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New Flavanol–Anthocyanin Condensed Pigments and Anthocyanin Composition in Guatemalan Beans (*Phaseolus* spp.)

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It has long been considered that the pigments resulting from direct condensation between anthocyanins and flavanols were formed exclusively during storage and processing, both in plant-derived foods and in drinks. Recently, however, the minor presence of this type of pigment has been shown in different plant extracts, among them beans. In this work we have studied this family of pigments in beans from Guatemala belonging to two distinct species of the genus *Phaseolus*, confirming the presence of (epi)gallocatechin carbon−carbon linked to the aglycone of delphinidin and (epi)catechin-cyanidin-3-glucoside. Furthermore, for the first time in this foodstuff another three flavanol−anthocyanin condensed pigments formed by the linkage of (epi)catechin with the corresponding aglycones of cyanidin, petunidin, and peonidin have been described. Also, the natural occurrence of anthocyanidins in this matrix has been confirmed in some of the samples analyzed in this work, the majority being components of their anthocyanin composition. The corresponding aglycones of delphinidin, cyanidin, petunidin, pelargonidin, and malvidin have been identified as well as two aglycones with identical mass to those of peonidin and petunidin, but with different retention times, which leads to the supposition that they are probably methylated derivatives of cyanidin and delphinidin, respectively.

KEYWORDS: Anthocyanins; flavanols; condensed pigments; beans; *Phaseolus*

INTRODUCTION

In general, foods are complex matrixes containing a great diversity of chemical substances capable of interacting to produce new compounds. These new compounds, on numerous occasions, influence the stability of the food and its organoleptic characteristics; thus, their study has been of interest to researchers. One of the families in which this type of transformation has been most studied is that of the polyphenols, since they constitute a group of very reactive compounds due to the acid character of their hydroxyl groups and the nucleophilic properties of their phenolic rings (*1*−*3*). Among the many reactions that can occur, the processes of oxidation and the addition reactions between anthocyanins and tannins are outstanding. There are numerous studies, principally in wine, of the pigments derived from anthocyanins, both pyranoanthocyanins (*4*, *5*) and pigments resulting from condensation mediated by aldehydes between a flavanol and anthocyanins (*3*, *6*, *7*) and also pigments resulting from direct condensation between anthocyanins and flavanols (*8*). Although the existence of this last type of pigment was proposed in the 1960s, it is, perhaps, the group about which there are the least references.

For the pigment formation from direct condensation two mechanisms are proposed. The first is by nucleophilic addition of the flavanol (C-6 or C-8) on the C-4 of the anthocyanins in the flavylium form giving a colorless dimer in which the anthocyanin is in the flavene form. This structure can oxidize to give the corresponding flavylium form or rearrange to a yellow xanthylium ion (*9*, *10*). By this same mechanism it has been proved that colorless flavene−flavanol adducts undergoing cyclic condensation with an A-type interflavonoid bond can originate (*11*, *12*). The second mechanism proposed begins with the acid cleavage of the interflavanic bond of an oligomer flavanol giving the corresponding carbocation which acts as electrophile (C-4) with addition on the C-8 or C-6 of the anthocyanins, in their hydrated forms (leucoanthocyanins), giving the colorless adduct which easily dehydrates to the corresponding flavylium form of the anthocyanins with red color. All these pigments derived from anthocyanins are characterized by bathochromically displaced UV−vis maxima with regard to the original anthocyanins, having absorbance maxima around 540 nm and other maxima at 280 and between 440 and 450 nm (*13*).

For a long time it has been considered that these types of compounds were formed exclusively during the storage and processing both of plant-derived foods and drinks, wine being

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one of the best-studied matrixes. Different research groups have detected, in wine, the presence of adducts of the flavylium−flavanol and flavonol−flavylium types (*14*, *15*). At present, however, it is known that these pigments also appear naturally, although in small quantities in unprocessed plants and foods (*16*). Our research group has identified this type of pigment in different plant products species (*17*); the presence, in strawberries, of four different condensed pigments corresponding to links between derivatives of pelargonidin with glucose or rutinose linked to molecules of (epi)catechin or (epi)afzelechin is noteworthy. In this same study, it was also demonstrated that these types of compounds appeared in other matrixes, such as purple maize ((epi)catechins linked to cyanidin 3,5-diglucoside) or grapes (condensed pigments derived from peonidin or malvidin linked to (epi)catechins).

