

Stability of Pelargonidin 3-glucoside in Model Solutions in the Presence and Absence of Flavanols

F. Lopes-da-Silva, M.T. Escribano-Bailón and C. Santos-Buelga
Grupo de Investigación en Polifenoles, Unidad de Nutrición y Bromatología,
Facultad de Farmacia, Universidad de Salamanca, E-37007 Salamanca, Spain

Abstract: The stability of pelargonidin 3-glucoside (Pg3gluc), the main anthocyanin in strawberry, has been studied in model citrate solutions (pH 3.5, 25°C) in the absence and presence flavanols (catechin and procyanidin B3) and/or oxygen. Changes in the solutions were monitored by HPLC-DAD/MS and UV-visible spectra. A progressive decrease in the concentration of the anthocyanin was produced according to a first-order kinetics whose rate was influenced by the composition of the medium. The availability of oxygen was the most influential factor on the stability of the anthocyanin that decreased much slower in anaerobic conditions. On the contrary, the presence of flavanols constituted a destabilizing factor for the anthocyanin. In the absence of flavanols, the anthocyanin was mostly degraded to colourless compounds, although the formation of some unknown unstable pigments with maximum absorbance in the visible region around 400-460 nm was also observed. In the presence of oxygen the breakdown of the heterocycle was found to constitute a major pathway for anthocyanin disappearance, whereas this mechanism was hardly produced in anaerobic conditions. In the presence of flavanols the disappearance of the anthocyanin was mostly due to condensation reactions, yielding both colourless products and xanthylum pigments. Conclusions about the structure of some of the products and pigments formed on the solutions were obtained based on their mass spectra and fragmentation patterns.

Key words: Pelargonidin, catechin, procyanidin B3, anthocyanin stability, xanthylum pigments, Strawberry

INTRODUCTION

The colour is one of the main attributes through the consumer assesses food quality and that, therefore, influences food choice and acceptability. Anthocyanins constitute a widespread family of plant pigments that are responsible for the colour of a large number fruits and fruit-derived products. These compounds are unstable with regard to pH and temperature variations and other factors such as light exposure, oxidation and the presence of metals and other substances, giving place to colour changes during processing and storage, which reduce the commercial value and the life span of the products containing anthocyanins. The difficulty of strawberry-derived foods (jams, juices, concentrates or purées) to maintain their attractive red colour is particularly known. In spite of many studies have been carried out, there is still an insufficient knowledge of the mechanisms subjacent to the changes of colour in strawberry foodstuffs and consequently no definitive solutions to reinforce or maintain their colour exist. Similar changes of colour are produced during ageing and storage of red wine, which are attributed to the lost of original grape anthocyanins and their substitution by complex pigments through different reactions involving anthocyanins and flavanols, without (Liao *et al.*, 1992;

Corresponding Author: C. Santos-Buelga, Grupo de Investigación en Polifenoles,
Unidad de Nutrición y Bromatología, Facultad de Farmacia,
Universidad de Salamanca. E-37007 Salamanca, Spain
Tel: +34 923 294537 Fax: +34 923 294515

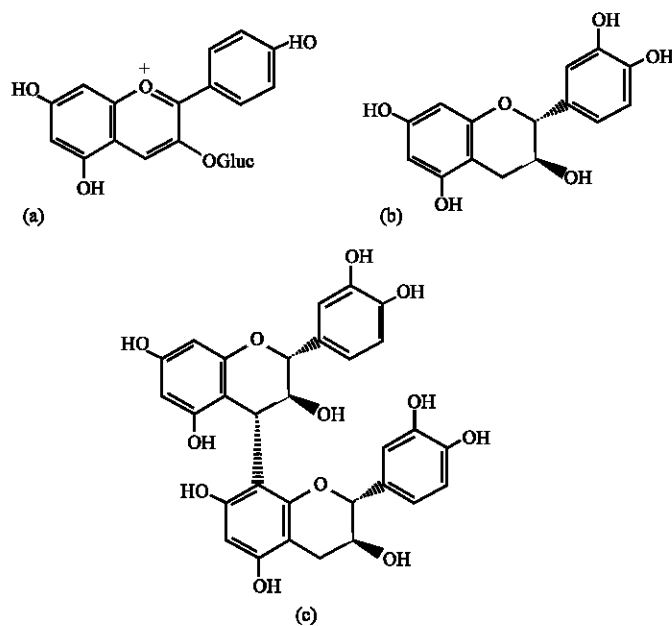


Fig. 1: Chemical structures of Pg3gluc (a), (+)-catechin (b) and procyanidin dimer B3 (c)

Somers, 1971; Boulton, 2001; Kunsági-Máté *et al.*, 2006; Hermosín-Gutiérrez *et al.*, 2005) or with the involvement of other compounds, such as acetaldehyde (Bakker *et al.*, 1993; Francia-Aricha *et al.*, 1997; Rivas-Gonzalo *et al.*, 1995; Timberlake and Bridle, 1976), pyruvic acid (Bakker *et al.*, 1997; Bakker and Timberlake, 1997; Fulcrand *et al.*, 1998; Romero and Bakker, 1999), vinylphenols (Cameira dos Santos *et al.*, 1996; Fulcrand *et al.*, 1996; Hayasaka and Asenstorfer, 2002), α -ketoglutaric acid (Benabdeljalil *et al.*, 2000), acetone (Hayasaka and Asenstorfer, 2002), or glyoxylic acid (Es-Safi *et al.*, 1999; Fulcrand *et al.*, 1997). As a result of these reactions, new flavylum-derived compounds, pyranoanthocyanins and xanthylum pigments with different chromatic features are produced. In addition to anthocyanins, the strawberry also contains flavanols and, therefore, it can be supposed that some of the previous processes may also occur in their derived products and contribute to their changes of colour. The anthocyanin composition in the strawberry is characterised by the presence of pelargonidin 3-glucoside (Pg3gluc, Fig. 1) as major pigment, constituting 77-95% of total anthocyanins (Goiffon *et al.*, 1999; Lopes da Silva *et al.*, 2007). Strawberry flavanols are mainly based on catechin (de Pascual-Teresa *et al.*, 2000b; Thompson *et al.*, 1972), being (+)-catechin the majority compound followed by the procyanidin dimer B3 (catechin-4,8-catechin, Fig. 1); these two compounds represent around 40 and 13% of the total content of flavanols of low degree of polymerisation in the strawberry, respectively (Lopes da Silva *et al.*, 2002). The aim of the present work was to study the behaviour of Pg3gluc in model assays in the absence and presence of oxygen and flavanols (catechin and procyanidin B3), so as to conclude about the mechanisms involved in their instability.

MATERIALS AND METHODS

Standards

(+)-Catechin, (\pm)-taxifolin, *p*-hydroxybenzoic acid and 2,4,6-trihydroxybenzaldehyde were purchased from Sigma Chemical Co. (St. Louis, MO).

Isolation of Pelargonidin 3-Glucoside

Strawberries of the *Camarosa* variety were homogenized in methanol containing 0.1% HCl, maintained at -30°C overnight and further centrifuged (4000 g, 15 min, 2°C). Water was added to the supernatant and the methanol removed in a rotary evaporator at 30°C. The aqueous extract obtained was purified by liquid chromatography in an open column filled with a mixed stationary phase composed of 20% Polyclar AT and 80% silica gel G60 to remove sugars and acids, as described by Escribano-Bailón *et al.* (2002). Isolation of Pg3gluc was further carried out by semipreparative-HPLC. An RP Nova-Pak C18 cartridge (6 µm, 60 Å, 25×100 mm) (Waters, Milford, MA) was used. Solvents were (A) 5% acetic acid and (B) 100% HPLC grade methanol. The elution profile was as follows: 0 min, 90% A, 10% B; 5-20 min, 85% A, 15% B; 20-25 min, 85% A, 15% B; 25-30 min, 82% A, 18% B; 30-50 min, 65% A, 35% B. The flow rate was 10 mL min⁻¹. Detection was carried out at 280 nm and the peaks were collected in a fraction collector. Fractions containing the anthocyanin were mixed, the solvents eliminated under vacuum and the pigment transferred to water and freeze-dried. The purity of the anthocyanin was checked by HPLC-DAD/MS.

