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Oxidative formation and structural characterisation of new α-pyranone (lactone) compounds of non-oxonium nature originated from fruit anthocyanins

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ABSTRACT

A new group of pyranoanthocyanin-derived polyphenolic compounds was recently found to occur in aged wines and named as oxovitisins, the spectrum of which displayed only a pronounced broad absorption peak around 370 nm in the UV region, being quite different from that of anthocyanin-like chromenylium pigments which present maximum absorption in the visible wavelength ranges. The possibilities and reaction conditions for the oxidative transformation of anthocyanin type flavylium cations into new type of stable pyranone structures of non-oxonium nature was studied through two-step reactions using anthocyanins obtained from different fruit extracts. The irreversible change of pyranoflavyliums to the neutral pyranone compounds by hydration and further oxidation reactions in the second step were strongly related to pH. The reaction took place only in mildly acidic solutions with the most favourable pH range 4.0–5.5, as the hemiacetal formation by the nucleophilic attack of water could be hindered at higher or lower pH. The reaction rate increased significantly with increasing temperature. The new non-oxonium compounds resulting from malvidin-3-coumaroylglucoside and cyanidin-3-rutinoside were structurally characterised by MS and NMR spectroscopy and shown similarly to possess the common α-pyranone (lactone) ring between C-4 and the hydroxy group at C-5 of the anthocyanin core, which confers them with unique spectrum characteristics. The interest in new anthocyanin derivatives of non-oxonium nature with additional pyranone (lactone) ring will go beyond wine chemistry and bring expectations concerning their use in the food and pharmaceutical industry due to their spectral and structural similarity to flavones as well as their naturally occurring nature.

1. Introduction

Anthocyanins constitute a major group of polyphenolic natural pigments widely distributed in the plant kingdom and are responsible for a variety of colours in flowers and fruits. These pigments are relatively unstable and may undergo several chemical reactions in anthocyanin-rich foodstuffs and beverages like red wine, leading to the formation of more stable condensation products (Jurd, 1969; Liao, Cai, & Haslam, 1992; Somers, 1971; Timberlake, 1980). One of the most interesting group of reaction products is pyranoanthocyanins, possessing an additional pyran ring structure (Fig. 1) between C-4 and the hydroxyl group at C-5 of the anthocyanin core (Bakker & Timberlake, 1997; Francia-Aricha, Guerra, Rivas-Gonzalo, & Santos-Buelga, 1997; Fulcrand, Benabdeljalil, Rigaud, Cheynier, & Moutounet, 1998; Fulcrand, Cameiro dos Santos, Sarni-Machado, Cheynier, & Moutounet, 1996; He, Santos-Buelga, Silva, Mateus, & de Freitas, 2006), which is thought to be responsible for the relatively higher stability of pyranoanthocyanins compared to that of the original anthocyanins (Bakker & Timberlake, 1997; Cameira dos Santos, Brillouet, Cheynier, & Moutounet, 1996; Fulcrand et al., 1998).

Amongst pyranoanthocyanins bearing different moieties of small molecular reactants, one of the most important groups was carboxy-pyranoanthocyanins (A-type vitisins), which resulted from the cycloaddition reaction of pyruvic acid with anthocyanins (Bakker et al., 1997; Fulcrand et al., 1998). Recently, a new class of pyranoanthocyanin dimers that present an outstanding turquoise blue colour was identified in aged Port wine and lees and shown to be formed by reactions of carboxy-pyranoanthocyanin with methyl-pyranoanthocyanin (Oliveira et al., 2010). More recently, another family of pyranoanthocyanin derived neutral pigments was also found to occur in aged old Port wines and named as oxovitisins (He, Oliveira, Silva, Mateus, & de Freitas, 2010). All these newly formed pigments point to a new pathway involving chemical transformations of pyranoanthocyanins into new types of structures contributing to the colour changes during wine ageing.

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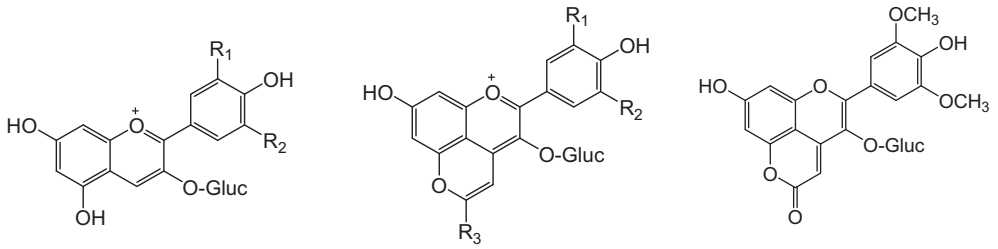

Fig. 1. Chemical structures of anthocyanins, pyranoanthocyanins and a new non-oxonium derivative detected in wine. R_1, R_2 = H, OH, or OCH_3; R_3 = H, COOH, CH_3, phenol or flavanol; Gluc, glucose.

The interest in anthocyanin derivatives goes beyond wine chemistry. The formation and occurrence of vitisins with different aglycons have been extensively studied during the last decades in table wine, Port wine or by-product like grape pomace, and fruit and vegetable juices (Anderson, Fossen, Torskangerpoll, Fossen, & Hauge, 2004; Fossen, & Anderson, 2003; Hillebrand, Schwarz, & Winterhalter, 2004; Mateus, Silva, Vercauteren, & De Freitas, 2001; Rentzsch, Quast, Hillebrand, Mehnert, & Winterhalter, 2007; Schwarz, Wary, & Winterhalter, 2004). For the newly discovered neutral wine pigment (not flavylium cations) which contains an additional pyran-2-one or lactone ring onto the anthocyanin molecules (Fig. 1), its spectral and structural similarities to flavones as well as its non-oxonium nature bring expectations concerning the use of these natural compounds in the food and pharmaceutical industry. Bearing this in mind, the aim of this work was to study the possibilities and reaction conditions for the oxidative formation of non-oxonium anthocyanin-derivatives through two-step chemical transformation of genuine anthocyanins obtained from different red fruits. The resulting new polyphenolic compounds were prepared and characterised by NMR and mass spectrometry so as to better understand their spectral characteristics and non-oxonium chemical nature as well as their oxidative formation mechanism, which could also help to explain their possible occurrence during storage and processing of anthocyanin-rich foodstuffs and beverages.

