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The stability of pelargonidin-based anthocyanins at varying water activity

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Abstract

The stability of pelargonidin 3-glucoside, pelargonidin 3-sophoroside and pelargonidin 3-sophoroside 5-glucoside acylated with malonic and cinnamic acids was determined at varying water activities. Model systems, containing purified anthocyanin in pH 3.4 citrate buffer and glycerol, were stored at 25°C in the dark for 242 days. Changes in pigment, degradation index, and anthocyanin profile, as monitored by high-performance liquid chromatography (HPLC), were studied. In general, anthocyanin degradation followed first order kinetics and the degree of anthocyanin degradation increased with water activity. Half lives of the anthocyanins ranged from 56 to 934 days. Changes in the chromatographic profile showed hydrolysis of pelargonidin 3-sophoroside to pelargonidin 3-glucoside and production of malonic acid from the acylated anthocyanin.

Keywords: Anthocyanin; Pigment; Water activity; Rate of degradation; Half life

1. Introduction

Pelargonidin 3-glucoside (pgd 3-glu), which is the major anthocyanin (ACN) present in strawberries, is responsible for the attractive, bright red colour of this fruit. Colour of food is an important factor influencing consumers' acceptability; consequently, the loss of this quality reduces the marketability of strawberry products. The short half life ($t_{1/2}$) of pgd 3-glu, and consequent appearance of undesirable brown substances upon processing of the fruit, has been well documented (Abers & Wrolstad, 1979; Erlandson & Wrolstad, 1972; Garzón & Wrolstad, 2001; Kertesz & Sondheimer, 1948; Mackinney & Chichester, 1952; Pilando, Wrolstad, & Heatherbell, 1985; Wrolstad, Lee, & Poei, 1980; Wrolstad, Putnam, & Varseveld, 1970). It has been concluded from these studies that the prompt hydrolysis of glucose from the ACN molecule is responsible for the pigment degradation during processing. In addition, composition of the system can lead to Maillard browning, enzymic browning, ascorbic acid degradation, and polymerization of reactive phenolics. All the mentioned reactions can contribute to browning of strawberry products.

Garzón and Wrolstad (2001) observed that stability of pgd 3-glu also depends to a large extent on the composition of the system in which ACNs are present. Strawberry juice and strawberry concentrate spiked with the same pelargonidin derivatives, at the same concentration levels, displayed $t_{1/2}$ values of the monomeric pigment of 3.5–5 days in strawberry concentrate and 8–12 days in strawberry juice.

The chemical structure of ACNs is believed to be a major factor influencing the stability of these pigments. ACNs may be glycosylated and acylated by different sugars and acids. Increase in glycosidic substitution and the presence of aromatic acyl groups are reported to give more stability to the ACN molecule (Markakis, 1982; Mazza & Miniati, 1993). The higher colour stability of red raspberry wine, than strawberry wine, is believed to be due to the diglycosidic nature of sophorose present in raspberry ACN (Rommel, Heatherbell, & Wrolstad 1990; Rommel, Wrolstad, & Heatherbell, 1992). Acylation of the ACN molecule is reported to improve stability to these pigments (Asen & Norris,

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1977; Brouillard, 1981; Goto, Hoshino, & Takase, 1979; Hoshino, Matsumoto, & Goto, 1980). Studies on colour and pigment stability of cherries coloured with radish ACN report a $t_{1/2}$ between 29 and 33 weeks for pelargonidin 3-sophoroside 5-glucoside acylated with cinnamic and malonic acids (acyl-pgd 3-soph 5-glu), the main ACN present in radish (Guisti & Wrolstad, 1996b).

Water activity ($A_{w)}$ is another factor influencing the stability of the ACNs. Markakis, Livingston, and Fellers (1957) observed that ACNs were stable when stored in dry crystalline form or on dry paper chromatograms, which resulted in the hypothesis that water was involved in discolouration reactions. Erlandson and Wrolstad (1972) found that degradation of ACNs in freeze-dried strawberry puree increased when stored at high relative humidity (RH) conditions; however, Kearsley and Rodriguez (1981) reported that ACN powder, dissolved in different concentrations of glycerol–water solutions, was relatively stable to changes in $A_{w.}$ Brønnum and Flink (1985) showed that loss of freeze-dried elderberry ACNs was significant only when A_w values were above 0.51 and Thakur and Arya (1989) found that increase in A_w produced loss of grape ACNs adsorbed onto microcrystalline cellulose. Studies conducted by Sian and Soleha (1991) resulted in a slight increase of retention of pineapple and papaya ACNs as the percentage of glycerol increased from 10 to 20%, but a drop in retention was observed when the percentage of glycerol increased from 20 to 30%.

Due to the complexity of chemical reactions taking place in natural systems, e.g. juice, concentrate, jams, and jellies, it is difficult to isolate a single factor that explains changes in ACNs. Therefore, evaluation of a particular parameter requires model systems in order to closely control the composition and factors influencing pigment stability.

The objective of this project was to isolate and evaluate the effects of chemical structure and A_w on the stability of purified pelargonidin ACNs. ACNs with mono, di and acylated-triglycosidic substitution were evaluated in model systems in which composition factors could be closely controlled. A second objective was to compare the stability of the pelargonidin derivatives in model systems with the stability of the same derivatives when they are present in natural systems, as previously reported by Garzón and Wrolstad (2001).

2. Materials and methods

2.1. Plant materials

Individually quick-frozen (I.Q.F.) strawberries (*Fragaria anannassa* cv., Totem) were supplied by Conroy Packing Inc. (Salem, OR). Orange nasturtium flowers (*Tropaeolum majus*) were collected from local gardens during the summer season (Corvallis, OR). Stems and leaves were separated from the petals, which were then frozen with liquid nitrogen. Red radishes (*Raphanus sativus* L. cv., fuego) were obtained from the local market in Corvallis, OR. The radishes were peeled manually; the peel was washed and frozen under liquid nitrogen. All frozen materials were stored at −23°C until further processing.

2.2. Chemicals and reagents

Glycerol was supplied by Mallinckrodt Chemical, Inc. (Paris, KY), citric acid monohydrate was purchased from J.T. Baker (Phillipsburg, NJ), sodium citrate was obtained from Archer Daniels Midland (Faries Parkway, Decatur, IL), sodium benzoate was purchased from Chemical Works (St. Louis, MO), and potassium sorbate was acquired from Monsanto Company (St. Louis, MO).

2.3. Pigment extraction and isolation

Extraction and isolation of the ACNs from strawberries, nasturtium flowers, and radish peel were performed by the method described by Guisti and Wrolstad (1996b). The amount of frozen material used for extraction was approximately 2 kg of frozen strawberries, 2 kg of frozen petals, and 250 g of frozen radish peel. Individual samples were ground to a powdery state with liquid nitrogen using a stainless steel Waring blender. The powder was blended with acetone (2 l/kg powder) for extraction of the ACNs, which were then separated from the cake by filtration on a Buchner funnel. Total extraction of the pigments from the cake residue was achieved with aqueous acetone (30:70 v/v). Filtrates were combined and shaken in a separatory funnel with chloroform (1:2 acetone: chloroform v/v) and stored at 10°C overnight for clean separation between the aqueous and organic phases.

