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Stabilisation of natural anthocyanins by micellar Systems

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Abstract

In this paper, we discuss the influence of different micellar systems on the degradation of natural anthocyans, either glycosides and aglycones, at pH values ranging from 2.8 to 6.0. The interaction of anthocyanins, in suitable dispersed systems such as negative micelles of sodium dodecylsulphate (SDS), consistently increased their chemical stability in aqueous solutions. The results of these experiments point out how both the number of available negative charges and the presence of an organised distribution of the negative charges on the micellar surface appear to be necessary conditions to achieve the anthocyanins' stability and colour retention. The sodium dodecylbenzensulphonate (SDBS), containing an aromatic ring near the negative surface of the micelle, seems to increase the rate of decomposition. Preliminary findings of circular dicroism (CD) investigation allowed us to hypothesise that these pigments undergo an intermolecular self-association process induced by the SDS micelles and this phenomenon presumably contribute to increase stability.

Keywords: Anthocyanins; Micelles; Sodium dodecylsulphate; Stability; UV-Vis

1. Introduction

Anthocyanins are a group of reddish-blue water-soluble pigments that are widespread in plants and they significantly contribute to the red-blue coloration of many flowers, fruits and vegetables. These pigments display a remarkable number of biochemical and pharmacological activities and many of their pharmacological properties have been correlated to the scavenging ability of oxygen-generated free radicals and to the inhibition of lipid peroxidation (Tsuda et al., 1994; Satué-Garcia et al, 1997). Therefore, antioxidant properties (Frankel et al., 1998; Martin Aragon et al., 1998), together with low toxicity, make anthocyanins an interesting class of natural pigments to use in the pharmaceutical and alimentary.

In these last years, considerable interest in replacing synthetic colorants with natural pigments has developed, nevertheless the main problem related to their utilisation is the very low stability in aqueous media at pH values above 2.0. An increase of the chemical stability of anthocyanins is

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conferred by the *O*-glucoside linkage, by the presence of acyl groups on the sugar moiety (Francis, 1989) and by inter and intra molecular co-pigmentation processes. Many studies have been performed to evaluate the influence of pH, glycosidation and co-pigmentation phenomenon on the chemical stability of these pigments. Brouillard (1988) has investigated the effect of pH for slightly diluted anthocyanin in acid and neutral aqueous solutions. At pH values ranging from 3 to 6, typical of many foods, anthocyanins are almost completely hydrated to obtain the colourless carbinol pseudobases, while the flavilium ions exist mostly below pH 2.0.

Asen et al. (1972) first put forward a self-association phenomenon to explain the deviation from the Beer–Lambert law associated with increasing concentration of cyanidin, at pH 3.16. The intra and inter molecular co-pigmentation is a predominant process in reddish-blue flowers or fruits, and this phenomenon seems to be essential to partially or totally prevent the anthocyanins' hydration reaction in the vegetal vacuoles (Brouillard, 1988; Goto and Kondo, 1991). Some Japanese researchers have developed most of the investigations on self-association involving natural anthocyanins at pH value around neutrality (Hoshino and Matsumoto, 1980; Hoshino et al., 1981). ^{1}H-NMR (Goto and Kondo, 1991) experiments and circular dicroism (CD) measurements have been also applied to demonstrate that the self-association process occurs in malvidin aqueous solutions leading to a vertical stacking of the neutral bases (Hoshino et al., 1981; Hoshino, 1991). This phenomenon may be of biological significance since the neutral quinonoidal bases are the anthocyanin coloured forms that prevail in most fruit and flower cell vacuoles and it could explain the stability of these pigments in vivo. Recently, the interaction between malvin and different flavonoids has been investigated by in vitro experiments in order to study the kinetic and thermodynamic parameters of the co-pigmentation reaction (Baranac et al., 1997, 1997a,b).

Until today, only a few studies have been made to verify the ability of micellar systems to stabilise anthocyanins. Micellar solutions are widely used as host systems for synthetic and natural organic compounds and basically three differently charged surfactants can be used to produce micelles, anionic, cationic and non-ionic. The reactivity of organic molecules and enzymes in direct and reverse micellar systems has been shown to be dependent on many factors. The main ones are the surface charge, the sign of this charge, the micelle size, the presence of added salts and/or co-surfactant (alcohols), and the size of the water pool of reverse micelles.

