins occur in nature as glycosides of anthocyanidins and may have aliphatic or aromatic acids attached to the glycosidic residues. Acylation with aromatic acids including *p*-coumaric, caffeic, ferulic, sinapic, gallic, or *p*-hydroxybenzoic acids has an important stabilizing effect on anthocyanins (*3*).

The anthocyanins present in black carrots, which are derivatives of cyanidins, are cyanidin-3-xylosyl-galactoside and cyanidin-xylosyl-glucosyl-galactoside; the latter also further monoacylated with ferulic, sinapic, and *p*-coumaric acids (*3*, *6*).

Factors that affect the stability of anthocyanins include structure, pH, temperature, light, copigments, self-association, metallic ions, oxygen, ascorbic acid, sugars and their degradation products, proteins, and sulfur dioxide (*7*). The type of anthocyanidin, its glycosylation pattern, and its acylation with aromatic and/or aliphatic acids have an important effect on anthocyanin stability (*6*, *8*−*10*).

Several studies regarding the storage stability of anthocyanin pigments have been conducted in model beverages (*11*−*15*), juice/juice extracts (*16*−*18*), and jams (*19*). The storage temperature was found to be the main factor responsible for anthocyanin loss. Degradation kinetics was found to be of first order. The higher heat stability of diglucosides as compared to monoglycosides was observed upon storage (*20*). The activation energies have been calculated as 27−28 kcal/mol for strawberry (*21*), 23.1 kcal/mol for sunflower hull (*22*), 28 kcal/mol for grape (*22*), 13−15 kcal/mol for Hibiscus (*23*), and 17.5−21.4 kcal/mol for blood orange anthocyanins (*24*). Anthocyanins acylated with aromatic acids showed a higher storage stability as compared to nonacylated counterparts (*8*−*9*, *17*). It was shown that the concentration of polymeric pigments increases with temperature and storage time (*19*).

The objective of this research was to investigate the stability of anthocyanins of shalgam at three different storage temperatures (4, 25, and 40 °C). Changes in monomeric and individual anthocyanins were determined during 90 days of storage. The stability of shalgam beverage anthocyanins during storage was compared for control, pasteurization, and addition of sorbate.

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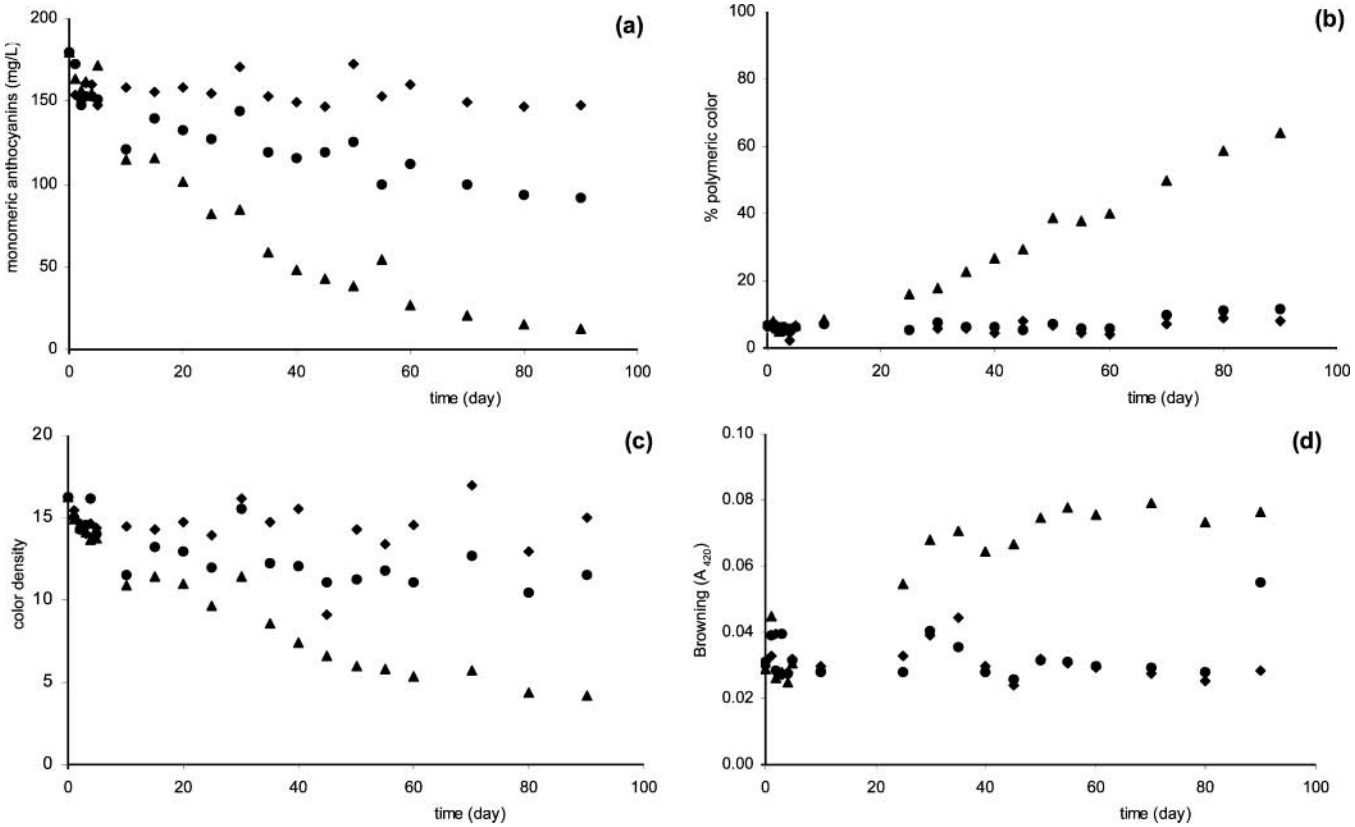