2.4. Anthocyanin purification

The aqueous extract was transferred to a Bucchi rotary evaporator to remove residual acetone at 40°C. The ACNs were subsequently purified using a C-18 cartridge (high load C-18 tube), 20 ml capacity (Alltech Assoc., Inc., IL). The cartridge was washed with 0.01% HCl upon loading with pigment, and the ACNs were eluted with methanol, acidified with 0.01% HCl; elimination of methanol and concentration of pigments were achieved by additional rotary evaporation at 40°C. In all cases, the aqueous extract was diluted to a known volume with distilled water. Total monomeric ACN content was determined as mg pgd 3-glu/l with a molar absorptivity of 22,400 and molecular weight of 433.2 using the pH differential method as described by

Wrolstad (1976). Colour density and polymeric colour were calculated as the sum of the absorbance at 420 nm and that at the absorbance maximum of an untreated or of a bisulfite treated sample, respectively.

2.5. Semipreparative HPLC

Separation and collection of pelargonidin derivatives was done using semipreparatory high-performance liquid chromatography (HPLC) techniques. Pgd 3-glu from strawberries, pelargonidin 3-sophoroside (pgd 3-soph) from nasturtium flowers and acyl-pgd 3-soph 5-glu from radish peel were obtained using a Dynamax SD-300 chromatograph (Raining Instrument Company, Inc., Woburn, MA) equipped with a Hewlett-Packard 1040A photodiode array detector and a Hewlett-Packard 9000 computer. A reverse phase Supelcosil PLC-18 column (25 cm×21.2 mm; Supelco Inc., Bellefonte, PA) was used for preparative isolation. Three different mobile phases were used for separation.

Mobile phase I was used for collection of pgd 3-glu: 12% acetonitrile, 10% acetic acid, 1% phosphoric acid under isocratic conditions. Flow rate was 20 ml/min, injection volume 1 ml. Primary detection was at 520 nm.

Mobile phase II was for collection of pgd 3-soph: 8% acetonitrile, 10% acetic acid, 1% phosphoric acid under isocratic conditions. Flow rate was 20 ml/min, injection volume 1 ml. Primary detection was at 520 nm.

Mobile phase III was for collection of acyl-pgd 3-soph 5-glu: 18% acetonitrile, 10% acetic acid, 1% phosphoric acid under isocratic conditions. Flow rate was 20 ml/min, injection volume 1 ml. Primary detection was at 520 nm.

Purity of the three ACN fractions was checked by analytical HPLC.

2.6. Analytical HPLC

The HPLC system consisted of a Perkin–Elmer Series 400 Liquid Chromatograph (Perkin–Elmer Corp., Norwalk, CT) equipped with the detector and computer described in the semipreparatory HPLC section. The analytical column was a ODS C-18 column (25 cm×4.6 mm), 5 μ particle size (PolyLC Inc., Columbia, MD). The mobile phase composition was: solvent A: 100% acetonitrile, B: 5% acetonitrile, 10% acetic acid, 1% phosphoric acid. Separations were achieved with a linear gradient from 2 to 13% A in 15 min, 13 to 15% A in 5 min, holding at 15% A for 15 min. Elution was at 1 ml/min, Injection volume was 100 μl, primary detection was at 520 nm. UV–vis spectra were taken directly from chromatographic runs.

2.7. Preparation of model systems

Model systems used for studies on ACN degradation were prepared at five A_w levels, which were obtained using glycerol as liquid humectant according to the proportions listed in Table 1. A_w levels were calculated according to the method of Norrish (1966):

$$\mathrm{Ln}\, A_w - \mathrm{Ln}\, X_2 = K_2(X_1)^2$$

where A_w = water activity, X_2 = mole fraction of water, X_1 = mole fraction of glycerol, and K_2 = a constant related to the free energy change in a binary system (for water-glycerol = −0.38).

The calculated A_w values were confirmed by Food Products Laboratories (Portland, OR) where measurements were carried out in a SC-3 thermocouple psychrometer (Decagon Devices, Inc., Pullman, WA 99163). The experimental A_w values were 1.0, 0.90±0.009, 0.89±0.009, 0.66±0.025 and 0.44±0.08 as reported in Table 1. A randomized block design with two replicates was used for each level of pigment; purified ACN, ranging from 240 to 320 mg/l was added to 0.1 M pH 3.4 citrate buffer/glycerol solutions. Potassium sorbate 0.1% (w/w) and sodium benzoate 0.1% (w/w) were used as antimicrobial agents. Twenty millilitres of each preparation were placed into vials which,

Table 1
Composition of model systems used for evaluation of anthocyanin stability at varying A_w

Glycerol (g)	pH 3.4 Citrate buffer (g)	Calculated A_w	Experimental[a] A_w	pH after pigment addition[a]			Monomeric anthocyanin[a] (mg/l)		
				Model system					
				pgd 3-glu	pgd 3-soph	acyl-pgd 3-soph 5-glu	pgd 3-glu	pgd 3-soph	acyl-pgd 3-soph 5-glu
0	100	1.0	1.0	2.4 (0.23)	2.3 (0.29)	2.5 (0.12)	294 (2.10)	250 (0.30)	251 (0.00)
20	80	0.90	0.90±0.009	2.3 (0.11)	2.2 (0.30)	2.4 (0.09)	320 (7.64)	240 (0.60)	255 (2.40)
32.3	67.7	0.86	0.89±0.009	2.5 (0.08)	2.6 (0.04)	2.6 (0.25)	288 (5.10)	251 (3.00)	263 (2.25)
70	30	0.63	0.66±0.025	2.4 (0.04)	2.3 (0.23)	2.4 0.28)	319 (8.54)	241 (1.65)	267 (7.49)
85	15	0.37	0.44±0.08	2.3 (0.20)	2.2 (0.07)	2.4 (0.04)	298 (2.10)	263 (17.99)	271 (1.05)

Values in parentheses are standard errors.

[a] The values reported are averages of two replicates.

together with the teflon-lined caps, had been previously sterilized at 125°C for 15 min. After pasteurization for 30 s at 85°C, the vials were placed in a dark room at 25°C for storage.

2.8. Pigment analysis

Total monomeric ACN was determined as described in the ACN purification section. The methods described by Somers and Evans (1977) were used to determine polymeric colour, colour density and degradation index. Polymeric colour (%) was calculated as the ratio between polymeric colour and colour density $\times 100$. Degradation index was calculated as the ratio of the absorbance maximum of the ACN/absorbance at 420 nm. Determinations were made on a Shimadzu UV visible spectrophotometer, model UV 160 U, using 1 cm disposable cells. Previously described analytical HPLC techniques were applied periodically to monitor changes in chromatographic profile during storage.

2.9. Statistical analysis

Pigment degradation was plotted to represent first order kinetics and linear regression analysis was used to determine adequacy of the model. Rate constants were calculated from slopes of the lines plotted, and half lives ($t_{1/2}$) were calculated from the equation: $t_{1/2} = \text{Ln } 0.5/k$ where k = rate constant.

Statistical analysis of variance (ANOVA) was applied. Significant ($P < 0.05$) differences between means were identified using the least significant difference procedure (LSD). The analyses were performed with Statgraphics plus, version 3.