Sadlowski (1986) demonstrated that an equilibrium colour stabilisation of malvin occurred in micellar solutions of the anionic surfactant sodium dodecylsulphate (SDS). It was hypothesised that the presence of surfactant could retard the hydration process about seven-fold with respect to the same reaction in pure water. A patent was also registered about the stabilisation of red cabbage extract containing anthocyanins in a candy preparation by adding SDS. It was observed how the pigment preparation appeared more resistant to light, pH changes and heating after addition of SDS 0.1% (Kobayashi et al., 1988).

In this paper, we investigate the influence of neutral and charged micelles on colour retention of some widely diffused anthocyanins and their corresponding aglycones. The chemical structures of the six investigated pigments, anthocyanidins and their glycosides, are shown in Fig. 1. To prepare micellar solutions, ionic and non-ionic surfactants were tested. The chemical degradation was correlated to the amount of the flavilium forms in solution, thus evaluating the absorbance decrease of each sample over time by spectrophotometric measurements.

2. Materials and methods

2.1. Materials

Pelargonidin, cyanidin, malvidin, pelargonidin 3-*O*-glucoside or callistephin, cyanidin 3-*O*-rutinoside or keracyanin and malvidin 3,5-*O*-diglucoside or malvin were purchased from Extrasynthése (Genay Cedex, France); SDS was

purchased from Eastman-Kodak (Rochester, New York, USA); 4-ter-octilfenossipolietilene (Triton X-100) was purchased from Aldrich-Chimica (Milano, Italy); sodium dodecylbenzensulphonate (SDBS) was purchased from Carlo Erba (Milano, Italy). The water was Milli-Q grade (Millipore, Milano, Italy)

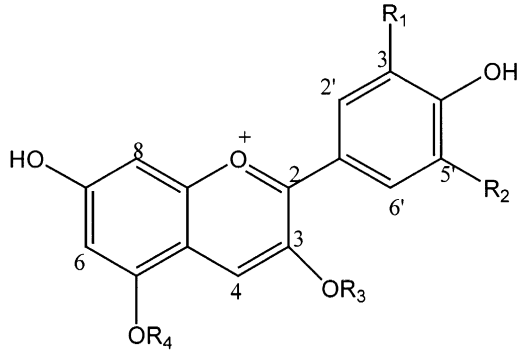
2.2. Micellar solutions

The tested surfactants were SDS, SDBS for micellar systems with negative charge and Triton X-100, for non-ionic micelles. In SDS, SDBS and Triton X-100 the different pH values of the samples were obtained by adding HCl to Milli-Q water before the addition of the surfactants. All the samples were prepared at concentration values over their specific CMC to be sure to obtain micellar solutions. Mother solutions of the pigment were prepared by dissolving the samples in water/methanol 5:1 and immediately freezing at − 25° C. All samples, at different pH values, were prepared by adding different volumes of mother solutions to the surfactant solution.

2.3. Methods

The absorption spectra were recorded on a Perkin–Elmer 502 spectrophotometer and the percentage decomposition of each pigment was evaluated measuring the colour decrease in different microenvironments. The measurements were performed at the maximum absorption wavelength of each sample between 496 and 548 nm. For each sample the decomposition was evaluated by spectrophotometric measurements over time. The data reported in all figures and tables are the average of two determinations with a S.D. between 3 and 5%.

CD spectra were registered by a JASCO J-500C instrument, equipped with a data processor DP-500. Ke, Ma and Ca solutions were tested in acetic buffer (CH_3COOH/CH_3COONa 1:1 both 0.01 M) for samples at pH values near 4.0.



	R_1	R_2	R_3	R_4
Pelargonidin	H	H	H	H
Cyanidin	OH	H	H	H
Malvidin	OCH_3	OCH_3	H	H
Callistephin	H	H	Glucose	H
Keracyanin	OH	H	Rutinose	H
Malvin	OCH_3	OCH_3	Glucose	Glucose

Fig. 1. Chemical structure of anthocyanidins and their glycosides.

