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Food Chemistry 94 (2006) 448–456
Food Chemistry
www.elsevier.com/locate/foodchem

# Natural occurrence of free anthocyanin aglycones in beans (*Phaseolus vulgaris* L.)

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Received 20 September 2004; accepted 19 November 2004

**Abstract**

The application of an improved method of extraction and purification has allowed the characterization of anthocyanins and free anthocyanidins in beans, *Phaseolus vulgaris* L. using LC-MS determination.

HPLC analysis of the final extract showed the existence of seven compounds when 520 nm was the selected wavelength. Major peaks were identified, according to their retention times and UV–Vis spectra, as cyanidin and pelargonidin monoglucosides when they were compared to those of the in-house library. Taking into account mass spectrometric data, together with elution time and spectral features, it was possible to identify peaks corresponding to cyanidin and pelargonidin diglucosides. As far as we know, these anthocyanins have not been detected in *P. vulgaris* L. before.

The last three peaks in the chromatograms were thought to be acylated anthocyanins, due to their smaller polarity. However, MS spectra showed signals at *m/z* 287, 301 and 271, which would be in accordance with molecular ions corresponding to anthocyanidins: cyanidin, peonidin and pelargonidin, respectively. Moreover, these compounds had the same retention times and UV–Vis spectral features as those of aglycones arising from acid hydrolysis of the final extract.

These results demonstrate, for the first time, the presence, in natural form, of free aglycones in vegetable samples. Until now, the bibliographical data have indicated that, in nature, this type of compound is only found in heteroside forms.


*Keywords:* Anthocyanins; Anthocyanidins; Beans; Legumes; *Phaseolus*; LC-MS analysis

## 1. Introduction

The anthocyanins constitute one of the major groups of natural pigments and are responsible for many of the colours of both fruits and vegetables as well as flowers (Rivas-Gonzalo, 2003; Strack & Wray, 1993). In recent years numerous studies have been carried out whose main objective has been to characterise the profiles of anthocyanins of different natural products, among them beans (*Phaseolus vulgaris* L.), to use this type of phenolic compounds as an alternative to the synthetic colorants used in the food industry (Harbone & Grayer, 1988; Hong & Wrolstad, 1990).

The anthocyanins and anthocyanidins, aglycones of the anthocyanins, have also aroused the interest of researchers, having shown, principally in "in vitro" studies, beneficial effects for health. There are numerous bibliographical data in which the anti-inflammatory, vasotonic, and anti-oxidant properties of the anthocyanins and their respective aglycones are cited (Cardador, Loarca, & Domah, 2002; García, de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2004; Tsuda, Ohshima,

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doi:10.1016/j.foodchem.2004.11.038

Kawakishi, & Osawa, 1994; Wang et al., 1997). They may, therefore, play an important role in the prevention of degenerative illnesses such as cancer, Alzheimer's disease or cardiovascular illnesses (Markham & Bloor, 1998).

From a technological point of view, one of the main advantages of the anthocyanins is their hydro-solubility, which facilitates their incorporation in different foodstuffs. Nevertheless, they present inconveniences with regard to stability, since, in solution, they are affected by light, changes in pH and especially by their combination with sulphur dioxide, which is used as a food preservative (Brouillard, 1982; Coutalte, 1986; Harbone & Grayer, 1988).

The anthocyanidins have limited solubility in water, are rapidly destroyed by alkalis and are photolabile. Moreover, they have a very short half-life in relation to their glycosylated derivatives. Due to this instability the anthocyanidins rarely appear in nature (Dao, Takeota, Edwards, & Berrios, 1998) and, up to the present time, no author has described the presence of these compounds in foodstuffs or any other natural source. Some authors have studied the determination and quantification of anthocyanidins in different foodstuffs. It must be borne in mind that these assays have always been performed by causing the formation of the anthocyanidins by acid hydrolysis of the base material and that, in these foodstuffs, they never appear naturally, but only as their corresponding glycosylated derivatives (Merken, Merken, & Beecher, 2001; Nyman & Kumpulainene, 2001).