The presence of naturally occurring aglycones in the samples of Spanish beans, all of them belonging to the genus *Phaseolus* (*18*), suggests the possibility of finding, in this foodstuff, products of direct flavanol−anthocyanidin condensation, which was confirmed in samples of *Phaseolus* in which derivatives of cyanidin linked to(epi)gallocatechin or (epi)catechins were detected (*17*). The principal objective of this work was to increase our knowledge in the study of the presence of this type of pigment, derived from direct condensation between flavanols and anthocyanins, in beans from Guatemala belonging to two distinct species of the genus *Phaseolus* and also the presence of aglycones in the species analyzed.

MATERIALS AND METHODS

Samples. The samples analyzed in this study were collected in the region of Alta Verapaz (Guatemala). Samples A and B belong to the species *P. coccineus* (scarlet red runner beans), whereas C and D correspond to seeds of *P. vulgaris* L. (kidney bean). The determination of the species was based on botanical analysis.

Method of Extraction and Purification. The seed coat of 2 g of beans was manually separated and ground until a fine homogeneous powder was obtained. The powder obtained was submitted to successive extraction with MeOH which contained 0.5% HCl. To favor the extraction of the anthocyanins the sample was kept in an ultrasonic bath for 15 min and then submitted to continual mechanical shaking, completing a total of 26 h of extraction (*18*). The methanolic extract was evaporated in a vacuum, yielding an aqueous phase which was purified by successive extractions with hexane and a second process of purification using Strata C18-E SepPak (Phenomenex, Torrance, CA). Later, the anthocyanins retained in the cartridge were eluted by MeOH containing 0.01% HCl, and the eluent was evaporated and made up to 2 mL with ultrapure water.

Acid Hydrolysis. A solution of 6 N HCl was added to an aliquot of purified extract in a N_2 inert atmosphere. The resulting solution was kept in a boiling water bath for 40 min. It was then rapidly cooled in crushed ice and chromatographically analyzed (*19*).

Reaction with Aluminum Chloride. The solution was prepared by carefully adding 100 mL of MeOH to 5 g dry weight of $AlCl_3$. Six drops of the solution (*20*, *21*) were added to the isolated anthocyanin compound, and the UV−vis spectrum was obtained immediately with a Hewlett-Packard 8453 spectrophotometer.

Anthocyanin Standards. Commercially available standards of 3-*O*-*β*-glucopyranosides of delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin (Polyphenols Labs., Sandnes, Norway) were dissolved in aqueous HCl (pH 0.5) and used as standard stock solution.

Analysis by HPLC. The identification of the anthocyanins present in the sample was carried out using the Hewlett-Packard 1100 chromatographic system coupled to a Finnigan LCQ mass spectrometer with an ESI-type ionization source and ion trap detector. The separation of the anthocyanins was performed using a 150 mm × 4.6 mm i.d., 5 *μ*m Aqua C18 column (Phenomenex).

The chromatographic conditions were as follows: flow 0.5 mL/min; injection volume 100 *μ*L, column temperature 35 °C; and solvents A, 0.1% trifluoroacetic acid (TFA), and B, HPLC grade acetonitrile. The gradient consisted of 10−15% B for 20 min, 15−18% B for 10 min, and 18−35% B for 20 min. Detection was carried out with a Hewlett-Packard diode array detector at 280, 320, and 520 nm as preferred wavelengths (*22*).

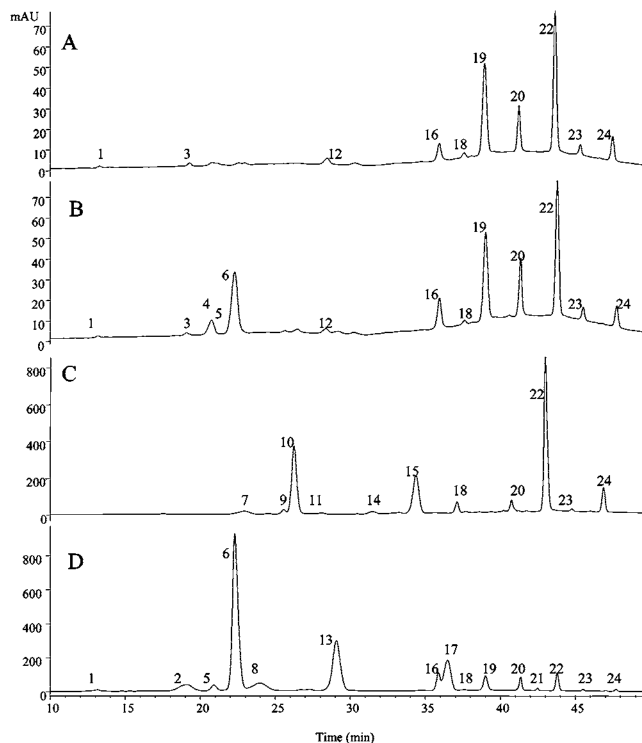


Figure 1. Chromatograms recorded at 520 nm, corresponding to a methanolic extract of the samples: (**A** and **B**) *Phaseolus coccineus* L, (**C** and **D**) *Phaseolus vulgaris* L.