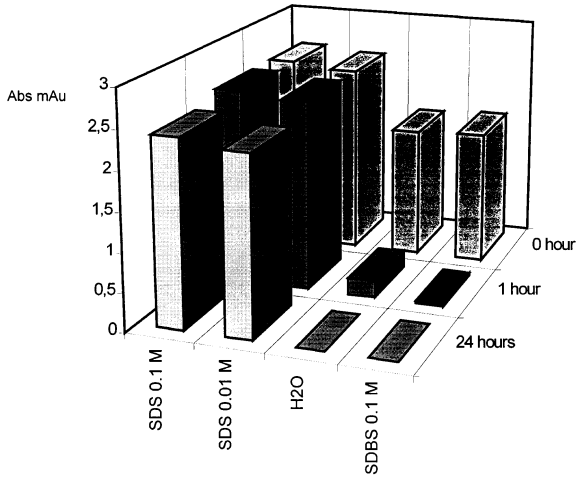


Fig. 2. Behaviour of M in two different micellar systems, SDS and SDBS. Pigment concentration 1.1×10^{-4} M, pH range 2.9–3.0.

3. Results

3.1. Anthocyanidins: pelargonidin (P), malvidin (M) and cyanidin (C)

Three anthocyanidins with a different substitution pattern on ring B were investigated to test their chemical stability in different microenvironments.

For testing the anthocyanidins' colour stability, only the anionic surfactants, SDS and SDBS, were used as micellar solutions because the red colour quickly disappeared in Triton X-100 micelles.

Fig. 2 shows for M the absorbance values in SDS, SDBS (548 nm) and in water solutions (520 nm) at pH values ranging between 2.8 and 3.0. Both in SDS solution 0.1 and 0.01 M, the stability of M is greater than in other microenvironments and its colour intensity is almost unchanged up to 24 h. In water and in SDBS the decomposition is almost total after only 1 h. For P and C, in SDS 0.1 and 0.01 M, the same behaviour was also observed.

A large bathochromic shift (from 520 to 548 nm) between micellar solutions and water solution was observed for M, and analogous shifts were also registered for P (from 504 to 520 nm) and for C (from 516 to 532 nm) in the same experimental conditions. The SDS micelles showed the greatest ability of all these compounds to retain colour.

In Fig. 3 the percent decomposition values of P, C and M coloured forms in different SDS concentrations are compared over time. For P, C and M the percent decompositions continued to increase linearly up to 8 days. It is worthy to note that the samples with lower micellar concentration (SDS 0.01 M) were more unstable.

3.2. Anthocyanins: callistephin (Ca), keracyanin (Ke) and malvin (Ma)

The following glycosides: callistephin (Ca), keracyanin (Ke) and malvin (Ma), were investigated.

Ca absorbance values, at pH 2.8–3.8, in micellar solutions and in water, are reported in Fig. 4. For SDBS, a stabilising effect was first observed with respect to water media, which significantly decreased after one day while in Triton X-100, at the same pH, no effect was observed. Greater pigment stability in SDS 0.1 M with respect to all

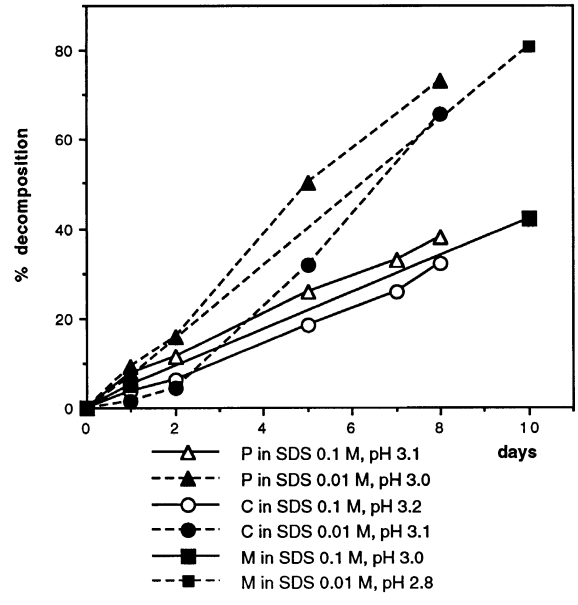


Fig. 3. Anthocyanidins at 1.1×10^{-4} M in SDS 0.1 and 0.01 M. Comparison of their stability over time, pH range 2.8–3.1. The absorbance registered at time 0 in SDS 0.1 M was chosen as decomposition 0%, for each anthocyanidin. The maximum wavelengths were, 520 nm for P, 532 nm for C, 548 nm for M.

