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Review

Anthocyanins in cereals

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Abstract

The anthocyanic composition of some pigmented cereals is still not well established, neither in relation to some of their components, nor from the quantitative point of view. Nonetheless, the use of analytical techniques, such as diode array spectroscopy and mass spectrometry (MS, PDMS, MALDI) coupled or not to liquid chromatography, are permitting, in recent years, the confirmation of the structure of some of the principal anthocyanins and a knowledge of those which are present in minor proportion. In this article, firstly, a review of the principal methods of analysis of anthocyanins is made. This is followed by a review of the most significant advances achieved in the last years in the field of the identification and quantification of these pigments in cereals and the present uses of the commercial extracts of anthocyanins obtained from these sources and the perspectives for their use is included.
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Keywords: Anthocyanins; Cereals; Maize; Rice; Wheat; Sorghum; Analysis; Reviews

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1. Introduction

The anthocyanins compose a group of intensely coloured pigments responsible for the orange, red, purple and blue colours of many fruits, vegetables, flowers, leaves, roots and other storage organisms of plants. They are found in nature in the form of polyhydroxylated and or methoxylated heterosides which derive from the flavylium ion or 2-phenylbenzopyrilium (Fig. 1). Aglycon (anthocyanidin) is found united to one or various sugars, which, in turn, can be acylated with different organic acids. The presence of these hydroxyl groups on the rings, as well as one or several sugar molecules, make these compounds quite soluble in water, ethanol, and methanol [1].

According to the literature, 21 anthocyanidins have been fully described, of which only 6 are widespread and usually found in fruits and vegetables (Table 1). They are: pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. These are based on the same 2-phenylbenzopyrilium (flavylium) skeleton hydroxylated in 3, 5, and 7 positions, and differing in the number and position of hydroxyl and methoxyl groups in the B-ring. Derived from these anthocyanidins, a wide range of anthocyanins exist which differ in the nature and number of sugars attached to the aglycon and the position of the attachment and in the nature and number of aliphatic or aromatic acids attached to the sugar residues. The most commonly found sugars are xylose, arabinose and rhamnose among pentoses and galactose and glucose among hexoses. They are linked to the aglycon by an α or β linkage of the free anomeric hydroxyl. Di- and trisaccharide functional groups are also common, among which the most usual are rutinose (6-*O*-α-rhamnosyl-D-glucopyranose), sophorose (2-*O*-β-glucopyranosyl-D-glucopyranose), 2^G xylosylrutinose and glucosylrutinose. Sugar moieties are always found attached to the hydroxyl group at position 3 of the anthocyanidin. When additional sugar groups exist, they occupy positions 5 and/or 7, and less frequently 3′and 5′. Sugars may be substituted by aliphatic, hydroxybenzoic or hydroxycinnamic acids, the most usual being acetic, malonic, *p*-coumaric, and caffeic acids [1].

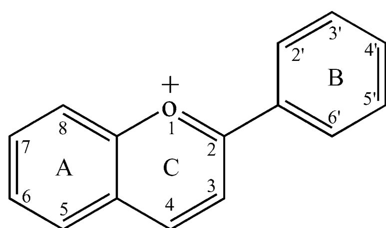


Fig. 1. The structure of the flavylium ion or 2-phenylbenzopyrilium.

Table 1
Common anthocyanidins present in nature

Anthocyanidins	Substitution pattern
Delphinidin	3,5,7,3′,4′,5′-OH
Cyanidin	3,5,7,3′,4′-OH
Pelargonidin	3,5,7,4′-OH
Petunidin	3,5,7,4′,5′-OH; 3′-OMe
Peonidin	3,5,7,4′-OH; 3′-OMe
Malvidin	3,5,7,4′-OH; 3′,5′-OMe

In relation to the stability, anthocyanins are compounds that may suffer reactions that alter their structures through the action of different agents due to the electronic deficiency of their flavylium nuclei. Anthocyanin stability increases with the number of methoxyls in the B ring and decreases as hydroxyls increase. Thus, among the most common anthocyaninidins, the most stable is malvidin, followed by peonidin, petunidin, cyanidin and delphinidin. In general, anthocyanins are more stable at an acid pH. Glycosylation and acylation of the sugars also increases stability and, therefore, the diglycosides are more stable than their corresponding monoglycosides [2,3].

During analysis, it is important to control the temperature since the rate of degradation of anthocyanins increases as temperature rises and is more accused at higher pH values [4–7].

Furthermore, luminous radiation accelerates the degradation of anthocyanins [7,8], acylated diglucosides being more stable than monoglucosides [9].

The functions of the anthocyanins in plants are similar to the general functions of all flavonoids: antioxidant functions, photoprotectors, defence mechanisms, and other ecological functions (symbiotic phenomena). Since they give colour to different parts of plants, they also play an interesting role in reproductive mechanisms: found in flowers, they serve to attract pollinators and in seeds and fruits to attract seed disseminators. It has also been observed that cyanide-3-glucoside inhibits larval growth in tobacco worms (*Heliothis viriscens*) and thus, anthocyanins may be considered agents of biological control [10].

In the last decades there has been considerable interest in the chemistry of the anthocyanins, since, on the one hand, their use as a possible alternative to artificial food colorants has been posited, and, on the other, it seems they present

bio-active properties. The consumption of foodstuffs rich in anthocyanins has been associated with an increase in visual acuteness [11], anti-oxidant capacity [12–21], vasoprotective and anti-inflammatory effects [22], inhibition of platelet aggregation [23], etc.