Figure 1. Monomeric anthocyanin degradation (**a**), polymeric color formation (**b**), color density change (**c**), and browning (**d**) in shalgam (control lot) at 4 (◆), 25 (●), and 40 (▲) °C.

MATERIALS AND METHODS

Chemicals and Materials. All chemicals used were purchased from Merck KgaA (Darmstadt, Germany). Black carrots, bulgur, salt, and sourdough were obtained from local markets.

Production of Shalgam. Shalgam production comprises two stages: a prefermentation step for the enrichment of lactic acid bacteria and a main fermentation step at which black carrot fermentation takes place. For the prefermentation step, 1 kg of bulgur flour, 0.1 kg of sourdough (produced from bakery dough fermented overnight at room temperature), 0.1 kg of salt, and adequate water were mixed. This dough mixture was fermented at 20 °C for 5 days in a temperature-controlled oven. The fermented bulgur dough was then extracted twice with 5 and 3 L of water, respectively. The extracts were combined for the main fermentation step. In this step, 2 kg of sliced black carrots and 0.2 kg of salt were added into a plastic bottle filled with the extract. The plastic bottle was sealed, and anaerobic fermentation was carried out for 1 week at 20 °C.

Experimental Design. The produced shalgam was divided into three lots. One lot was used as the control, the second lot was pasteurized at 90 °C for 1 min and then cooled rapidly, and the third one was treated with 1000 mg/L potassium sorbate. Each lot was filled into 250 mL sterilized glass bottles with a sampling port equipped with a 0.45 μm air filter and a 2 mL sterile injector. Duplicate bottles of each lot were stored at 4, 25, and 40 °C for 90 days. Samples were taken every day for 5 days and then in 5 day intervals until 60 days of storage and finally in 10 day intervals until the end of storage. Two milliliters of samples was taken aseptically and centrifuged (Hettich Micro 22R, Germany) in Eppendorf tubes at 36 220*g* at 10 °C for 15 min. The samples were then diluted with 4% phosphoric acid (if necessary) and filtered through a 0.45 μm nylon filter (Millipore) before injection. Twenty microliters of filtered sample was directly injected into a high-performance liquid chromatograph (HPLC).