2. Materials and methods

2.1. Reagents

TSK Toyopearl gel HW-40(s) was purchased from Tosoh (Tokyo, Japan); pyruvic acid (d = 1.267; 97%) was purchased from Sigma–Aldrich (Madrid, Spain). All solvents were HPLC grade.

2.2. Anthocyanin extracts preparation

A total of 1 kg of sweet cherries (*Prunus avium*) and red grapes (*Vitis vinifera*) was extracted with a solution of 50% aqueous ethanol (pH 1.5, acidified with HCl) for 1 day at room temperature. The anthocyanin extracts were purified by a TSK Toyopearl gel column (250 × 16 mm i.d.) chromatography to obtain the major anthocyanin malvidin-3-*O*-coumaroyl-glucoside 1 from grapes and cyanidin-3-*O*-rutinoside from cherries 2 according to the procedures described previously (Pissarra, Mateus, Rivas-Gonzalo, Santos-Buelga, & de Freitas, 2003).

2.3. Oxidative formation of anthocyanin-derived pyranone compounds of non-oxonium nature

The formation of non-oxonium pyranone derivatives was achieved through two-step chemical reactions. The two original anthocyanins were firstly converted into respective carboxy-pyranoanthocyanins by reactions with pyruvic acid (molar ratio pyruvic acid/anthocyanin of 50:1) in water (pH 2.6, 35 °C) during 5 day according to the method of Oliveira et al. (2006). The resulting carboxy-pyranoanthocyanins recovered were dissolved in 20% aqueous ethanol in amber-glass jars with screw-cap. The solutions were then adjusted to different pH values from 1.0 to 7.0 and enriched with air/oxygen through mildly fortified oxygenation periodically (2 min treatment every week). After treatment, the jars were closed and sealed with a septum and the solutions were incubated at 35 °C. The formation of new compounds was monitored periodically (every 3 days) by high performance liquid chromatography with diode array detection (HPLC-DAD).

The effect of temperature on the reaction was studied by incubation of solutions at ambient temperature (25 °C), 50 and 70 °C, respectively. For the comparison of oxidising conditions, another two sets of sample solutions at pH 3.8 without periodical oxygenation treatment or in the presence of additional 5 ppm hydrogen peroxide were also carried out.

2.4. Preparation and purification of anthocyanin-derived pyranone compounds

Under the optimised reaction conditions, when the newly formed pyranone-anthocyanin derivatives detected at 370 nm reached its maximum concentration the reaction was stopped and the major compounds were purified. Each reaction mixture of different anthocyanin origin was applied directly onto a TSK Toyopearl gel HW-40(s) column chromatography. The fraction containing the derived pigment eluted with 40% aqueous methanol was collected and then submitted to semi-preparative HPLC using the below indicated reversed-phase C18 column using an injection volume of 500 μl and the same gradient programme. The isolated compound was concentrated under vacuum and then submitted to further purification with distiled methanol, which consisted of a final elution on silica gel 100 C18-reversed phase using a vacuum filtration system.

2.5. Monitoring of reaction products

2.5.1. HPLC-DAD analysis

A Knauer K-1001 HPLC with a 250 × 4.6 mm i.d. reversed-phase C18 column (Merck, Darmstadt, Germany) was used to monitor the reactions. The detection was carried out using a Knauer K-2800 diode array detector. An HPLC pump Knauer K-1001 was used together with a Knauer K-3800 autosampler. The solvents were A: H_2O/HCOOH (9:1), and B: HCOOH/H_2O/CH_3CN (1:1:8). The gradient consisted of 15–35% B over 70 min, 35–80% B over 5 min, and then isocratic for 10 min at a flow rate of 1.0 ml/min. The column was then stabilised with the initial conditions for another 10 min.

2.5.2. HPLC-electrospray ionisation mass spectrometry (ESI-MS) analysis

For the LC–MS analysis was used a Finnigan Surveyor series liquid chromatograph, equipped with a 150 × 4.6 mm i.d., 5 μm LicroCART® reversed-phase C18 column thermostated at 35 °C. The mass detection was carried out by a Finnigan LCQ DECA XP MAX (Finnigan Corp., San José, CA) mass detector with an API (Atmospheric Pressure Ionisation) source of ionisation and an ESI (ElectroSpray Ionisation) interface. Solvents were A: aqueous 0.1% trifluoroacetic acid, and B: acetonitrile, establishing the HPLC gradient as reported elsewhere (He et al., 2006). The capillary voltage and temperature were 4 V and 190 °C, respectively. Spectra were recorded in positive ion mode between m/z 120 and 1500. The mass spectrometer was programmed to do a series of three scans: a full mass, a zoom scan of the most intense ion in the first scan, and a MS–MS of the most intense ion, using relative collision energy of 30 and 60 V.

2.6. NMR analysis

^{1}H NMR (500.13 MHz) and ^{13}C NMR (125.77 MHz) spectra were measured in CD_3OD/TFA (98:2) and $CDCl_3$ on a Bruker-Advance 500 spectrometer at 303 K with TMS as internal standard. 1H chemical shifts were assigned using 1D and 2D ^{1}H NMR (gCOSY and NOESY). ^{13}C resonances were assigned using 2D NMR techniques (gHMBC and gHSQC) (Bax & Subramanian, 1986; Bax & Summers, 1986). The delay for the long-range C/H coupling constant was optimised to 7 Hz.