Mass spectrometric analysis was performed using an electrospray ionization (ESI) probe. The capillary temperature and voltage used were 195 °C and 4 V, respectively, and spectra were obtained in positive ion mode. Both the auxiliary and the sheath gas were nitrogen at a flow rate of 6 and 1.2 L/min, respectively. The MS detector was programmed to perform a series of three consecutive scans: a full scan, an MS^2 scan of the most abundant ion in the first scan, and an MS^3 of the main fragment ion in the MS^2 spectrum, using a normalized collision energy of 45%.

Semipreparative HPLC. Some pigments present in the beans were isolated by semipreparative HPLC using a Waters 600 chromatograph. The column was an Ultracarb ODS column (250 mm × 10 mm i.d., 5 *μ*m particle size, Phenomenex). Solvents were 5% acetic acid (A) and HPLC grade methanol (B) with the following gradient: from 15% to 20% B for 10 min, from 20% to 25% B over 10 min, from 25% to 35% B over 10 min, isocratic 35% B for 10 min, from 35% to 15% B over 10 min, and from 15% to 50% B over 5 min at a flow rate of 3 mL/min. Detection was carried out at 520 nm using a UV−vis detector (Waters 486), and the peaks were collected, each min, in a fraction collector (FRAC-100, Pharmacia).

RESULTS AND DISCUSSION

The chromatographic profiles of the four samples analyzed are shown in **Figure 1**. A total of 24 different compounds were detected whose UV−vis characteristics and mass spectra data are presented in **Table 1**. Of these 24 compounds, 12 were glycosides, 5 minor pigments resulting from direct condensation between flavanol−anthocyanins, and 8 were identified as anthocyanidins, aglycones of the anthocyanins. The spectrum characteristics and the retention times, coincident with those of the standards used in our laboratory, permitted the identification of the corresponding monoglucosides of the anthocyanins

Table 1. List of Compounds Detected in the Group of Samples

peak	compounds	[M+] (m/z)	MS2 (m/z)	MS3 (m/z)
1	(epi) gallocatechin-delphinidin	607	439[a], 345[b], 481[c], 303[d]	421, 377
2	petunidin-3,5-diglucoside	641	317, 479, 302	317, 302
3	(epi)catechin-cyanidin-3-glucoside	737	575[e], 329[b], 423[a], 449[c]	557[e,f], 287[d]
4	delphinidin-3-glucoside-5-pentoside	597	303, 465	303
5	delphinidin-3-galactoside	465	303	303
6	delphinidin-3-glucoside	465	303	303, 275
7	peonidin-3,5-diglucoside	625	301, 463	285, 301
8	malvidin-3,5-diglucoside	655	331, 493, 475	331, 315
9	cyanidin-3-glucoside-pentoside	581	287, 241	287, 193
10	cyanidin-3-glucoside	449	287	287
11	(epi)catechin-cyanidin	575	423[a], 329[b], 435[g], 287[d], 449[c]	361, 405, 287[d]
12	(epi)catechin-petunidin	605	359[b], 421, 453[a], 587[f], 465[g]	359, 317[d]
13	petunidin-3-glucoside	479	317	317
14	pelargonidin-3-glucoside	433	271	271
15	peonidin-3-glucoside	463	301, 257	285, 301
16	delphinidin	303	303, 275, 257	303, 257
17	malvidin-3-glucoside	493	331, 316	331, 316
18	(epi)catechin-peonidin	589	437[a], 343[b], 449[g], 463[c]	405, 301[d]
19	methyldelphinidin	317	317, 301, 275	317, 301
20	cyanidin	287	287, 259, 219	287, 231
21	petunidin	317	302, 317, 271	274, 227
22	methylcyanidin	301	285, 301, 257	258, 285
23	pelargonidin	271	271, 239, 225	271
24	malvidin	331	331, 316, 299	331, 316

[a] RDA fission. [b] Partial loss of flavanol. [c] Loss of A ring in the flavanol. [d] Loss of complete flavanol. [e] [M+] – glucose. [f] [M+] – H_2O. [g] [M+] – 140 amu.

delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin (peaks 6, 10, 13, 14, 15, and 17, respectively). The mass spectrometric data of these peaks corroborated the proposed identification, always considering, when carrying out the MS-MS analysis, the loss of the corresponding glucose (−162 amu). The presence of these compounds in different species of *Phaseolus* has already been described by other authors (*23*, *24*).

