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Review

Chemical studies of anthocyanins: A review

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ABSTRACT

Anthocyanins are natural colorants which have raised a growing interest due to their extensive range of colours, innocuous and beneficial health effects. Despite the great potential of application that anthocyanins represent for food, pharmaceutical and cosmetic industries, their use has been limited because of their relative instability and low extraction percentages. Currently, most investigations on anthocyanins are focused on solving these problems, as well as their purification and identification.

In this paper, the most recent advances in the chemical investigation of the anthocyanins are summarised, emphasising the effects of pH, co-pigmentation, metal ion complexation and antioxidant activity on their stability.

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Contents

1. Introduction

The study of natural colorants is an extensive and active area of investigation due to the growing interest of substituting synthetic colorants with toxic effects in humans (Chou, Matsui, Misaki, & Matsuda, 2007). Carotenoids and anthocyanins are amongst the most utilised vegetable colorants in the food industry (International Food Information Council and Foundation US Food & Drug Administration, 2004). Carotenoids are liposoluble, stable and able to colour food products from yellow to red (Buchanan, Gruissem, & Jones, 2002; Potter & Hotchkiss, 1995); they are obtained mostly from carrots, tomatoes and peppers (Branen, 2001). On the other hand, the anthocyanins are water-soluble and less stable than carotenoids; they are extracted from grapes, berries, red cabbage, apples, radishes, tulips, roses and orchids, amongst others (Dey & Harborne, 1993).

Anthocyanins (of the Greek *anthos* = flower and *kianos* = blue) are the most important pigments of the vascular plants; they are harmless and of easy incorporation in aqueous media, which makes them interesting for its use as natural water-soluble colorants (Pazmiño-Durán, Giusti, Wrolstad, & Glória, 2001). These pigments are responsible of the shiny orange, pink, red, violet and blue colours in the flowers and fruits of some plants.

Another significant property of anthocyanins is their antioxidant activity, which plays a vital role in the prevention of neuronal and cardiovascular illnesses, cancer and diabetes, among others (Konczak & Zhang, 2004). There are several reports focused on the effect of anthocyanins in cancer treatments (Lule & Xia, 2005; Nichenametla, Taruscio, Barney, & Exon, 2006), human nutrition (Stintzing & Carle, 2004), and its biological activity (Kong, Chia, Goh, Chia, & Brouillard, 2003).

Therefore, due to the enormous potential of natural anthocyanins as healthy pigments, there is an increasing number of reports found in the literature on diverse fields such as: development of analytical techniques for their purification and separation (Antolovich, Prenzler, Robards, & Ryan, 2000; Robards & Antolovich, 1997), applications in food (Giusti & Wrolstad, 2003), identification and distribution in plants (Cooper-Driver, 2001; Harborne & Williams, 1998; Harborne & Williams, 2000), tracking colour and pigment changes (Brouillard, Chassaing, & Fougerousse, 2003; Wrolstad, Durst, & Lee, 2005), biosynthesis (Martens et al., 2003; Springob, Nakajima, Yamazaki, & Saito, 2003), quantitative analysis using chromatographic and electrophoretic techniques (da Costa, Horton, & Margolis, 2000) and effect in the plants stress (Simmonds, 2003).

In this review, the most recent anthocyanin investigations are summarised with emphasis in the prominent information related to stability, extraction, purification and identification methods.

2. Anthocyanins chemistry

The anthocyanidins are the basic structures of the anthocyanins (Table 1). The anthocyanidins (or aglycons) consist of an aromatic ring [A] bonded to an heterocyclic ring [C] that contains oxygen, which is also bonded by a carbon–carbon bond to a third aromatic ring [B] (Konczak & Zhang, 2004). When the anthocyanidins are found in their glycoside form (bonded to a sugar moiety) they are known as anthocyanins.

The colour exhibited by these molecules was first explained by Pauling in 1939, who proposed that the resonant structure of the flavylium ion caused the intensity of their colour (Wrolstad et al., 2005).

There is a huge variety of anthocyanins spread in nature (names and abbreviations of the most common are listed in Table 1). The main differences between them are the number of hydroxylated groups, the nature and the number of bonded sugars to their structure, the aliphatic or aromatic carboxylates bonded to the sugar in the molecule and the position of these bonds (Kong et al., 2003). Up to now there are reports of more than 500 different anthocyanins (Andersen & Jordheim, 2006) and 23 anthocyanidins (Andersen & Jordheim, 2006; Kong et al., 2003; Rein, 2005) of which only six are the most common in vascular plants, Pg, Pn, Cy, Mv, Pt and Dp (Clifford, 2000).

The glycoside derivatives of the three non-methylated anthocyanidins (Cy, Dp and Pg) are the most common in nature, being found in 80% of pigmented leaves, 69% in fruits and 50% in flowers (Dey & Harborne, 1993).

The distribution of the six more common anthocyanidins in fruits and vegetables is: Cy 50%, Dp 12%, Pg 12%, Pn 12%, Pt 7% and Mv 7%. The glycoside derivatives more widespread in nature are 3-monosides, 3-biosides, 3,5- and 3,7-diglucosides. The presence of the 3-glucoside derivatives is 2.5 more frequent than the 3,5-diglucosides and the most common anthocyanin is the Cy-3-glucoside (Kong et al., 2003).

The enormous variety of anthocyanins found in nature makes them a very complex and interesting group. Recently, the anthocyanic patterns exhibited by different species of fruits, have been very useful for chemotaxonomic investigations and wine adulterations (García-Benítez, Cabello, & Revilla, 2003; Goiffon, Mouly, & Gaydou, 1999; Nørbæk, Brandt, Nielsen, Ørgaard, & Jacobsen, 2002; Revilla, García-Beneytez, Cabello, Martín-Ortega, & Ryan, 2001).

3. Anthocyanins stability

The isolated anthocyanins are highly instable and very susceptible to degradation (Giusti & Wrolstad, 2003). Their stability is affected by several factors such as pH, storage temperature, chemical structure, concentration, light, oxygen, solvents, the presence of enzymes, flavonoids, proteins and metallic ions (Rein, 2005).

The anthocyanins chemical stabilisation is the main focus of recent studies due to their abundant and potential applications, their beneficial effects and their use as alternative to artificial colorants (Rein, 2005).

3.1. Solvent and anthocyanin concentration effects

Recent investigations with synthetic flavylium salts (FVs) in solutions of different nature (acetonitrile:water, ethanol, propilenglycol, dioxane and 2-butanone) have shown that changes on colour depend on the solvent and the FVs concentration (Ito, Tanaka, Katsuki, & Fujii, 2002). In protic solvents the FVs exhibit red colouring, whilst in aprotic solvents the solutions are yellow. This fact has been explained by proposing that the red and yellow species correspond to a monomer and dimer respectively; therefore, when increasing FVs concentration, the red colouring is favoured.