Preparation of Procyanidin B3

It was synthesized as described by de Freitas (1995). The method is based on the reduction of (±)-taxifolin by sodium borohydride followed by the condensation between the carbonium ion (C⁴⁺), formed in acidic media and (+)-catechin. Procyanidin B3 was isolated from the reaction mixture by fractionation on a Sephadex LH-20 column and further purified by semi-preparative HPLC as described by de Pascual-Teresa *et al.* (1998). The compound was freeze-dried and its purity checked by HPLC-DAD/MS.

Model Solutions

Five solutions were prepared in citrate buffer 0.1M at pH 3.5. Solutions A and B contained 2 mM Pg3gluc; solutions C and D contained a mixture of Pg3gluc (2 mM), catechin (1.5 mM) and procyanidin B3 (1.0 mM) and solution E contained Pg3gluc (2 mM) and procyanidin B3 (1.5 mM). All the solutions were kept in a glass vial closed with a septum and stored in dark at 25°C. Solutions B and D were purged with N₂ during 2 min before sealing the vial. Oxygen exposure was not avoided when vials of solutions A, C and E were closed. Samples were taken periodically over 3 months by puncturing the septum and analysed by HPLC-DAD/MS. Solution B was followed during 6 months. The study was conducted in 2004 at the laboratory of Food Science, University of Salamanca (Spain).

HPLC-DAD/MS Analysis

Analyses were carried out using a Hewlett-Packard 1100 chromatograph with a quaternary pump and a Diode Array Detector (DAD) coupled to an HP Chem Station. An Aqua C18, 5 µm (4.6×150 mm) (Phenomenex, Torrance, CA) column was used thermostated at 30°C. The mobile phase was (A) 0.1% trifluoroacetic acid in water and (B) HPLC-grade acetonitrile. The elution gradient established was 0 min, 90% A, 10% B; 5-20 min, 85% A, 15% B; 20-25 min, 85% A, 15% B; 25-30 min, 82% A, 18% B; 30-50 min, 65% A, 35% B. The flow rate was 0.5 mL min⁻¹. Detection was carried out at 280, 330, 440 and 500 nm. MS detection was also performed using a Finnigan LCQ detector (San Jose, CA) equipped with an ESI source and anion trap mass analyser, which were controlled by the LCQ Xcalibur software. The mass spectrometer was connected to the HPLC system via the DAS cell outlet. Nitrogen was used as both auxiliary and sheath gas at flow rates of 6 and 1.2 L min⁻¹, respectively. The capillary voltage was 4V and the capillary temperature 195°C. Spectra were recorded in positive ion mode between m/z 150 and 1500. The MS detector was programmed to perform a series of three consecutive scans: a full mass scan, a zoom scan of the most abundant ion in the first scan and an MS-MS scan of the most abundant ion, using a normalized collision energy of 45%

RESULTS AND DISCUSSION

Kinetics of Pg3gluc Disappearance

The changes in the composition of the solutions were followed by analysis of HPLC-DAD/MS. In all cases a progressive decrease in the content of Pg3gluc was produced, although at a different rate depending on the solution. The disappearance of the anthocyanin took place according to an apparent first-order kinetics ($C = C_0 \cdot e^{-kt}$), as previously observed for the degradation of Pg3gluc in juices (Garzon and Wrolstad, 2001) and concentrates of strawberry (Garzon and Wrolstad, 2002; Özkan *et al.*, 2002), as well as in model solutions of different anthocyanins (Baublis *et al.*, 1994; Cemeroglu *et al.*, 1994; Özkan, 2002; Giusti and Wrolstad, 1996). The Table 1 shows the reaction rate constants (k) and the half-life time ($t_{1/2}$) of Pg3gluc in the different solutions determined using linear regression from plot of $\ln[C]$ vs time. For this calculation only the two first months of storage were considered, since, except for the solution B, small or no amounts of anthocyanin were detected after that time. The rates of disappearance were of the same order of magnitude than the ones observed by Dueñas *et al.* (2006) for the disappearance of malvidin 3-glucoside (Mv3gluc) in the presence of equimolar concentrations of epicatechin in model solutions prepared in 20 mM ammonium acetate at the same pH value ($k = 4.92 \times 10^{-2} \text{ days}^{-1}$, $t_{1/2} = 14.1$ days).

As it could be expected, the disappearance of the anthocyanin was much slower when oxygen was avoided. The faster degradation in the presence of oxygen was already reported either for pure anthocyanins (Poei-Langston and Wrolstad, 1981; Kallio *et al.*, 2000) or food matrixes (Clydesdale *et al.*, 1978). The adjustment to a first-order kinetics was worse in the solutions containing catechin and/or procyanidin B3, suggesting that other processes may be contributing to the disappearance of Pg3gluc. Indeed, the presence of flavanols in the medium constitutes a destabilizing factor for the anthocyanin. In the conditions of the assay, the effect of the flavanols even counteracts the protection offered by the anaerobic storage, reducing the half-life of the anthocyanin to values closed to the one observed in the solutions prepared without avoiding oxygen. Poei-Langston and Wrolstad (1981) also found a destabilizing effect of the flavanols in model solutions containing Pg3gluc and catechin in a molar ratio of 1:6.7, greater than the one used in our solutions. In the assays carried out by those authors, the decrease of the anthocyanin was even faster in the mixtures Pg3gluc:catechin maintained in the absence of oxygen than in the solutions containing only anthocyanin in the presence of oxygen.

The rate of disappearance of the flavanols in the solution is slower than that of the anthocyanin (Table 1) and did not fit well a first-order kinetics, as also observed by Dueñas *et al.* (2006). The time of half-life was greater for catechin than for procyanidin B3, which may be explained by the lability of the interflavanic linkage and easier oxidation in the case of the dimer (Oszmianski *et al.*, 1985; de Freitas *et al.*, 1998; Francia-Aricha *et al.*, 1998). Furthermore, the interflavanic cleavage of the procyanidin releases free catechin, thus contributing to moderate the decrease of this latter.

Changes in the Solutions Containing Only Pg3gluc

In the solutions containing only Pg3gluc, the disappearance of the anthocyanin is accompanied by the formation of new compounds (Fig. 2). The Table 2 shows the characteristics (wavelength of

Table 1: Rate of disappearance of pelargonidin 3-glucoside (Pg3gluc), catechin (c) and procyanidin B3 in the different model solutions assayed

Solution	Composition	Pg3gluc			Catechin $t_{1/2}$ (days)	Procyanidin B3 $t_{1/2}$ (days)
		k (days^{-1})	$t_{1/2}$ (days)	R^2		
A	Pg3gluc	5.07×10^{-2}	13.6	0.9788		
B	Pg3gluc (anaerobic)	1.44×10^{-2}	48.0	0.9817		
C	Pg3gluc + C + B3	5.52×10^{-2}	12.5	0.9079	17.8	12.3
D	Pg3gluc + C + B3 (anaerobic)	4.28×10^{-2}	16.9	0.8249	35.9	29.6
E	Pg Pg3gluc 3g + B3	4.88×10^{-2}	14.2	0.8929		16.7