The blue, red and purple varieties of some cereals are drawing the attention of scientists and the food industry, since they are potential sources for the obtention of extracts rich in anthocyanins. These compounds are found in some cereals, such as in purple corn, in such quantities as to make the obtention and commercialisation of the extracts viable, moreover, the fact that they are frequently located in external tissues of the plant greatly facilitates their production.

In spite of that, for some cereals, the anthocyanic composition is still not well established, neither in relation to some of their components nor from the quantitative viewpoint. At present, a rapid advance is taking place favoured by the development of more precise and selective analysis techniques, such as diode array spectroscopy and mass spectrometry (MS, PDMS, MALDI) coupled or not to liquid chromatography, which facilitate the detection and characterisation of pigments. These techniques have, in recent years, permitted the confirmation of the structure of some of the principal anthocyanins and the knowledge of the others present in lesser proportion in the pigmented cereals.

In this article, firstly, a review of the principal methods of analysis of anthocyanins is made. This is followed by a review of the most significant advances achieved in the last years in the field of the identification and quantification of these pigments in cereals. The present uses of the commercial extracts of anthocyanins obtained from these sources and the perspectives for their use are also included.

2. Analysis of anthocyanins

2.1. Extraction and isolation

Extraction of anthocyanins is commonly carried out under cold conditions with methanol or ethanol containing a small amount of acid with the objective of obtaining the flavylium cation form, which is red and stable in a highly acid medium [24]. However, acid may cause partial hydrolysis of the acyl moieties in acylated anthocyanins, especially in anthocyanins acylated with dicarboxylic acids such as malonic acid. For the extraction of anthocyanins with the dicarboxylic substituents intact, the use of weak acids is advisable, such as tartaric [25,26] or citric acid [27–29].

Recently new techniques have been introduced for the extraction of phenolic compounds which can be used for the extraction of anthocyanins, such as pressurized liquid extraction (PLE) [30] and supercritical fluid extraction (SFE) [31–44].

For use in food, it is preferable to use ethanol, as it is less toxic, even though it is less efficient in extraction and more difficult to eliminate later. With ethanol, if the extraction process is carried out in the presence of heat, artifacts may be produced through esterification reactions with free carboxylic groups, for example: in malonyl conjugate anthocyanins from maize [45].

When it is suspected that the extract could contain lipidic material or liposoluble substances (waxes, chlorophylls, etc.), these can be eliminated by washing the extract with an organic solvent such as hexane. For this it is necessary to previously incorporate water to the extract and eliminate the alcohol by evaporation.

The selected techniques to carry out the isolation and purification of the anthocyanins are the chromatographic ones. Initially the techniques of *paper chromatography* (PC) [46–49] or *thin layer chromatography* (TLC) [24,50], permitted not only isolating, but also identifying, and quantifying certain anthocyanins. At present, others, which achieve greater efficacy and yield, are more used. Thus, column chromatography (CC) techniques began to be used because of the need to obtain pure compounds in sufficient quantities for subsequent identification and characterization and to be used as reference substances in qualitative and quantitative analyses. Many different chromatographic supports have been tried, Amberlite GC-50 [51], Amberlite XAD-7 [52,53] Toyopearl [54], Sephadex [55,56], Polyamide [57,58], polyvinylpirrolidone (PVP, Polyclar AT) [59–62] and resin mixtures [63–67]. In CC, the best resolution is achieved with reverse phase columns of the type Lichroprep-RPS [68] which offer a quick and efficient separation of the anthocyanins.

The technique of choice today is *high performance liquid chromatography* (HPLC) [54,69,70], since it permits simultaneous separation, identification and quantification of anthocyanin compounds, without requiring excessive purity in the extracts. Nevertheless, solid-phase extraction (SPE) on C_{18} cartridges or Sephadex is commonly used for the initial purification of the crude anthocyanin extracts before carrying out the HPLC method. The anthocyanins are bound strongly to these adsorbents through their unsubstituted hydroxyl groups and are separated from unrelated compounds by using a series of solvents of increasing polarity.

As it is well known, the anthocyanidins become more polar as the number of hydroxyl groups in the B-ring ring increases and more apolar as the number of methoxyl groups increases. Thus, the following order of elution for the six most widespread anthocyanidins is observed [71]: delphinidin → cyanidin → petunidin → pelargonidin → peonidin → malvidin.

This same elution order is maintained for anthocyanins bearing identical sugar substituents. Sugar substitution increases the polarity and, therefore, anthocyanins elute at shorter retention times than their parent anthocyanidins. The influence of the sugar moieties on the polarity varies depending on the type of sugars and their position of substitution. When considering the more usual 3-glycoside derivatives, the elution observed for anthocyanins derived from the same

anthocyanidin is as follows [71]:

Monosaccharides: galactoside → glucoside → arabinoside → xyloside → rhamnoside.

Disaccharides: sophoroside → sambubioside → rutinoside.

The anthocyanins may also bear additional sugar moieties, usually attached to their 5, 7 or 3′ position. In general, the introduction of a second sugar moiety in the anthocyanin molecule increases the polarity, and, thus, for instance, 3,5-diglucosides elute before the corresponding 3-glucosides. It has also been indicated that the increase of polarity is greater in the case of 3,5-derivatives than in that of 3,7-derivatives bearing identical sugars [72]. On the other hand, disaccharide moieties in position 3 increase the polarity less than the presence of same two monosaccharides in 3,5-positions [71].