Monomeric Anthocyanin Content, Polymeric Color, Color Density, and Browning Measurements. The monomeric anthocyanin content, polymeric color, color density, and browning index of samples were determined using pH differential and bisulfite bleaching methods (*25*). A UV−visible spectrophotometer (Perkin-Elmer Lambda EZ-201) and 1 cm path length glass cells were used for spectral measurements at 420, 520, and 700 nm, respectively. The pigment content was calculated as cyanidin-3-glucoside using an extinction coefficient of 26 900 L cm^{-1} mg^{-1} and a molecular weight of 449.2. All of the measurements were duplicated.

Alkaline and Acid Hydrolysis of Anthocyanins. Two milliliters of centrifuged shalgam was passed through a C-18 Sep-Pak (AccuBond^{II} SPE ODS-C18, 100 mg) cartridge, previously activated with one volume of methanol and followed by one volume of 0.01% aqueous HCl (*26*). Anthocyanins and other phenolics were adsorbed onto the cartridge; whereas sugars, acids, and other water soluble compounds were removed with 5 mL of 0.01% aqueous HCl. Then, anthocyanins were subsequently removed with methanol containing 0.01% HCl (v/v). The methanolic extract was then concentrated under vacuum (Büchi Rotavapor R 3000, Switzerland) at 35 °C.

The purified anthocyanin pigment (ca. 10 mg) was hydrolyzed in a screw cap test tube with 10 mL of 10% NaOH for 8 min at room temperature in the dark as described by Guisti and Wrolstad (*27*). The solution was neutralized using 2 N HCl, and then, the hydrolysate was purified using a C-18 Sep-Pak cartridge as described above.

Acid hydrolysis was performed using the method of Guisti and Wrolstad (*27*). Fifteen milliliters of 2 N HCl was added to ca. 1 mg of saponified pigment in a screw cap test tube, which was then flashed with nitrogen. The pigment was hydrolyzed at 100 °C for 30 min and was cooled in an ice bath immediately. The hydrolysate was purified as previously described. Twenty microliters of alkaline and acid-hydrolyzed samples was injected into the HPLC.

HPLC. *Apparatus.* The HPLC system used was an Agilent 1100 Series equipped with a variable wavelength UV−vis detector set to 520 nm and ChemStation (Agilent Tech. 1999−2000) software for liquid chromatography.

Column and Mobile Phase. A C-18 column (250 mm × 4.6 mm i.d. ACE 5 μm A114422) was used. Solvent A: 100% acetonitrile; solvent B: 4% phosphoric acid in water or 10% acetic acid, 5%