3. Results and discussion

3.1. Changes in total anthocyanin pigment during storage at different A_w

Linear regression analysis showed that ACN degradation, for the systems containing pgd 3-glu and pgd 3-soph, followed first order kinetics for all A_w levels and pigments tested. A first order reaction is described by the equation:

$$C/Co = \exp \quad (-kt),$$

where C = pigment concentration at time t; Co = initial pigment concentration; k = reaction rate constant; t = time. The linear regression model corresponding to this equation is defined as:

$$\text{Ln}(C/Co) = -\beta_1(\text{time}) + \beta_0$$

where C/Co = monomeric ACN concentration at any time/initial monomeric ACN concentration, β_0 = inter- = intercept, and β_1 = slope. First order kinetics for degradation of ACNs has been reported on black raspberry (Daravingas & Cain, 1968), sour cherry (Cemeroglu, Velioglu, & Isik, 1994), concord grape, red cabbage, and ajuga ACNs (Baublis, Spomer, & Berber-Jimenez, 1994), radish (Guisti & Wrolstad, 1996b), and strawberry (Garzón & Wrolstad, 2001).

When linear regression analysis was applied to the data from the samples at high A_w (1, 0.90, 0.89) to which acyl-pgd 3-soph 5-glu had been added, high correlation coefficients (r) were obtained. This indicates a relatively strong relationship between the variables Ln of the ACN concentration and time; however, the lack of fit test was significant (P-value < 0.01). This, along with some curvature observed on the plot representing the two variables, suggested that a linear regression model describing first order kinetics was not an adequate model for the observed data. If $t_{1/2}$ of the ACN present in these particular samples were calculated, based on linear regression analysis, which involves extrapolation, the real $t_{1/2}$ of the pigment could be underestimated. Therefore, comparison of the stability at these particular A_w levels was based on the time at which 15% of the initial ACN was degraded during storage; this calculation involves interpolation where reasonable straight lines can be drawn and values are known. The lack of linearity between the variables Ln ACN concentration and time, of some of our samples, is in good agreement with results obtained by Erlandson and Wrolstad (1972). During the study of degradation of freeze-dried strawberry ACNs, stored at six levels of RH, they found that none of the samples showed first order kinetics throughout the entire storage time. Our findings along with the results from Erlandson and Wrolstad (1972) seem to support the hypothesis by Labuza (1980). He stated that a major effect of modification of A_w on reaction rates could be to change the order of the reaction, but that unfortunately, most studies on food reactions did not allow degradation to proceed far enough to determine the true order. This is because most reactions are very slow or significant loss of quality occurs at a low extent of reaction (Labuza, 1979).

Degradation parameters, calculated from linear regressions, are listed in Tables 2, 3 and 4. Analysis of the three samples containing acyl-pgd 3-soph 5-glu, that did not follow linearity, are reported in Table 5.

Table 2 shows that the extent of ACN degradation increased with A_w in model systems containing pgd 3-glu. Reaction rate constants decreased from -3.74×10^{-3} at A_w 1 to -7.48×10^{-4} at A_w 0.66. Reduction of the A_w from 1 to 0.66 caused a five-fold increase in $t_{1/2}$ from 186 to 934 days for pgd 3-glu. No significant difference in rate of degradation was observed between the sample at A_w 0.44 and the sample at A_w 0.66.

Table 2
Linear regression and first order reaction parameters for the degradation of model systems containing pelargonidin 3-glucoside

A_w	β_0 (intercept)	β_1 (slope) Rate of degradation (K) days^{-1}	r^2	$t_{1/2}$ (half life days)
1	$-2.72\times10-^{2}$	-3.74×10^{-3} a	0.97	186
	(1.91×10^{-2})	(1.62×10^{-4})		(14.4)
0.90	-2.74×10^{-2}	-2.08×10^{-3} b	0.95	332
	(1.37×10^{-2})	(1.16×10^{-4})		(2.44)
0.89	-1.28×10^{-2}	-2.49×10^{-3} b	0.93	278
	(1.99×10^{-2})	(1.68×10^{-4})		(5.64)
0.66	3.41×10^{-3}	-7.48×10^{-4} d	0.97	934
	(3.74×10^{-3})	(3.15×10^{-5})		(83.9)
0.44	1.27×10^{-2}	-1.15×10^{-3} d	0.77	690
	(1.84×10^{-2})	(1.55×10^{-4})		(234.9)

Values in parentheses are standard errors of two duplicates. Means in the same column with different letters indicate significant difference ($P<0.05$).

Table 3
Linear regression and first order reaction parameters for the degradation of model systems containing pgd 3-soph

A_w	β_0 (intercept)	β_1 (slope) Rate of degradation (K) days^{-1}	r^2	$t_{1/2}$ (half life days)
1	-2.66×10^{-2}	-3.02×10^{-3} a	0.95	232
	(2.01×10^{-2})	(1.74×10^{-4})		(24.3)
0.90	-1.18×10^{-2}	-1.67×10^{-3} b	0.96	416
	$(9.63\times10-^{3})$	(8.11×10^{-5})		(1.78)
0.89	-4.43×10^{-2}	-2.02×10^{-3} b	0.94	345
	(1.56×10^{-2})	(1.31×10^{-4})		(23.2)
0.66	$8.78\times10-^{3}$	-9.46×10^{-4} c	0.97	733
	(5.29×10^{-3})	(4.46×10^{-5})		(7.47)
0.44	-5.14×10^{-3}	$-1.39\times10-^{3}$ b c	0.77	500
	(2.28×10^{-2})	(1.92×10^{-4})		(40.9)

Values in parentheses are standard errors of two replicates. Means in the same column with different letters indicate significant difference ($P<0.05$).

Table 4
Linear regression and first order reaction parameters for the degradation of model systems at A_w 0.66 and 0.44 containing acyl-pelargonidin 3-sophoroside 5-glucoside

A_w	β_0 (intercept)	β_1 (slope) Rate of degradation (K) days^{-1}	r^2	$t_{1/2}$ (half life days)
0.66	2.21×10^{-3}	-2.49×10^{-3} a	0.96	278
	(1.49×10^{-2})	(1.25×10^{-4})		(9.05)
0.44	8.23×10^{-2}	-1.23×10^{-2} b	0.99	56
	(3.26×10^{-2})	(2.75×10^{-4})		(1.76)

Values in parentheses are standard errors of two replicates. Means within a column with different letters are significantly different ($P<0.05$). Means within a row with different letters in parentheses for each A_w level are significantly different ($P<0.05$).

Table 5
Time for 15% of the initial anthocyanin to degrade during storage of model systems at high A_w levels (1, 0.90, 0.89)

A_w	Time (days)		
	pgd 3-glu	pgd 3-soph	acyl-pgd 3-soph 5-glu
1	28 a(a)	74 a(b)	15 a(a)
	(7)	(0)	(0)
0.90	60 ab(a)	49 a(a)	10 a(b)
	(10)	(10)	(2)
0.89	80 b(a)	50 a(b)	11 a(c)
	(6)	(0)	(4)

Values in parentheses are standard errors of two replicates. Means within a column with different letters are significantly different ($P<0.05$). Means within in a row with different letters in parentheses for each A_w level are significantly different ($P<0.05$).