Acylation of the sugar moieties of the anthocyanins involves a loss of polarity that is reflected in an increase of the retention time. The extent of this polarity decrease depends on the type of acyl substituent. For the most usual aliphatic acids' residues, the elution order would be as follows: malic → acetic → malonic → succinic.

High-speed countercurrent chromatography (HSCCC) and *high-performance centrifugal partition chromatography* (HPCPC) have been used recently for separating and purifying anthocyanins from plants and red wine [73–76]. As an all-liquid chromatographic technique, CCC uses no solid support and allows complete recovery of the sample, since no irreversible adsorption on active surfaces can occur [73]. *Capillary electrophoresis* (CE) is a relatively new analytical tool with excellent mass sensitivity, high resolution, low sample consumption, and minimal generation of solvent waste. Very few reports have appeared in the literature on the application of capillary zone electrophoresis methods (CZE) for separating anthocyanins [77–79]. In the case of anthocyanins, the applicability of this technique is limited, in part, by the fact that anthocyanins are not stable under alkaline conditions. This is why anthocyanins are separated using an acidic running buffer [80]. The use of *micellar electrokinetic chromatography* (MEKC) with SDS solution in a phosphate buffer at pH 7.0, produced excellent results for separating anthocyanins from elderberry juice [81].

2.2. Detection and identification

2.2.1. UV–vis spectroscopy

Spectral properties are often very useful for characterizing anthocyanins, especially for identifying the anthocyanidin type [64,72,82–85]. Ring B functional groups may have some influence on the wavelength of maximum absorption in the visible, so the anthocyanins that possess two functional groups in that cycle (cyanidin and peonidin) have absorption maxima 11 nm lower than those with three functional groups (delphinidin, petunidin and malvidin) [15]. The presence of methoxyl groups on the B ring provokes a certain movement of colour toward the red zone, lending, at the same time, greater stability to the molecule. Also, delphnidin, petunidin and cyanidin, unlike malvidin and peonidin undergo bathochromic shift in the presence of $AlCl_3$, a fact that has been used occasionally as criterion for differentiating between them.

Glycosylation produces a displacement of the absorption maximum in the visible region of some 10 nm toward lower wavelengths, in relation to the corresponding aglycon and, at the same time, decreases the molar absorption coefficient. Diglycoside anthocyanins present absorption maxima toward lower wavelengths than the corresponding monoglycosides and the presence of a shoulder in the visible region at about 440 nm in the 3-monoglycosides is not seen in the 3,5-diglycosides.

The presence of acyl groups in the molecule may cause the appearance of an additional maximum (or shoulder) in the UV spectrum. Thus, sterification with *p*-coumaric acid increases absorption approximately at 308–313 nm and with caffeic acid at 326–329 nm. With aliphatic acids no modifications in this range of the spectrum are produced.

The introduction of diode array detection systems (DAD) and their utilisation jointly with HPLC opened new perspectives for anthocyanin separation, identification and quantification in that they made it possible to acquire on-line UV–vis spectra during chromatographic analysis [64,72]. Today, DAD is the usual technique used for analysing these compounds.

2.2.2. Mass spectrometry

Mass spectrometry is a technique that permits anthocyanin identification thanks to determining the mass of the molecular ions in the sample and of the fragments from the rupture of these compounds through the application of higher ionization energies. The application of HPLC–DAD–MS using electrospray ionization (ESI) has facilitated the identification of the anthocyanins in numerous plant samples such as, for example, black carrots [86], grapes [87], strawberries [88], corn cobs [45], red wines [89,90].

Anthocyanidins in their flavylium cation form are relatively stable and do not have an easily broken linkage site, consequently, their spectra present an *m*/*z* signal which corresponds to a molecular ion with very little fragmentation. To provoke fragmentation, electrospray ionization in combination with collision-induced dissociation (CID) and/or tandem mass spectrometry (MS–MS) are used.

In anthocyanins, the number and type of fragments formed will vary depending on whether the substance is monoglycoside, diglucoside, or bioside. With anthocyanins substituted on position 3 with a single sugar, the ion mainly detected will correspond to aglycon in addition to the peak at *m*/*z* of the molecular ion. In the case of a diglucoside with sugars on positions 3 and 5, several ions will be detected in the spectrum, the ion corresponding to aglycon alone, the ions corresponding to anthocyanin substituted

on positions 3 and 5 and the anthocyanin molecular ion [91,92].

If the two sugars are the same, only one signal appears, apart from the molecular ion and the aglycon ion, so that the elimination sequence cannot be characterized. In the case of a 3-bioside, only one fragment will be formed, since the glucosidic bond between the two sugar units does not usually break. There is an exception, when the bioside on 3 is a rutin derivative there will be a rupture in the glycosidic linkage leading to an additional fragment corresponding to the loss of the rhamnose. This 1–6 linkage between rhamnose and glucose permits free rotation and greater accessibility of the gas used in fragmentation [91]. In this type of compound, the formation of an ion has also been observed due to loss of the rhamnose terminal and of oxygen from the glucosidic bond.

In the case of acylated anthocyanins, there will not be any breaking of the ester bonds and the sugar and acid will be lost together.

Gas chromatography combined with mass spectrometry have been used in some cases, though this proves to be a difficult and costly application due to the structural lability of the anthocyanins with regard to ionization techniques using electronic impact, and due to the necessity of derivatizing them [16,93,94].