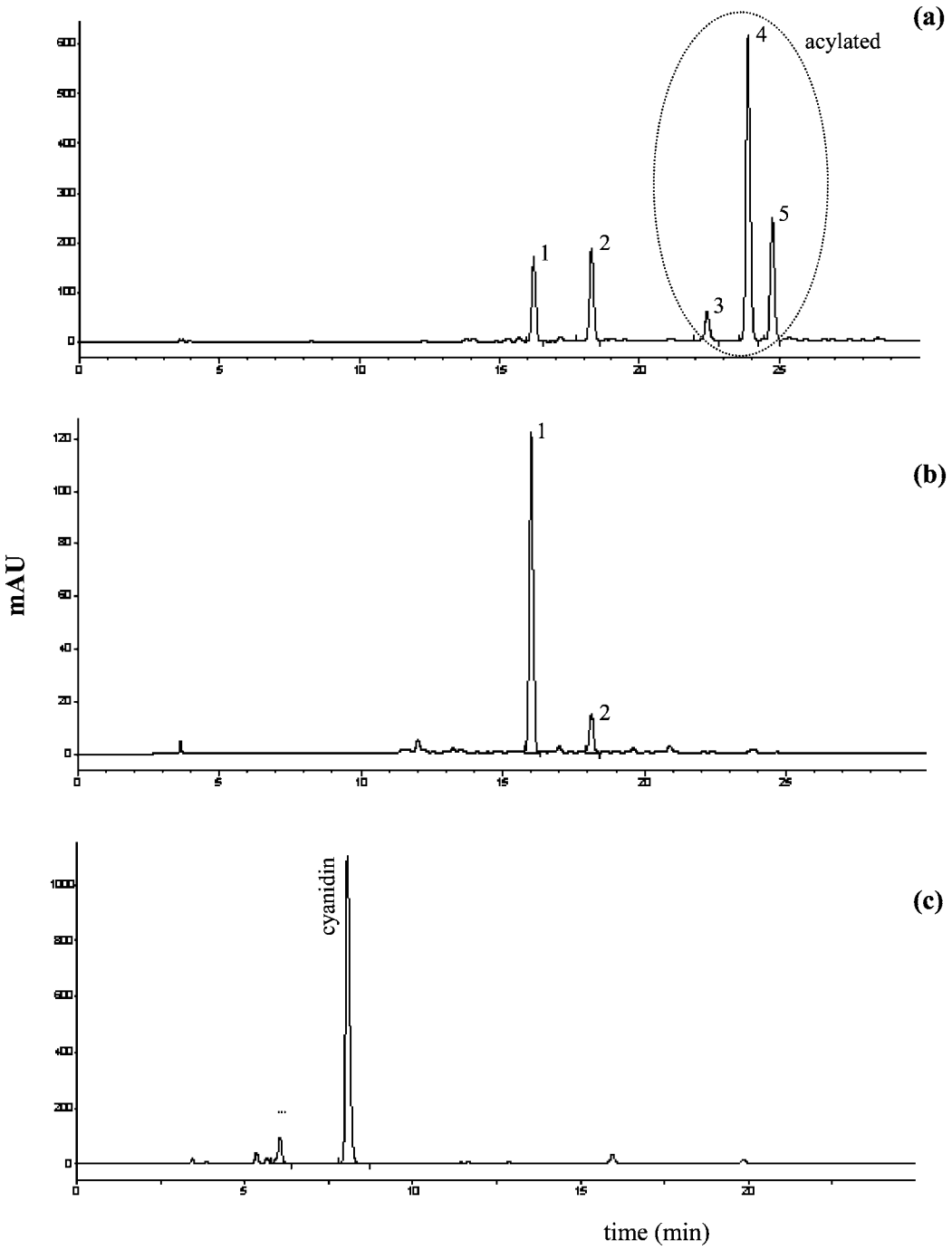

Figure 2. HPLC separation of purified (**a**), saponified (**b**), and acid-hydrolyzed (**c**) shalgam anthocyanins.

acetonitrile, 1% phosphoric acid in water depending on the analysis protocol used. The flow rate of the mobile phase was 1.00 mL/min.

Analytical Conditions. For directly injected shalgam beverage samples and alkali-hydrolyzed anthocyanins, the alternate protocol was used, and for the acid-hydrolyzed anthocyanins, the basic protocol 2 was used (*28*).

Statistical Analysis. Statistical analysis of variance was applied, and the significant ($p < 0.005$) difference between the means was determined using the Tukey's classification. The analyses were performed with SPSS statistical program version 11.0.

RESULTS AND DISCUSSION

The pH values of all samples were recorded throughout storage in order to follow pH changes, since pH has a significant impact on the pigment stability (*29*−*31*). The initial pH of shalgam was 3.5. Pasteurization and sorbate treatment did not affect the pH of the samples. Minor pH changes were observed during storage in all samples, ranging from 3.54 to 3.76 after 90 days of storage.

Monomeric Anthocyanin Content, Polymeric Color, Color Density, and Browning. The initial monomeric content, polymeric color, color density, and browning of shalgam were determined as 180 mg/L, 1.0, 16.26, and 0.030, respectively, prior to storage.