In the cases of samples B and D a compound (peak 5) appears with similar characteristics, both in the UV−vis and the mass spectrometric analysis, to those of delphinidin-3-glucoside. Taking into account its shorter retention time, this compound has been tentatively identified as delphinidin-3-galactoside. Recently, Wu and Prior (*25*) indicated the presence in beans of galactosides of delphinidin, petunidin, and malvidin.

In samples C and D, both from *P. vulgaris*, the presence of 3,5-diglucosides of petunidin (peak 2), peonidin (peak 7), and malvidin (peak 8) was detected. The diglucosides of petunidin and malvidin have already been described by Yoshida et al. (*23*) in this same species, whereas the presence of peonidin-3,5-diglucoside is described, as far as we are aware, for the first time in beans. When our research group analyzed the anthocyanin profile in *P. vulgaris* var. Tolosa (*18*), none of these diglucosides appeared; however, the diglucosylated derivatives of cyanidin and pelargonidin did, which indicates that the anthocyanin profile does not depend exclusively on the species, but is controlled by other factors, among them the variety. None of these diglucosides appeared in the samples of *P. coccineus* analyzed.

In one of the samples of *P. vulgaris* (sample C) a compound (peak 9) appeared with spectrum characteristics similar to those of cyanidin, which showed a molecular ion $[M]^+$ at *m/z* 581 that, when fragmented, gave an ion at *m/z* 287, which would correspond to the loss of a disaccharide constituted by hexose (162 amu) + pentose (132 amu). As it was a minor compound, it has not been possible to isolate it to perform an analysis which would permit the confirmation of the identity of the sugars that constitute it, nor their exact position. Nonetheless, the data obtained from the mass spectrum are identical to those described by Giusti et al. (*26*) for the identification of cyanidin-3-xylose-glucose (cyanidin-3-sambubiose) present in *Hibiscus sabdariffa* L. Although, until now, the presence of the sambubioside of cyanidin in beans has not been described, Wu and Prior (*25*) suggest the existence of pelargonidin-3-sambubioside in small red bean. The evidence indicates that this compound can be identified as cyanidin-3-sambubiose.

In sample B a compound (peak 4), overlapping peak 5, appeared, whose MS characteristics were similar to those of peak 9. This compound showed a molecular ion at *m/z* 597 which fragments to give ions at *m/z* 465 (−132 amu) and 303 (−294 amu) which would correspond to the loss of a pentose and a hexose plus pentose. When the sugars were linked to the same position, the MS^2 spectra shows only the fragment ion corresponding to the aglycone. In this case we observed two sequential losses of 132 (pentose) and 294 (hexose + pentose) mass units, which would indicate that the sugars are linked to different positions. This peak probably corresponds to delphinidin-3-glucose-5-pentose and is the only diglucoside present in samples of *P. coccineus*. As far as we know, the presence in beans of diglucosides having a hexose and a pentose situated in different positions has not been described.

In the samples, using mass spectrometric analysis data (**Table 1**) and the spectrum characteristics (**Figure 2**), it has been possible to identify minor compounds produced by direct condensation between (epi)catechin and cyanidin, petunidin, and peonidin aglycones (peaks 11, 12, and18) and the previously described (*17*) (epi)gallocatechin(4α→8)delphinidin and (epi)-catechin(4α→8)cyanidin 3-glucoside (peaks 1 and 3). This type of compound has characteristics, both in the UV−vis spectra and in the MS, common to all of them, with a bathochromic displacement of the spectra maxima in a range of 10−15 nm in the visible region in relation to that of the parent anthocyanins base. With regard to MS analysis, all of them have a common pattern of fragmentation. In **Figures 3** and **4** the mass spectrum and the fragmentation scheme for pigment 18, detected in the four samples, are presented.