2.2.3. Nuclear magnetic resonance

This technique has been used for identifying anthocyanins, and it undoubtedly permits correct identification. The use of 1D and 2D ^{1}H and ^{13}C NMR spectroscopy with large signal suppression methods enable reliable assignments for ^{1}H and ^{13}C resonance signals of individual anthocyanins in CD_3OD [95].

The NMR technique, in homo- and heteronuclear experiments, such as COSY, TOCSY, NOESY, HMQC and HMBC is commonly used for identifying anthocyanins and their derivatives in natural plant sources and in industrial products as well.

2.2.4. Hydrolysis techniques

These techniques can serve as a complement to the more modern methods of identification previously described. These consist of the performance of selective hydrolyses and later identification of the resulting compounds. Thus, *acid hydrolysis*, which is usually carried out in a concentrated hydrochloric medium at boiling temperature, provokes the rupture of the heteroside linkages, leading to the appearance of the corresponding sugars and aglycones [69,96–99]. Aglycon identification may be carried out by HPLC [97] and sugars may be identified by classic paper, thin layer, or gas chromatography techniques.

Furthermore, *alkaline hydrolysis* provokes the rupture of the bonds between the anthocyanin heterosides and the acylating acids. The analysis of these last can be carried out after solvent extraction by spectroscopic and chromatographic techniques [83,96,100,101].

3. Occurrence in cereals

3.1. Maize

Purple corn is a pigmented variety of *Zea mays* L. cultivated in Latin America, principally in Peru and Bolivia, where the traditional drink “Chicha Morada”, to which beneficial effects for health are attributed, is prepared cooking the corn with pineapple and quincepeel, cinnamon and cloves [102]. Purple maize is also used for the production of purple tortilla chips and the extracts are used as food colorants.

In the plant, the anthocyanins appear in epidermal cells, where it is believed they exercise, among others, a protective function against UV-B radiation [103].

3.1.1. Identification of anthocyanins in purple corn

Maize is one of the cereals whose anthocyanic composition is better defined. In our laboratory [45], liquid chromatography coupled to diode array spectrometry and mass spectrometry detection (LC–DAS–MS) was used to identify the anthocyanins present in a commercial extract of purple corn cob used as an additive in the food industry. Thus, in the extract, which contained 34% of anthocyanins (expressed as cyanidin-3-monoglucoside), nine anthocyanins were identified of which six were present in the original cob and three were generated during the process of industrial extraction and were identified as ethylmalonyl derivatives. A similar event has been indicated by Fossen et al. [104], since these authors, on identifying the anthocyanins present in flowers of *Z. mays*, found that during the isolation and later storage of the anthocyanins in the RMN solvent, methyl esterification of the free acid function of the malonyl units of the pigments was produced.

Previously, in maize, cyanidin-3-glucoside had been identified and various glycosides of cyanidin, pelargonidin, peonidin and delphinidin insufficiently described [105–112]. The presence of cyanidin-3-galactoside in Peruvian dark-seeded corn and in leaves of a *Z. mays* hybrid has been suggested [109–113]. Cyanidin-3-(6″-malonylglucoside) and cyanidin-3-dimalonylglucoside were identified in maize leaves [111]. Nonetheless, the pattern of substitution of this diacylated anthocyanin was not determined. Recently, this dimalonylated anthocyanin, present in the flowers and leaves of maize, has been identified by homo and heteronuclear two-dimensional NMR as cyanidin-3-(3″,6″-dimalonylglucoside) [104]. Later, Schwarz et al. [102] also analysed, by HSCCC, the commercial extract of corn cob, proving the presence of the anthocyanins previously indicated by de Pascual-Teresa et al. 2002 [45] and contributed, moreover, the presence of trace quantities of dimalonylated monoglucosides of cyanidin and peonidin. Recently, our research group has identified the compound corresponding to the dimer formed by the condensation between a flavan-3-ol and cyanidin-3,5-diglucoside [114].

In Fig. 2 we show the chromatogram registered at 520 nm, obtained on injecting in HPLC a sample corresponding to

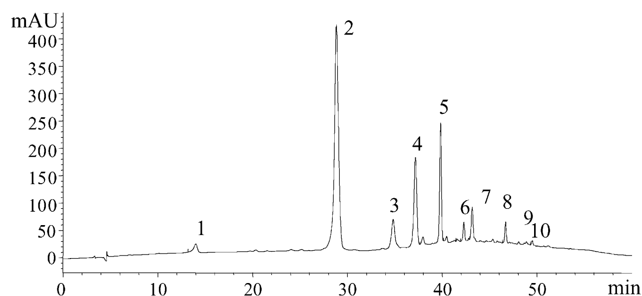


Fig. 2. HPLC chromatogram recorded at 520 nm corresponding to original cob extract [45]. Peak identification in Table 2.

the original extract of cob corn and in Table 2 the identity of the pigments which have been identified together with their chromatographic characteristics and molecular ions and fragments found in MS and MS^2.

The anthocyanins identified in flowers and leaves [104] are shown in Table 3 together with the percentage that each of the anthocyanins represents. Both contain the same anthocyanins in very similar relative proportions. The derivatives of cyanidin represent more than 90% in both parts of the plant and only small quantities of peonidin derivatives were detected. With regard to the acylated anthocyanins, they are found in a high proportion, constituting more than 40% of the total of anthocyanins.