3. Results and discussion

3.1. Reaction pathway for the oxidative formation of pyranone compounds derived from fruits anthocyanins

Anthocyanins obtained from different red fruit extracts were used to produce the respective new derivatives of non-oxonium nature with additional pyranone ring (Fig. 2). Two anthocyanins malvidin-3-*O*-coumaroylglucoside **1** and cyanidin-3-*O*-rutinoside **2** were chosen aiming to obtain the anthocyanins with different aglycons and different types of glycosyl and acyl moiety. The original anthocyanins were firstly converted into respective pyranoanthocyanins **3** (**4**) by reactions with pyruvic acid under the conditions described previously (Fulcrand et al., 1998; Oliveira et al., 2006). The occurrence of carboxy-pyranoanthocyanins with different aglycons has been extensively studied over recent years in wine and fruit juices (Hillebrand et al., 2004; Mateus et al., 2001; Rentzsch et al., 2007; Schwarz et al., 2004), as well as in fresh fruits and vegetables (Anderson et al., 2004; Fossen & Anderson, 2003) and are believed to result from reactions of original anthocyanins with pyruvic acid, a ubiquitous intermediate of metabolism like aerobic glycolysis and anaerobic fermentations during storage and processing.

When fruit anthocyanins were transformed into carboxy-pyranomalvidin-3-*O*-coumaroyl-glucoside **3** and carboxy-pyranocyanidin-3-*O*-rutinoside **4**, the progressive changes in the concentration of pyranoanthocyanins and the appearance of new derivatives were monitored by HPLC-DAD/MS during further incubation of the solutions under oxidative conditions. Fig. 3A and B showed the formation of new products after 20 days of further incubation of the reaction solutions at pH 3.8, typical pH for many fruit juices and wine. A significant increase in the intensity of the new peaks 5 and 6, concurrent with the decrease of the pyranoanthocyanin peaks 3 and 4, respectively, was observed in the UV region of the HPLC chromatograms.

The mass spectra of newly formed compounds 5 and 6 were obtained by LC-ESI/MS in the positive ion mode. The MS data of peak 5 showed a *quasi* molecular ion $[M+H]^+$ at *m/z* 679 and a major fragment ion $[M+H-308]^+$ in MS^2 at *m/z* 371 (Fig. 4A and B), which could correspond to the loss of a coumaroylglucoside moiety originated from the parent anthocyanin. Whilst the minor fragment ion $[M+H-146]^+$ in MS^2 at *m/z* 533 could result from the loss of the coumaroyl moiety. The mass of peak 5 is 29 amu less than the precursor pyranomalvidin-3-coumaroylglucoside **3** and is consistent with the molecular structure where an oxo-group replaced the carboxyl group in the C-10 position of pyran ring D to give rise to an α-pyranone (lactone) ring, as shown in Fig. 2. The MS^3 spectrum of the aglycon ion at *m/z* 371 in MS^2 revealed the presence of a major fragment ion at *m/z* 343 (Fig. 4C), corresponding to the further loss of the C-10 carbonyl (C=O) group (equal to 28 amu),

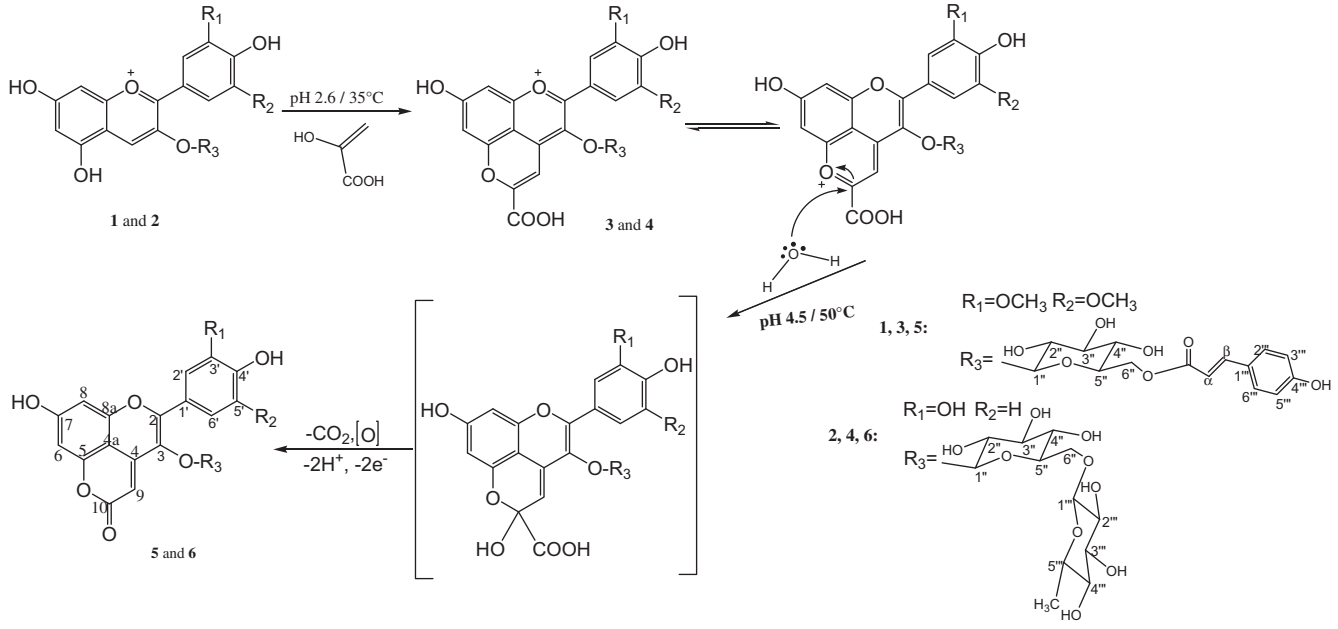

Fig. 2. Reaction pathway for the formation of non-oxonium pyranone compounds **5** and **6** derived from anthocyanins **1** and **2**, respectively.