Table 1
Structural identification of anthocyanidins (aglycons). (Castaneda-Ovando, et al.)
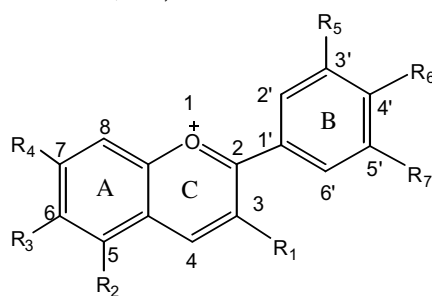
General anthocyanins structure

Name	Abbreviations	Substitution pattern							Colour
------	---------------	R_1	R_2	R_3	R_4	R_5	R_6	R_7	
Apigeninidin	Ap	H	OH	H	OH	H	OH	H	
Arrabidin	Ab	H	H	OH	OH	H	OH	OMe	N.R.[a]
Aurantinidin	Au	OH	OH	OH	OH	H	OH	H	
Capensinidin	Cp	OH	OMe	H	OH	OMe	OH	OMe	Blue-red
Carajurin	Cj	H	H	OH	OH	H	Ome	OMe	N.R.[a]
Cyanidin	Cy	OH	OH	H	OH	OH	OH	H	Orange-red
Delphinidin	Dp	OH	OH	H	OH	OH	OH	OH	Blue-red
Europinidin	Eu	OH	OMe	H	OH	OMe	OH	OH	Blue-red
Hirsutidin	Hs	OH	OH	H	OMe	OMe	OH	OMe	Blue-red
3'-HydroxyAb	3'OHAb	H	H	OH	OH	OH	OH	OMe	N.R.[a]
6-HydroxyCy	6OHCy	OH	OH	OH	OH	OH	OH	OH	Red
6-HydroxyDp	6OHDp	OH	OH	OH	OH	OH	OH	OH	Blue-red
6-HydroxyPg	6OHPg	OH	OH	OH	OH	H	OH	H	N.R.[a]
Luteolin	Lt	H	OH	H	OH	OH	OH	H	
Malvidin	Mv	OH	OH	H	OH	OMe	OH	OMe	Blue-red
5-MethylCy	5-MCy	OH	OMe	H	OH	OH	OH	H	Orange-red
Pelargonidin	Pg	OH	OH	H	OH	H	OH	H	
Peonidin	Pn	OH	OH	H	OH	OMe	OH	H	Orange-red
Petunidin	Pt	OH	OH	H	OH	OMe	OH	OH	Blue-red
Pulchellidin	Pl	OH	OMe	H	OH	OH	OH	OH	Blue-red
Riccionidin A	RiA	OH	H	OH	OH	H	OH	H	N.R.[a]
Rosinidin	Rs	OH	OH	H	OMe	OMe	OH	H	Red
Tricetinidin	Tr	H	OH	H	OH	OH	OH	OH	Red

[a] N.R: not reported.

Also it was observed that when increasing the water proportion, in acetonitrile:water mixtures, the monomer is transformed into a green colour dimer (monomer with charge-transfer character). Thus, the water plays a fundamental role in the dimerisation of flavylium salts due to the fact that these molecules require neutralising their own electrostatic repulsions with water molecules so that the dimerisation can be carried out.

3.2. pH influence

Anthocyanins can be found in different chemical forms which depend on the pH of the solution (Fig. 1) (da Costa, Nelson, Margolis, & Horton, 1998; Fleschhut, Kratzer, Rechkemmer, & Kulling, 2006; Heredia, Francia-Aricha, Rivas-Gonzalo, Vicario, & Santos-Buelga, 1998; Kennedy & Waterhouse,2000). At pH 1, the flavylium cation (red colour) is the predominant species and contributes to purple and red colours (Fig. 1A). At pH values between 2 and 4, the quinoidal blue species are predominant (Fig. 1B–D). At pH values between 5 and 6 only two colourless species can be observed, which are a carbinol pseudobase (Fig. 1E) and a chalcone (Fig. 1F), respectively. At pH values higher than 7, the anthocyanins are degraded depending on their substituent groups (Fig. 1, degradation reaction).

At pH values between 4 and 6, four structural forms of the anthocyanins coexist: flavylium cation, anhydrous quinoidal base, colourless carbinol base and the pale yellow chalcone. The equilibrium between the quinoidal bases and carbinol occurs via the flavylium cation just as shown in Fig. 1 (D, A and E structures).

When the pH is increased, also the amount of anhydrous base increases and under more acidic conditions, the predominant species is the red flavylium ion (Cooper-Driver, 2001).

The anthocyanidins stability is influenced by the ring B substituents and the presence of additional hydroxyl or methoxyl groups which decrease the aglycon stability in neutral media; therefore, Pg is the most stable anthocyanidin (Fleschhut et al., 2006). In contrast with aglycons, monoglycosides, and mostly, diglycosides derivatives are more stable in neutral pH conditions (Fleschhut et al., 2006). This behaviour is explained because the sugar molecules avoid the degradation of instable intermediaries into phenolic acid and aldehyde compounds (Fleschhut et al., 2006) (Fig. 1, degradation reaction).

Investigations about anthocyanins stability and the colour variation with pH conclude that the changes in the colour of these compounds are more significant in the alkaline region due to their instability (Cabrita, Fossen, & Andersen, 2000).

3.3. Co-pigmentation effect

Co-pigmentation is a phenomenon in which the pigments and other colourless organic compounds, or metallic ions, form molecular or complex associations, generating a change or an increment in the colour intensity (Boulton, 2001). In food science, this phenomenon is considered a very important interaction because colour is one of the main quality factors crucial in a product acceptance (Eiro & Heinonen, 2002). Some investigations suggest that the co-pigmentation of anthocyanins with other compounds
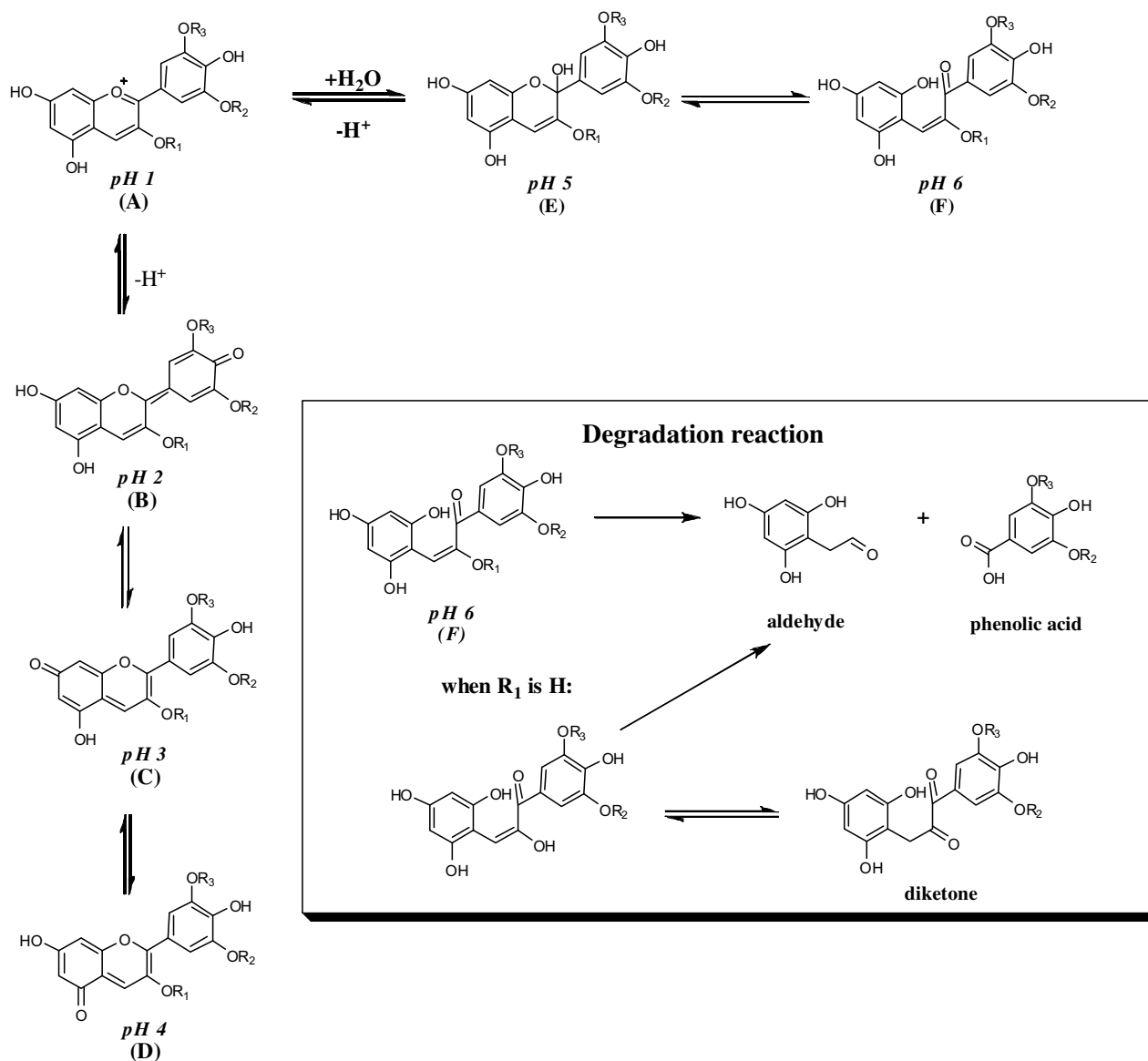
Fig. 1. Anthocyanins chemical forms depending on pH and degradation reaction for anthocyanins. Where R_1 = H or saccharide, R_2 and R_3 = H or Methyl (Castañeda-Ovando et al.).