In seeds of purple corn (Table 3), Aoki et al. [115] identified, by MS and ^{1}H, ^{13}C NMR, the glucosylated derivatives and the 3-(6″-malonylglucoside) corresponding to cyanidin, peonidin and pelargonidin. The relative content of derivatives of acylated anthocyanins, around 40%, was similar to that found in leaves and flowers and notably superior to that found in the commercial extract of cob corn. The proportion of the derivatives of cyanidin was inferior to that in leaves and flowers and similar to that found in the commercial extract of cob corn, around 70%.

Regarding the total content of anthocyanin present in purple corn, it is notably high. It has been found that, for whole purple corn, the content is 1642 mg/100 g in humid base and 1779 mg/100 g in dry base expressing the concentration in mg of cyanidin-3-glucoside/100 g [116], which makes this cereal a good source for the obtention of extracts rich in anthocyanins.

3.1.2. Utilisation of the anthocyanins of purple corn

3.1.2.1. Activity against plant pathogens. *Aspergillus flavus* and *Aspergillus parasiticus* are pathogens of food crops such as maize. Some strains of these fungi produce aflatoxin B1, a potent hepatotoxin and carcinogen. The contamination of maize is still endemic in many regions and could affect public health as well as causing important economic losses. Furthermore, it appears that the anthocyanins are involved in the resistance of the maize to diverse pathogens [117–119]. Given that the anthocyanins are present in kernels of some maize lines, Norton [120] studied the possibility that the kernel components or their precursors could inhibit the production of aflatoxins, which would make it interesting to pose the development of maize lines rich in anthocyanins which could be well resistant to infection by *A. flavus* or inhibit the production of aflatoxins. These authors demonstrated that both the glycosylated forms and the aglycons of the majority anthocyanins present in maize, pelargonidin-3-glucoside and cyanidin-3-glucoside, present a high inhibitory activity of the production of the toxin.

Table 2
Identity, chromatographic characteristics and molecular ions and fragments found in a commercial extract of cob corn. Adapted from [45]

Peak	Compound	R_t	$[M]^+$ (amu)	Fragments $[M+H]^+$ (amu)	Relative abundance (%)
1	Dimer[a]	14.0	899	737, 575, 423	1.8
2	Cyanidin-3-glucoside	28.8	449	287	54.3
3	Pelargonidin-3-glucoside	34.8	433	271	6.1
4	Peonidin-3-glucoside	37.2	463	301	14.7
5	Cyanidin-3-(6″-malonylglucoside)	39.8	535	449, 287	11.6
6	Pelargonidin-3-(6″-malonylglucoside)	42.3	519	433, 271	3
7	Peonidin-3-(6″-malonylglucoside)	43.2	549	463, 301	5.5
8	Cyanidin-3-(6″-ethylmalonylglucoside)	47.7	563	449, 287	2.6
9	Pelargonidin-3-(6″-ethylmalonylglucoside)	48.9	547	433, 271	0.2
10	Peonidin-3-(6″-ethylmalonylglucoside)	49.5	577	463, 301	0.1

[a] Dimer formed by direct condensation between a flavan-3-ol and cyanidin-3,5-diglucoside [114].

Table 3
Identification and relative abundance (%) of the anthocyanins present in flowers and leaves [104] and in seeds [115] of *Zea mays*

	Cy-G	Cy-MG	Cy-DMG	P-G	P-MG	P-DMG	Pl-G	Pl-MG
Flowers	49	26	17	2	t	1	n.d.	
Leaves	48	26	16	2	t	1	n.d.	
Seeds	44	23	3	11	6	n.d.	5	4

Cy-G: cyanidin-3-glucoside; Cy-MG: cyanidin-3-(6″-malonylglucoside); Cy-DMG: cyanidin-3-(3″,6″-dimalonylglucoside); P-G: peonidin-3-glucoside; P-MG: peonidin-3-(6″-malonylglucoside); P-DMG: peonidin-3-dimalonylglucoside, without determination of the bonds between the malonyl units and glucose; Pl-G: pelargonidin-3-glucoside; Pl-MG: pelargonidin-3-(6″-malonylglucoside).

3.1.2.2. Source of natural extracts of anthocyanins. Interest in foodstuffs and extracts rich in anthocyanins has intensified in recent times. On the one hand, there is a considerable demand for food colorants from natural sources which can serve as an alternative to the use of synthetic colorants, due both to existing legal restrictions and the rejection by the consumer of the use of synthetic additives. On the other, the anthocyanins are potent anti-oxidants which could also have possible beneficial effects for health [2,3,7–9,12,14,121]. Nevertheless, they present an important limitation which has greatly restricted their use in food systems: their relatively low stability against some processes, formulations and storage conditions. However, thanks to the stabilising effect of the intramolecular copigmentation [122–124], polyglycosilated and polyacylated anthocyanins present greater stability against changes of pH, thermal treatment and exposure to light [125–128].

Among the highly pigmented plant products which have been suggested as sources of anthocyanic colorants purple corn (*Z. mays*) is included [13,99,110]. The presence of acyl groups in the molecules of the anthocyanins which contain them, together with the fact of the high concentration they present, makes the extract of purple corn a good alternative as a food colorant. The anthocyanins of purple corn have a long history as colorants, since it seems they were already utilised by Inca civilisations. At present there are several patents which describe preparations and processes of application for their use as colorants.