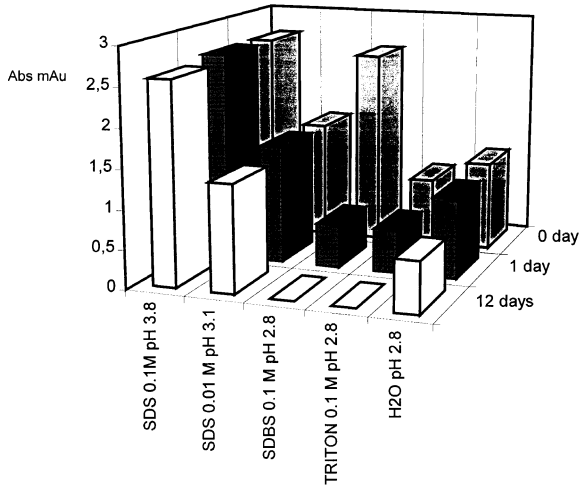


Fig. 4. Behaviour of Ca, 1.1×10^{-4} M, in different micellar systems and in water; pH range 2.8–3.8. The maximum wavelengths were respectively registered at 496 nm for H_2O, 504 nm for Triton X-100, 520 nm for SDS and SDBS.

other systems appears evident, particularly at pH 3.8. This sample was very stable over time. In fact, the flavilium concentration remained nearly unchanged up to 3 months with a percent of decomposition, evaluated at 512 nm, below 8%.

Ke behaviour in micellar solutions and in water (pH range 2.8–3.2) was the same as observed for Ca (Fig. 4) and the greatest pigment stability in SDS over time was confirmed as shown in Fig. 5a. In fact, in each micellar system, as well as in water, the highest percent decomposition of the coloured forms was immediately obtained after dissolution (time 0) it then remained almost unchanged up to 14 days. For all the Ke samples in SDS, the percent decomposition remained under 20% and particularly for three samples it never exceeded 2%. Ke colour stability immediately after dissolution was also tested at 'prohibitive' pH values for water solutions. The same pigment amount (7.0×10^{-5} M) as in Fig. 5a gave very different absorbance values depending on micellar type, micellar concentration and pH as shown in Table 1. It is worthy to note that Ke in SDS 0.1 M, also at pH 5.5, gave higher absorbance values with respect to Ke in water and Triton X-100 at pH 3.1. Fig. 5b points out that higher pigment concentration (1.1×10^{-4} M) in SDS 0.1 M allowed maintenance of reddish colour over time, also at weakly acid pH (5.5). This considerable

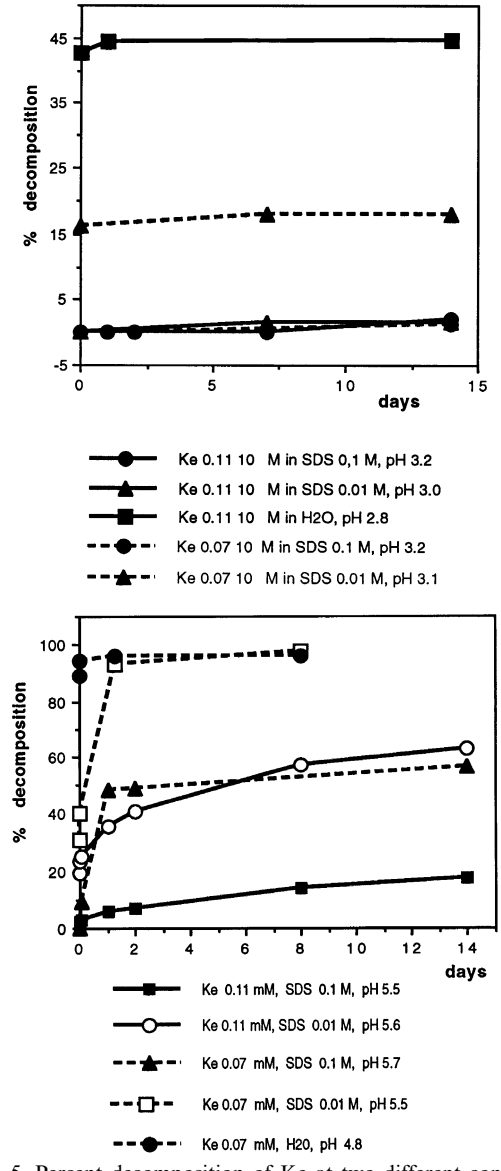


Fig. 5. Percent decomposition of Ke at two different concentrations in SDS micelles. A comparison with water is also reported. For all Ke samples the absorbance values registered in SDS 0.1 M at time 0, were chosen as references (0%). The maximum Abs values were registered at 528 nm for SDS and 512 nm for H_2O. (a) Ke decomposition at pH range 2.8–3.2; (b) Ke decomposition at pH values 5.5–5.7 and pH 4.8 for water.