The monomeric anthocyanin content, polymeric color, color density, and browning of the samples stored at 4, 25, and 40 °C were plotted against time (**Figure 1**). The monomeric anthocyanin content of the control lot of shalgam decreased with increasing time as a function of increasing storage temperature (**Figure 1a**). The same degradation pattern was also observed in pasteurized and sorbate-treated samples. The monomeric

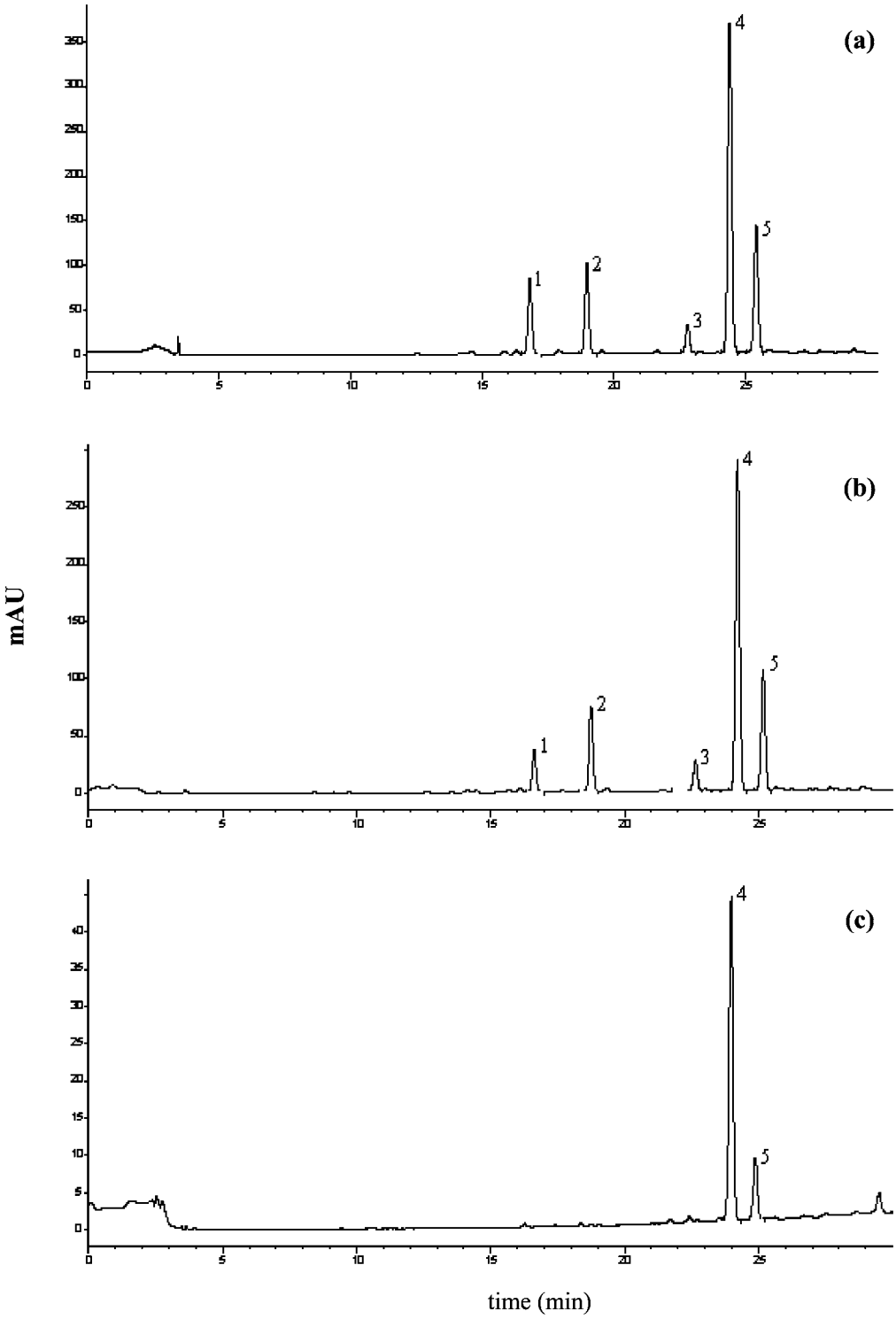

Figure 3. HPLC profile of shalgam (control lot) anthocyanins at 4 (**a**), 25 (**b**) and 40 °C (**c**) after 90 days of storage (1/2 dilution).