Fig. 1 shows the change in monomeric ACN for the pgd-3-glu systems at different A_w. There is an apparent increase in the rate of degradation for the sample at A_w 0.44 at day 242 of the study. Changes in monomeric pgd 3-glu are in accordance with changes in percentage of polymeric colour during storage (Fig. 2). Model systems at A_w 1 presented higher percentage of polymeric colour than samples with other A_w levels ($P-<0.01$). Although the sample at A_w 0.44 showed an increase in percentage of polymeric colour, at the end of the storage, this sample, along with the one at A_w 0.66, presented the lowest amount of polymers. No difference in percentage of polymeric colour was found among samples at $A_w<1$ ($P->0.1$). Similar trends were observed with regard to effects of A_w on the rate of ACN degradation for the

model systems containing pgd 3-soph. Increase in A_w caused an increase in ACN degradation (Table 3). A threefold increase in $t_{1/2}$ of pgd 3-soph from 232 to 733 days was observed. These numbers correspond to rates of degradation ranging from -3.02×10^{-3} at A_w 1 to -9.46×10^{-4} at A_w 0.66. In Fig. 3, the sample at A_w 0.44 shows a sharp drop in monomeric ACN concentration at the latter stages of storage. Because of this, no significant

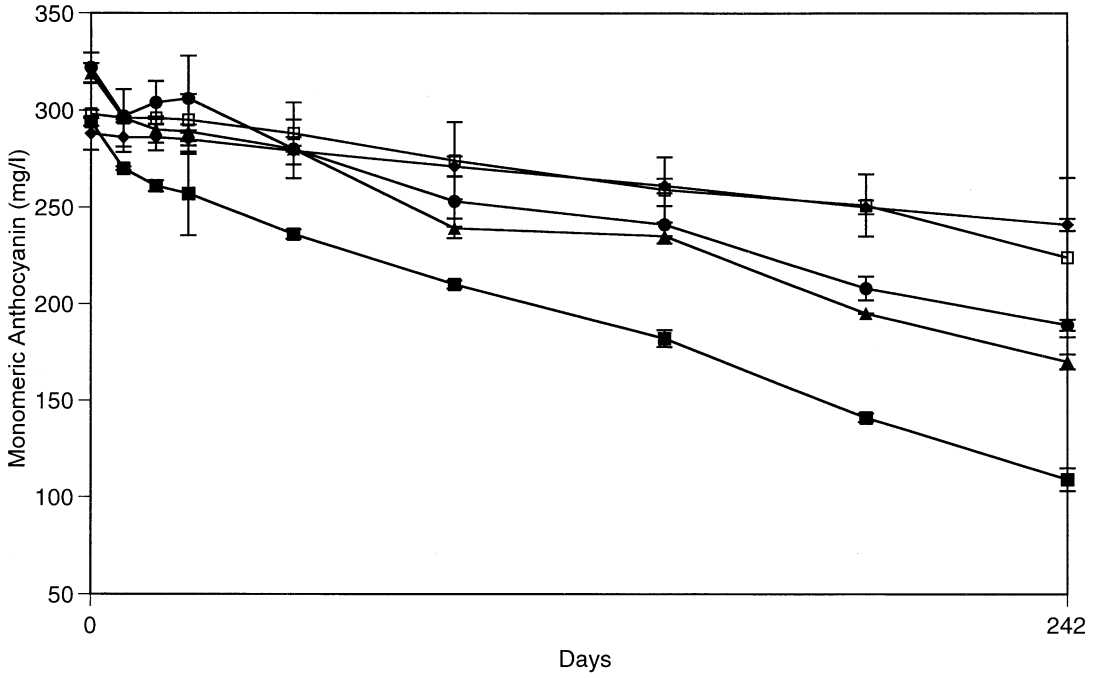

Fig. 1. Changes in monomeric anthocyanin colour during storage of model systems containing pelargonidin 3-glucoside. ■ A_w 1, ● A_w 0.90, ▲ A_w 0.89, ◆ A_w 0.66, □ A_w 0.44.

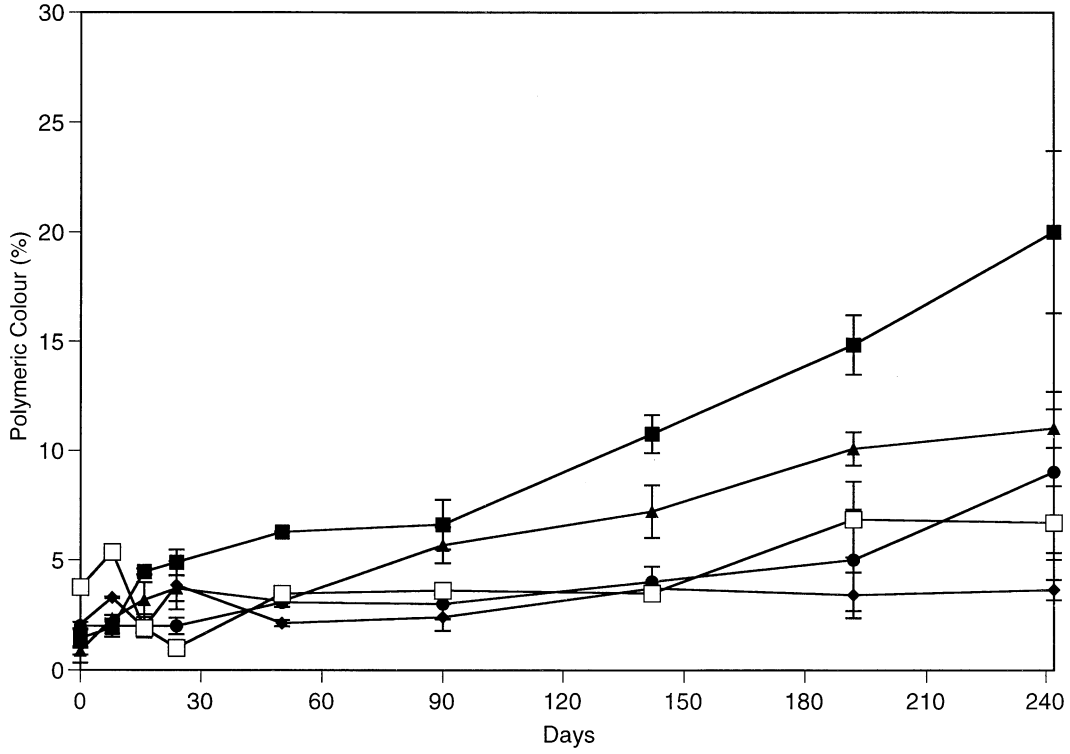

Fig. 2. Changes in percentage of polymeric colour during storage of model systems containing pelargonidin 3-glucoside. ■ A_w 1, ● A_w 0.90, ▲ A_w 0.89, ◆ A_w 0.66, □ A_w 0.44.

difference was found between this sample and the sample at A_w 0.9 ($P->0.1$).