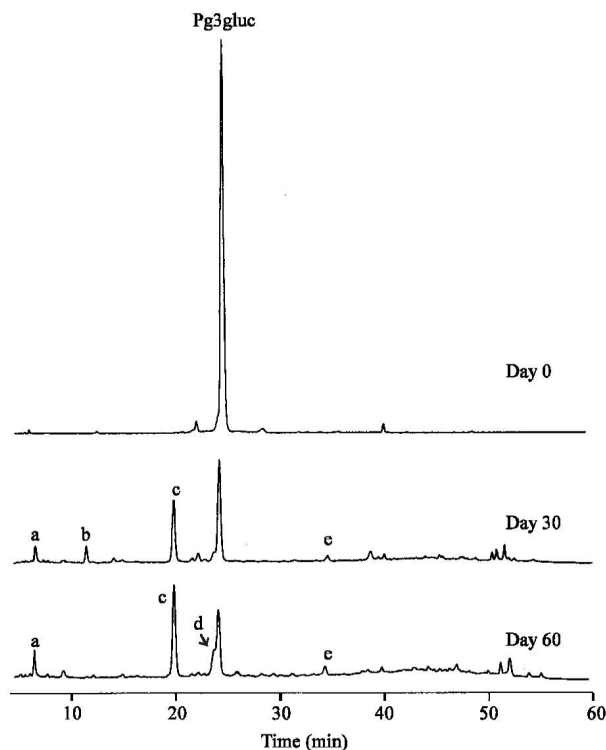


Fig. 2: HPLC chromatogram recorded at 280 nm of solution A at days 0, 30 and 60 of assay. The scale (mAbs units) is the same in all cases

Table 2: Absorption and mass spectral characteristics of the products found in solutions A and B

Peak	λ max (nm)	Molecular ion (m/z)	MS ² fragment ions (m/z)*	Peak identity
a	290	487	457, 325, 317	unknown
b	254			p-hydroxybenzoic acid
c	291	155	137	2,4,6-trihydroxybenzaldehyde
d	292	257		unknown
e	340	865	703, 541	Anthocyanin dimer

*Fragment ions are indicated in decreasing order of relative abundance

maximum absorption, molecular ion and MS² fragments) of peaks a-e for which information could be obtained.

Compound a showed a molecular ion at m/z 487, to which no suitable structure could be fitted. One of its MS² fragments corresponds to the loss of glucose (m/z at 325, -162 amu), indicating that the compound directly derives from the anthocyanin. Its higher mass in relation to Pg3gluc suggests that it could be formed by the reaction of the anthocyanin with a compound of low molecular weight, which may derive from the citric acid used in the preparation of the solutions or a product from the degradation of the anthocyanin.

Peaks b and c were observed in solution A and identified as p-hydroxybenzoic acid (p-HB) and 2,4,6-trihydroxybenzaldehyde (THB), respectively, by comparison of their UV spectra and retention time with standards. The identity of THB could also be confirmed by its mass spectrum (molecular ion at m/z 155), whereas no ionisation of p-HB was produced in the MS conditions used. These compounds would derive from the fragments corresponding to the anthocyanin rings A (THB) and B (p-HB) released after breakdown of the heterocycle, according to the mechanism proposed by Furtado *et al.* (1993) and Piffaut *et al.* (1994). The presence of the aglycone (Pelargonidin, Pg) was

irregularly detected in the chromatograms during the first of month of assay (Fig. 3), suggesting that the separation of the glucose constitutes a previous step in the anthocyanin degradation. The levels of the aglycone detected were always low in agreement with its assumed instability. THB and p-HB were not stable in the solution and progressed to the formation of other unidentified structures. The amounts of p-HB accumulated were lower and its disappearance faster than those of THB, indicating a greater reactivity. In the absence of oxygen (solution B) hardly formation of THB and p- HB was detected, denoting that the anthocyanin breakdown does not represent a relevant mechanism for anthocyanin disappearance in anaerobic conditions.

Another major product found in the solutions was peak d whose formation was observed both in the presence and absence of oxygen. In fact, this was the only important peak detected in the chromatograms of solution B maintained in anaerobic conditions (Fig. 4). This compound showed a molecular ion at m/z 257 that produced no MS^2 fragments in the conditions employed. No suitable structure could be assigned to this ion. The compound e mostly accumulated in the solutions when low levels of Pg3gluc existed, suggesting that it does not derive directly from the anthocyanin but an intermediate product should exist that could not be detected.

Peak e showed a molecular ion at m/z 865 twice the mass of Pg3gluc and in its fragmentation the successive loss of two hexose residues is produced (fragments at m/z 703 and m/z 541). These characteristics are coherent with a dimer constituted by two anthocyanin moieties. In assays carried out with Mv3gluc other authors (Salas *et al.*, 2005; Vidal *et al.*, 2004) had already observed the formation of anthocyanin dimers, for which flaven-flavylium C-C linked or flavan-flavylium doubly linked structures were proposed. The compound e did not show an absorption maximum in the region of 500 nm pointing out that it did not contain flavylium units. Thus, two possible structures, i.e., flaven-flaven (Fig. 5a) and doubly-linked flavan-flaven (Fig. 5b) can be suggested as possible.

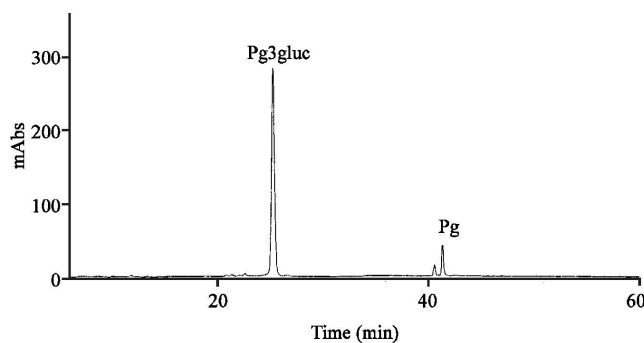


Fig. 3: HPLC chromatogram recorded at 520 nm of solution A at day 30 of assay where the presence of pelargonidin (Pg) can be seen

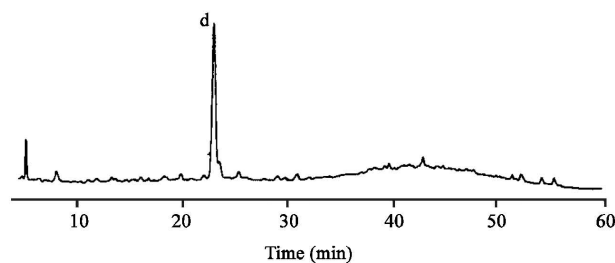


Fig. 4: HPLC chromatogram recorded at 280 nm of solution B at day 180 of assay

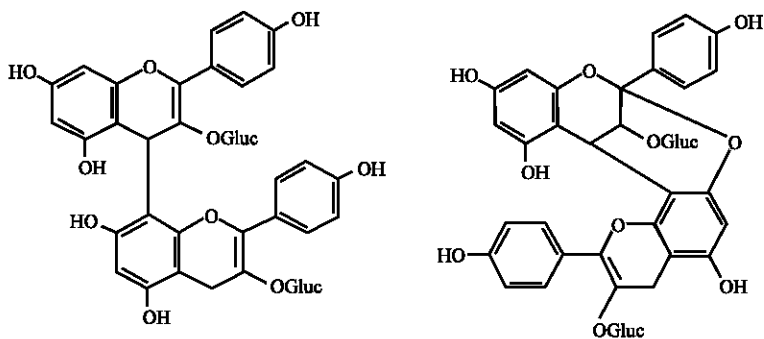


Fig. 5: Suggested structures for compound e (m/z at 865)