(co-pigments) is the main mechanism of stabilisation of colour in plants (Davies & Mazza, 1993; Mazza & Brouillard, 1990). The co-pigments are rich systems in π-electrons which are able to associate with flavylium ions, which are rather poor in electrons. This association gives protection for the water nucleophilic attack in the 2 position of the flavylium ion (Matsufuji, Otsuki, Takeda, Chino, & Takeda, 2003) and for other species such as peroxides and sulphur dioxide in the 4 position (García-Viguera & Bridle, 1999; Mazza & Brouillard, 1987).

Co-pigments are generally colourless, but when mixed with an anthocyanic solution, an interaction is carried out producing a hyperchromic effect and a bathochromic shift in the absorption spectra (UV–Vis region). The co-pigments can be flavonoids, alkaloids, amino acids, organic acids, nucleotides, polysaccharides, metals or another anthocyanin.

The anthocyanin-co-pigment interaction can be carried out in five different ways depending on the interacting species (Fig. 2). If the co-pigment is other anthocyanin, a self association or an intramolecular co-pigmentation is formed (Fig. 2A and B); when the interaction is with a metal, a complexation is carried out

(Fig. 2C, see Section 3.4); in the case of co-pigments with free electron pairs, an intermolecular co-pigmentation takes place (Fig. 2D); finally, in the most complex case, the co-pigmentation can be carried out by aglycon, sugar, co-pigment and protons all at the same time.

When the co-pigment is another phenolic compound, the interaction is transitory because of the lack of chemical bonds. This behaviour is the result of the chemical phenomena known as charge-transfer complex formation or π–π interactions (Foster, 1969); this phenomenon occurs when opposite charge compounds interact. Thus, in the rings linked by a weak bond, the electronic density is transferred from the rich to the poor ring; so, as the flavylium ion in the anthocyanin is positively charged, it is then, a suitable candidate for the formation of complexes by charge transfer with rich electron substrates.

The total resulting co-pigmentation is based in two effects: (Dangles & Brouillard, 1992): (1) the formation of the π–π complex which causes changes in the spectral properties of the molecules in the flavylium ion, increasing the absorption intensity (hyperchromic effect) and its wavelength (bathochromic shift); and (2) the
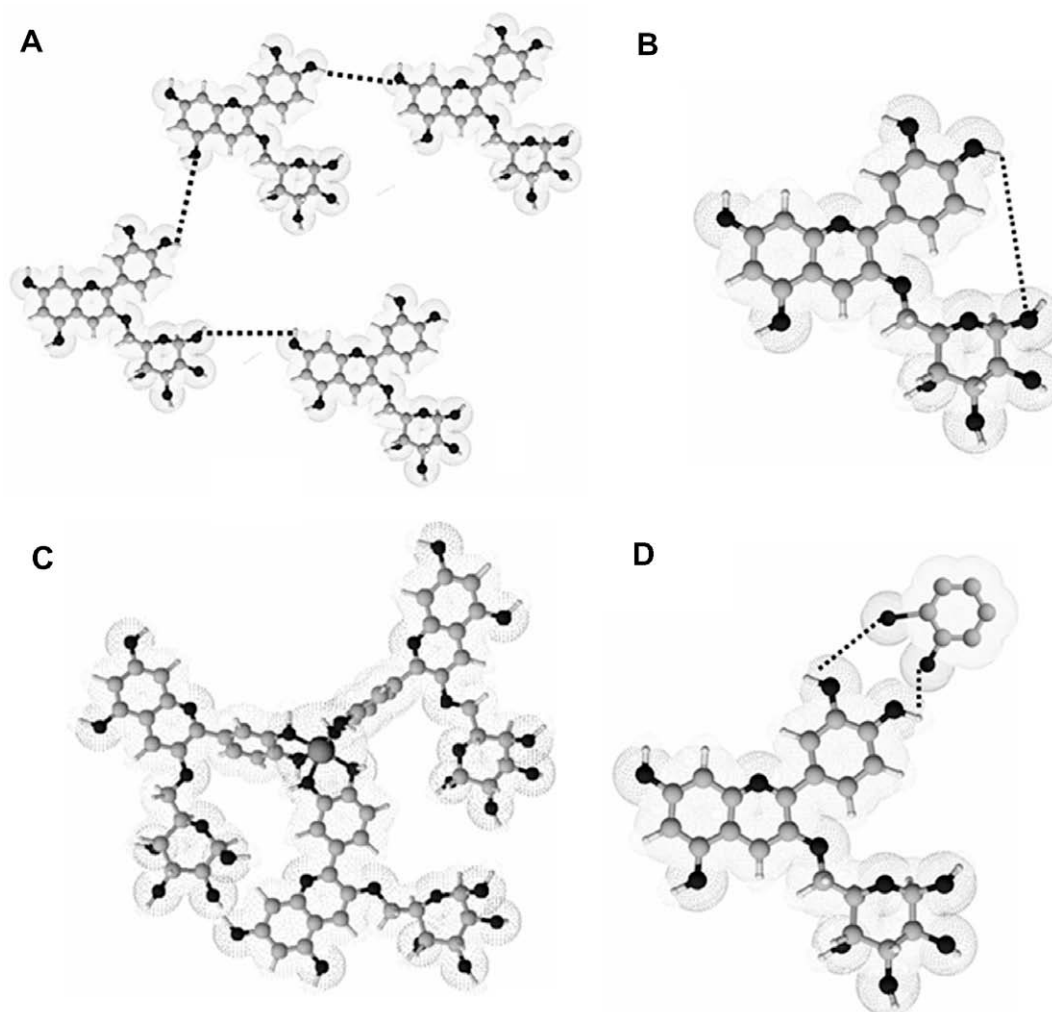
Fig. 2. Anthocyanins interaction. (A) self-association, (B) intramolecular copigmentation, (C) metal complexation, (D) intermolecular copigmentation (Castañeda-Ovando et al.).

stabilisation of the flavylium form by the π complex displaces the equilibrium in such way that the red colour increases (Fig. 3). Therefore, the magnitude of the co-pigmentation effect is pH dependent, because at low pH values, all the anthocyanin molecules are in flavylium form, and at high pH values, the anthocyanin is in its carbinol pseudobase form, which is colourless.

The co-pigmentation effect is evident under weakly acid conditions (pH 4–6) where anthocyanins exist in its colourless forms. Recently, it has been proposed that the formation of the π–π complex induces the reactions between anthocyanins and tannins in wines, producing covalent bonds which generate pigmented tannins (Mirabel, Saucier, Guerra, & Glories, 1999). The chemical coupling between anthocyanins and tannins is not well known; nevertheless, some researchers have suggested that the mechanism involves acetaldehyde, tannins and anthocyanins (Timberlake & Bridle, 1976); similar reactions have also been observed between tannins and flavan-3-ols (Salas et al., 2004; Saucier, Guerra, Pianet, Laguerre, & Glories, 1997).

3.4. Metallic ions interaction

The variety of colours in flowers initially was explained by the formation of chelates between metals and flavylium salts (Clifford, 2000). Despite the low interest in the food industry about the

anthocyanin-metal complexations, this interaction constitutes a viable alternative for colour stabilisation; particularly if the metals involved do not imply a risk for the health or even they are part of the essential minerals in diet.