3.2. Rice

Anthocyanins are found in red rice and black rice grain in husk. Coloured rice has been consumed traditionally in Asian countries where, what is more, the anthocyanin pigments it contains are used as food colorants in the elaboration of alcoholic beverages [129].

3.2.1. Identification of anthocyanins in rice

The principal anthocyanin in rice is cyanidin-3-glucoside, followed, in minor proportion by peonidin-3-glucoside [112,130–136]. Small quantities of other derivatives of cyanidin have also been found: cyanidin-3-gentiobioside [134], cyanidin-3-rhamnoside, cyanidin-3,5-diglucoside and cyanidin-3-rhamnoglucoside [112,131]. In some crops malvidin-3-galactoside [131,137], peonidin-3-rhamnoglucoside and a derivative of delphinidin [112,131] have been described.

With regard to the total content of anthocyanins, it varies greatly according to the species. Ryu et al. [133] quantified the two principal anthocyanins present in 10 black rice crops: Jawangdo, Sanghaehyeolla, Hongmi, Heugjinmi, Suwon #405, Suwon #415, Suwon #420, Suwon #425 and Kilimheugmi. In Table 4 the contents for these anthocyanins found in the varieties analysed are shown.

Table 4
Anthocyanin contents in pigmented rices [133]. Expressed as mg of anthocyanin/100 g of grain

Varieties	Cyandin-3-glucoside	Peonidin-3-glucoside	Total	Cyandin-3-glucoside (%)
Suwon #415	470	23	493	95
Kilimheugmi	240	26	266	90
Suwon #425	206	40	246	84
Heugjinmi	200	32	232	86
Sanghaehyeolla	50	5	55	91
Hongmi	30	6	36	83
Suwon #405	16	4	20	80
Suwon #420	10	n.d.	10	100
Jawangdo	10	t	10	100

n.d.: not detected; t: traces.

Recently, Hu et al. [136] isolated the pigmented fraction of an extract of black rice by Bio-Gel P-2 column chromatography and determined the content of anthocyanins. They found that the content of total anthocyanins in the whole grain was 0.16% and of the aleurone layer 1.36%. This latter presents 85% of the total anthocyanins contained in the whole rice.

3.2.2. Perspectives

3.2.2.1. Utilisation of the anthocyanins present in pigmented rices. As a consequence of the antioxidant activity and scavenging capacity of the anthocyanins, diverse authors have proposed the utilisation of pigmented rices as an extra source of anthocyanins in the diet and even as a potential source of them in the elaboration of nutraceutical or functional food formulations [135,136].

A recent study [138] shows that supplementation of atherogenic diets with black rice reduces the oxidative stress, thus the anthocyanins present could provide cardiovascular protection. Hu et al. [136] investigated the capacity of the anthocyanic extract of black rice to neutralise reactive nitrogen and oxygen species (free radicals) in model cell cultures. These authors demonstrated that, as a consequence of a marked antioxidant activity and a capacity to capture free radicals in vitro, the pigmented fraction of black rice prevents DNA scission and the deterioration of human LDL induced by reactive oxygen species. The extract of black rice suppresses the formation of nitric oxide in activated macrophage without producing cell toxicity [136].

Morimitsu et al. [135] assayed the anthocyanins of rice as potential inhibitors of the formation of cataracts in diabetics. They used rat lens organ culture and observed inhibitory activity for lens opacity of extracts from different strains of red rice and black rice. Of them, two strains, *asamurasaki-2* (**AS2**; Japanese black rice) and *chikushi-akamochi-2* (**CH2**; Japanese red rice) were selected as potential inhibitory strains for rat lens opacity.

3.2.2.2. Utilisation of varieties of rice resistant to diseases. Padmavati et al. [139], observed that anthocyanic extracts from the leaves and the pericarpium of a crop of pigmented rice inhibit the growth of one of the principal pathogens of rice, *Xanthomonas oryzae* pv. Oryzae, at a concentration of 100 μg disc^{-1}. This fact led to the proposal of the utilisation of metabolic engineering with the objective of favouring,

Table 5
Content in anthocyanins (mg/100 g), in whole meal, flour and bran of Blue wheat, Purple wheat and Red wheat [143,144]

Wheat product	Blue wheat	Purple wheat	Red wheat
Whole meal	16	9	0.5
Flour	2	0.7	0.2
Bran	46	24	1

in the plant, determined biosynthetic routes, particularly the routes of secondary metabolites which give rise to the synthesis of molecules, such as anthocyanins, which serve as chemical defence against the attack of pathogens.

3.3. Wheat (Triticum spp.)

3.3.1. Identification of anthocyanins in wheat

The anthocyanin composition of pigmented wheat is not well characterised. Little is known about the identity of the majority of the compounds present and those which are known have been identified by comparison with standards. According to our knowledge, for the moment, in wheat, the presence of glucosides and rutinosides of cyanidin and peonidin has been indicated as well as acylated derivatives of them [140,141]. The presence of cyanidin-3-gentiobioside [142] has also been indicated.

Recently, Abdel-Aal and Hucl [143] studied the anthocyanic composition of blue aleurone spring wheat (*Triticum aestivum* L. cv. Purendo 38), purple wheat (*T. aestivum* L. cv. Konini) and red wheat (*T. aestivum* L. cv. Katepwa) during three harvests. The average contents of anthocyanins found in whole meal, flour and bran are shown in Table 5. The anthocyanins are found in noteworthy concentrations in purple wheat and above all in blue wheat whole meals (16 mg/100 g) and brans (46 mg/100 g) [144]. This fact leads to the thought of the possible utilisation of this last variety as a source for the commercial obtention of anthocyanins or for the elaboration of functional foods. On the contrary, as can be seen in Table 5, red wheat hardly contains anthocyanins.