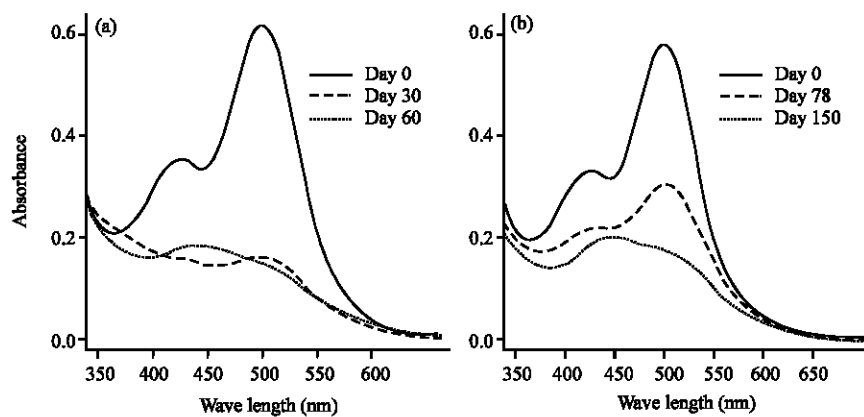


Fig. 6: Visible absorption spectra in solutions A (a) and B (b) recorded at different days of the assay

During storage, a progressive change in the colour of the solution from bright red to red orange and yellow orange was produced (Fig. 6). The formation of turbidity was also observed in the second month of assay and further accumulation of a brown sediment. The compounds detected in the chromatograms above discussed were mostly colourless and, therefore, other substances must have been contributing to the colour when low or no levels of the anthocyanin exist in the solution. As it can be seen in Fig. 2, in addition to peaks a-e the appearance of other peaks and a hump were also observed in the end part of the chromatograms. Those peaks, some of which showed absorption in the region of 400-460 nm, were not reproducible in the successive analyses, indicating that they corresponded to transient substances, quite possibly evolving to the formation of polymers responsible for the brown precipitate. No reliable information about those compounds could be obtained by mass spectrometry and, therefore, their nature remains unknown. The greater formation of these compounds was observed when hardly Pg3gluc existed in the medium, suggesting that they rather derived from products of the degradation of the anthocyanin and/or the citric acid used in the preparation of the model solutions. The evolution of the colour in solution B was much slower than in solution A, in accordance with the increased stability of the anthocyanin due to the restriction of oxygen. The red colour was maintained during a substantial part of the storage and only in the fourth month, when the levels of Pg3gluc were already low, a change to orange started to be appreciated. The formation of a light precipitate was also observed at the end of the six months of assay. In this solution, the products responsible for the colour when the anthocyanin decreased could not be detected in the chromatograms.

Changes in the Solutions Containing Pg3gluc and Flavanols

In solutions C-E different pattern of changes was observed with regard to A and B. The formation of four colourless compounds (P1, P2, P5 and P6) and four pigments (P3, P4, P7 and P8) was detected (Fig. 7) about which information (UV-visible and mass spectra) could be obtained (Table 3). The same compounds were observed in the presence and absence of oxygen, although in the latter case the composition changes took place more slowly.

Peak P5 corresponded to the unknown product d found in solutions A and B. This was the only important compound derived from the degradation of Pg3gluc detected in solutions C-E, where no significant formation of THB and p-HB was observed, suggesting that the heterocycle breakdown is not a preferential pathway for the anthocyanin loss in the presence of flavanols. The remaining compounds detected in solutions C-E derived from different processes of condensation between the anthocyanin and the flavanols, which constituted the most important mechanisms for the anthocyanin disappearance in these solutions, either in the presence and absence of oxygen.

The compounds P1 and P2 had the same molecular ion (m/z at 577) and similar fragmentation pattern. Their presence in the solution E that contained the dimer B3 as the only flavanol confirmed the implication of this procyanidin in their formation. The molecular ion would fit A-type

Table 3: Absorption and mass spectral characteristics of the products found in solutions C-E

Peak	λ max (nm)	Molecular ion (m/z)	MS ² fragment ions (m/z)*	Peak identity
P1	281	577	425, 407, 299, 409, 559	Flavanol-anthocyanidin condensed dimer
P2	274	577	425, 407, 299, 409	Flavanol-anthocyanidin condensed dimer
P3	276, 447	741	527, 375, 363, 405	Xanthylum pigment
P4	273, 436	453	301, 435, 257, 283	Xanthylum pigment
P5	292	257		Unknown (peak d)
P6	275	723	571, 561	Anthocyanin-flavanol condensed dimer
P7	271, 434	861	723, 741, 553, 571, 419	Xanthylum pigment
P8	267, 425	573	435, 453, 283	Xanthylum pigment

*Fragment ions are indicated in decreasing order of relative abundance

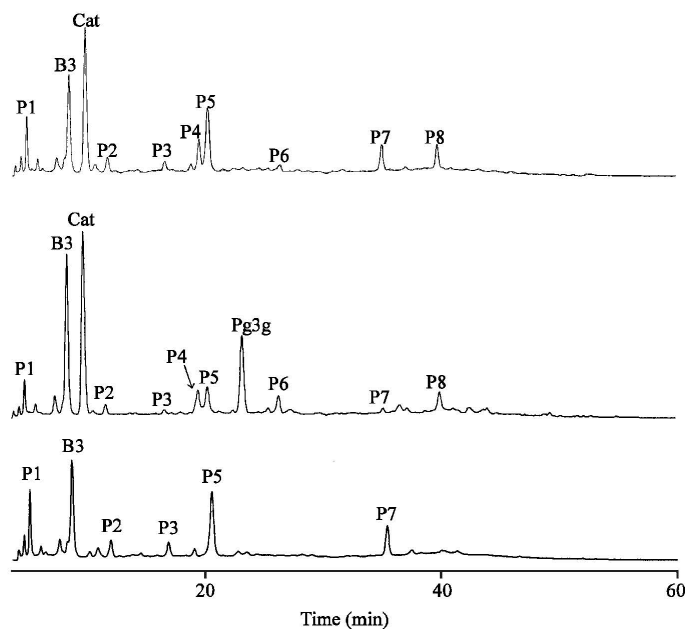


Fig. 7: HPLC chromatograms recorded at 280 nm of solution C (upper chromatogram), solution D (middle) and solution E (bottom) after 80 days of storage. The scale (mAbs units) is the same in all cases

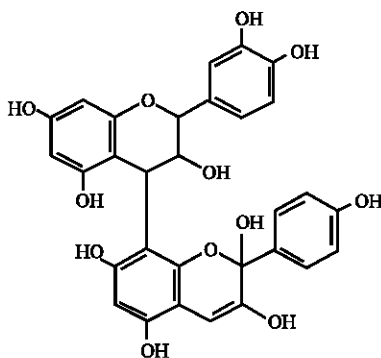


Fig. 8: Suggested structure for peak P1 (m/z at 577). P2 would have similar structure but C4-C6 linked

proanthocyanidin dimers, which might derive from the structural rearrangement of B3 following breakage of its interflavanic bond. However, this possibility was discarded, since such type of dimers should elute later than procyanidin B3 in the chromatograms using reversed-phase columns (Santos-Buelga *et al.*, 2003). Another possibility is that they result from the direct condensation of the carbocation $C4^+$, released after the interflavanic breakdown of procyanidin B3, over the nucleophilic positions C8 and C6 of the carbinol form of Pg3gluc to yield F-A dimers where the anthocyanin moiety is a flavene hydrated form (Fig. 8). The MS^2 fragmentation the compounds would be coherent with such a type of structure. The main fragment (m/z at 425, -152 amu) would correspond from the Retro Diels-Alder decomposition (RDA) of catechin, which has been seen to take place preferentially on the upper unit (de Pascual-Teresa *et al.*, 2000a; Friedrich *et al.*, 2000). Further fragmentation of this ion would give rise to the fragments at m/z 407 (loss of water) and m/z 299 (-126 amu, loss of phloroglucinol after heterocyclic ring fission of the catechin unit). Minor signals at m/z 559 and m/z 409 are interpreted as the loss of water from the hydroxyl group at C3 of the flavanol moiety (-18 amu) and catechin RDA together with the loss of the hydroxyl group at C2 of the anthocyanin moiety (-152-16 amu), respectively.