One of the main characteristics of anthocyanins and anthocyanidins with *o*-di-hydroxyl groups in the B ring (Cy, Dp, Pt), is their ability to form metal-anthocyanin complexes (Boulton, 2001) (Table 1). Some studies about the colour stability in plants, suggest that the blue colours are due to a complexation between anthocyanins and some metals such as Al, Fe, Cu and Sn (Starr & Francis, 1973) or Mg and Mo (Hale et al., 2001).

In the Al(III)-anthocyanin interaction, the complexation was carried out with Cy and other flavonoid derivatives, finding out that this process stabilises the blue quinoidal base by avoiding its oxidation (Moncada et al., 2003). Other authors were studying the variation of the colour of a Hindu cabbage tissue adding Mo solutions (IV and VI), where, in both cases the blue colour was stabilised. This fact suggests the possible anthocyanin-molybdenum complexation (Hale et al., 2001).

More recent studies have shown that the complexation between *o*-di-hydroxyl anthocyanins and Fe (III) or Mg (II) ions at pH 5 are essential for the formation of blue colour in plants, mostly if the stoichiometrical ratio anthocyanin: Fe (III) is 1:6, or higher for the Mg (II) (Yoshida, Kitahara, Ito, & Kondo, 2006).
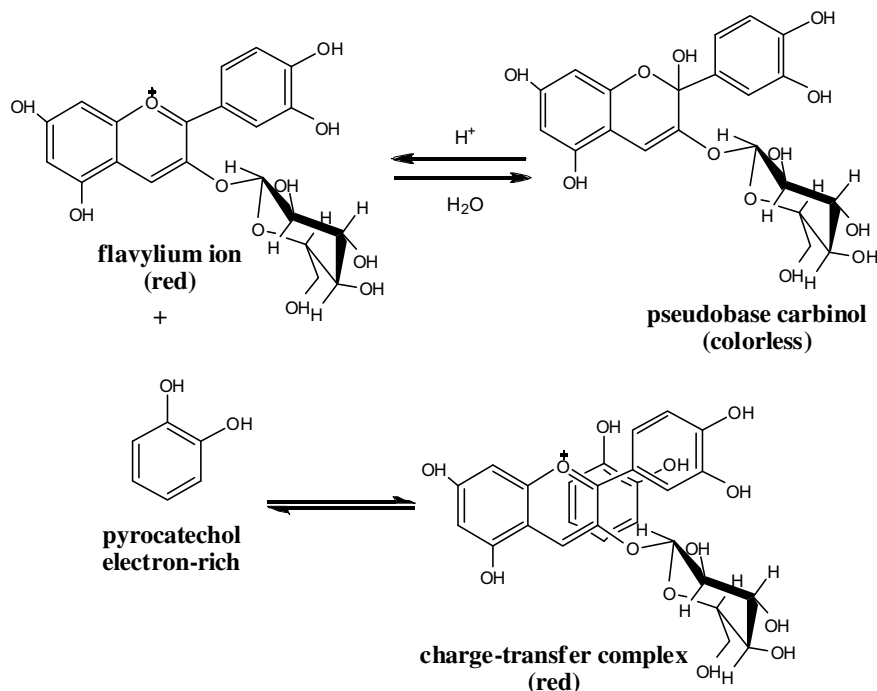
Fig. 3. Example of anthocyanins stabilisation via formation of the complex by charge transfer (interaction of the anthocyanin with a phenolic compound) (Castañeda-Ovando et al.).

3.5. Antioxidant activity

The compounds which are easier to oxidise are often the best antioxidants (molecules which can donate a free electron or hydrogen atoms to reactive free radicals). Several studies have suggested that the anthocyanin content and their corresponding antioxidant activity, contribute to the fruits and the vegetables protective effect against degenerative and chronic diseases (Heinonen, Meyer, & Frankel, 1998; Record, Dreosti, & McInerney, 2001). Some plants and fruit extracts with high phenolic compounds content, have been reported to act as mutagenesis and carcinogenesis inhibitors (Kumpulainen & Salonen, 1998; Macheix, Fleuriet, & Billot, 1990). The phytochemical substances that exhibit an antioxidant activity include, besides the phenolic compounds (flavonoids), nitrogenous compounds (chlorophyll derivatives), tocopherols, carotenoids and ascorbic acid (Nichenametla et al., 2006).

In catechols the oxidation is carried out via free radicals until forming a very stable semiquinone. The compounds with catechol groups or 1,4-hydroquinone are especially easy to oxidise because the phenoxyl radical can be stabilised with oxygen. This species is quite stable and does not "extract" hydrogen from other substances, lasting enough time to react with another semiquinone and originate a deprotonation reaction that generates a quinone and a phenol group by using two radicals (Waterhouse, 2001).

If an analogy between the catechol groups and the most common anthocyanidins is done, it would be expected that those with an o-dihydroxyl substitution (Cy, Dp and Pt) were the most susceptible to oxidation. In the case of Pg, Pn and Mv oxidation is not easy because they are not o-dihydroxyl substituted. From the six most common anthocyanidins, Pg is the most stable in neutral pH conditions (Fleschhut et al., 2006).

Considering the stabilisation of the radical semiquinone in the catechol (Waterhouse, 2001), in Fig. 4 it is proposed a free radical mechanism for the semiquinone stabilisation formed from the Cy oxidation. Assuming also this supposition for o-di-hydroxyl anthocyanidins with metals, it is probable that the complex reduce the antioxidant capacity of the anthocyanin or anthocyanidin, because

oxygen atoms are bounded to the metal ion, as in the Al-anthocyanin complex (Moncada et al., 2003) and they would no longer be available for oxidation reactions.

The anthocyanidins and anthocyanins have shown a higher antioxidant activity than vitamins C and E (Bagchi et al., 1998). These compounds are able to capture free radicals by donation of phenolic hydrogen atoms (Chen, Chan, Ho, Fung, & Wang, 1996; Rice-Evans, Miller, & Paganga, 1996); this is the reason for its anticarcinogenic activity (Kamei et al., 1995). It has also been reported, a linear correlation between the values of the antioxidant capacity and the anthocyanins content in blackberries, red raspberries, black raspberries and strawberries; additionally, it has been described that the berry extracts posses a high scavenging activity towards reactive oxygen species chemically generated (Wang & Lin, 2000). The antioxidant activity of berries is directly proportional to the anthocyanins content (Heinonen et al., 1998).

3.6. Pyranoanthocyanins

First pyranoanthocyanins report dates back to 1996 when a new class of pigments was detected in red wines filtrates (Cameira-dos Santos, Brillouet, Cheynier, & Moutounet, 1996). These molecules have got increasing attention during the last ten years because they are more stable at various pH values than anthocyanins. They are formed by the reaction of anthocyanins and molecules of low molecular weight such as 4-vinylphenol, pyruvic acid and flavonols. Also, some pyranoanthocyanins have been detected in wine model solutions studies and in wines. These different attributes of pyranoanthocyanins have raised some questions about their possible contribution to the colour of aged red wines.