Changes in percentage of polymeric colour in systems containing pgd 3-soph, are shown in Fig. 4. Formation of polymers increased correspondingly with ACN pigment degradation. The highest percentage of polymers was found in the sample at A_w 1, which was significantly different from the others ($P-<0.01$). Samples with close levels of A_w did not present differences in the amount of percentage of polymeric colour, e.g. A_w 0.89 and 0.9, A_w

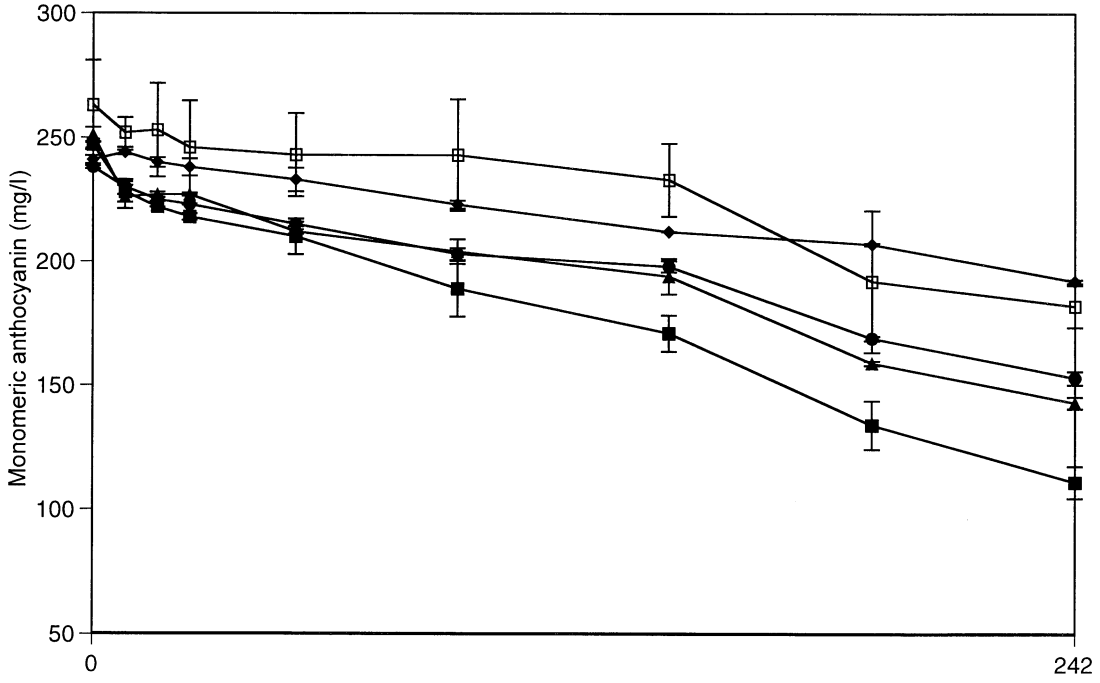

Fig. 3. Changes in monomeric anthocyanin anthocyanin colour during storage of model systems containing pelargonidin 3-sophoroside. ■ A_w 1, ● A_w 0.90, ▲ A_w 0.89, ◆ A_w 0.66, □ A_w 0.44.

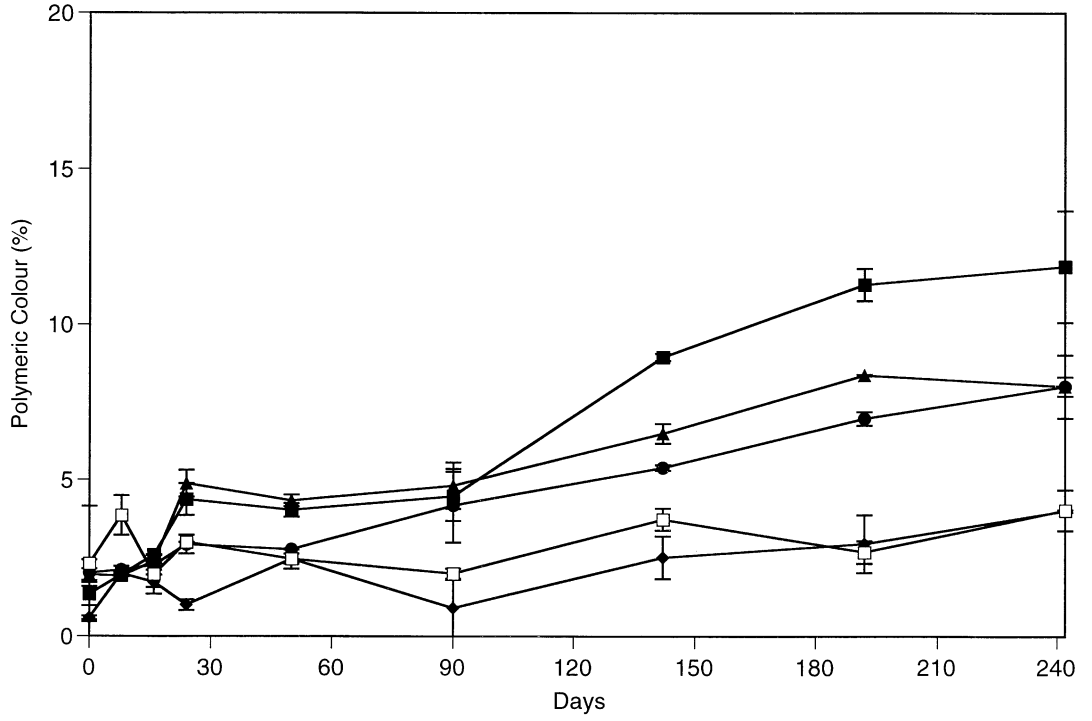

Fig. 4. Changes in percentage of polymeric colour during storage of model systems containing pelargonidin 3-sophoroside. ■ A_w 1, ● A_w 0.90, ▲ A_w 0.89, ◆ A_w 0.66, □ A_w 0.44.

0.66 and 0.44 ($P-<0.1$). Under the conditions of our experiment, the model systems containing pgd 3-glu and pgd 3-soph presented very similar trends during storage. The highest retention of monomeric ACN, for both samples, was observed at A_w 0.66. However, there was no significant difference in the rate of degradation between the samples at A_w 0.66 and A_w 0.44 ($P->0.1$). The monomeric pigment, in both systems, presented the highest rate of degradation at A_w 1 and significant variation was found when comparing all the samples with the one at A_w 1 ($P-<0.01$). The similar behaviour of these two pigments was attributed to their similar molecular structures.

Fig. 5 illustrates changes in monomeric ACN in model systems to which acyl-pgd 3-glu 5-soph had been added. A direct relationship was found between ACN degradation and *A*w, except for the sample at 0.44, which exhibited a pronounced decrease in monomeric ACN during the entire period of storage. Table 4 reports the linear regression parameters for the degradation of the samples at intermediate and low A_w, e.g. 0.66 and 0.44, which presented good fitness for the linear regression model. The rate of ACN degradation between these two samples was significantly different, ranging from -2.49×10^{-3} at A_w 0.66 to -1.23×10^{-2} at A_w 0.44. This represents a five-fold increase in $t_{1/2}$ from 56 to 278 days. Table 5 reports the time for 15% of the initial ACN concentration to degrade in samples at high A_w (1, 0.90, 0.89); for the samples spiked with acyl-pgd 3-soph 5-glu, the times ranged from 10 days for the sample at A_w 0.90 to 15 days for the sample at A_w 1. No significant differences between the times of degradation were detected among these samples ($P->0.1$).