anthocyanin content decreased 17.8, 49.2, and 93.3% in the control samples stored for 90 days at 4, 25, and 40 °C, respectively. The percent polymeric color increased significantly in all samples stored at 40 °C, whereas only a minor increase was observed in samples stored at 4 and 25 °C as shown in **Figure 1b** plotted for the control samples. The color density decreased 7.8, 29.0, and 74.2% in the control samples stored at 4, 25, and 40 °C, respectively, after 90 days (**Figure 1c**). A similar trend was observed in pasteurized and sorbate-treated samples. Almost no increase in browning was observed in the control and sorbate-treated samples stored at 4 and 25 °C, whereas the pasteurized samples showed a slight increase in browning when stored at 25 °C. Browning increased rapidly after 24 days in all samples stored at 40 °C (**Figure 1d**). Statistically, a 40 °C storage temperature had a significant impact ($p < 0.05$) on the monomeric anthocyanin content, polymeric color, color density, and browning while no significant difference ($p < 0.05$) was observed between 4 and 25 °C. Furthermore, no significant difference ($p < 0.05$) was found in terms of the above-mentioned items when pasteurization and sorbate treatments were compared with the control. Surface mold growth was observed only in control samples at 25 °C after 20

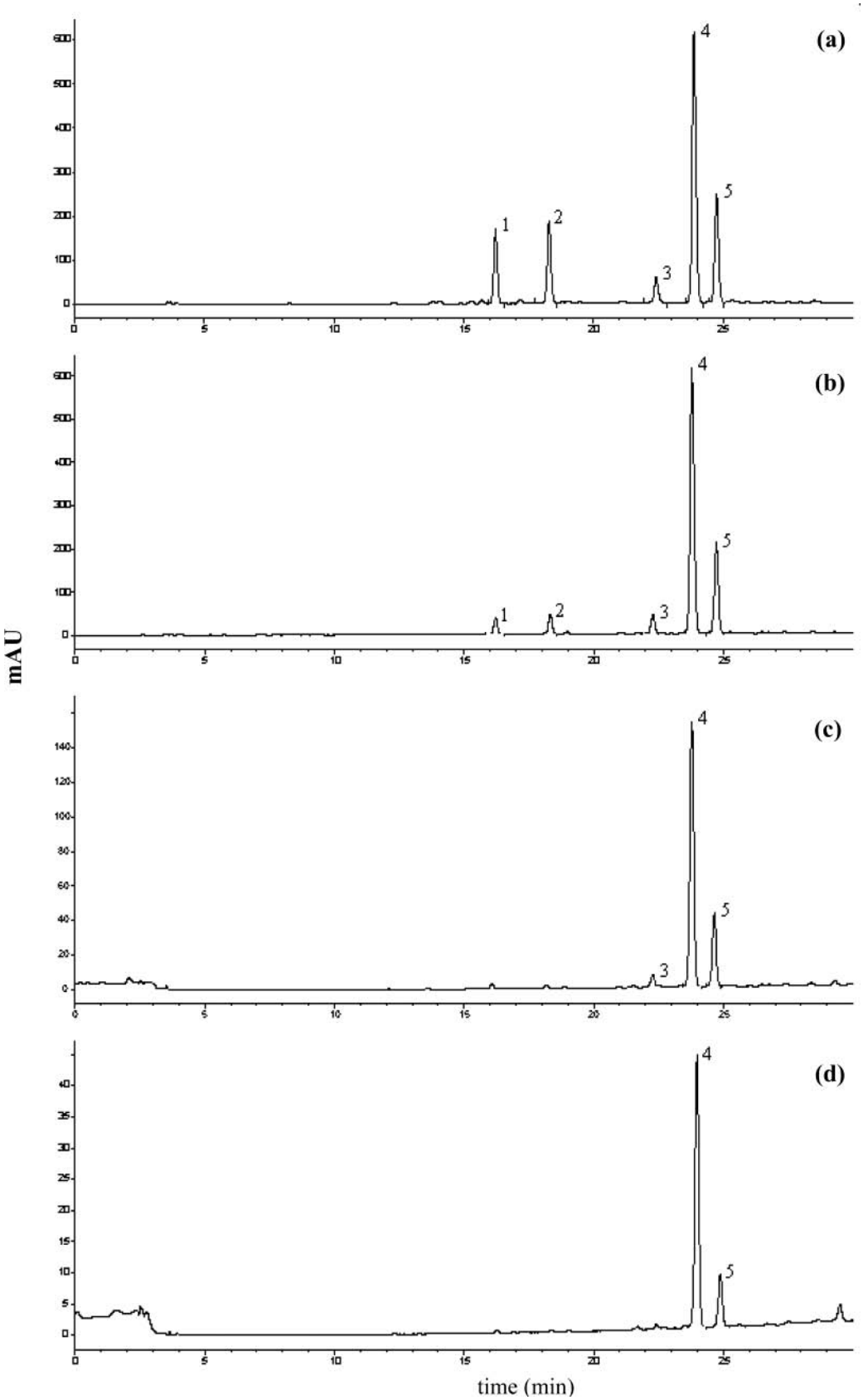

Figure 4. HPLC profile of shalgam beverage (control lot) anthocyanins during storage at 40 °C; (**a**) 0, (**b**) 30, (**c**) 60, and (**d**) 90 days of storage.

days of storage and at 4 °C after 50 days of storage. These results were in agreement with the literature data (*7*−*9*, *11*−*13*, *15*−*17*, *19*, *20*). The effect of temperature on the storage stability of shalgam anthocyanins was further evaluated applying the first-order degradation kinetics to the experimental data.

HPLC Separation of Shalgam Anthocyanins. Direct injection of shalgam resulted in five anthocyanins separated by HPLC (**Figure 2a**). Peak 4 represented about 48.4% of the total area at 520 nm. Peaks 1−3 and 5 represented 12.3, 14.5, 5.1, and 19.7% of the total area, respectively. Only peaks 3−5 showed