The structure of pyranoanthocyanins results from the cyclisation between C-4 and the hydroxyl group at C-5 (Table 1) of the original flavylium moiety yielding a fourth ring [D], which has been referred to be responsible for their higher stability. The well-known structures of main pyranoanthocyanins are shown in
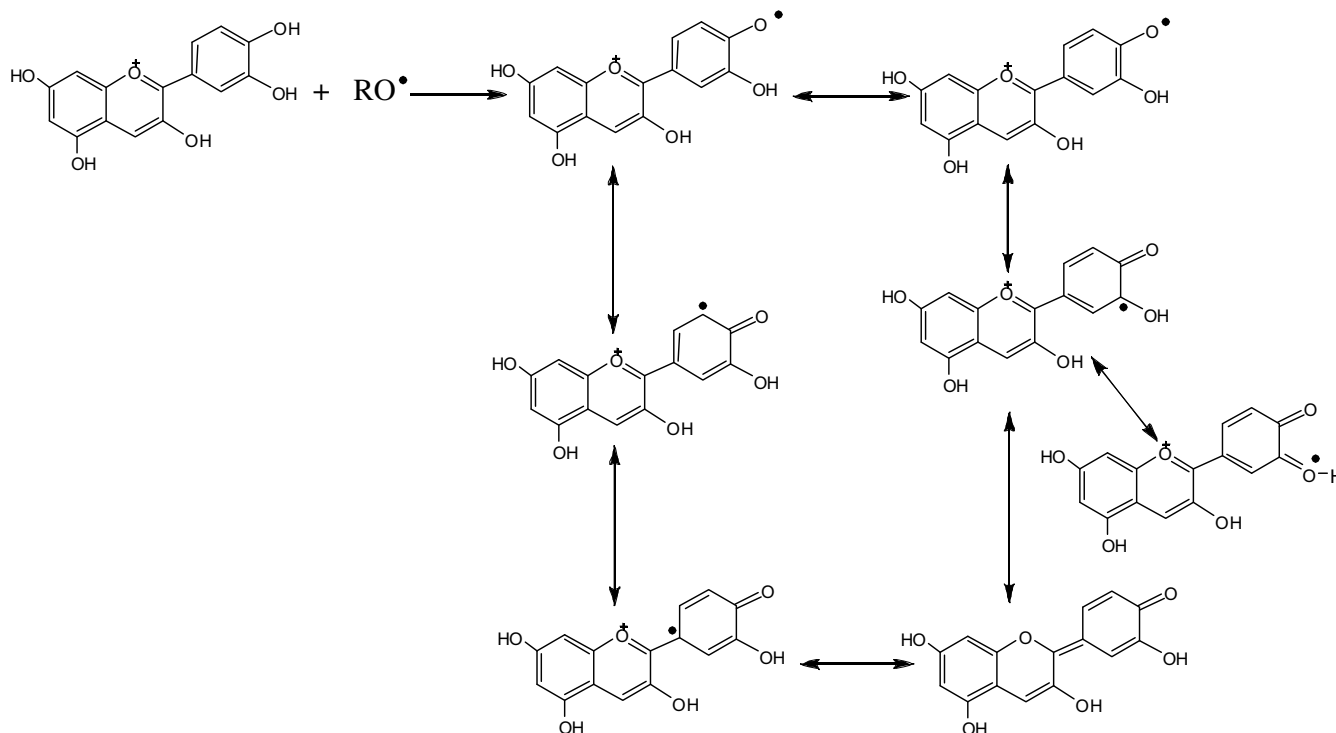
Fig. 4. Proposed mechanism for the stabilisation of the Cy semiquinone radical (resonance) (Castañeda-Ovando et al.).

Fig. 5. Studies performed in model solutions have shown that their concentrations depend on several factors including pyruvic acid, acetaldehyde, anthocyanin levels, pH and temperature. The mechanism proposed (Schwarz, Wabnitz, & Winterhalter 2003) suggested a reaction of wine anthocyanins with intact simple and free hydroxycinnamic acids, like coumaric, caffeic, ferulic, and sinapic acid. They found that the reaction is quite fast in model solutions but it depends on the storage conditions. It was also observed that the concentration of these newly formed pigments increased with the storage time of red wines. UV–Vis, ^{1}H and ^{13}C NMR, ESI–MS and LC–ESI–MS were used to analyse pure compounds solutions (Schwarz, Hofmann, & Winterhalter, 2004; Schwarz, Wray, & Winterhalter, 2004).

Some pyranoanthocyanins have been identified also in black carrot juice (Schwarz et al., 2004) and in blood orange juices, although the structure of the blood orange anthocyanins is different from the pigments commonly encountered in red wines or black carrots, formation of such pyranoanthocyanin pigments in blood orange juice was likely to occur (Hillebrand, Schwarz, & Winterhalter, 2004).

4. Extraction and purification methods
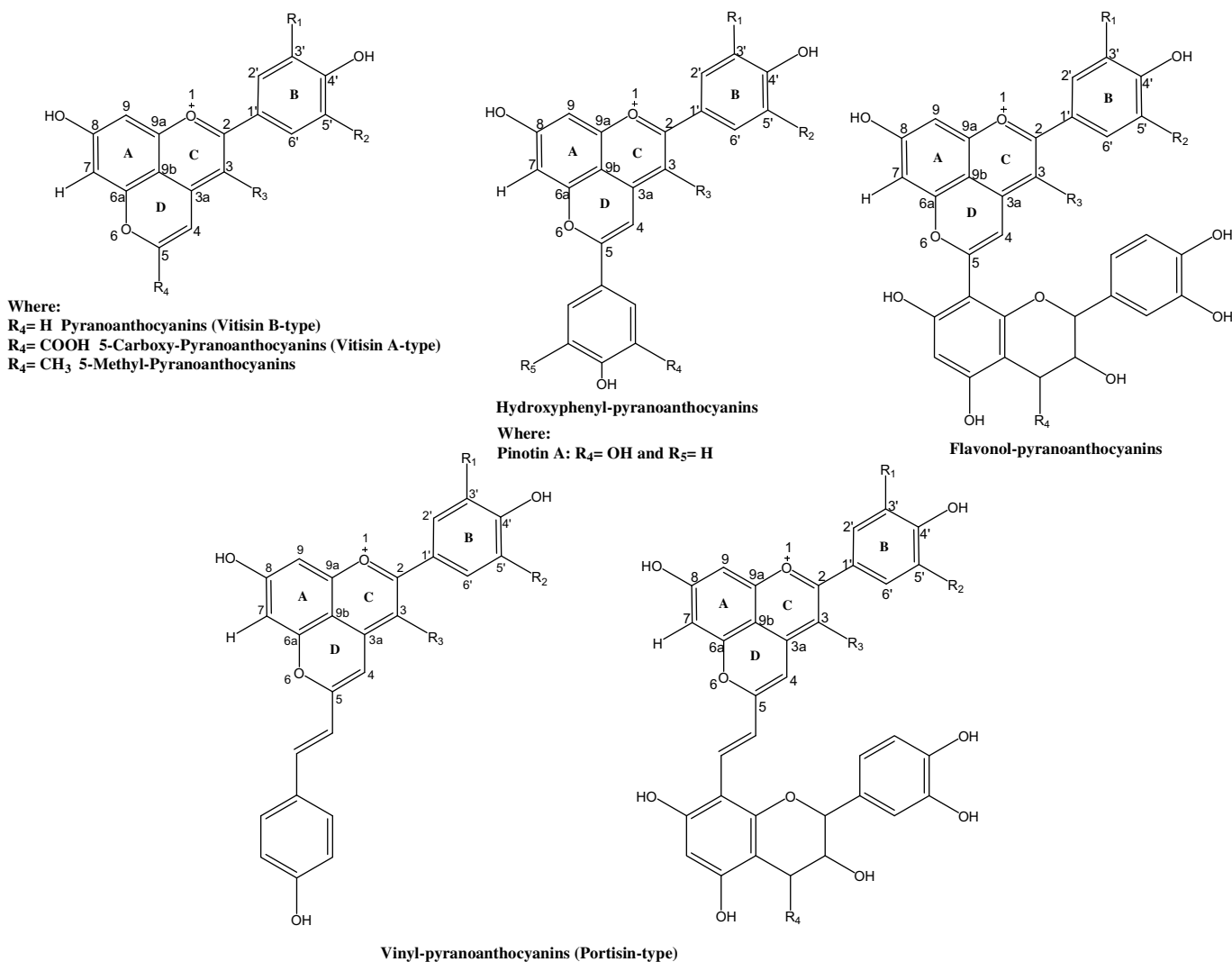
4.1. Extraction methods

The solvent extraction has been the most common method for extraction of diverse compounds found in fruits, including flavonoids. The phenolic compounds have been extracted by grinding, drying or lyophilising fruits, or only by soaking fresh fruits with subsequent solvent extraction (Merken & Beecher, 2000). Anthocyanins are polar molecules, thus the most common solvents used in the extractions are aqueous mixtures of ethanol, methanol or acetone (Kahkonen, Hopia, & Heinonen, 2001). These methodologies imply the co-extraction of non-phenolic substances such as sugars, organic acids and proteins requiring subsequent purification processes (for example extraction in solid phase, SPE).