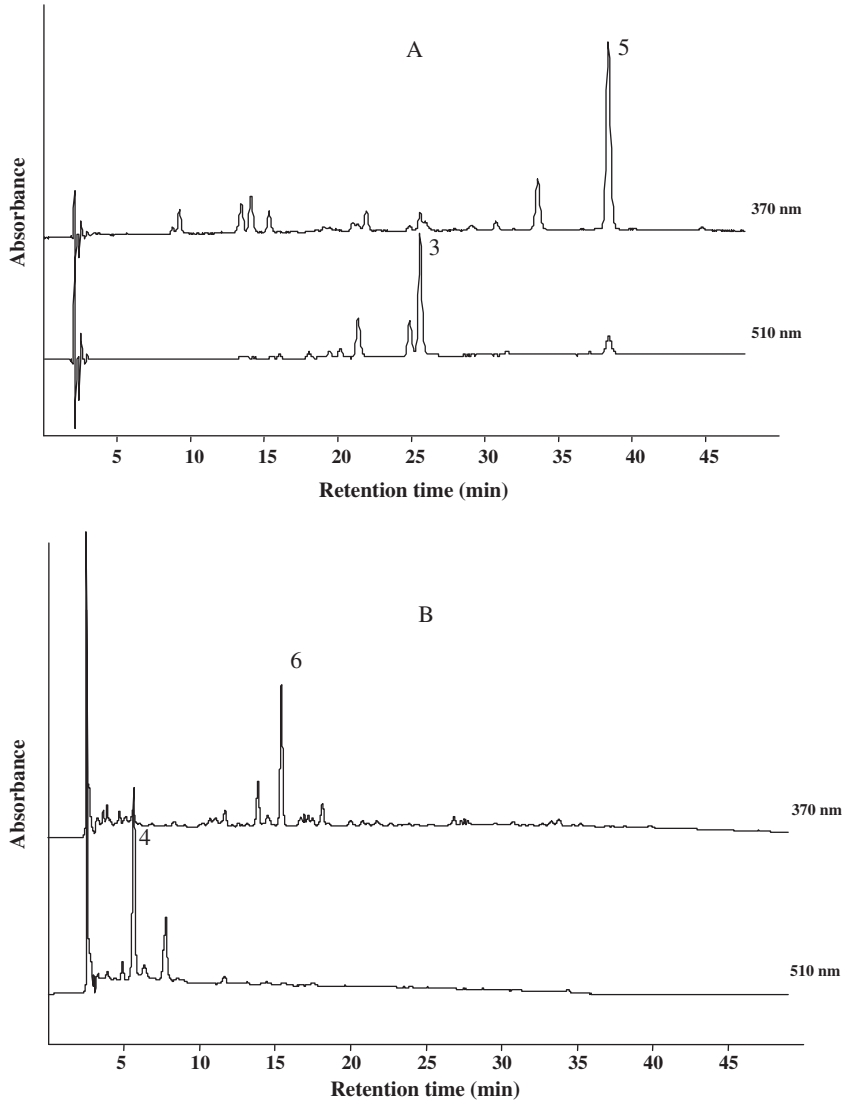

Fig. 3. HPLC chromatograms recorded at 510 and 370 nm showing the formation of anthocyanin-derived new pyranone compounds **5** and **6** during further oxidative incubation of the reaction solutions containing pyranoanthocyanins **3** and **4**.

characteristic fragmentation pattern of the α-pyranone ring as observed for oxovitisin A and the coumarins (He et al., 2010; Xie, Zhao, Zhou, Fan, & Wu, 2010). Additionally, fragmentation of the aglycon ion also produced other fragment ions at *m/z* 338, 327, 310 and 282, the same phenomena observed by Hayasaka and Asenstorfer (2002) who found this fragmentation pathway to be typical for malvidin-derived compounds. All these results indicated that the newly formed peak 5 in Fig. 3A could correspond to the non-oxonium derivative of malvidin-3-*O*-coumaroylglucoside with additional pyranone ring, as proposed compound **5** in Fig. 2. The *quasi* molecular ion $[M+H]^+$ at *m/z* 635 of the peak 6 in Fig. 3B and its similar MS/MS fragmentation pattern (data not shown) are consistent with the proposed cyanidin-3-*O*-rutinoside derivative **6** in Fig. 2. Therefore the original fruit anthocyanins could be converted into respective new derivatives with pyranone ring by two-step reactions through the proposed pathway in Fig. 2.

3.2. Effect of reaction conditions on the oxidative transformation of pyranoanthocyanins

Factors influencing the formation of pyranoanthocyanins from anthocyanins and pyruvic acid have been studied previously (Mateus, Oliveira, Pissarra, & de Freitas, 2003; Romero & Bakker, 1999). The optimised reaction conditions in the second step for the further transformation of pyranoanthocyanins were explored at different temperatures and various pH values so as to better understand the oxidative formation mechanism of new pyranone derivatives. HPLC-DAD analysis showed that the production of new pyranone-anthocyanins was very slow at room temperature as presented in Fig. 5 based on the study of malvidin-3-*O*-coumaroylglucoside, but increased significantly when the temperature was raised to 50 °C. At the higher temperature of 70 °C, the appearance of pyranone-anthocyanins was faster but a lower amount of derivatives was observed probably resulting from a higher degradation. Hence, the following reactions were performed at 50 °C due to the relatively higher stability and reaction rate of pyranoanthocyanins.