When examining Fig. 6, a marked increase in percentage of polymeric colour was noticed in the sample at A_w 0.44 containing acylated pigment. This was the only sample significantly different from the others ($P-<0.01$).

Parameters comparing the stability of the three pgd derivatives at the same A_w level are summarized in Tables 5 and 6. The time needed for the model systems at A_w 1, 0.90 and 0.89, to lose 15% of the initial pigment, ranged between 15 and 74 days. Statistical analysis showed that this time was significantly higher for the system containing pgd 3-soph than for the other two systems ($P-<0.01$). Comparison of the rate of degradation among samples with different pigments at A_w 0.66 and A_w 0.44 showed a significantly higher rate of degradation of the sample containing acyl-pgd 3-glu 5-soph than the other two (Table 6). These results differ from the ones obtained by Garzón and Wrolstad (2000) when comparing the stability of the same pgd derivatives in strawberry juice and concentrate. No difference, either in k or $t_{1/2}$ was found among these pigments; nevertheless, the ACNs were more stable in juice; the $t_{1/2}$ of all pgd derivatives was 12 days in juice, as compared with 4 days in the concentrate. From these results, it is clear that the components of the environment in which ACNs are present are definitely involved in their degradation.

The systems that contained acyl-pgd 3-glu 5-soph did not present higher stability, in spite of the acylated nature

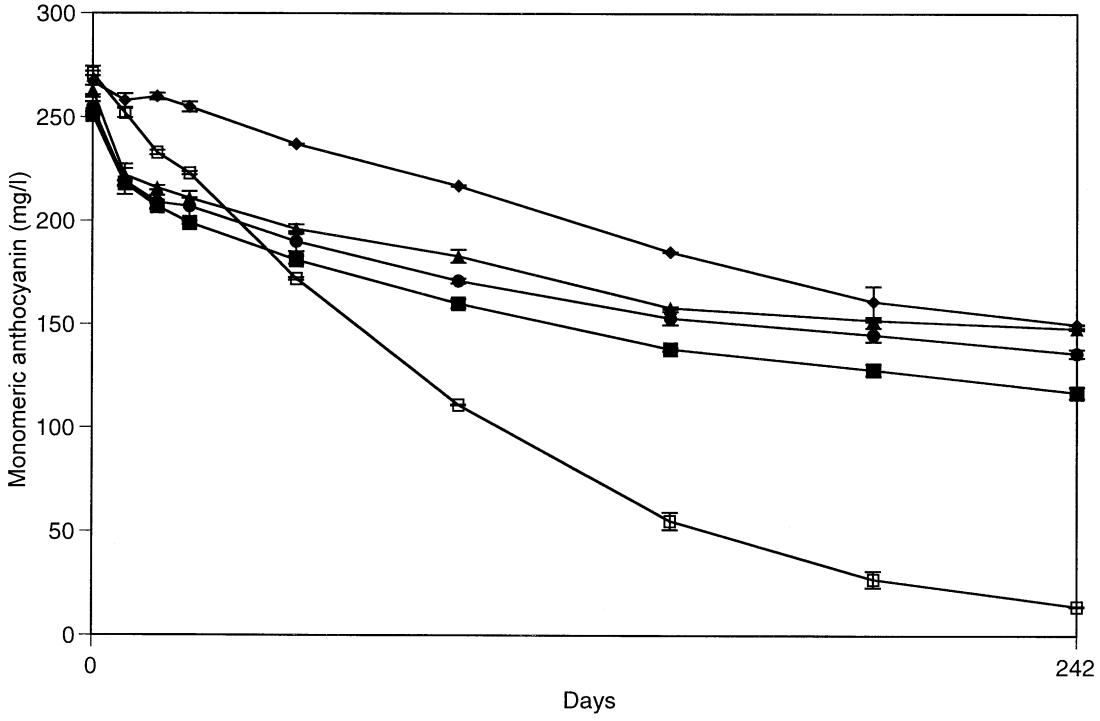

Fig. 5. Changes in monomeric anthocyanin colour during storage of model systems containing acyl-pelargonidin 3-sophoroside 5-glucoside. ■ A_w 1, ● A_w 0.90, ▲ A_w 0.89, ◆ A_w 0.66, □ A_w 0.44.

of the molecule. This was especially true for the sample at A_w 0.44 whose $t_{1/2}$ was only 56 days. These results suggest that the acylated ACN exhibits an unusual behaviour when present in model systems containing glycerol, which confirms the theory proposed by Brouillard, Figueiredo, Elhabiri, and Dangles (1997) concerning colour stabilities of ACNs being deeply affected by the nature of their physico-chemical environment. Acylation of the ACN molecule lends stability in most cases (Bassa & Francis, 1987). However, some model systems have not shown increase in stability with increase in acylation. As an example, comparison of the ACNs of grape, red cabbage, and *Ajuga reptans*, in pH 3.5 citrate buffer solutions, did not present differences in the rates of degradation among the three pigments in spite of their different chemical configurations (Baublis et al., 1994). ACNs from grapes include mono and diglucosides of five different aglycones with the addition of monoacylation (Lea, 1988), red cabbage contains diacylated triglucosides of cyanidin (Mazza & Miniati, 1993), and *Ajuga reptans* contains glucosylated cyanidin, acylated with p-hydroxycinnamic acid, ferulic acid, and malonic acid (Callebaut, Hendrickx, Voets, & Motte, 1990). Similar findings were reported by previous studies comparing the stability of pgd 3-glu, pgd 3-soph, and acyl-pgd 3-soph 5-glu and showed no significant differences in remaining ACN, $t_{1/2}$ or rate of degradation in spiked strawberry juice or spiked strawberry concentrate (Garzón & Wrolstad, 2001).

The unusual behaviour of the samples containing acylated ACN, along with the accelerated degradation of monomeric ACN at latter stages of the storage of model systems at A_w 0.44, suggests some kind of interaction between the pigment and glycerol. Labuza (1980) mentioned that glycerol has water-like properties that produce a plasticizing effect and increase reactant

Table 6
Rate constants for anthocyanin degradation during storage of model systems containing pelargonidin-based anthocyanins at intermediate and low A_w levels (0.66, 0.44)

A_w	Rate of degradation constant (K) $days^{-1}$		
	pgd 3-glu	pgd 3-soph	acyl-pgd 3-soph 5-glu
0.66	-7.48×10^{-4} a(a) (3.15×10^{-5})	-9.46×10^{-4} a(a) (4.46×10^{-5})	-2.49×10^{-3} a(b) (1.25×10^{-4})
0.44	-1.15×10^{-3} a(a) (1.55×10^{-4})	-1.39×10^{-3} a(a) (1.92×10^{-4})	-1.23×10^{-2} b(b) (2.75×10^{-4})