Among the most common methods are those which use acidified methanol or ethanol as extractants (Amr & Al-Tamimi, 2007; Awika, Rooney, & Waniska, 2005; Cacace & Mazza, 2003; Donner, Gao, & Mazza, 1997; Fossen & Andersen 2003; Phippen & Simon, 1998). From these methods, the extraction with methanol is the most efficient (Kapasakalidis, Rastall, & Gordon, 2006); in fact, it has been found that in anthocyanin extractions from grape pulp, the extraction with methanol is 20% more effective than with ethanol, and 73% more effective than only water (Metivier, Francis, & Clydesdale, 1980); nevertheless, in food industry ethanol is preferred due to the methanol toxicity.

In acidified solvent extractions, special care should be taken to avoid strong acid media because the acylated anthocyanins might be degraded (hydrolysis reaction) and in the case of 3-monoside anthocyanins the glycoside bonds could be destroyed (Kapasakalidis et al., 2006). Besides the acidified methanol and ethanol extractions, there are also reports with sulphur derivatives as extractants (Cacace & Mazza, 2002; Gil, Holcroft, & Kader, 1997; Romani, Mulinacci, Pinelli, Vincieri, & Cimato, 1999) using an aqueous SO_2 solution (HSO_3^-) which can react with anthocyanins causing a nucleophilic attack in the molecule (García-Viguera & Bridle, 1999; Mazza & Brouillard, 1987) and causing that the monomeric anthocyanins form colourless adducts (Berké, Chèz, Vercauteren, & Deffieux, 1998). Soaking has also been extensively used for anthocyanins extraction; in the wine industry it is the most recurrent method that consists in grinding the fresh fruit and putting in contact the grape juice with the skins to extract the pigments (Ella Missang, Guyot, & Renard, 2003).

In the anthocyanins extractions from black currants, an aqueous solution of 80% EtOH saturated with SO_2 was used as extractant (Romani et al., 1999), observing that the type of solvent, SO_2 concentration and the temperature affected the extraction process; and that the mass transfer was higher in sulphurated water that in ethanolic solution (Cacace & Mazza, 2003).

The anthocyanins from black sorghum were extracted with HCl 0.1 % in MeOH and 70% aqueous acetone (Awika et al., 2005).

Where:
R₄= H Pyranoanthocyanins (Vitisin B-type)
R₄= COOH 5-Carboxy-Pyranoanthocyanins (Vitisin A-type)
R₄= CH₃ 5-Methyl-Pyranoanthocyanins

Hydroxyphenyl-pyranoanthocyanins
Where:
Pinotin A: R₄= OH and R₅= H

Flavonol-pyranoanthocyanins

Vinyl-pyranoanthocyanins (Portisin-type)

In all cases R₃ is either glucose or its acetylated form

Fig. 5. Chemical structures of the main pyranoanthocyanins (Castañeda-Ovando et al.).

Acidified methanol resulted in significantly higher values for total anthocyanins than aqueous acetone, the extraction with acidified methanol was twice more efficient than aqueous acetone (Lee, Finn, & Wrolstad, 2004).

The anthocyanins in aqueous acetone extract (black sorghum) were not identified; apparently (Awika et al., 2005), the anthocyanin molecules undergo significant structural modification in aqueous acetone, a phenomenon that did not occur in acidified methanol. This observation agrees with other investigations (Lu & Foo, 2001) which were able to confirm formation of pyranoanthocyanins from anthocyanins through oxidative addition mediated by acetone. Therefore aqueous acetone is not an appropriate solvent for extracting sorghum anthocyanins.

According to the extraction methods reported in literature, it can be observed that in order to extract acylated anthocyanins and 3,5-diglc, it is necessary to use weakly acid media to avoid their hydrolysis (formic or acetic acid); in the case of the extraction with HCl 0.1% in MeOH also aglycons have been obtained, but under these conditions it is not possible to know if the aglycons were on the sample or if hydrolysis occurred during the extraction. It can also be concluded that when using weakly acid media it is possible to extract more and different anthocyanins (methanol with TFA, MFW, MAW).

4.2. Purification methods

The extraction methods proposed up to now are not selective for anthocyanins, since they are able to co-extract a great number of other compounds, such as sugars or organic acids (Coutinho, Quadri, Moreira, & Quadri, 2004). Consequently, it is necessary to implement new purification techniques in order to isolate the anthocyanins of interest.

In this sense, it has been proposed a wide variety of techniques; from extractions in solid phase (SPE) and liquid–liquid (LLE) (Donner et al., 1997; Fossen & Andersen, 2003; Romani et al., 1999) up to the use of sophisticated chromatographic techniques like countercurrent chromatography (CCC) (Schwarz, Hillebrand, Habben, Degenhardt, & Winterhalter, 2003), medium pressure liquid chromatography (MPLC) (Vivar-Quintana, Santos-Buelga, & Rivas-Gonzalo, 2002) and the high performance liquid chromatography (HPLC) (Alcalde-Eon, Escribano-Bailón, Santos-Buelga, & Rivas-Gonzalo, 2004; Berente, D., Reichenbacher, & Danzer, 2000; Gao & Mazza, 1994; Kennedy & Waterhouse, 2000; Mataix & Luque de Castro, 2001; Nyman & Kumpulainen, 2001).

SPE commonly is carried out in C18 or Sephadex cartridges (Donner et al., 1997), in which the anthocyanins are strongly bonded through its hydroxyl groups and separated subsequently

from other compounds by increasing the polarity with different solvents (da Costa et al., 2000). The CCC and the MPLC are used as purification methods with subsequent analysis by HPLC for structural elucidation, with the advantage of minimising the separation time and mobile phase solvents (Mikanagi, Saito, Yokoi, & Tatsuzawa, 2000; Vivar-Quintana et al., 2002). The most common method used for anthocyanins separation is HPLC with UV–Vis or photodiode array (PDA) detectors (Ella Missang et al., 2003; Mikanagi et al., 2000).

4.3. Pilot-plant scale processing

Although the importance of natural pigments is known, there are only few reports on the use of plants or fruits for anthocyanin extraction in plant scale. This might be caused by three limiting factors, often overlooked in scientific studies: (i) the effectiveness of recovery and extraction, (ii) the marketability of resulting extracts and (iii) the practical suitability for food and pharmaceutical products.

Another important factor is the stability and intensity of coloration provided by anthocyanins, which depends on their molecular structures and their intra- and inter-molecular associations. Anthocyanins are also known for their heat sensitivity, in an effort to preserve the properties of these compounds some pilot-plant strategies has been explored. Lee, Durst, and Wrolstad (2002) carried out some experiments for anthocyanins extraction from frozen blueberries. They used food-grade acetone extraction with SO_2 addition as extractant. Then a purification step was done by re-extracting with a chloroform:acetone solution (1:2 acetone chloroform v/v) and concentrating this with a thermal treatment. The final extract contained 465.0 mg kg^{-1} of total anthocyanins which corresponded to a recovery rate from 13% to 23%.

In another study, Kammerer, Kljusuric, Carle, and Schieber (2005) evaluated an ion exchange resin (Amberlite XAD 16HP resin) for recovery and concentration of anthocyanins from grape pomace extracts. The aqueous extract was prepared by adding water to fresh grapes skins (1:3 sample:water at 80 °C). The crude extract was passed through the resin and eluted with methanol. The temperature of adsorption and desorption were 25 and 50 °C, respectively. Recovery rates reported ranged from 96% to 100%. The recovery rates values in pilot-plant scale were compared to bench-lab experiments and the results revealed no significant difference between the recoveries provided by both experiments.