The pH dependency of the transformation reactions was also investigated (Fig. 6). In very acidic medium (pH < 2), the reaction did not take place during the incubation period. With the increase of pH to higher values in the range 3.0–6.0 the reaction rate was improved significantly. However, at a pH above 6, the open chalcone form of pyranoanthocyanins was expected to occur and therefore decreased the amount of new derivatives. The favourable

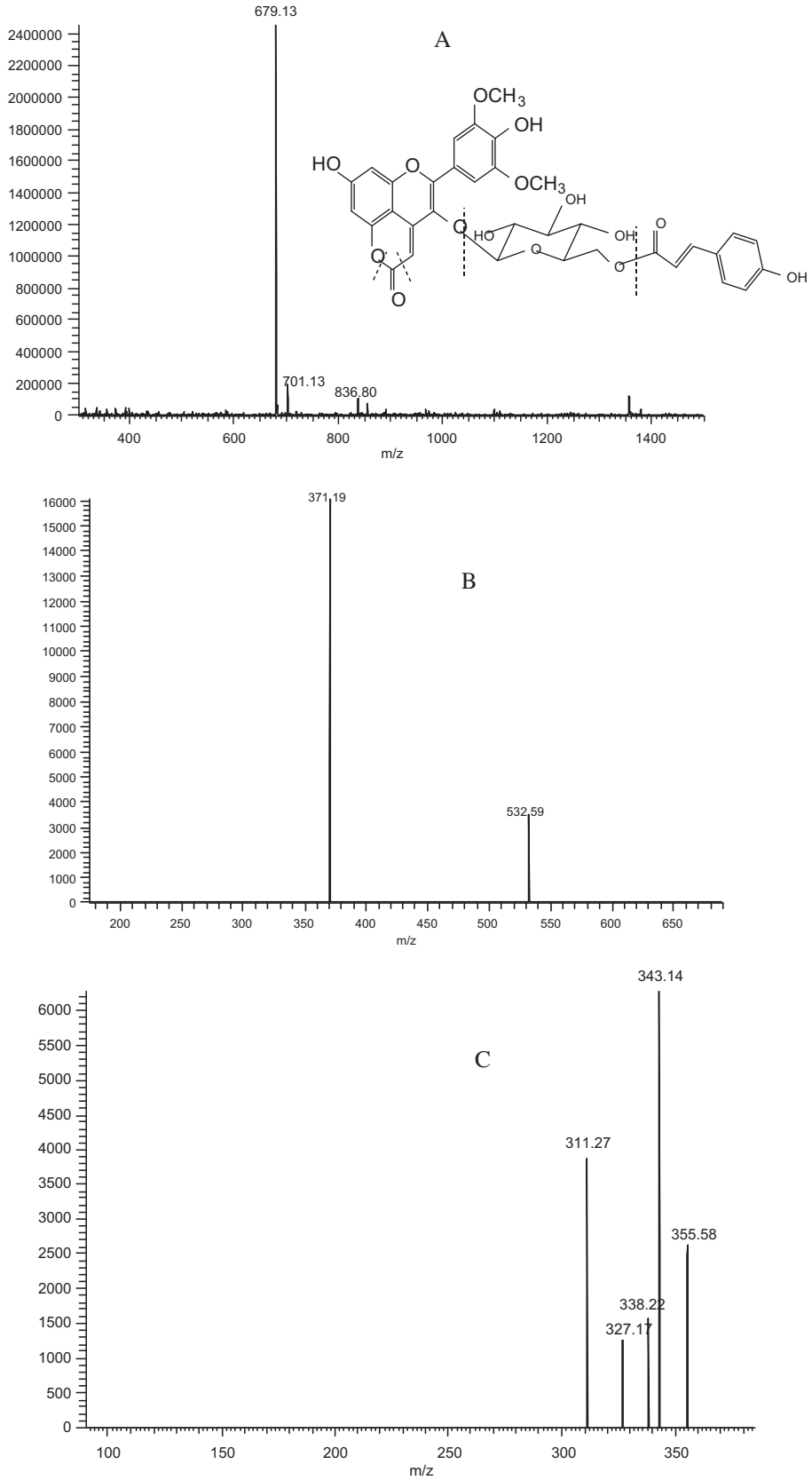

Fig. 4. LC/ESI/MS analysis of the newly formed peak 5 in Fig. 3 originated from malvidin-3-coumaroylglucoside: A, full mass spectrum (quasi molecular ion); B, MS^2 spectrum, and C, MS^3 spectrum of the major ion fragment in the last spectrum.

pH for the formation of new pyranone-anthocyanins ranged between 4.0 and 5.5. The rate of formation under other oxidative conditions was also studied. The reaction in the presence of hydrogen peroxide proceeded slightly faster, but at the same time a higher degradation of pyranoanthocyanin precursors was also observed, which considerably lowered the reaction yields to less than 10%.

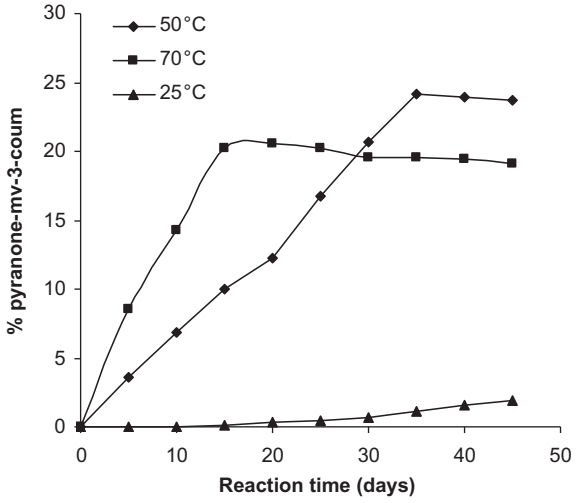

Fig. 5. Influence of temperature on the rate of formation of new pyranone compound **5**.

Without the periodical oxygenation treatment, the formation of new pyranone compounds was negligible during the incubation period and could not be detected under the present analysis conditions.

The transformation of anthocyanins into α-pyranone-anthocyanins via pyranoanthocyanins apparently involves the hydration reactions of the pyranoflavylium cations followed by hemiacetal formation as shown in Fig. 2. Subsequent decarboxylation and further oxidation of the pyran moiety bearing an α-hydroxyl substituent to the aromatic lactone or pyran-2-one structure led to the formation of the final product, a stabilised neutral (non-flavylium cation) pyranone-anthocyanin derivative. The hydration reaction by the nucleophilic attack of water should be the key step for the irreversible change of chromenylium pigments to the neutral pyranone compounds, which also explained the negative reaction at pH below 2 as observed in Fig. 6, where hemiacetal formation by the nucleophilic attack of water was hindered in very acidic medium.