significant absorbance at 320 nm indicating that these anthocyanins were acylated with hydroxylated aromatic acids (*32*). Acylation was further confirmed with saponification. The retention times of saponified anthocyanins coincided with those of peaks 1 and 2 (**Figure 2b**). Acid hydrolysis of anthocyanins yielded one anthocyanidin showing exactly the same retention time as that of cyanidin from acid-hydrolyzed strawberry anthocyanins (**Figure 2c**). These results all together showed that two nonacylated (peaks 1 and 2) and three acylated (peaks 3−5) anthocyanins based on cyanidin aglycone were present in shalgam. The ratio of acylated anthocyanins to nonacylated ones was 2.7. Stintzing et al. (*6*) identified four anthocyanins present in black carrot: cyanidin 3-xylosyl-galactoside, cyanidin 3-xylosyl-glucosyl-galactoside, and cyanidin 3-xylosyl-glucosyl-galactoside acylated with sinapic acid or ferulic acid. Furthermore, a fifth anthocyanin, cyanidin 3-xylosyl-glucosyl-galactoside acylated with *p*-coumaric acid, was reported by Guisti and Wrolstad (*3*). Moreover, studies with carrot cell cultures resulted in only cyanidin 3-xylosyl-glucosyl-galactoside and its derivates, some of which acylated with cinnamic acids (*33*, *34*). Combining the information from the literature and our data obtained from the saponified and acid-hydrolyzed pigments, it may be proposed that peak 1 is cyanidin 3-xylosyl-glucosyl-galactoside and peak 2 is cyanidin 3-xylosyl-galactoside since HPLC analysis of saponified anthocyanins showed an increase in the relative amount of peak 1 as compared to peak 2 (**Figure 2b**); furthermore, triglycosides have shorter retention times than diglycosides (*35*). Peaks 3−5 are probably acylated with one of the following aromatic acids: sinapic, ferulic, or *p*-coumaric acids. Further identification studies on these black carrot anthocyanins are required.