Table 1
Comparison among the Abs values of Ke 7.0×10^{-5} M, at time 0, in different microenvironments and pH values

Samples	mAu	
	pH 3.1	pH 5.5
SDS 0.1 M	1.94	1.45
SDS 0.01 M	1.80	1.00
SDBS 0.1 M	1.82	1.25
Triton X-100 0.1 M	0.59	0.29
Water	1.02	0.16

enhancement of the chemical stability in SDS micelles is particularly accentuated if compared with Ke behaviour in water at pH 4.8.

Analogously to Ca and Ke, Ma was tested. In Fig. 6 the behaviour of the pigment at 7.0×10^{-5} M is compared in SDS, SDBS and water. After only 24 h, the colour almost disappeared both in SDBS 0.1 M and in water. The decomposition of the samples in SDS 0.1 M and 0.01 M at pH around 3.0, was monitored up to 30 days showing decomposition values below 20 and 40%, respectively.

In Fig. 7 the CD spectra of Ma, Ke and Ca in SDS 0.1 M at buffered pH 4.0 are compared. All three pigments showed a negative Cotton effect with the maxima in the range of 500–522 nm and some differences in their ellipticity. CD spectra of

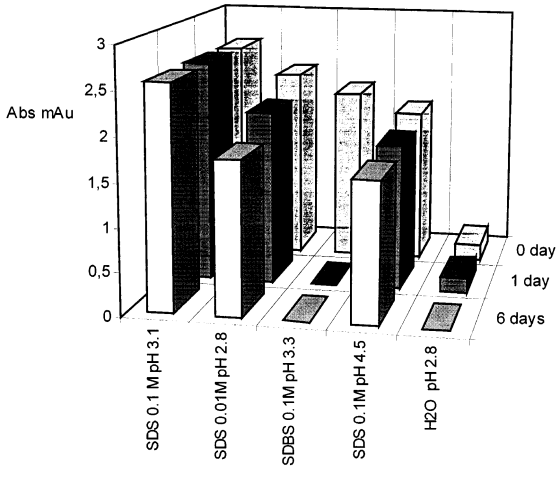


Fig. 6. Decomposition of Ma 7.0×10^{-5} M in SDS 0.1 M and 0.01 M, SDBS 0.1 M and in water, pH range 2.8–4.5.

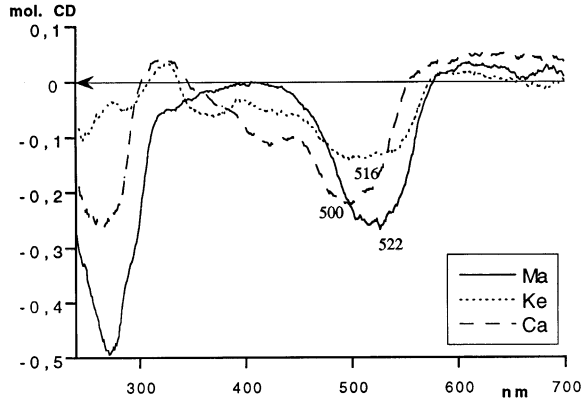


Fig. 7. CD spectra of Ma, Ke and Ca (1.18×10^{-4} M), recorded within few minutes after dissolution.

dilute solutions of Malvin in SDS 0.1 M were also performed (Fig. 8). Negative cotton effects were again observed in the range of 450–600 nm, but the shapes and λ_{max} absorptions were changed. This behaviour was in accord with the correspondent UV-Vis spectra, thus, indicating a deviation from the Lambert Beer law. This result suggests the occurrence of a self-association phenomenon previously described for the anthocyanidins (Hoshino et al., 1981; Hoshino, 1986; Hoshino and Goto, 1990).

Moreover, the CD spectra of Ma, Ke and Ca in SDBS at the same experimental conditions were also performed. These spectra were almost overlapped with that obtained in SDS at time 0, but after 24 h the dichroic effect completely disappeared and the solutions were colourless.

The chemical stability of the samples reported in Fig. 7 was compared with that of solutions of Ma, Ke, Ca, in HCl 0.12 N at the same pigment concentration. In Table 2 the results related to the monitoring up to 50 days are summarised. The highest percent decomposition was obtained for Ma in HCl solution while the other values never went over 22%.