In order to minimise undesirable natural microbial decomposition of anthocyanins during the extraction step, Meng, Lozano, Bombarda, Gaydou, and Li (2006) proposed a methodology including cross flow microfiltration with a ceramic type membrane and reverse osmosis. This process was applied in *Perilla frustescens* with 0.01 mol l^{-1} nitric acid as extractant. The acidic final extract was concentrated 9.4 times and contained 422.0 mg l^{-1} of total anthocyanins, representing 77% of the total extracted.

5. Identification methods

The identification of anthocyanins has a critical role in taxonomic and adulteration studies besides in the quality evaluation of crude and processed food. Because of anthocyanins spectral characteristics provide very useful qualitative and quantitative information; actually the spectroscopy is the main technique used due to its simplicity and low cost. Giusti and Wrolstad (2001) and Wrolstad et al. (2005) published excellent reviews about the main methods used in the characterisation and quantification of anthocyanins by UV–Vis. Additionally, HPLC with photodiode array detector (PDA) has been also used in the anthocyanins identification and quantification (da Costa et al., 2000; Hong & Wrolstad,

1990), but the difficulty to obtain reference compounds and the spectral similarities of the anthocyanins represent and important drawback. Therefore, mass spectrometry (MS) and nuclear magnetic resonance (NMR) of ^{1}H and ^{13}C have become the preferred techniques for anthocyanins identification.

5.1. Mass spectrometry (MS)

Anthocyanins analyses by MS have been carried out for different purposes, among them: structural elucidation (Ando et al., 1999; Dugo, Mondello, Errante, Zappia, & Dugo, 2001; Hillebrand et al., 2004; Kuskoski et al., 2003; Longo & Vasapollo, 2005b; Longo, Vasapollo, & Rescio, 2005; Markham et al., 2000; Matsumoto, Hanamura, Kawakami, Sato, & Hirayama, 2001; Saito, Toki, Moriyama, Shigihara, & Honda, 2002; Williams et al., 2002; Wu, Gu, Prior, & McKay, 2004; Wu & Prior, 2005a; Berardini et al., 2005; Wu & Prior, 2005b; Wang, Race, & Shrikhande, 2003a; Vidal, Hayasaka, Meudec, Cheynier, & Skouroumounis, 2004a; Kim, Iwai, Onodera, & Matsue, 2003), study of the transformations during red wine ageing (Baldi, Romani, Mulinacci, Vincieri, & Casetta, 1995; Wang, Race, & Shrikhande, 2003b; Schwarz, Wabnitz et al., 2003; Schwarz et al., 2004) and polymerisations and reactions with other flavonoids (Remy-Tanneau, Le Guerneveä, Meudec, & Cheynier, 2003; Vidal, Meudec, Cheynier, Skouroumounis, & Hayasaka, 2004b). In Table 2 the main references about using MS in the anthocyanins studies are presented.

Recently, hyphenated techniques such as HPLC coupled to MS APCI-MS, FAB-MS and ESI–MS have become very powerful tools for the anthocyanins identification. In the past two decades, numerous ionisation methods have been developed for non-volatile or thermodynamically unstable samples, such as the anthocyanins. These techniques avoid the volatilisation and subsequent ionisation and they supply energy to the solid sample or liquid in different ways, so that the direct formation of gaseous ions is produced. As a consequence, it is possible to obtain very simplified spectra. Among these desorption sources it is found FAB, ESI and MALDI, which are considered smooth ionisation sources, because they cause very low fragmentation and allow exact molecular weight determinations.

In Table 2 it can be observed that by using these soft ionisation techniques it is possible to analyse molecules which are sensitive to suffer major fragmentations, such as anthocyanins and their respective acylated derivatives.

Wang, Kalt, and Sporns (2000) reported the use of MALDI-ToF-MS for identification of blueberries extract. The main advantage of this technique, according to the authors, was that the analyses were carried out in approximately 4 min per sample.

5.2. Nuclear magnetic resonance (NMR)

NMR technique has been used for the anthocyanins structural elucidation, as in the case of acylated anthocyanins found in "pompadour" (Williams et al., 2002), *Acalypha hispida* (Reiersen, Kiremire, Byamukama, & Andersen, 2003), *Zea mays*, *Phalaris arundinacea* (Fossen, Slimestad, & Andersen, 2001), anthocyanins with rhamnoside derivatives found in some fruits like blackberries (Longo & Vasapollo, 2005a) and minor compounds in the wine analysis (Košir & Kidrič, 2002). By carrying out COSY correlation experiments, it was possible to identify the acylated anthocyanins in *Rose* flowers, observing that the main anthocyanins were Cy-3-cum-glc-5-glc, Pn-3-cum-glc-5-glc and Cy-3-sop (Ando et al., 1999).

As for the identification of anthocyanins in strawberries some correlation experiments such as HMBC, HSQC, DQF-COSY were done by Frøytlog, Slimestad, and Andersen (1998). In this study, a new aglycon was found: the 5-carboxypyranpelargonidin. The

Table 2
Anthocyanins analyses by MS[a]

MS Mode	Main anthocyanins	References
APCI	3-gal (Dp, Cy), 3-glc (Dp, Cy, Pt, Pn, Mv), 3-ara (Dp, Cy), aglycons (Dp, Cy, Pt, Pn, Mv)	Zhang, Kou, Fugal, and McLaughlin (2004)
FAB	Acylated anthocyanins (based on mal), anthocyanins 3-glc (Dp, Cy) and 3,5–di-glc, 3-rut (Dp, Cy)	Ando et al. (1999), Markham et al. (2000), Saito et al. (2002) Williams et al. (2002)
ESI	3-glc (Dp, Cy, Pt, Pn, Mv, Pg), 3-ace-glc (Dp, Cy, Pt, Pn, Mv), 3-gal (Mv) 3-ace-gal (Mv), 3-caf-glc (Pn, Mv, Pt, Cy), 3-cum-glc (Pt, Pn, Mv), 3-cum-gal (Mv), Mv-3-glc-epi-catechin, 3,5-diglc (Dp, Cy, Pt, Pn, Mv), 3-glc-pir (Dp, Pt, Mv), 3-(6"ace)-glc (Pn, Mv), 3-(6")-cum)-glc (Pn, Mv), 3-cum-5-diglc (Dp, Cy, Pt, Mv), 3-cum-glc (Cy, Pt, Pn, Mv, Dp), Mv-3-ace-5-diglc, Cy-3-sam, 3-p-cum-glc (Dp, Cy, Pt, Pn, Mv), 3-glc-4-vinylphenol (Pt, Mv), Mv-3-ace-glc-pir, Mv-3-glc-acetone, Mv-3-glc-Et-epi-catechin, Mv-3-cum-glc-pir, Mv-3-ace-glc-Et-flavonol, Mv-3-glc-4-vinyl-epi-catechin, Mv-3-glc-4-vinylcatechol, Mv-3-ace-glc-4-vinylphenol, Mv-3-cum-glc-4-vinylphenol, Mv-3-glc adducts with: 4-vinylphenol, 4-vinylcatechol, 4-vinylguaiacol, 4-vinyl-syringol, 4-dimethylaminostyrene; Mv 3-glc-(-)-epicatechine, MvCy, MvPn, MvDp, MvPt, MvMv, MvCy-glc, MvPn-glc, MvDp-glc, MvPt-glc, MvMv-glc, MvCy-diglc, MvPn-diglc, MvDp-diglc, MvPt-diglc, MvMv-diglc, MvCy-glc-p-cum-glc, MvMvPn-glc-p-cum-glc, MvMvDp-glc-p-cum-glc, MvMvCy-triglc, MvMvPt-glc-p-cum-glc, MvMvPn-triglc, MvMvDp-3-glc, MvMvMv-glc-p-cum-glc, MvMvPt-triglc, MvMvMv-triglc, MvMvCy-diglc-p-cum-glc, MvMvPn-diglc-p-cum-glc, MvMvDp-diglc-p-cum-glc, MvMvPt-diglc-p-cum-glc, MvMvMv-diglc-p-cum-glc	Ella Missang et al. (2003), Schwarz, Hillebrand et al. (2003), Kuskoski et al. (2003), Wu et al. (2004), Hillebrand et al. (2004), Longo et al. (2005), Wu and Prior (2005a), Dugo et al., 2001, Longo et al. (2005), Berardini et al. (2005), Wu and Prior (2005b), Wang et al. (2003a), Vidal et al. (2004), Kim et al. (2003), Wang et al. (2003b), Schwarz et al. (2004), Schwarz, Wabnitz et al. (2003b), Remy-Tanneau et al. (2003), Vidal et al. (2004), García-Benítez et al. (2003)
MALDI	3,5-diglc (Cy, Pn), 3-glc (Dp, Cy, Pt, Pn, Mv), 3-glc-cum (Dp, Cy, Pt, Pn, Mv), 3-glc-cum-5-glc (Dp, Cy, Pt, Pn, Mv)	Wang and Sporns (1999)
MALDI-ToF	3-glc (Pg, Cy, Pn, Mv)	Wang et al. (2000)