The structure proposed in Fig. 8 for P1 and P2 lacks the glucose attached to the anthocyanidin moiety. The glucosylated precursor of such a structure could be expected to be stable, however, it was not detected in any of the HPLC-DAD/MS analyses made. Thus, if the proposed structure is confirmed, it might have formed from the direct reaction of the flavanol carbocation with the anthocyanin aglycone (Pg). Taking into account that the production of the aglycone constitutes a first step in the anthocyanin breakdown (Piffaut *et al.*, 1993), its possible reaction with the flavanol might explain the low rate of products from Pg degradation (THB and p-HB) observed in the solutions containing flavanols.

Compound P6 showed a molecular ion at m/z 723 with MS^2 fragments at m/z 571 and m/z 561 corresponding to the RDA fission of catechin and the loss of a glucose moiety respectively. These characteristics fit a dimer containing catechin and Pg3gluc residues. This compound was not detected in the solution E (Pg3gluc+procyanidin B3), pointing out that the monomeric catechin was the flavanol involved in its formation. The possibility that P6 was a catechin-Pg3gluc dimer C6'-C8 linked was discarded, since no RDA fission of catechin could have been produced. On the other hand, the formation of such a type of dimers would require the previous oxidation of catechin to an *o*-quinone (Sarni-Manchado *et al.*, 1997) that would not be expected to occur in the solution D (prepared avoiding oxygen) in which P6 is a relevant product. This observation linked to the fact that flavanol monomers are unable to produce a $C4^+$ carbocation, strongly suggested that catechin was located in the lower unit of the condensed dimer. Thus, two possible structures that fit the molecular ion found for P6 can be

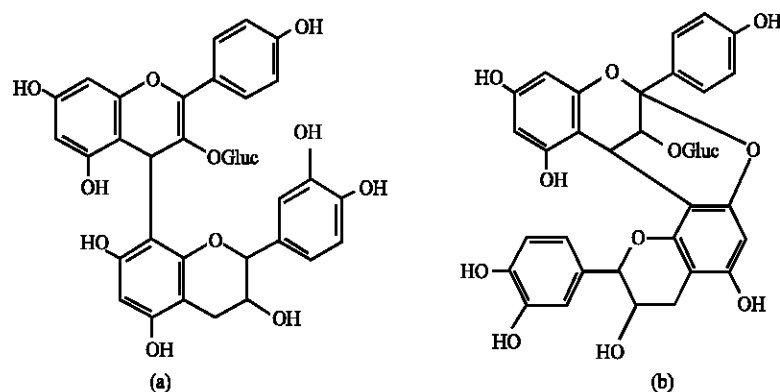


Fig. 9: Suggested structures for product P6 (m/z at 723)

proposed corresponding to A-F dimers in which anthocyanin and catechin would be either C-C linked (Fig. 9a) or doubly-linked like in A-type proanthocyanidins (Fig. 9b).

In the fragmentation of anthocyanin-flavanol singly-linked species in positive ion mode, the preferential loss of the glucose moiety and further catechin RDA of the released aglycone would be expected (Gonzalez-Paramas *et al.*, 2006; Salas *et al.*, 2003). However, the production of a fragment from the direct catechin RDA that could even predominate over the fragment corresponding to the loss of glucose was observed for A type dimers (Dueñas *et al.*, 2006; Remy-Tanneau *et al.*, 2003). In our case, the main fragment produced in the MS² fragmentation of P6 corresponded to the direct catechin RDA fission (m/z at 571), whereas a minor signal was observed for the fragment derived from the loss of the glucose (m/z at 561, 10% of relative abundance), which might support an A-type nature for P6 (Fig. 9b).

A flaven-flavan dimer like the one in Fig. 9a would be expected to be the first species formed after the condensation anthocyanin-catechin. Further rearrangement of this adduct could be produced to either a coloured xanthylium structure (Jurd, 1969; Liao *et al.*, 1992; Somers, 1971) or a colourless A-type derivative (Jurd, 1969; Jurd and Waiss, 1965). The preferential formation of this latter in anaerobic media was found by Bishop and Nagel (1984) and Remy-Tanneau *et al.* (2003). In our assays P6 accumulated in higher amounts in solution D that had been purged with N₂ than in solution C where oxygen was not avoided and only traces of P6 were detected. This set of observations would contribute further support to the A-type nature of this compound. On the other hand, greater formation of xanthylium pigments was observed in the solution C, suggesting that in oxidative conditions a rearrangement of the A-F adducts towards xanthylium derivatives is produced.

The products P4 (m/z at 453) and P8 (m/z at 573) showed absorption spectra corresponding to xanthylium structures (Fig. 10). In previous studies of our group the formation of a compound with the same molecular ion and similar UV-visible spectrum as P4 was found in wine model assays containing catechin and Mv3gluc (Santos-Buelga *et al.*, 1999). That pigment was suggested to derive from the glyoxylic acid-mediated condensation between catechin and phloroglucinol moieties, in accordance with the mechanism described by Fulcrand *et al.* (1997). In that case, the glyoxylic acid would result from the oxidation of the tartaric acid used in the preparation of the model solutions. In the current assays the presence of glyoxylic acid in the solutions would not be expected and suggest that other mechanisms are involved in the formation of P4.

Xanthylium pigments can also be formed from the direct condensation between flavylium cations and structures containing phloroglucinol nuclei (like flavanols) as early described by Jurd (1969). Such a mechanism was also used by Liao *et al.* (1992) and Santos-Buelga *et al.* (1995) to explain the

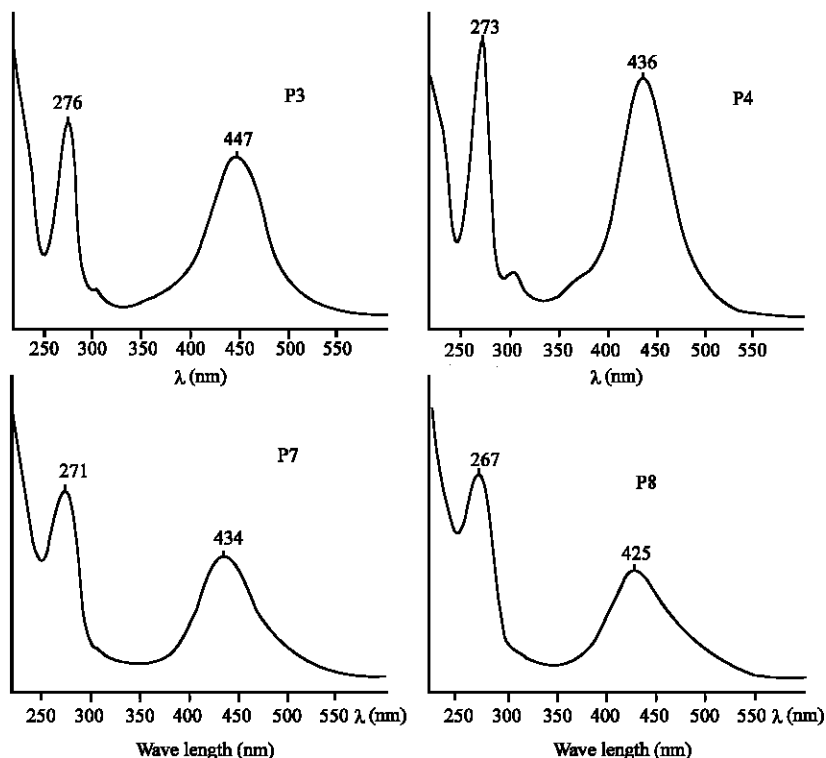


Fig. 10: UV-visible spectra of new xanthylum pigments formed in solutions C-E

formation of yellow products in wine model solutions. Quite recently Dueñas *et al.* (2006), in model solutions of Mv3gluc and epicatechin in ammonium formate, found the formation of a xanthylum derivative with same molecular ion (m/z at 453) and similar UV-visible spectrum as P4 and also suggested a mechanism based on the direct condensation anthocyanin-flavanol to explain its formation. The mechanism proposed by these authors would also involve the C2-C3 oxidative cleavage of the anthocyanin heterocycle to yield an intermediate malvone, as previously described by Hrazdina and Franzese (1974). The process would require an acidic pH since under neutral or alkaline conditions the separation of the anthocyanin B-ring with formation of a coumarin would be expected (Hrazdina and Franzese, 1974). Based on the proposal of Dueñas *et al.* (2006), a possible pathway for the formation of pigments P4 and P8 in our solutions is suggested in Fig. 11.