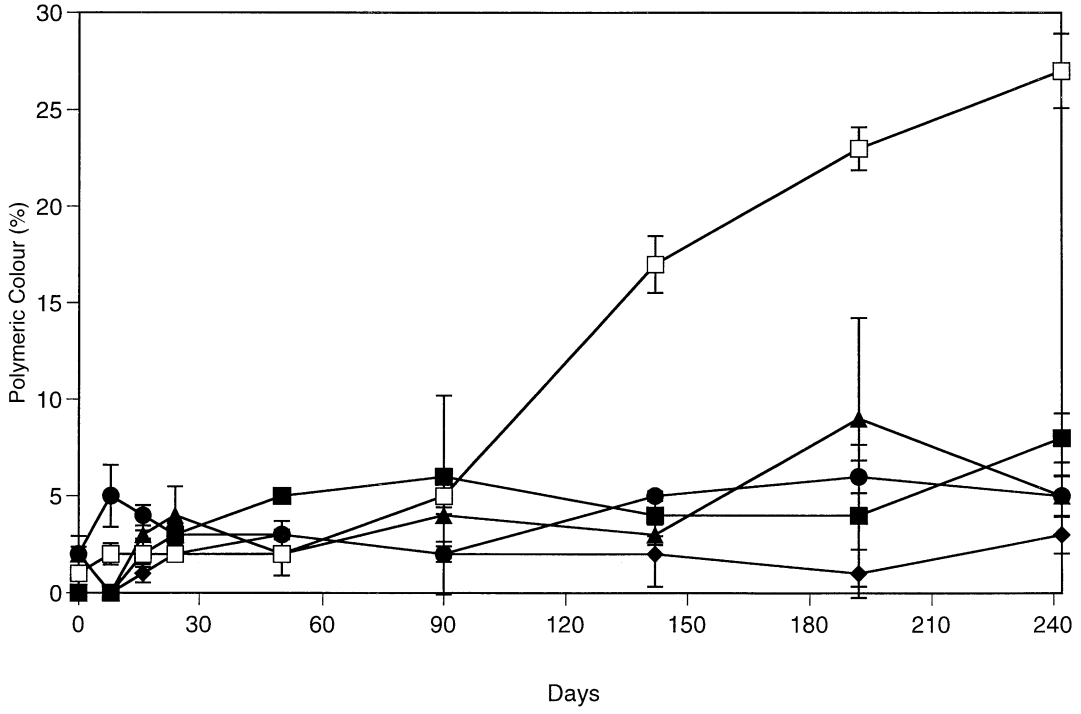

Fig. 6. Changes in percentage of polymeric colour during storage of model systems containing acyl-pelargonidin 3-sophoroside 5-glucoside. ■ A_w 1, ● A_w 0.90, ▲ A_w 0.89, ◆ A_w 0.66, □ A_w 0.44.

mobility and/or solubility at A_w levels below which most water-soluble reactions occur very slowly. Previously reported results on stability of ACNs at varying A_w, using glycerol as a humectant, are comparable with our general findings and support Labuza's theory. Soleha, Sian, and Mamat (1990) lowered the A_w of a tropical dried fruit mix with glycerol, based on its humectant property. These studies showed that the addition of glycerol increased the moisture content of the fruit and that the increase was directly proportional to the glycerol concentration. Similarly, evaluation of papaya and pineapple ACNs, after fruit cubes were soaked overnight in 10, 20 and 30% glycerol solutions, by Sian and Soleha (1991), showed that the concentration of ACN increased slightly as the percentage of glycerol increased from 10 to 20%, but dropped when 30% glycerol was added. This behaviour was explained as a result of hydrolysis and release of the sugar moeity of the ACN that takes place at high water content, such as in a system with 30% glycerol. Kearsley and Rodriguez (1981) compared absorbance readings at λ_{max} of model systems containing water, ACN powder, and glycerol at A_w levels ranging from 0.37 to 1. After incubation of the samples at 60, 90 and 160°C for 170 min, they found that, in all cases, there was an inverse relationship between absorbances and there was no difference between absorbance of the ACNs at A_w 0.63 and A_w 0.47.

The importance of the physico-chemical environment in which ACNs are present, even at the same A_w level, has been demonstrated. Systems using solutes other than glycerol to decrease A_w have also found a direct relationship between A_w and ACN degradation; however no accelerated degradation has been observed at low A_w levels. Thakur and Arya (1989) tested the degradation of grape ACNs in isolated model systems made of 80% (v/v) aqueous methanol and micro-crystalline cellulose at 0, 0.33, and 0.73 A_w levels. Results showed that, after 100 days of storage, the percentage of ACN retention was higher in the samples with lower A_w. When Brønnum and Flink (1985) stored freeze-dried elderberry ACNs in desiccators at different relative humidity levels, they observed that loss of ACN was significant only at A_w levels above 0.51.

Storage did not cause significant changes in the degradation index (A_{510}/A_{420}) of the samples. As reported in Table 7, only a slight drop in this parameter was observed, except for systems containing pgd 3-glu and pgd 3-soph at A_w 0.89, and for the system containing acyl-pgd 3-soph 5-glu at A_w 1 and 0.44 ($P<0.01$). Decrease in degradation index indicates development of brown pigments.

3.2. Changes in HPLC profile during storage

HPLC analysis of systems containing pgd 3-glu showed presence of only that pigment throughout the

Table 7
Changes in degradation index (A_{510}/A_{420}) during storage of model systems spiked with pelargonidin-based anthocyanins

System																			
A_w (days)	pgd 3-glu					pgd 3-soph					acyl-pgd 3-glu 5-glu								
	1	0.90	0.89	0.66	0.44	1	0.90	0.89	0.66	0.44	1	0.90	0.89	0.66	0.44
0	1.71 (0.03)	1.84 (0.02)	1.78 (0.01)	1.82 (0.03)	1.37 (0.39)	1.81 (0.03)	1.84 (0.03)	1.82 (0.01)	1.85 (0.01)	1.83 (0.04)	3.41 (0.01)	3.36 (0.20)	3.56 (0.04)	3.92 (0.51)	4.07 (0.03)
8	1.91 (0.13)	1.82 (0.02)	1.78 (0.01)	1.78 (0.02)	1.66 (0.03)	1.83 (0.01)	1.86 (0.00)	1.83 (0.03)	1.78 (0.01)	1.81 (0.04)	3.31 (0.07)	3.41 (0.05)	3.46 (0.07)	3.69 (0.49)	3.56 (0.04)
16	1.76 (0.01)	1.84 (0.01)	1.72 (0.01)	1.81 (0.01)	1.80 (0.01)	1.82 (0.04)	1.50 (0.29)	1.80 (0.03)	1.87 (0.01)	1.87 (0.00)	3.20 (0.09)	3.27 (0.04)	3.39 (0.03)	3.61 (0.02)	3.79 (0.01)
24	2.02 (0.01)	1.83 (0.02)	2.02 (0.00)	1.84 (0.05)	1.82 (0.00)	2.00 (0.01)	2.08 (0.01)	1.92 (0.08)	1.85 (0.01)	1.88 (0.01)	3.32 (0.11)	3.41 (0.04)	3.33 (0.06)	3.76 (0.00)	3.80 (0.01)
50	1.70 (0.02)	1.80 (0.02)	1.66 (0.03)	1.81 (0.01)	1.77 (0.01)	1.72 (0.01)	1.77 (0.02)	1.74 (0.00)	1.86 (0.00)	1.87 (0.02)	3.07 (0.01)	3.43 (0.02)	3.43 (0.01)	3.68 (0.07)	3.38 (0.07)
90	1.74 (0.01)	1.78 (0.02)	1.73 (0.04)	1.73 (0.01)	1.79 (0.00)	1.82 (0.02)	1.83 (0.01)	1.81 (0.04)	1.83 (0.02)	1.82 (0.04)	3.05 (0.14)	3.31 (0.05)	2.94 (0.02)	3.31 (0.07)	3.21 (0.14)
142	1.56 (0.04)	1.73 (0.02)	1.64 (0.00)	1.76 (0.02)	1.72 (0.02)	1.60 (0.01)	1.72 (0.02)	1.66 (0.02)	1.53 (0.29)	1.80 (0.00)	2.82 (0.08)	3.08 (0.06)	3.11 (0.02)	3.37 (0.00)	2.31 (0.06)
192	1.47 (0.02)	1.69 (0.02)	1.57 (0.03)	1.72 (0.03)	1.63 (0.01)	1.53 (0.02)	1.69 (0.01)	1.64 (0.02)	1.80 (0.02)	1.65 (0.20)	2.89 (0.03)	2.89 (0.16)	3.01 (0.10)	3.33 (0.02)	1.48 (0.10)
242	1.56 (0.04)	1.70 (0.07)	1.53[a] (0.05)	1.73 (0.02)	1.65 (0.04)	1.62 (0.04)	1.73 (0.00)	1.69[a] (0.00)	1.84 (0.00)	1.75 (0.04)	2.51[a] (0.05)	3.22 (0.03)	3.18 (0.07)	3.47 (0.01)	1.55[a] (0.04)