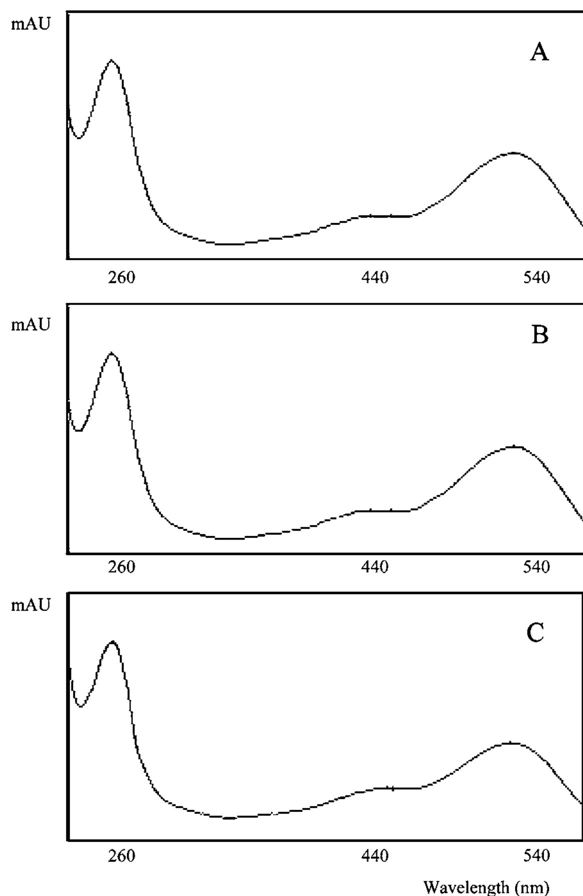


Figure 2. UV–vis spectra of the three condensed pigments: (**A**) (epi)-catechin-cyanidin, (**B**) (epi)catechin-petunidin, and (**C**) (epi)catechin-peonidin.

Pigment 18 shows a molecular ion [M^+] at *m/z* 589. In the MS^2 spectrum different fragments are observed, the majority being the ion of *m/z* 437 (−152) that corresponds to a retro Diels−Alder cleavage of the flavanol. This cleavage involves a loss of 152 amu if it is a unit of (epi)catechin (peaks 3, 11, 12, and 18) or 168 amu if the flavanol that constitutes the structure is an (epi)gallocatechin, as in peak 1. The second important fragment is the ion at *m/z* 343, and that corresponds to the partial elimination of the flavanol unit which gives rise to a fragment with loss of 246 amu, when the flavanol is (epi)-catechin, or of −262 if it is (epi)gallocatechin. The ion of *m/z* 571 (−18 amu) is detected with similar intensity, which corresponds to a loss of a molecule of water.

It was also observed that in all the pigments of this type the complete loss of the flavanol (ion at *m/z* 301 in peak 18) that produces fragments with −288 amu when the flavanol is (epi)-catechin, or −304 (peak 1) if it is (epi)gallocatechin. The loss of 288 amu occurs when the (epi)catechin is situated in the upper unit; if the flavanol is found in the terminal unit the loss that would be observed would be of 290 amu (*27*). In our case losses of 290 amu have never been observed, which confirms that the flavanol is always found as the upper unit.

In most of these types of pigments, including the one being described here, the appearance of a fragment corresponding to the loss of 140 amu is observed, and that, we believe, is due to the cleavage of the flavanol, producing the loss of dihydroxy-benzaldehyde from the B ring to give a fragment at *m/z* 449. As noted by Friedrich et al. (*28*) and also observed by Salas et al. (*15*) for flavanol−flavylium adducts found in red wines, the loss of a $C_6H_6O_3$ fragment is characteristic of the upper flavanol unit, which would, once more, confirm that the anthocyanin is always found in the lower unit in this type of pigment.

The MS data do not permit us to obtain information about whether the flavanol anthocyanin bond occurs in position C4−C8 or at C4−C6. Fossen et al. (*16*) in pigments of strawberries, similar to those described here, established by NMR that the union between the flavanol and the anthocyanin was always of the C4−C8 type; thus, it is probable that in our case it is the