The structures proposed for pigments P4 and P8 in Fig. 11 fit their MS² fragmentation patterns. Regarding P4 (m/z at 453) the main fragment at m/z 301 is attributed to catechin RDA fission $[M+H-152]^+$; the signal at m/z 257 $[M+H-152-44]^+$ would correspond to the additional loss of the carboxyl group. The fragments at m/z 435 and m/z 283 would derive from the loss of water $[M+H-18]^+$ and further RDA $[M+H-18-152]^+$, respectively. In the case of P8 (m/z at 573) the main fragment (m/z at 435) would result of the loss of the *p*-hydroxybenzoyl moiety (-138 amu) corresponding to anthocyanin ring-B and the ion at m/z at 453 from the alternative breakdown of the same ring (-120 amu). Further RDA of the ion at m/z 435 would yield the fragment at m/z 283. Pigments P3 (m/z at 741) and P7 (m/z at 861) would be similar to P4 and P8, but derived from procyanidin B3 instead of catechin.

The formation of xanthylum pigments according to the mechanism of Fig. 11 involves oxidative steps, which makes it difficult to justify their presence in the solution D prepared in anaerobic

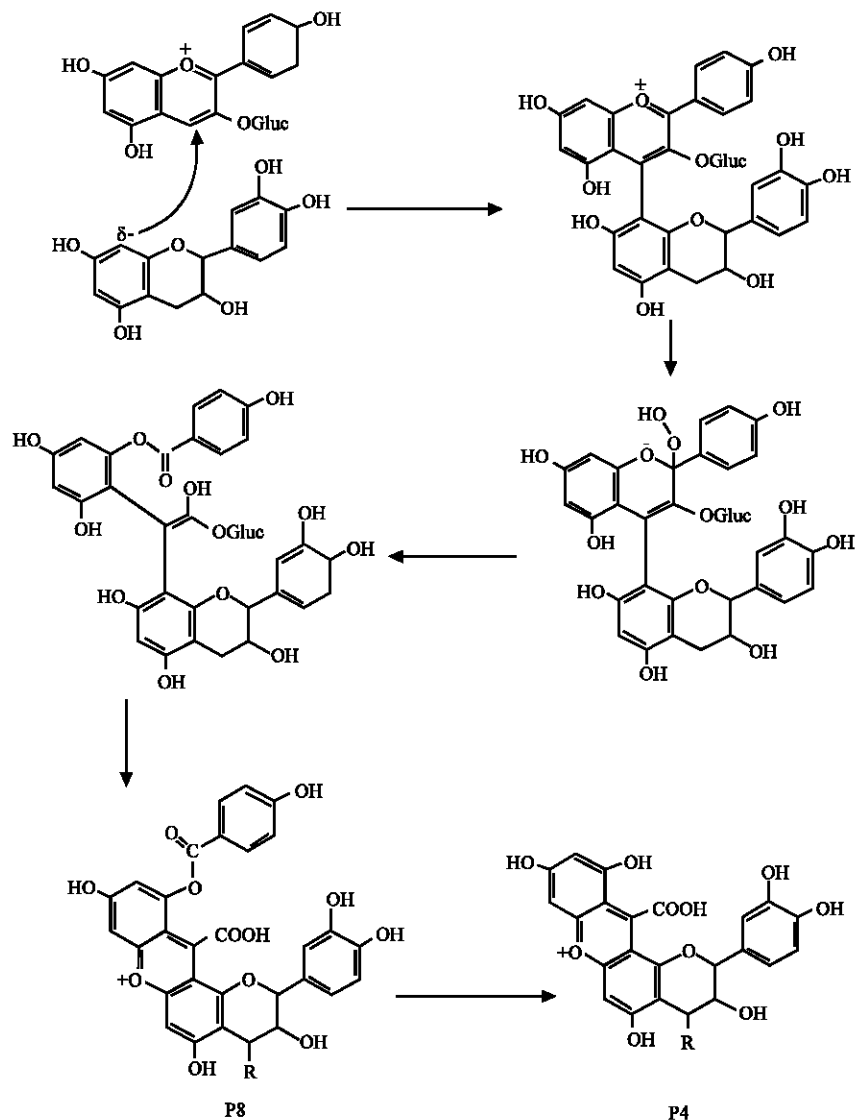


Fig. 11: Proposed pathway for the formation of compounds P4 and P8

conditions. Obviously the incorporation of small amounts of oxygen to the vials during sampling might be an explanation. On the other hand, Somers and Evans (1986) observed that similar pattern of changes were produced in the colour composition of red wines conserved in the absence and presence of oxygen and Jurd (1967) had also showed that the flavylum cation could act as an oxidant in the process of formation of new pigments derived from the condensation between flavylum salts (like the anthocyanins) and catechin. These observations might support the formation of yellow pigments in the solution D even if oxygen was not available.

In the solutions containing anthocyanins and flavanols a change of colour was produced from bright red to brown-orange. Furthermore, the accumulation of a light brown precipitate was also observed at the end of the assay. In the visible spectra of the solutions a progressive decrease in the absorbance at 500 nm and an increase in the region of 430 nm was produced (Fig. 12), attributable to

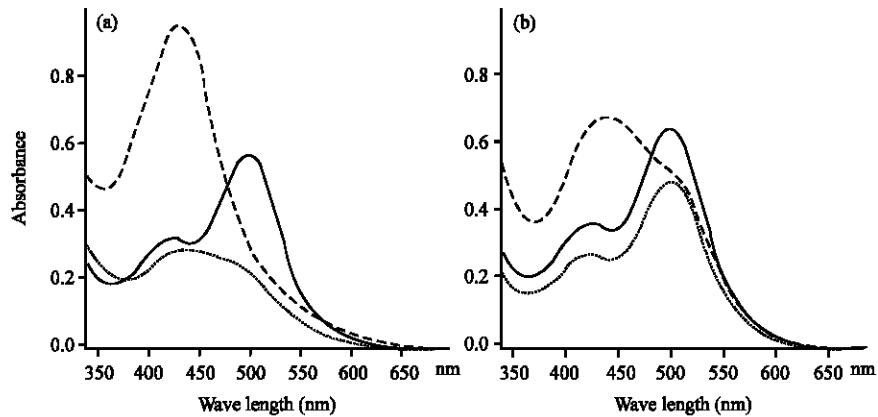


Fig. 12: Changes in the visible absorption spectra in solutions C (a) and D (b) at days 0 (—), 24 (---) and 80 (....) of the assay

the disappearance of the anthocyanin and the progressive accumulation of xanthylum pigments. The colour changes observed were similar in the three solutions containing flavanols, although they were faster in solutions C and E than in solution D, in agreement with its slower decrease of the anthocyanin and accumulation of xanthylum pigments due to the restriction of oxygen.