Values in parentheses are standard errors.
[a] Denotes significant difference with value at zero time in the same column ($P<0.05$).

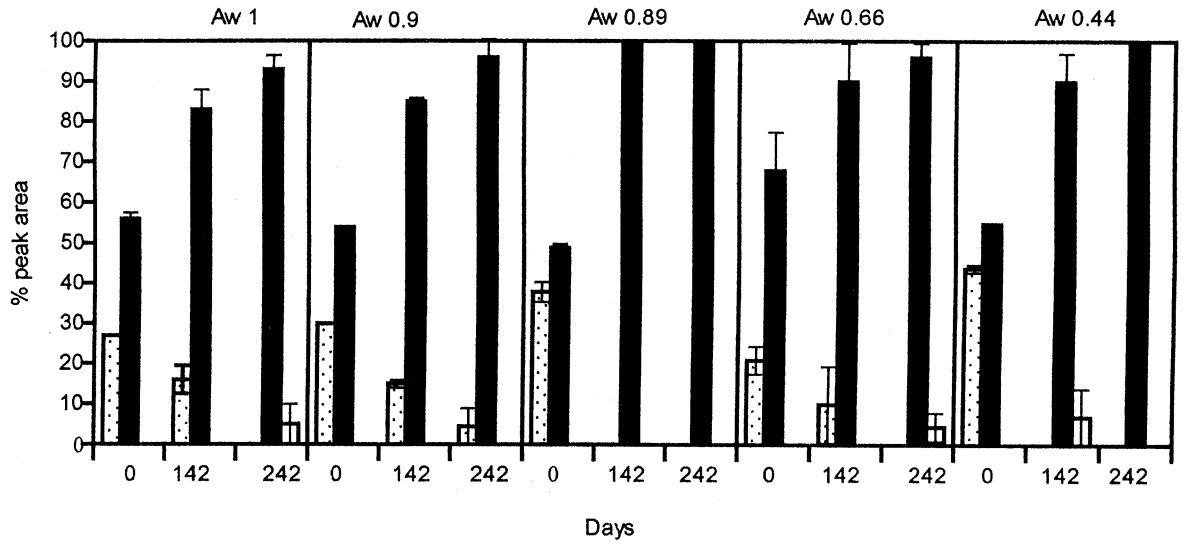

Fig. 7. Changes in relative peak area during storage of model systems spiked with acyl-pelargonidin 3-sophoroside 5-glucoside. ■ pgd 3-soph 5-glu + cinnamic and malonic acids, ■ pgd 3-soph 5-glu + cinnamic acids, □ pgd 3-soph 5-glu.

storage study. The aglycone pelargonidin was not detected; thus it must have been polymerized or degraded to other forms if glycoside hydrolysis occurred.

Systems containing pgd 3-soph at A_w 1 and A_w 0.90 had levels of pgd 3-soph representing 100% of the total ACN at time zero while, after 242 days, a small amount (approximately 5%) of pgd 3-glu was detected. This can be explained by the hydrolysis of the diglycoside sophorose to form pgd 3-glu.

Changes in the HPLC profile of systems spiked with acyl-pgd 3-soph 5-glu are consistent with changes in monomeric pigment (Fig. 7). The relative concentration of the ACN acylated with malonic and cinnamic acids decreased with time in all the samples, while the area of the ACN acylated with only cinnamic acids increased. Samples at A_w 1 and A_w 0.44 showed the highest amount of degradation. At zero time in the system at A_w 1, acyl-pgd 3-soph 5-glu, with both cinnamic and malonic acids, accounted for 57% of the total area; production of malonic acid is believed to have occurred during removal of solvents by rotary evaporation during sample preparation. At day 242, total release of malonic acid was evident; small amounts of pgd 3-soph 5-glu and pgd 3-soph were found. Spectral analysis and comparison of retention times with the corresponding purified ACNs made identification of these two peaks possible. Harborne (1967) and Timberlake and Bridle (1975) reported that 3,5-diglycosides exhibit only 50% of the absorption at 440 nm (when compared to the colour maximum absorbance), as do the 3-glycosides, which is in agreement with our findings (data not shown).

ACN degradation was more pronounced in the sample at A_w 044. At day 142 of storage, total cleavage of malonic acid from the acylated ACN accompanied by small amounts of pgd 3-soph 5-glu and pgd 3-soph from hydrolysis of the latter was observed. At the end of the storage, pgd 3-soph 5-glu, acylated with cinnamic acids, accounted for 100% of the pigment present in the system. Guisti and Wrolstad (1996a) observed similar changes during acid hydrolysis of radish ACN and storage of model systems containing acyl-pgd 3-soph 5-glu. After a short period (10 min) of acid hydrolysis with 2N HCl 60% of the malonic acid was released, resulting in formation of pgd 3-soph 5-glu acylated with cinnamic acids as the major pgd derivative and small amounts of pgd 3-soph 5-glu. In pH 3.5 model systems studied by Guisti and Wrolstad (1996b), pgd 3-soph 5-glu, acylated with cinnamic and malonic acids, represented 20% of the total ACNs at the beginning of the storage, but after 10 weeks, release of malonic acid was evident, and pgd 3-soph 5-glu, acylated with only cinnamic acids, became the major ACN present, representing about 80%.

4. Conclusions

A direct relationship between increasing A_w and ACN degradation rate has been demonstrated. Investigation also showed that accelerated ACN degradation may occur at low A_w where glycerol has been used to adjust A_w. This phenomenon is more pronounced in model systems containing acylated pigments. A dramatic finding is the remarkable stability of the pelargonidin derivatives in model systems as compared with the same pigments in strawberry juice and concentrate. For example, the $t_{1/2}$ increased from 12 days in strawberry juice (Garzón & Wrolstad, 2000) to 934 days in the model systems. Factors in addition to molecular structure have a marked influence on pigment stability.

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