3.3. Structural analysis of anthocyanin-derived new pyranone compounds

The structure of newly formed pyranone derivatives 5 and 6 originated from malvidin-3-*O*-coumaroylglucoside and cyanidin-3-*O*-rutinoside respectively was elucidated by ^{1}H and ^{13}C NMR analysis using 1D and 2D techniques (Table 1).

For compound **5**, the protons H-6 and H-8 of ring A were assigned to two *meta*-coupled doublets ($J = 2.0$ Hz) at 6.44 and 6.41 ppm, respectively. Protons H-2′, 6′ and those of the methoxyl groups at ring B were situated at 7.36 and 3.85 ppm, respectively. Proton H-9 was assigned to the singlet at 5.97 ppm. Concerning the glucosyl moiety, the β-anomeric proton H-1″ was attributed to a doublet ($J = 7.7$ Hz) at 4.77 ppm, all other protons were situated in the 3.28–3.52 ppm region, except for the last two protons H-6a″ and H-6b″ that were assigned at 4.22 and 4.15 ppm, respectively. With respect to the coumaroyl group, protons H-α and H-β were assigned to two doublets ($J = 15.8$ Hz) at 7.38 and 6.05 ppm, respectively. Protons H-2‴, 6‴ and H-3‴, 5‴ were readily attributed to two doublets ($J = 8.6$ Hz) at 7.31 and 6.80 ppm, respectively.

The carbon resonances were attributed using two dimensional techniques (HSQC and HMBC) (Fig. 7). Carbons C-6, C-8, C-9, C-2′,6′ and OCH_3 were assigned to 99.2, 99.8, 92.7, 108.2 and 57.1 ppm, respectively through their direct ^{1}H–^{13}C correlations with protons H-6, H-8, H-9, H-2′,6′ and H-OCH_3. Carbons C-7 and C-4a were assigned to 163.8 and 102.7 ppm, respectively by their correlations in the HMBC spectrum with protons H-6 and H-8. Carbon C-5 and C-8a were attributed to 155.4 and 151.9 ppm, respectively by their long distance correlations with proton H-6 and H-8, respectively. Carbons C-2, C-1′, C-3′, 5′ and C-4′ were attributed to 153.2, 122.4, 148.5 and 139.8 ppm, respectively, by their long distance correlation with protons H-2′, 6′. Carbon C-3 was assigned to 132.3 ppm through its correlations in the HMBC spectrum with proton H-9 and the anomeric proton H-1″. Carbon C-10 was attributed at 165.5 ppm by its long distance correlation with proton H-9. The higher chemical shift observed for this carbon, comparing with other pyranoanthocyanins described in literature (He et al., 2006; Mateus et al., 2001; Oliveira, de Freitas, & Mateus, 2009), can be due to the presence of an electronegative group, such as an oxo group, attached to this carbon. In fact, the ESI/MS fragmentation of the aglycon by elimination of a carbonyl (C=O) group in the

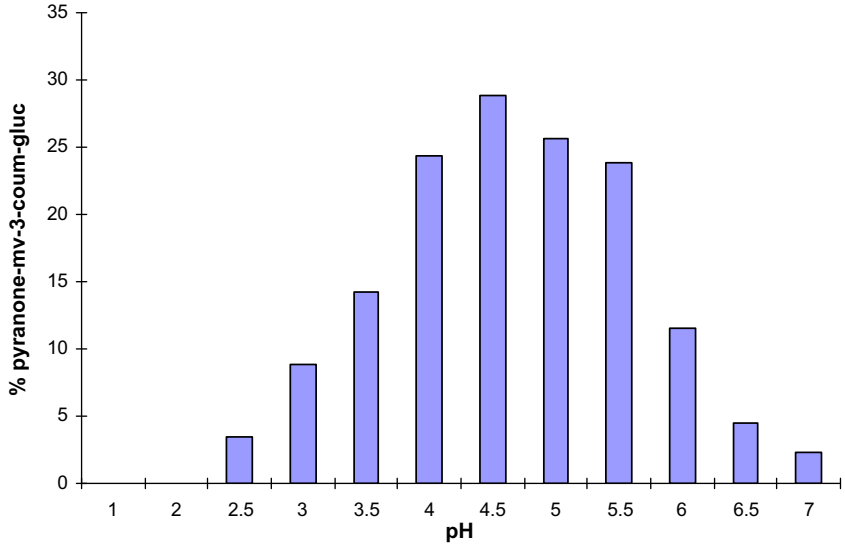

Fig. 6. Influence of pH on the rate of formation of new pyranone compound **5**.

Table 1
^{1}H and ^{13}C chemical shifts of compound **5** (pyranone-malvidin-3-coumaroylglucoside) and **6** (pyranone-cyanidin-3-rutinoside), determined in CD_3OD/TFA (98:2)[a].

Pigment	**5**		**6**	
Position	δ ^{1}H (ppm); *J* (Hz)	δ ^{13}C	δ ^{1}H (ppm); *J* (Hz)	δ ^{13}C
Pyranone-anthocyanidin moiety				
2	–	153.2	–	154.1
3	–	132.3	–	132.2
4	–	n.a.	–	102.8
4a	–	102.7	–	102.8
5	–	155.4	–	152.3
6	6.44; d, 2.0	99.2	6.55; d, 2.0	98.7
7	–	163.8	–	163.6
8	6.41; d, 2.0	99.8	6.51; d, 2.0	99.6
8a	–	151.9	–	155.5
9	5.97; s	92.7	6.16; s	94.0
10	–	165.5	–	166.0
1′	–	122.4	–	123.0
2′	7.36; s	108.2	7.66; d, 2.1	118.2
3′	–	148.5	–	145.6
4′	–	139.8	–	149.2
5′	–	148.5	6.87; d, 8.4	117.2
6′	7.36; s	108.2	7.44; dd, 8.4/2.1	123.5
3′, 5′ – OMe	3.85; s	57.1	–	–
Glucose moiety				
1″	4.77; d, 7.7	103.7	4.63; d, 7.7	102.8
2″	3.52; dd, 9.2/7.7	75.9	3.77*	78.2
3″	3.42; t, 9.1	76.5	3.53*	72.3
4″	3.28; t, 9.1	72.0	3.75*	69.0
5″	3.46; m	78.5	3.80–3.82; m	83.3
6a″	4.22; dd, 11.8/2.3	64.9	3.93; dd, 11.8/2.2	62.4
6b″	4.15; *	64.9	3.79*	62.4
Coumaroyl group				
$R_1CO_2R_2$	–	169.2	–	–
$CH{=}CH_\alpha CO_2R$	7.38; d, 15.8	115.3	–	–
$CH_\beta{=}CHCO_2R$	6.05; d, 15.8	147.1	–	–
1‴E	–	127.1	–	–
2‴,6‴E	7.31; d, 8.6	131.5	–	–
3‴,5‴E	6.80; d, 8.6	117.3	–	–
4‴E	–	161.4	–	–
Rhamnosyl moiety				
1‴	–	–	4.44; d, 1.6	102.5
2‴	–	–	3.66; dd, 3.3/1.6	72.2
3‴	–	–	3.38*	69.8
4‴	–	–	3.16*	69.0
5‴	–	–	3.26*	73.9
CH_3	–	–	1.13; d, 6.2	17.9