These same authors separated, by HPLC, the anthocyanins present in the extracts corresponding to blue and purple wheat. Cyanidin-3-glucoside was the principal one in the samples corresponding to purple wheat, whereas it was the second principal in samples of blue wheat, which seems to indicate that each type of wheat has a characteristic anthocyanic profile. Regarding the principal anthocyanin present in blue wheat, at present it remains without having been identified.

Significant differences were found in the content of total anthocyanins from one year to another, indicating that their synthesis is influenced by environmental factors. In fact it has been demonstrated by diverse authors that determined environmental factors, such as the quantity of UV-B received by the plant, alter the expression of defence genes giving rise, among other changes, to an increase in the synthesis of anthocyanins [145]. The magnitude of the environmental effects was observed to be greater in purple wheat than in blue wheat, which was attributed to the different localisation of the pigments in the wheat grain [143]. The purple pigments are localised in the pericarpium (outer cover), whereas the blue pigments are found in the aleurone layer [146].

3.4. Sorghum

As a response against situations of stress, such as fungal infections, some plants produce phytoalexins [147–151]. At present, more than 300 phytoalexins have been identified and characterised [149,152]. These compounds are secondary metabolites which act as antimicrobials forming part of an active defence mechanism which protects the plant from the infection [152–154]. Since it was discovered that the phytoalexins served as factors of resistance in the defence of the plants against microorganisms [155,156], diverse investigations have questioned when, where and how these compounds are produced and act.

Few species of monocotyledons are capable of synthesising phytoalexins, among them, sorghum (*Sorghum bicolor* L.) produces pigmented phytoalexins after inoculation with various fungi, both pathogenic and non-pathogenic [147,150,157–162].

The phytoalexins of sorghum are 3-deoxyanthocyanidins, an unusual type of anthocyanins which present a structure in which position 3 of the oxygen heterocycle of the flavylium cation is not substituted. In lower plants 3-deoxyanthocyanidins have been found in musci [163] and pteridophytes [164,165] and in few species of higher plants, among them in *Sorghum* sp. Here, the 3-deoxyanthocyanidins seem only to be formed after fungal infection, accumulating in cellular inclusions [147,166] which are presented in vesicles derived, probably, from the ER-Golgi apparatus [147,166] and which are localised around the area of infection in the cells which are under the fungal attack.

It appears that the host tissue can rapidly detect the presence of the pathogen and as soon as the fungus begins to penetrate, the host initiates the defence. A rise in the phenylalanine ammonia-lyase (PAL) has been observed and the accumulation of PAL transcripts in response to infection with a non-pathogen, *Bipolaris maydis* [167]. This enzyme is necessary for the synthesis of phenols in general and particularly anthocyanins [168–172] and it has been frequently demonstrated that a rise in the level of its activity is produced as an early response of plants against pathogens [173–178] or environmental stress [179–181]. In the tissues of sorghum affected by the infection, an accumulation of transcripts that codify chalcon synthase, a key enzyme in the synthesis of flavonoids, is observed [148,182–184] and an increase in the activity of the enzyme [185].

Aguero et al. [186], observed that the concentration of 3-deoxyanthocyanidins of sorghum inoculated with *Cochliobolus heterotrophus* presents a accumulation curve with a maximum rate between 10 and 32 h after the inoculation. These same authors demonstrated that the 3-deoxyanthocyanidins begin to be produced hours before what had been previously assumed [158]. The analyses carried out by HPLC showed

the presence of phytoalexins 4 h after the inoculation, which confirms that the host can rapidly detect the presence of the infecting agent.

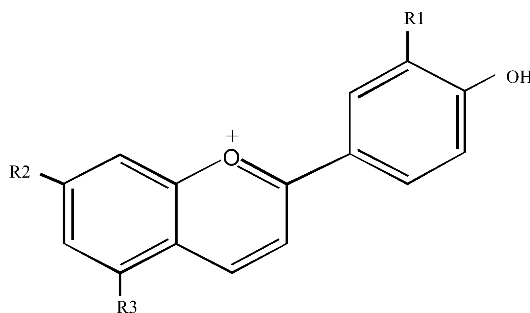
With regard to the expression of the fungotoxicity, this takes place when the compounds are released from the inclusions [147,186]. It seems that these pigments are toxic for the pathogens at concentrations of less than 10 μM [158,159], although they accumulate in the infected tissue at much higher concentrations than those necessary to exercise fungotoxicity [166]. This often makes these compounds toxic for the plant, killing the cell that synthesised them at the same time as they restrict the growth of the pathogen.

3.4.1. *Identification of anthocyanins in sorghum*

The induction of the formation of deoxyanthocyanidins inoculating fungal conidia in sorghum is a habitually used practice with the objective of obtaining sufficient quantities to allow them to be identified [159,160,187]. Once the compounds are synthesised by the plant, their extraction is usually performed incorporating the habitual solvents of anthocyanins, methanol or ethanol, slightly acidified on occasions, to the samples of tissue, the extraction being favoured if the contact between the material and the solvent is maintained at low temperature for 12–24 h [160,186,188,189]. Other authors [190], due to the lesser polarity of the 3-deoxyanthocyanidins, prefer extraction with chloroform, although they use other plant sources.