4. Discussion

It is worthy to note that all the tested surfactants form, at the concentrations used, spherical

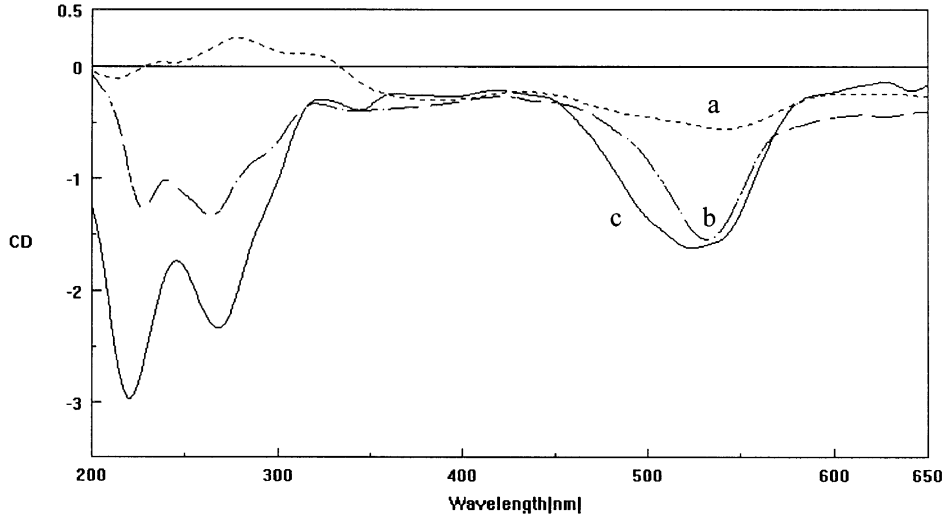


Fig. 8. CD spectra of Ma in SDS 0.1 M at pH 4.0 by acetic buffer. (a) 0.59×10^{-4} M; (b) 077×10^{-4} M; (c) 1.18×10^{-4} M.

or slightly ellipsoidal micelles with about the same aggregation number to the order of 100. Nevertheless, the highest stability of the coloured forms both of aglycones and glycosides, was obtained only with negative micelles of SDS. Due to the lypophilic inner part of all these micelles, it is not possible to hypothesise an inclusion of the pigments, especially for the more polar pigments, Ca, Ma and Ke. As a consequence, only a kind of surface interaction between the dyes and micelles can be postulated for all three tested surfactants.

The coloured forms, either of aglycones and glycosides, exhibit bathochromic shifts going from water solutions to negatively charged micellar solutions (SDS and SDBS) working at the same pH values and this phenomenon could be related to the presence of organised systems on the micellar surface.

Owing to the higher instability of the anthocyanidins with respect to their corresponding glycosides at acidic pH values in water media (Brouillard, 1988), pelargonidin, malvidin and cyanidin were tested only at pH values not over 3.0. All the samples were prepared at suitable concentration, 1.1×10^{-4} M, to increase their stability and to not exceed the maximum absorbance values for instrument evaluation.

When the colour stability of a pigment at a fixed concentration in both SDS 0.1 and 0.01 N was compared, the absorbance value obtained with the highest surfactant concentration was chosen as 0% decomposition. This procedure guarantees the best method accuracy (see Table 1).

For the anthocyanidins the stability of the flavilium forms was not influenced by the different substitution pattern, in fact comparing all data, any significant difference was evidenced in the behaviour of P, M and C in the tested micellar

Table 2
Absorbance values (mAu) and percent decomposition of Ca, Ke and Ma (1.18×10^{-4} M) in SDS 0.1 M and HCl 0.12 M up to 50 days. The pH value of SDS solution was 4.0

Samples	mAu		% decomposition
	Time 0	50 days	
Ca–SDS at 508 nm	2.53	1.97	22.1
Ca–HCl at 500 nm	2.62	2.32	11.5
Ke–SDS at 530 nm	2.82	2.74	2.8
Ke–HCl at 510 nm	3.00	2.66	11.3
Ma–SDS at 530 nm	2.89	2.40	16.9
Ma–HCl at 516 nm	2.98	1.63	45.3

systems over time. Furthermore, the samples in SDS 0.01 M decompose more rapidly showing that higher micellar concentrations improve anthocyanidin' stability (Fig. 3).