[a] Key: s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet; *, unresolved; n.a., not assigned.

MS^3 spectrum (Fig. 4) also supports the structure of a pyranone-anthocyanin molecule, which possesses an oxo group at C-10. With respect to the carbons of the glucose molecule, they were assigned by their ^{1}H–^{13}C direct correlation in the HSQC spectrum. The Carbons C-α, C-β, C-2‴,6‴ and C-3‴,5‴ of coumaroyl group were also assigned at 115.3, 147.1, 131.5, 117.3 ppm, respectively by their direct ^{1}H–^{13}C correlations with protons H-α, H-β, H-2‴,6‴ and H-3‴,5‴, respectively. Carbon C-CO_2 was attributed at 169.2 ppm by its long range correlation with protons H-α and H-β. Carbons C-1‴ and C-4‴ were attributed by their correlations in the HMBC spectrum with protons H-2‴, 6‴ and H-3‴, 5‴ at 127.1 and 161.4, respectively.

The structure of newly formed pyranone compound **6** derived from cherry anthocyanin was also confirmed by NMR analysis (Table 1), similar to the analysis obtained for **5**. The major differences concerned the ring B and the sugar moiety that in this case is rutinose which is composed by a molecule of glucose linked to a molecule of rhamnose by an α-(1,6) linkage. The protons H-2′, H-5′ and 6′ of ring B were attributed at 7.66, 6.87 and 7.44 ppm as doublet (*J* = 2.1 Hz), doublet (*J* = 8.4 Hz) and double doublet (*J* = 8.4, 2.1 Hz), respectively. The glucose β-anomeric proton was assigned to the doublet (*J* = 7.7 Hz) at 4.63 ppm. All of the other glucosyl protons were situated in the 3.53–3.80 ppm region, except for the last two protons H-6a″ and H-6b″ that were assigned at 3.93 and 3.79 ppm, respectively. This suggests that the rhamnosyl moiety is linked to C-6 of the glucosyl moiety. The anomeric proton of the rhamnosyl moiety corresponds to the signal at 4.44 ppm with a small coupling constant (*J* = 1.6 Hz), suggesting an α configuration of the rhamnose molecule. Protons of the rhamnosyl moiety were situated in the 3.16–3.66 ppm region. The methyl group of the rhamnose was assigned to a doublet (*J* = 6.2 Hz) at 1.13 ppm. All carbons of the rutinosyl moiety were assigned through direct ^{1}H–^{13}C correlations in the HSQC spectrum.

3.4. UV–Vis spectrophotometry

The pyranone structure and non-oxonium nature of the newly formed compounds confer them with unique spectral features. Indeed, the UV–Vis spectrum of new derivatives displayed only a pronounced broad peak at 370 nm for compound **6** derived from cyanidin-3-*O*-rutinoside (Fig. 8A). For compound **5** derived from malvidin-3-*O*-coumaroylglucoside the UV–Vis spectrum revealed a maximum absorption peak at 316 nm and also a broad absorption band at 373 nm (Fig. 8B). Except for the absorption at 316 nm resulting from the coumaroyl group of the molecules, the two new anthocyanin-derivatives presented similar spectra characteristics, a broad absorption peak only in the UV region around 370 nm, being quite different from that of original fruit anthocyanins and all other anthocyanin-derived chromenylium pigments which present maximum absorption in visible wavelength ranges, as shown in Fig. 8C, thus coinciding with its non-flavylium cation nature of the molecular structures. The small spectral difference (3 nm) between the two neutral pyranone aglycons of compounds **5** and **6** lies in the substitutive groups of

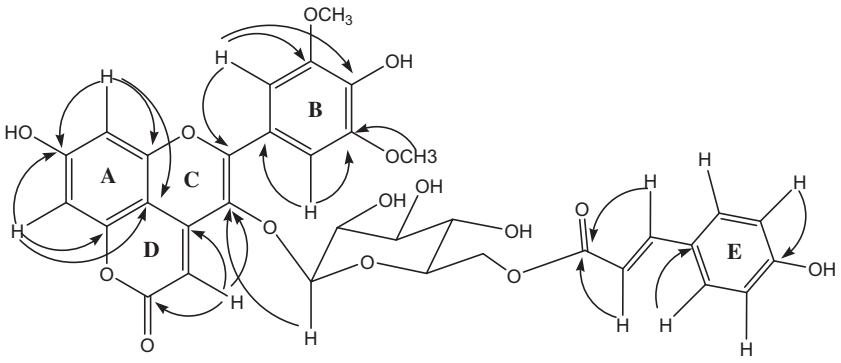

Fig. 7. The important long range ^{1}H–^{13}C correlations observed in the HMBC spectrum of pyranone-malvidin-3-coumaroylglucoside **5**.