[a] *Notes:* acids (acyl substitution): acetic (ace), malonic (mal), piruvic (pir), (gao), p-coumaric (cum), caffeic (caf),. Saccharides: arabinose (ara), galactose (gal), glucose (glc), sambubiose (sam), rutinose (rut).

correlation experiments have been used by several researchers mostly in order to determine the glycoside position in the anthocyanins (Bloor & Abrahams, 2002; Kim et al., 2003; Remy-Tanneau et al., 2003).

In a similar manner, some NMR correlation experiments have been done, such as DQF-COSY, ROESY, HSQC and HMBC, for anthocyanin adducts (with pyruvic acid) found in red wine (Mateus, Silva, Vercauteren, & de Freitas, 2001). In this study it is proposed a mechanism for the formation of two pigments obtained from the same product (Mateus, Silva, Rivas-Gonzalo, Santos-Buelga, & de Freitas, 2003b), which are Mv-3-glc-pyruvic acid and Mv-3-cum-glc-pyruvic acid adducts; such studies were carried out with ^{1}HNMR and with 2D-NMR (COSY).

NMR has also been very useful to identify the reaction products of anthocyanins with other compounds such as cinnamic acid derivatives (Schwarz et al., 2003), peroxyl radicals (Matsufuji et al., 2003), catechins (Pissarra et al., 2005) and flavonols (Mateus et al., 2003a).

6. Other studies

6.1. Capillary electrophoresis (CE)

The use of CE in the separation of anthocyanins is quite recent, scarce and promising due to the high hydrosolubility of these compounds. CE is a suitable technique for anthocyanins separation, identification and quantification.

The first anthocyanin analysis by CE was reported in 1996 (Bridle, García-Viguera, & Tomas-Barberan, 1996). In this study it was used a fused silica capillary tube, borate buffer (pH 8) and PDA detector. Due to the instability of the anthocyanins at basic pH, the resolution and sensitivity of the method were very low and the authors were able to get results only for high anthocyanin concentrations. Later on, the same researchers proposed another methodology by using phosphate buffer at pH 2.1 and CTAB as cationic surfactant (Bridle et al., 1996). The CTAB was used to coat the capillary surface in order to avoid wall-anthocyanin interactions

and change in the EOF direction which affects the separation and quantification results (Bicard, Fougerousse, & Brouillard, 1999).

Other CE variants have been used for quantitative determination of anthocyanins in wine as an HPLC alternative (Saenz-Lopez, Fernandez-Zurbano, & Tena, 2003; Saenz-Lopez, Fernandez-Zurbano, & Tena, 2004); the study was done using a fused silica capillary and a $Na_2B_4O_7$ buffer at pH 8.4 with 15% of MeOH as modifier. The electropherograms were obtained at 599 nm and for quantification purposes the calibration curves were done using Mv-3-glc as standard. In this report, the results were better than those published in 1996, since a preconcentration of the sample was done, besides adding organic modifiers to enhance the selectivity of the method and the stability of the anthocyanins.

A similar method has been proposed for acylated and non-acylated anthocyanins and 3-glucoside derivatives. Calvo, Saenz-Lopez, Fernandez-Zurbano, and Tena (2004), carried out some CE experiments for pigments that were separated by LC, concentrated and freeze-dried with the purpose of identifying the compounds, the migration order and to determine the migration time, their explanations are based on the mass to charge ratios, and in the possibility of a complex with tetraborate buffer molecules.

Another CE mode that has been used in the analysis of anthocyanins is the micellar electrochromatography [MEKC] (Bednar et al., 2003). By using this mode, it was developed a method to separate simultaneously 6 anthocyanins (Mv-3,5-diglc, Mv-3-glc, Mv-3-gal, Pg-3-glc, Cy-3,5-diglc and Cy-3-gal). The conditions of analysis were: buffer 30 mM phosphate + 400 mM borate-TRIS + 50 mM of SDS, pH 7.0. The high borate concentration was very important to separate the diasteromeric pairs such as the Mv-3-glc and Mv-3-gal.

As for the SDS, it is known that the formation of negatively charged micelles provide chemical stability to the anthocyanins in aqueous solutions (Mulinacci et al., 2001). The results of these investigations indicate that the number of negative charges in the surface and an organised distribution of these charges on the micelle surface are determining factors to allow the stability and the retention of colour in the anthocyanins.

CE has also been used to create correlations between the anthocyanins content and the ageing of the red wine (Saenz-Lopez et al., 2004). The CE with ESI–MS coupling has been used for the anthocyanins and flavonoids monitoring in wine. The experimental conditions were: ammonium chloroacetate buffer (pH 2) and ammonium borate buffer (pH 9, with high selectivity toward the derivatives with hydroxyl groups). The MS method was optimised and the anthocyanins fragmentations were studied in detail (Bednar et al., 2005).

6.2. Theoretical calculations

Some researchers have carried out theoretical calculations in order to predict the chemical behaviour of anthocyanins in different solutions. The reports of theoretical calculations for anthocyanins are scarce, but one of the studies that has been based on molecular orbital (MO) calculations, was done in order to determine the interaction of some organic acids with anthocyanins and the way they affect their coloration (co-pigmentation effect) (Ferreira da Silva et al., 2005).

The MO calculations and energy states were done using the density functional theory (DFT) approach, concluding that the anthocyanins form complexes in the basal state with a number of natural polyphenols, giving place to a charge-transfer from the polyphenol to the cationic form of the anthocyanin (Pg).

Additionally, some *ab initio* studies have been done for the structural and electronic characteristics of four anthocyanidins, Pg, Cy, Dp and Au (Sakata, Saito, & Honda, 2006). For these molecules, the planar and non-planar structures were determined in a level of theory B3LYP/D95. The results indicated that the planar structure is slightly more stable than the non-planar, and the authors showed that the hydroxylation in the phenyl group causes a change in the excitation energy without considering the effects of the solvent; this fact is in agreement with previous spectral experiments and with the MO calculations (Sakata et al., 2006).

7. Conclusions

The focus of recent investigations allows us to predict a notable increment in the use of anthocyanins as natural colorants in diverse products during the next years. Considering the beneficial effect for the health of these molecules, their incorporation in food and beverages industries will represent an important value. The implementation of better extraction, purification and identification methodologies first in a lab-bench and then in pilot-plant scale, will have an impact in the cost of these colorants and their standards in the near future; as well as in the establishment of new tools for the food products authentication and the discovery of sophisticated adulterations.

Acknowledgements

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