Anthocyanin Profile during Storage. The anthocyanin profiles of shalgam samples after 90 days of storage at different temperatures are given in **Figure 3**, which are in accordance with the monomeric anthocyanin data. The anthocyanin profile of shalgam changed during storage with the highest degradation rates observed in the samples stored at 40 °C. In all samples, the highest retention was observed for peak 4. The major acylated anthocyanin (peak 4) of control samples retained 91.2, 64, and 5% of its initial level when stored at 4, 25, and 40 °C for 90 days (**Figure 3**). While acylated anthocyanins retained 48.9, 32.3, and 7.9% of their initial level when stored for 30, 60, and 90 days, respectively, nonacylated anthocynins in control samples retained only 11.1% of their initial level upon 30 days and were completely degraded after 60 days of storage at 40 °C (**Figure 4**).

Statistical evaluations showed that there were no significant positive or negative effects ($p < 0.05$) of pasteurization and sorbate treatment on total anthocyanin content nor on the anthocyanin profile with respect to control at any storage temperature. The effect of 40 °C storage temperature on the anthocyanin content was significant ($p < 0.05$) while there was no significant difference between 4 and 25 °C. Acylated antocyanins were significantly more stable ($p < 0.05$) than the nonacylated anthocyanins at all storage temperatures.

Kinetics of Pigment Stability. The reaction rate constants (k) were determined taking the reaction rate as first-order. Similar kinetic responses for several anthocyanins have been reported (*21*−*24*, *36*).

The following first-order degradation reaction equation was used to evaluate the reaction rate constants:

$$C/C_0 = \exp(-kt)$$

where C is the pigment concentration at time t, C_0 is the initial pigment concentration (mg/mL), k is the first-order reaction rate constant (days^{-1}), and t is time (days).

Table 1. Degradation Reaction Rate Constants (k), Correlation Coefficients (in Parantheses), Half-Life Periods ($t_{1/2}$), and Activation Energies (E_a) for Monomeric Anthocyanins in Shalgam (Control, Pasteurized, and Sorbate Added) Stored at Different Temperatures

	temp (°C)	k (days^{-1})	$t_{1/2}$ (days)	E_a (kcal/mol)
control	4	0.0029 (0.9640)	239	
	25	0.0087 (0.7220)	80	11.11 (0.9707)
	40	0.0311 (0.9950)	22	
pasteurized	4	0.0030 (0.5327)	231	
	25	0.0089 (0.9383)	78	11.24 (0.9664)
	40	0.0332 (0.9814)	21	
sorbate added	4	0.0029 (0.7286)	239	
	25	0.0126 (0.9472)	55	11.64 (0.9999)
	40	0.0331 (0.9930)	21	

The half-life periods of pigments were calculated from the following equation:

$$t_{1/2} = \frac{-\ln(0.5)}{k}$$

The temperature dependence of the reaction rate constants followed an Arrhenius type relation:

$$k = k_0 \exp(E_a/RT)$$

where E_a is the activation energy (kcal/mol), R is the universal gas constant (kcal/mol K), and T is the absolute temperature (K).

The half-life values of anthocyanins in shalgam samples ranged between 231 and 239, 55 and 80, and 21 and 22 days when stored at 4, 25, and 40 °C, respectively. The E_a values for the control, pasteurized, and sorbate-added samples were calculated as 11.11, 11.24, and 11.64 kcal/mol, respectively (**Table 1**). The statistical analysis of the results showed no significant ($p < 0.05$) differences among the rate constants and also half-life values of the control, pasteurized, and sorbate-added samples.

In this study, the effect of storage temperature on the stability of the total and individual anthocyanins in shalgam was investigated. Anthocyanin degradation in terms of monomeric, total, and individual anthocyanins was measured during storage at different temperatures. Pasteurization and sorbate addition did not affect the stability of anthocyanins as compared to control samples. Anthocyanin degradation was affected by structure. Nonacylated anthocyanins were more sensitive to temperature changes than acylated ones. The anthocyanin degradation was taken as first-order kinetics, and the dependence of reaction rates on storage temperature was calculated using an Arrhenius relationship. The half-life values obtained from the first-order degradation kinetics suggest that commercial shalgam products should be kept at refrigeration temperature for long-term storage and could be stored at room temperature for short-term consumption.

The definite anthocyanin profile of shalgam can be an indicator of the product quality and authenticity. Moreover, the degradation pattern of the anthocyanin profile could serve as a good indicator of the storage time−temperature history of the commercial shalgam product.

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