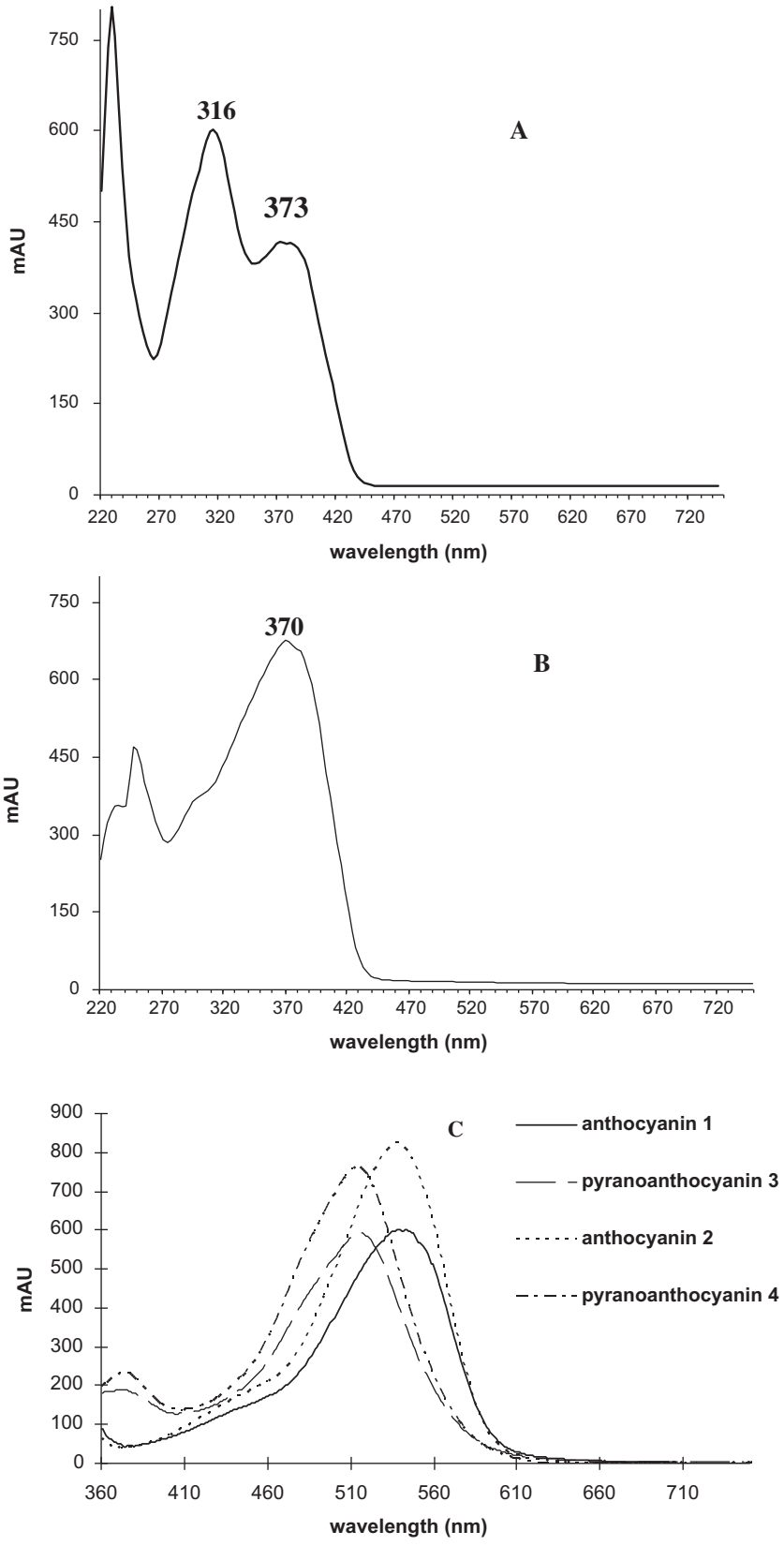

Fig. 8. UV–Vis spectrum of anthocyanin-derived new pyranone compounds **5** (A) and **6** (B) of non-oxonium nature; and UV–Vis spectra of genuine anthocyanins and anthocyanin-derived chromenylium pyranoanthocyanins (C).

ring B, which contains two methoxy groups in the former, indicating the origin of fruit anthocyanin aglycons has minimal influence on the characteristic λ_{max} of the new non-oxonium derivatives which possess commonly an additional α-pyranone (lactone) ring between C-4 and the hydroxyl group at C-5 of the anthocyanin core.

4. Conclusions

The results obtained in this study show that anthocyanins obtained from different fruit extracts may be readily transformed into the respective new pyranone derivatives of non-oxonium nature through two-step chemical reactions in mildly acidic solutions (pH 3.0–6.0) like in fermented beverages. The irreversible change of chromenylium pyranoanthocyanins to the neutral pyranone compounds in the second step by hydration reactions and further oxidation are strongly related to pH, as the hemiacetal formation by the nucleophilic attack of water could be hindered at higher and lower pH. Additionally, the increase of temperature improves the reaction rate significantly.

The implications of the reaction pathway studied for the transformation of anthocyanin type flavylium cations into new type of more stable non-oxonium structures via pyranoanthocyanins are significant. As the occurrence of pyranoanthocyanins with different aglycons has been confirmed over recent years in wine, fermented and stored cherry juices and other processed foodstuffs, as well as in some fresh fruits and vegetables, the oxidative formation of new pyranone compounds could thus be expected to take place during processing and years of storage or ageing, as the oxovitisin A of this family discovered in aged wine (He et al., 2010), highlighting their relevance to anthocyanin reactivity and wine colour evolution during winemaking and aging. On the other hand, the interest in new anthocyanin derivatives of non-oxonium nature with additional pyranone (lactone) ring will go beyond wine chemistry as their spectral and structural similarity to flavones as well as their naturally occurring nature open new perspectives for potential applications of such compounds in the food and pharmaceutical industry. Nevertheless, further studies will be performed especially regarding their physicochemical properties and biological activities.

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