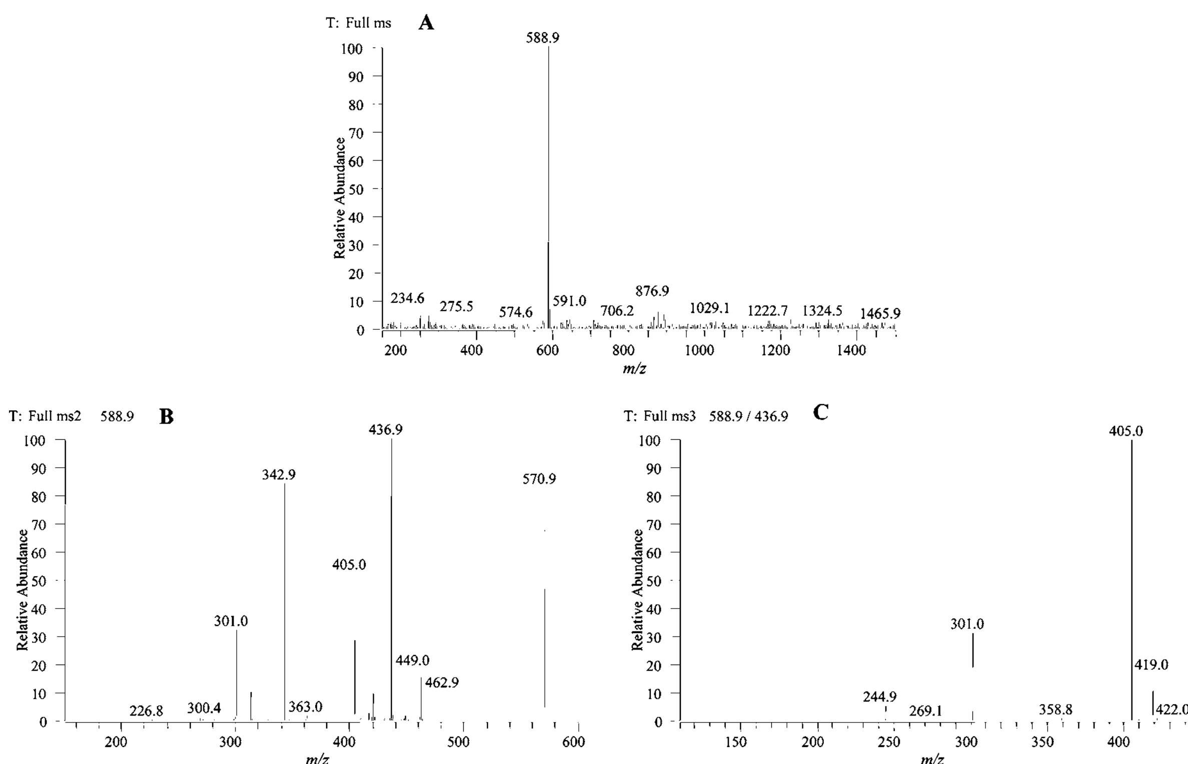


Figure 3. (**A**) MS, (**B**) MS^2, and (**C**) MS^3 spectra corresponding to (epi)catechin-peonidin (peak 18).

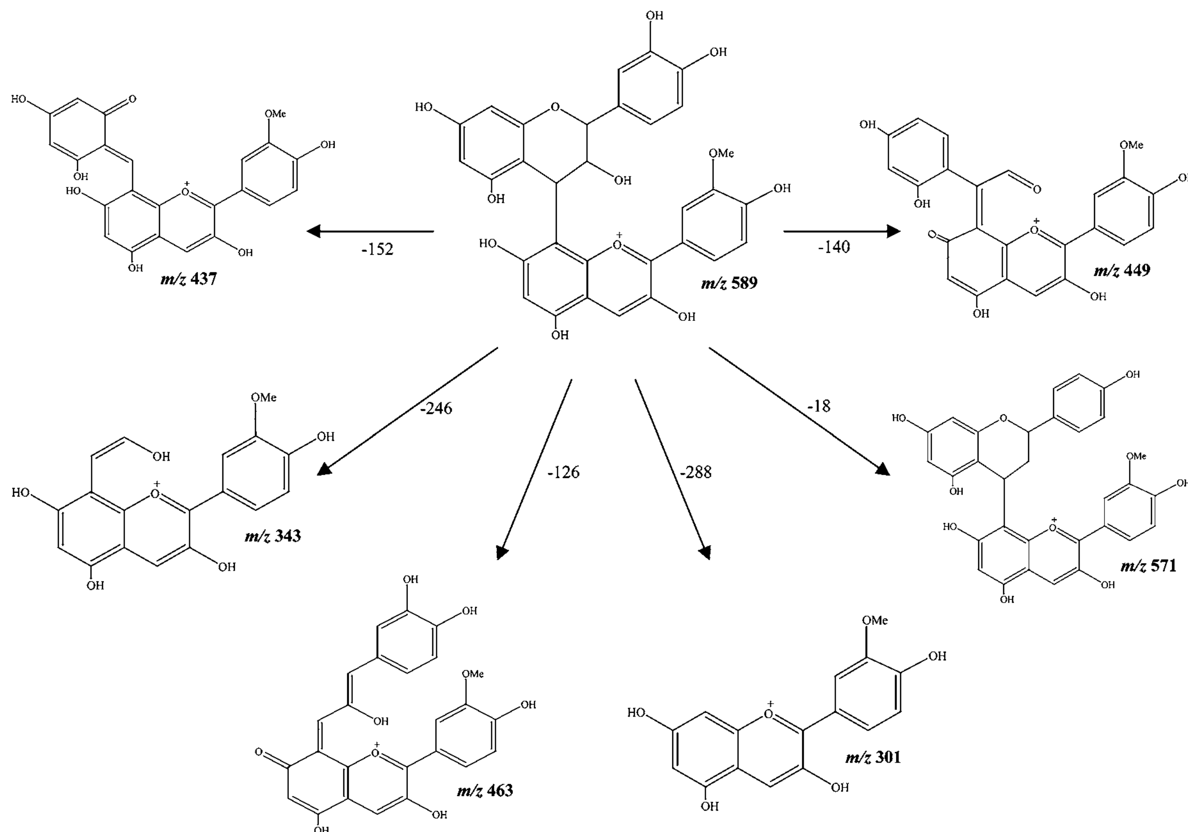
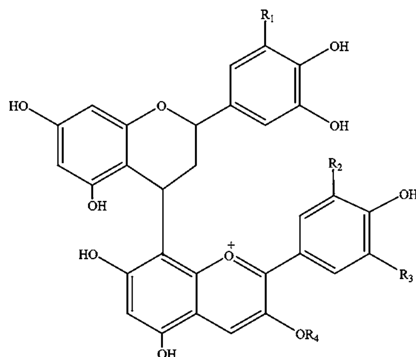