The glycoside, with respect to the aglycone, showed a higher variability in terms of percent decomposition as shown in Table 2, where Ma, Ke and Ca in the same experimental conditions are compared. For the anthocyanins the number and binding site for the sugar moieties seem to modulate the stability of the pigments in the micellar microenvironment. Particularly, malvidin 3,5 *O*-diglucoside is more unstable with respect to Ke and Ca at pH 4.0, as shown in Table 2. Ke and Ma were much more stable in SDS micelles compared with HCl solution, which is usually used to store these pigments over time.

The SDBS micelles were tested to verify if the presence of an aromatic ring, near the negative micellar surface, was able to increase the stability of the flavilium form (as previously observed for SDS) in which no aromatic system is present. Increments in reddish intensity immediately after pigment dissolution were observed for the three glycosides, but after 24 h the colour disappeared almost completely, as shown in Figs. 2, 4 and 6a. It can be hypothesised that the presence of benzene rings close to the micellar surface allows formation of Van der Waals interactions with the aromatic part of the flavilium molecule. These forces, increasing the negative charge delocalisation for SDBS, could reduce the strength of the possible ionic binding between the positive charge of flavilium molecules and negative charge of the micellar surface. Thus, a competitive effect between the ionic binding and the Van der Waals interactions can be postulated.

With respect to pure water at the same pH values, the neutral Triton X-100 micelles, with the presence of aromatic rings close to the micellar surface, seem to contribute to increase the hydration process (Table 1 Fig. 4), leading rapidly to the colourless forms of anthocyanins.

The particular distribution of SDS surfactant polar heads on the micellar surface and the ionic interaction between the negative charges of the SDS and the positive charge of flavilium ions seem to be the main causes for pigment stabilisation. The results of these experiments point out that both the number of available negative charges and the presence of an organised distribution of these charges on the micellar surface appear to be necessary conditions to improving colour stability. In fact, the presence of only negative charges, obtained by the dissolution of $Na_2SO_4^{2-}$ (0.1 M) in water, was unable to give any stabilising effect. An unspecific effect due only to the presence of a negatively charged surfactant can not be postulated. In fact, a very different behaviour was observed for the pigments dissolved in SDS and SDBS even if micellar concentration, micellar shape and aggregation number are substantially the same for the two surfactants.

Usually the storage of anthocyanins aqueous solution at temperatures below -20°C is an applied procedure able to stabilise the coloured forms of these molecules. Nevertheless, anthocyanins in SDS solutions were almost uncoloured at low temperatures (-18°C) where the micelles are strongly modified, but the samples rapidly recovered their colour by returning to room temperature. In addition, SDS solutions with higher concentrations of Ca, Ke and Ma maintain an intense reddish colour also after more than 12 months of storage at room temperature (data not shown).

To try to explain the higher stability of the coloured forms in SDS with respect to water solutions, preliminary CD analyses were also performed. This technique, being extremely sensitive to molecular asymmetry, represents an effective tool for evidencing self-association and chiral stacking of the anthocyanin chromophores (Hoshino 1986; Hoshino and Goto, 1990; Goto and Kondo, 1991). To show the existence of this phenomenon, CD investigations (mainly on the anthocyanins anhydrobases in aqueous neutral solutions) have been previously performed (Hoshino and Matsumoto, 1980). Furthermore, this technique has also been applied to closely examine the phenomenon relating to blue colour stability in flowers (Hoshino et al., 1981a; Goto et al., 1987). All these works have pointed out the existence of macro-molecular organisations showing large ellipticities attributed to the homo-

molecular association of the aromatic rings resulting in a vertical stacking, with the formation of chiral supra-molecular systems. The CD spectra showed in Figs. 7 and 8 support the hypothesis of a self association process of these pigments in SDS. To better elucidate the interaction between SDS micelles and anthocyanins other investigations should be carried out. Therefore, the self-association process must be taken into account to explain the anthocyanins' increased stability over time.

Several papers reporting the antioxidant activity of anthocyanins have been published in recent years (Tsuda et al., 1994; Satué-Garcia et al, 1997). The experimental conditions of all these texts apply a pH near neutrality and so it can be hypothesised that the active species are not the flavilium ones, but more often the chalcones formed after hydrolysis (Frankel et al., 1998; Martin Aragon et al., 1998; Lapidot et al., 1999).

The use of suitable micellar systems could be an interesting tool for monitoring real anthocyanins activity in some biological tests.

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