Various authors have identified some components of the phytoalexin complex from leaves and mesocotyls of infected bicolour sorghum. The most utilised techniques in the last years, with identification aims, are characterised by their great sensitivity. Plasma desorption mass spectrometry (PDMS), which has been used satisfactorily to analyse phenolic compounds [187,191], has also been used for the identification of phytoalexin deoxyanthocyanidins in sorghum plant tissue [159,187,191–193]. Matrix-assisted laser desorption ionization mass spectrometry (MALDI) permits the analysis of very small quantities of compound in purified samples, quantities between 5 and 15 pmol/μl, which makes this technique useful for the detection of these pigments in plant tissues [160]. Furthermore, MALDI has the advantage that the extracts can be analysed directly, previous purification being unnecessary, whether by HPLC or liquid–liquid extraction, which avoids the loss of sample during the pre-treatment.

The phytoalexin-3-deoxyanthocyanidins that have been identified in sorghum up to the present are shown in Fig. 3. The principal compounds present in the plant are apigenidin and luteolinidin [147,150,166,186,187,194–196], although if the infection is severe a caffeic acid ester of arabinosyl-5-*O*-apigenidin also accumulates [150,158,186,187]. Sugui et al.



	R1	R2	R3	$[M]^+$ (amu)
Apigenidin	H	OH	OH	255
Luteolinidin	OH	OH	OH	271
Caffeic acid ester of Arabinosyl 5-O-Apigenidin	H	OH	HO, HO, CH = CH – C(=O) – O – CH$_2$, O, O, OH, H, H, OH	549
7-Methoxy-Apigenidin	H	OCH_3	OH	269
5-Methoxy-Luteolinidin	OH	OH	OCH_3	285

Fig. 3. 3-Deoxyanthocyanidins identified in sorghum, substitution pattern and molecular ion (*m*/*z*) recorded in positive mode.

[160], using MALDI, found in the mass spectrum obtained for extracts corresponding to etiolated sorghum seedlings which had been inoculated with conidia of the fungus *Cochliobolus heterostrophus*, peaks at *m/z* 255, 269, 271 and 285. Ions which concord with the following deoxyanthocyanidins: apigenidin, 7-methoxy apigenidin [186,196], luteolinidin and 5-methoxy luteolinidin [150,159].

Moreover, the distinction between derivatives of luteolinidin and apigenidin can also be made by bathochromic shift analyses, since the derivatives of luteolinidin, after the addition of aluminium chloride, present a bathochromic shift of 10 nm as a consequence of a pattern of ortho dihydroxylation [159,197]. Similarly, which hydroxyl group (OH-5 or OH-7) of ring A of the deoxyanthocyanidin presents substitution can be determined, since the addition of sodium acetate causes an immediate bathochromic shift of approximately 70 nm when the molecule of the compound presents a hydroxyl group without substitution in position 7 [159].

Concerning the non-phytoalexin anthocyanidins present in sorghum, cyanidin-3-dimalonyl glucoside has been identified [160,189] and glycosides of cyanidin and pelargonidin not characterised [112]. The synthesis of these pigments is independent of the possible fungal infection of the plant, although it has been observed that red light and UV-A induce their formation [198].

3.4.2. *Perspectives of utilisation of the anthocyanins of sorghum*

3.4.2.1. Control of plagues. In vitro, the 3-deoxyanthocyanidins present toxicity against some bacteria (Stonecipher et al. [199]) and fungi, including *Colletotrichum sublineolum* [147,159,200].

This fungus, which is the agent that causes anthracnosa of sorghum [201–203], is extremely infective and produces important damage in susceptible crops. There are, nonetheless, strains of sorghum that are resistant to this fungus. Lo et al. [150] extensively studied the role of the phytoalexins of sorghum in the resistance to this disease and concluded that the evaluation of this response could contribute directions for the development of new strategies of control. They observed that, after the inoculation with *Colletotrichum sublineolum* in the plants, the resistant crop produced luteolinidin, 5-methoxyluteolinidin, apigenidin and a caffeic acid ester of 5-*O*-arabinosyl-apigenidin. Nevertheless, only apigenidin and its acyl ester derivative accumulated in the susceptible strain. In assays in vitro it has been demonstrated that luteolinidin and 5-methoxyluteolinidin present greater toxicity than the apigenidin derivatives against the pathogens [159]. Various studies have demonstrated that the methoxylated flavanoid compounds generally have greater anti-fungal capacity than the non methoxylated precursors [203]. Effectively, studies carried out by Lo et al. [159], showed that 5-methoxyluteolinidin presents greater fungotoxicity than luteolinidin, a non-methylated phytoalexin.

The difference between luteolinidin and apigenidin is based on the presence or absence of a hydroxyl group in position 3′, which is the result of the activity of a 3′-hydroxylase. Moreover, the formation of methoxyluteolinidin requires the *O*-methylation of luteolinidin, a process which generally occurs in the last stage in biosynthetic routes [204]. The isolation and characterisation of the genes involved in the synthesis of the enzymes responsible could permit metabolic engineering to manipulate the levels of these phytoalexins, achieving a greater resistance of the plant to diseases [205]. The transference of determined genes involved in the synthesis of phytoalexins could increase the resistance to some fungal pathogens in transgenic plants [150].

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