Dao et al. (1998) determined that the aglycones of delphinidin, petunidin and malvidin, in an acidic methanol solution, disappear completely after 48 h at room temperature or four days in refrigeration. These same authors observed, nonetheless, that when the anthocyanidins remain contained in a $C_{18}$ cartridge their stability increases considerably, their concentrations remaining invariable for the first 7 days and then decreasing progressively, although after 45 days they were still detectable. Some authors have explained this increase in stability (Kähkönen & Heinonen, 2003; Nyman & Kumpulainene, 2001; Rivas-Gonzalo, 2003) as the result of the association between different flavylium cations. The almost flat structure of the anthocyanidins, with extended electronic de-localisation, contributes to the possible formation of much more stable molecular complexes.

In spite of the importance of the world consumption of beans, the data related to the anthocyanin composition of these legumes are scarce. The first work, in this respect, was carried out by Feenstra in 1960 (cited in Mazza & Miniati, 1993), describing the presence of the corresponding 3-glucosides of malvidin, petunidin, delphinidin and the 3,5-diglucoside of delphinidin in beans corresponding to *P. vulgaris* L. Other authors report similar results, moreover detecting small quantities of other anthocyanins, such as the 3-monoglucosides and 3,5 diglucosides of cyanidin and pelargonidin (Takeota et al., 1997). The greater part of the studies coincide in indicating that the principal anthocyanin is always delphinidin 3-glucoside (Choung, Choi, An, Chu, & Cho, 2003; Romani et al., 2004), although there are important differences related to the variety, as, for example, in *Phaseolus lunatus* L., where the principal anthocyanin is peonidin 3-glucoside, followed by peonidin 3-rutinoside (Yoshida et al., 1996).

In preliminary studies carried out by our research group, it had been observed that part of the differences found by other authors could be attributed to the process of extraction and purification of the anthocyanin extracts. Furthermore, these studies revealed the presence, in the extracts, of compounds whose spectral characteristics, as well as their retention times, coincided with those of certain anthocyanidins, which should be found in the foodstuff in natural form, since in no case had the beans been submitted to previous hydrolysis.

Taking into account the qualitative and quantitative differences found by different authors with regard to the anthocyanin content in beans of the genus *Phaseolus*, and also the possible presence of anthocyanidins in the foodstuff, the objective of this work has been to establish an optimum method of extraction, identification and quantification of the anthocyanins and or anthocyanidins present in Spanish beans *P. vulgaris* L. var. Tolosa by LC-MS.

## 2. Materials and methods

### 2.1. Extraction of the sample

Approximately 2 g of grains of bean, a commercial sample of *P. vulgaris* L. var. Tolosa, were manually peeled, fractionating the coating until obtaining a fine and homogeneous powder which was submitted to the extraction process.

To the powder obtained and previously weighed, 50 ml of MeOH containing 0.5% HCl were added. The sample was then kept, for 15 min, in an ultrasonic bath (Branson 5200) to favour the extraction process. After this time the sample was continually mechanically shaken for 2 h. Later, it was centrifuged (Kokusan, series H-103N) and the supernatant was collected by vacuum filtering. This process was repeated five more times, varying the time of continuous shaking: three successive intervals of 3 h each, one interval of 13 h and the last of 2 h, completing a total of 26 h of extraction.

The groups of aliquots were evaporated in vacuum, below 25 °C, adding a small amount of ultra-pure water to avoid the drying of the sample and obtain an aqueous extract (A1) (Fig. 1).

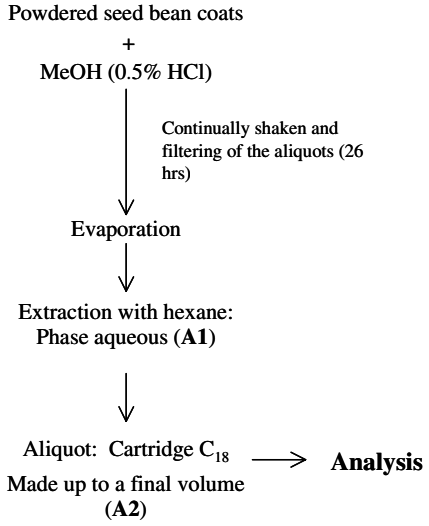


Fig. 1. Diagram of flow for the optimised method for the analysis of anthocyanins in beans.