Figure 4. Fragmentation scheme proposed for peak 18.



Peak	Compound	R_1	R_2	R_3	R_4
1	(epi)gallocatechin-delphinidin	OH	OH	OH	H
3	(epi)catechin-cyanidin 3-glucoside	OH	H	OH	Gluc
14	(epi)catechin-cyanidin	OH	H	OH	H
18	(epi)catechin-petunidin	OH	H	OMe	H
23	(epi)catechin-peonidin	OH	H	OMe	H
27	(epi)afzelechin -peonidin	H	H	OMe	H

Figure 5. Proposed structures for the condensed pigments detected in the group of samples studied.

same. The structures proposed for the five direct condensed pigments detected are presented in **Figure 5**.

Aglycones. The presence of the aglycones of anthocyanins has been confirmed in the samples analyzed (**Table 1**), this type of compound being in the majority in all except sample D. In the four samples analyzed the aglycones corresponding to cyanidin (peak 20), pelargonidin (peak 23), and malvidin (peak 24) appeared. The presence of these compounds in beans has been discussed previously (*18*), although in the study carried out with samples of *P. vulgaris* L. var Tolosa the presence of malvidin was not detected. In samples A, B, and D, moreover, the aglycone of delphinidin (peak 16) appeared, and the anthocyanidin petunidin (peak 21) appeared only in sample D. In the chromatograms, the presence of two compounds (peaks 19 and 22) was demonstrated, whose retention times and spectrum behavior corresponded to this group of compounds. When the mass analysis was performed for these compounds, in the majority of samples A, B, and C, the appearance of molecular ions at *m/z* 317 and 301 were observed, equal to those assigned to petunidin and peonidin, although the retention time does not coincide with that of these anthocyanidins. To confirm that the compounds were really different it was decided to carry out an acid hydrolysis on sample D in which all the anthocyanidins except peonidin were present. These data were compared

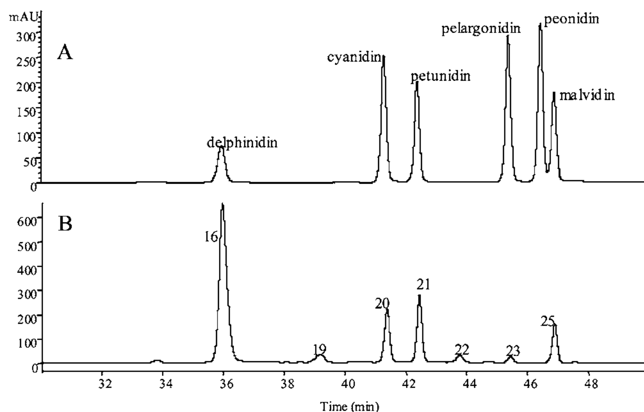


Figure 6. Chromatograms, recorded at 520 nm, corresponding to the hydrolysis performed on (**A**) a mixture of standards of anthocyanins and (**B**) extract of sample D.

with the characteristics resulting from a hydrolysis, in the same conditions, applied to a mixture of anthocyanin standards. As can be seen in **Figure 6**, on performing the hydrolysis of the sample, seven different peaks appeared, confirming that peaks 19 and 22 do not correspond to the aglycones peonidin and petunidin. Structures that would be in agreement with the *m/z* values observed could be those of the methylated derivatives, at positions 3, 5, or 7, of cyanidin and delphinidin. The methylcyanidin would have the same mass as peonidin and the methyldelphinidin the same as petunidin.