CONCLUSION

In this study further contributions are made to the understanding of the mechanisms involved in anthocyanin instability in food. The disappearance of Pg3gluc, by far the most important anthocyanin in strawberry, was studied in the absence and presence of flavanols and/or oxygen in aqueous acidic media. As it could be expected, the decrease of the anthocyanin was much slower when oxygen was avoided; however, the presence of flavanols constituted a destabilizing factor. Indeed, it was found that the effect of the flavanols even counteracted the protection offered by the anaerobic storage. In the solutions containing only Pg3gluc and oxygen, the anthocyanin breakdown to colourless products constituted the most important pathway for disappearance. However, in anaerobic conditions or in the presence of flavanols that mechanism does not represent a relevant way for anthocyanin loss. This, in the presence of flavanols, condensation with the anthocyanin yielding both colourless products and new xanthylum pigments was found to be the most important mechanism for anthocyanin disappearance. Conclusions about the identity of the products formed in all these reactions were obtained based on their mass spectra and MS² fragmentation patterns and the structure of some of the newly-formed compounds was tentatively established for the first time. The results obtained are expected to contribute not only to interpret the processes of anthocyanin disappearance but they might also provide the basis to define the conditions for a better preservation of the red colour in strawberry-derived products.

ACKNOWLEDGMENTS

Financial support of Junta de Castilla y Leon (Spain) through the grant ref. SA086/01 is greatly acknowledged. Author Lopes da Silva was funded by European Union and Government of Portugal through the Programme PRODEP III (2001-2003).

REFERENCES

- Bakker, J., A. Piccineli and P. Bridle, 1993. Model wine solutions: Colour and composition changes during ageing. *Vitis*, 32: 111-118.
- Bakker, J. and C.F. Timberlake, 1997. Isolation, identification and characterization of new color-stable anthocyanins occurring in some red wines. *J. Agric. Food Chem.*, 45: 35-43.
- Bakker, J., P. Bridle, T. Honda, H. Kuwano and N. Saito *et al.*, 1997. Isolation and identification of a new anthocyanin occurring in some red wines. *Phytochemistry*, 44: 1375-1382.
- Baublis, A., A. Spomer and M.D. Berber-Jimenez, 1994. Anthocyanin pigments: Comparison of extract stability. *J. Food Sci.*, 59: 1219-1221.
- Benabdeljalil, C., V. Cheynier, H. Fulcrand, A. Hakiki, M. Mosaddak and M. Moutounet, 2000. Identification of new pigments formed by reaction between anthocyanins and yeast metabolites (In French). *Sci. Aliments*, 20: 203-220.
- Bishop, P.D. and C.W. Nagel, 1984. Characterization of the condensation product of malvidin 3, 5-diglucoside and catechin. *J. Agric. Food Chem.*, 32: 1022-1026.
- Boulton, R., 2001. The copigmentation of anthocyanins and its role in the color of red wine: A critical review. *Am. J. Enol. Vitic.*, 52: 67-87.
- Cameira dos Santos, P.J., J.M. Brillouet, V. Cheynier and M. Moutounet, 1996. Detection and partial characterisation of new anthocyanin-derived pigments in wine. *J. Sci. Food Agric.*, 70: 204-208.
- Cemeroglu, B., S. Velioglu and S. Isik, 1994. Degradation kinetics of anthocyanins in sour cherry juice and concentrate. *J. Food Sci.*, 59: 1216-1218.
- Clydesdale, F.M., J.H. Main, F.J. Francis and J.R.R.A. Damon, 1978. *Concord* grape pigments as colorants for beverages and gelatin desserts. *J. Food Sci.*, 43: 1687-1692.
- de Freitas, V., 1995. Studies on condensed tannins: Structures and proprieties of grape and wine procyanidins (In French), Ph.D. Thesis, Université de Bordeaux II, France.
- de Freitas, V., Y. Glories and N. Laguerre, 1998. Incidence of molecular structure in oxidation of grape seeds procyanidins. *J. Agric. Food Chem.*, 46: 376-382.
- de Pascual-Teresa, S., Y. Gutierrez-Fernandez, J.C. Rivas-Gonzalo and C. Santos-Buelga, 1998. Characterization of monomeric and oligomeric flavan-3-ols from unripe almond fruit. *Phytochem. Anal.*, 9: 21-27.
- de Pascual-Teresa, S., J.C. Rivas-Gonzalo and C. Santos-Buelga, 2000a. Prodelphinidins and related flavanols in wine. *Int. J. Food Sci. Technol.*, 35: 33-40.
- de Pascual-Teresa, S., C. Santos-Buelga and J.C. Rivas-Gonzalo, 2000b. Quantitative analysis of flavan-3-ols in Spanish foodstuffs and beverages. *J. Agric. Food Chem.*, 48: 5331-5337.
- Dueñas, M., H. Fulcrand and V. Cheynier, 2006. Formation of anthocyanin-flavanol adducts in model solutions. *Anal. Chim. Acta*, 563: 15-25.
- Escribano-Bailón, M.T., C. Santos-Buelga, G.L. Alonso and M.R. Salinas, 2002. Anthocyanin composition of *Coriaria myrtifolia* L. fruit. *Phytochem. Anal.*, 13: 354-357.
- Es-Safi, N.E., C. Le Guernevé, V. Cheynier and M. Moutounet, 1999. New polyphenolic compounds with xanthylum skeletons formed through reaction between (+)-catechin and glyoxylic acid. *J. Agric. Food Chem.*, 47: 5211-5217.
- Francia-Aricha, E.M., M.T. Guerra, J.C. Rivas-Gonzalo and C. Santos-Buelga, 1997. New anthocyanin pigments formed after condensation with flavanols. *J. Agric. Food Chem.*, 45: 2262-2266.
- Francia-Aricha, E.M., J.C. Rivas-Gonzalo and C. Santos-Buelga, 1998. Effect of malvidin-3-monoglucoside on the browning of monomeric and dimeric flavanols. *Eur. Food Res. Technol.*, 209: 223-228.
- Friedrich, W., A. Eberhardt and R. Galensa, 2000. Investigation of proanthocyanidins by HPLC with electrospray ionization mass spectrometry. *Eur. Food Res. Technol.*, 211: 56-64.