Strack and Wray (*29*) reported, in a review on the presence of anthocyanins in nature, the existence of compounds derived from cyanidin methylated at position 5. Nonetheless, on reviewing the original work (*30*), in which the presence of 5-*O*-methylcyanidin 3-glucoside was described, no data was found that could be utilized in our study for the confirmation of the identities of the compounds.

To attempt to definitively discount that these compounds were derivatives of peonidin and petunidin it was decided to use chemical methods that permitted the elimination, at least in the case of peonidin, of the possible presence of a methyl group in ring B. The reaction was performed with $AlCl_3$ as described in Mabry et al. (*20*), according to which the ortho-dihydroxylated compounds form an acid-stable complex with the aluminum which causes a bathochromic displacement of the maximum in band I, close to 25 nm. (*21*). If peak 22 were a derivative of peonidin the reaction should be negative, whereas if, as we suppose, it is a methylated cyanidin (in position 3, 5, or 7) this reaction would be positive. In the case of peak 19, this test was not useful, since, independently of whether the compound was the anthocyanidin petunidin or a derivative of delphinidin, the reaction in both cases would be positive. The pigment corresponding to peak 22 was isolated as indicated in the Materials and Methods. The results of this test are presented in **Figure 7**, showing that after the addition of $AlCl_3$ the compound undergoes a displacement in the visible region, shifting the maximum absorbance from 519 to 542 nm. The same assay was performed with a standard of cyanidin and similar behavior being observed. Thus it was concluded that peak 22 could not be an aglycone of peonidin and was very probably a methylcyanidin. In the case of peak 19, bearing in mind its similar behavior, it is probable that it is methyldelphinidin.

As has been previously indicated (*30*) only the existence of a glucosylated derivative of cyanidin methylated at position 5 appears to have been described. To determine whether in our case the substitution was found in that same position both isolated compounds were incubated with pyruvic acid, as

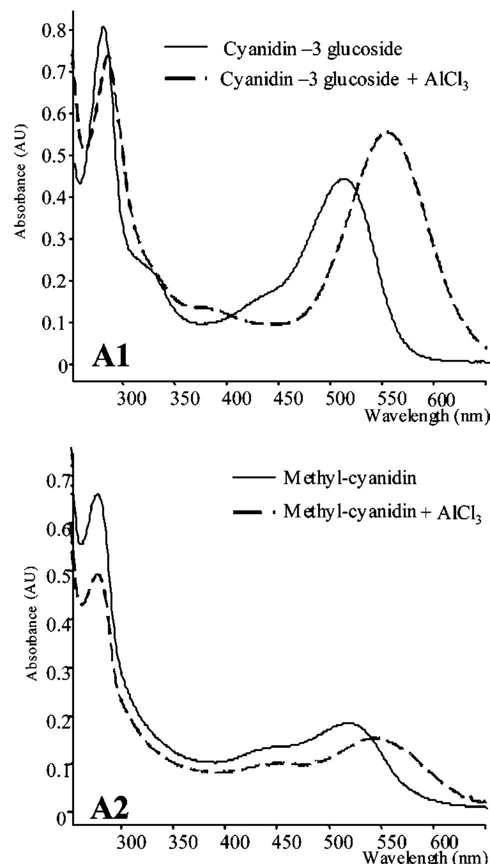


Figure 7. UV–vis spectra obtained in the reaction with $AlCl_3$ with the standard of cyanidin-3-glucoside **(A1)** and with peak 22 **(A2)**.

described by García-Alonso et al. (*31*). In these conditions, if there is a free hydroxyl group at position 5 the corresponding pyranoanthocyanin (vitisin A) should be formed, whereas if that position were occupied the condensation would not be possible. In both cases the corresponding vitisins were obtained, discounting the methylation in position 5. It seems probable that the methyl is at position 3, since when an anthocyanidin is glycosylated, the first position always substituted is the hydroxyl at position 3. Thus, in this case, bearing in mind the absence of this sugar, it would be foreseeable that the substituting methyl would occupy that position. On the basis of the data obtained peak 19 has been tentatively identified as 3-methyldelphinidin and peak 22 as 3-methylcyanidin, although it would be necessary to obtain a greater quantity of sample which would allow the isolation of these two compounds to clarify their structure exactly by an NMR study.

In summary, the data obtained confirm the natural presence of anthocyanidins in the samples of beans and their contribution to the formation of pigments resulting from the direct condensation between a flavanol which could be (epi)catechin or (epi)-gallocatechin and an anthocyanin or anthocyanidin, depending on the cases.

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