- Fulcrand, H., P.J. Cameira-dos-Santos, P. Sami-Manchado, V. Cheynier and J. Fabre-Bonvin, 1996. Structure of new anthocyanin-derived wine pigments. *J. Chem. Soc. Perkin Trans.*, 1: 735-739.
- Fulcrand, H., V. Cheynier, J. Oszmianski and M. Moutounet, 1997. An oxidized tartaric acid residue as a new bridge potentially competing with acetaldehyde in flavan-3-ol condensation. *Phytochemistry*, 46: 223-227.
- Fulcrand, H., C. Benabdeljalil, J. Rigaud, V. Cheynier and M. Moutounet, 1998. A new class of wine pigments generated by reaction between pyruvic acid and grape anthocyanins. *Phytochemistry*, 47: 1401-1407.
- Furtado, P., P. Figueiredo, H. Chaves-das-Neves and F. Pina, 1993. Photochemical and thermal degradation of anthocyanidins. *J. Photochem. Photobiol. A-Chem.*, 75: 113-118.
- Garzon, G.A. and R.E. Wrolstad, 2001. The stability of pelargonidin-based anthocyanins at varying water activity. *Food Chem.*, 75: 185-196.
- Garzon, G.A. and R.E. Wrolstad, 2002. Comparison of the stability of pelargonidin-based anthocyanins in strawberry juice and concentrate. *J. Food Sci.*, 67: 1288-1299.
- Giusti, M.M. and R.E. Wrolstad, 1996. Radish anthocyanin extract as a natural red colorant for maraschino cherries. *J. Food Sci.*, 61: 688-694.
- Goiffon, J.P., P.P. Mouly and E.M. Gaydou, 1999. Anthocyanic pigment determination in red fruit juices, concentrated juices and syrups using liquid chromatography. *Anal. Chim. Acta*, 382: 39-50.
- Gonzalez-Paramas, A.M., F. Lopes da Silva, P. Martin-Lopez, G. Macz-Pop, S. Gonzalez-Manzano, C. Alcalde-Eon and J.J. Perez-Alonso *et al.*, 2006. Flavanol anthocyanin condensed pigments in plant extracts. *Food Chem.*, 94: 428-436.
- Hayasaka, Y. and R.E. Asenstorfer, 2002. Screening for potential pigments derived from anthocyanins in red wine using nanoelectrospray tandem mass spectrometry. *J. Agric. Food Chem.*, 50: 756-761.
- Hermosín Gutiérrez, I., E. Sánchez-Palomo Lorenzo and A. Vicario Espinosa, 2005. Phenolic composition and magnitude of copigmentation in young and shortly aged red wines made from the cultivars, Cabernet Sauvignon, Cencibel and Syrah. *Food Chem.*, 92: 269-283.
- Hrazdina, G. and A.J. Franzese, 1974. Oxidation products of acylated anthocyanins under acidic and neutral conditions. *Phytochemistry*, 13: 231-234.
- Jurd, L. and A.C. Waiss, 1965. Anthocyanins and related compounds-VI: Flavylium salt-phloroglucinol condensation products. *Tetrahedron*, 21: 1471-1483.
- Jurd, L., 1967. Catechin flavylium salt condensation reactions. *Tetrahedron*, 23: 1057-1064.
- Jurd, L., 1969. Review of polyphenol condensation reactions and their possible occurrence in the aging of wines. *Am. J. Enol. Vitic.*, 20: 191-195.
- Kallio, H., M. Hakala, A.M. Pelkkikangas and A. Lapveteläinen, 2000. Sugars and acids of strawberry varieties. *Eur. Food Res. Technol.*, 212: 81-85.
- Kunsági-Máté, S., K. Szabó, M. Nikfardjam and L. Kollár, 2006. Determination of the thermodynamic parameters of the complex formation between malvidin-3-O-glucoside and polyphenols. Copigmentation effect in red wines. *J. Biochem. Biophys. Meth.*, 69: 113-119.
- Liao, H., Y. Cai and E. Haslam, 1992. Polyphenol interactions, Part 6, Anthocyanins: Copigmentation and colour changes in red wines. *J. Sci. Food Agric.*, 59: 299-305.
- Lopes da Silva, F., S. de Pascual-Teresa, J.C. Rivas-Gonzalo and C. Santos-Buelga, 2002. Identification of anthocyanin pigments in strawberry (cv Camarosa) by LC using DAD and ESI-MS detection. *Eur. Food Res. Technol.*, 214: 248-253.
- Lopes da Silva, F., M.T. Escribano-Bailon, J.J. Perez-Alonso, J.C. Rivas-Gonzalo and C. Santos-Buelga, 2007. Anthocyanin pigments in strawberry. *LWT-Food Sci. Technol.*, 40: 374-382.
- Oszmianski, J., J.C. Sapis and J.J. Macheix, 1985. Changes in grape seed phenols as affected by enzymic and chemical oxidation *in vitro*. *J. Food Sci.*, 50: 1505-1506.

- Özkan, M., 2002. Degradation of anthocyanins in sour cherry and pomegranate juices by hydrogen peroxide in the presence of added ascorbic acid. *Food Chem.*, 78: 499-504.
- Özkan, M., A. Yemencioğlu, N. Asefi and B. Cemeroglu, 2002. Degradation kinetics of anthocyanins from sour cherry, pomegranate and strawberry juices by hydrogen peroxide. *J. Food Sci.*, 67: 525-529.
- Piffaut, B., F. Kader, M. Girardin and M. Metche, 1994. Comparative degradation pathways of malvidin 3,5-diglucoside after enzymatic and thermal treatments. *Food Chem.*, 50: 115-120.
- Poei-Langston, M.S. and R.E. Wrolstad, 1981. Color degradation in an ascorbic-acid anthocyanin-flavanol model system. *J. Food Sci.*, 46: 1218-1222.
- Remy-Tarneau, S., C. Le Guernevé, E. Meudec and V. Cheynier, 2003. Characterization of a colorless anthocyanin-flavan-3-ol dimer containing both carbon-carbon and ether interflavanoid linkages by NMR and mass spectrometry. *J. Agric. Food Chem.*, 51: 3592-3597.
- Rivas-Gonzalo, J.C., S. Bravo-Haro and C. Santos-Buelga, 1995. Detection of compounds formed through the reaction of malvidin 3-monoglucoside and catechin in the presence of acetaldehyde. *J. Agric. Food Chem.*, 43: 1444-1449.
- Romero, C. and J. Bakker, 1999. Interactions between grape anthocyanins and pyruvic acid, with effect of pH and acid concentration on anthocyanin composition and color in model solutions. *J. Agric. Food Chem.*, 47: 3130-3139.
- Salas, E., H. Fulcrand, E. Meudec and V. Cheynier, 2003. Reactions of anthocyanins and tannins in model solutions. *J. Agric. Food Chem.*, 51: 7951-7961.
- Salas, E., M. Dueñas, M. Schwarz, P. Winterhalter, V. Cheynier and H. Fulcrand, 2005. Characterization of pigments from different High Speed Countercurrent Chromatography wine fractions. *J. Agric. Food Chem.*, 53: 4536-4546.
- Santos-Buelga, C., S. Bravo-Haro and J.C. Rivas-Gonzalo, 1995. Interactions between catechin and malvidin-3-monoglucoside in model solutions. *Z Lebensm Unters Forsch*, 201: 269-274.
- Santos-Buelga, C., E.M. Francia-Aricha, S. de Pascual-Teresa and J.C. Rivas-Gonzalo, 1999. Contribution to the identification of the pigments responsible for the browning of anthocyanin-flavanol solutions. *Eur. Food Res. Technol.*, 209: 411-415.
- Santos-Buelga, C., C. Garcia-Viguera and F.A. Tomas-Barberan, 2003. On-line identification of flavonoids by HPLC coupled to diode array detection. In: *Methods in Polyphenol Analysis*. Royal Society of Chemistry (Cambridge, UK), pp: 92-127.
- Sarni-Manchado, P., V. Cheynier and M. Moutounet, 1997. Reactions of polyphenoloxidase generated caftaric acid o-quinone with malvidin 3-glucoside. *Phytochemistry*, 45: 1367-1369.
- Somers, T.C., 1971. The polymeric nature of wine pigments. *Phytochemistry*, 10: 2175.
- Somers, T.C. and M.E. Evans, 1986. Evolution of red wines, I, Ambient influences on colour composition during early maturation. *Vitis*, 25: 31-39.
- Thompson, R.S., D. Jacques, E. Haslam and R.J.N. Tanner, 1972. Plant proanthocyanidins, Part I, introduction; the isolation, structure and distribution in nature of plant procyanidins. *J. Chem. Soc. Perkin Trans.*, 1: 1387-1399.
- Timberlake, C.F. and P. Bridle, 1976. Interactions between anthocyanins, phenolic compounds and acetaldehyde and their significance in red wines. *Am. J. Enol. Vitic.*, 27: 97-105.
- Vidal, S., E. Meudec, V. Cheynier, G. Skouroumounis and Y. Hayasaka, 2004. Mass spectrometric evidence for the existence of oligomeric anthocyanins in grape. *J. Agric. Food Chem.*, 52: 7144-7151.