### 2.2. Purification

Once the process of extraction is concluded, it is necessary to submit the extracts obtained to a process of purification, with the objective of eliminating substances such as lipids, carotenes or chlorophylls which could interfere in the later qualitative and/or quantitative analyses (Buldini, Ricci, & Sharma, 2002; Chandra, Rana, & Li, 2001; Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000). With this objective, the aqueous extract (A1) was purified by three successive extractions with hexane, discarding the organic phase.

The aqueous phase was newly evaporated in vacuum and made up to a final volume of 2 ml. An aliquot of 0.5 ml of this last extract was placed in a $C_{18}$ cartridge (Strata $C_{18}$ – E, Phenomenex.) which was cleaned up with 1.5 ml of ultra-pure water. Finally, the anthocyanins retained in the cartridge were eluted with MeOH containing 0.01% of HCl. The eluent solution was evaporated and newly made up to a final volume of 2 ml with ultra-pure water, obtaining the purified aqueous extract (A2) (Fig. 1).

### 2.3. Acid hydrolysis

With this technique, the sample was submitted to controlled acid conditions with the objective of breaking the *O*-glycoside bonds without affecting the flavylium ion structure; thus the molecule of the aglycone of the anthocyanin is released. 2 ml of 6 N HCl were added to an aliquot of 1 ml of purified extract (A2) in an inert atmosphere. The resulting solution was maintained boiling in a water-bath for 40 min (Gusti & Wrolstad, 1996). After this period, it was rapidly cooled in crushed ice and immediately analysed chromatographically.

### 2.4. HPLC analysis

The determination of the anthocyanins present in the sample of beans was performed using the *Hewlett–Packard 1100* chromatographic system (with control and data treatment station *HP Chem Station*) coupled to a Finnigan LCQ mass spectrophotometer with an API source, using an electrospray ionisation (ESI). The separation of the anthocyanins was carried out using an Aqua $C_{18}$ column (150 x 4.6 mm i.d., 5 μm of particle size, 125 Å; Phenomenex).

The chromatographic conditions were as follows: flow 0.5 ml/min; volume of injection 100 μl, the column temperature was set at 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 in a diode array detector (Hewlett–Packard 1100) using 280, 320 and 520 nm as preferred wavelengths.

Mass analysis was performed using an electrospray ionisation (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 $MS^2$ spectrum was obtained using a normalised energy of collision (with helium) of 45% (Lopes-da-Silva, De Pascual-Teresa, Rivas-Gonzalo, & Santos-Buelga, 2002).

### 2.5. Quantification

For the correct quantification, the equipment was calibrated with pure commercial patterns of monoglucosides of delphinidin (Dp), malvidin (Mv), petunidin (Pt), pelargonidin (Pg), cyanidin (Cy) and peonidin (Pn) (supplied by Polyphenols Laboratories As.) in a range of calibration from 0.1 to 0.001 mg/ml. The anthocyanidins were quantified as their corresponding glycosylated derivatives. The column used and the chromatographic conditions were identical to those for the qualitative analysis. The content of anthocyanins and/or anthocyanidins, the mean of three determinations, was expressed as mg/100 g of total grain.

## 3. Results and discussion

### 3.1. Method of extraction

Initially, the sample was submitted to the process of extraction and purification described by Takeota et al. (1997). In a first assay, the sample of whole grain was ground and MeOH containing 0.5% HCl was added to the resulting powder. The application of this process required around 60 h, yielding, as a final result, a turbid

solution, unstable and with the characteristic aroma of the legume after boiling.

Utilising, exclusively, the coat of the legume, without soaking (Yoshida et al., 1996), instead of the whole grain, the qualitative results improved significantly. When this coat was also submitted to a process of manual fragmentation, until a fine and homogeneous powder was obtained, the extraction of anthocyanins was 2.5 times greater than that obtained when the powder from ground whole grain was used. Moreover, the time employed for the complete process was reduced to 26 h.

### 3.2. Method of purification

The majority of the authors (Hebrero, Santos-Buelga, & Rivas-Gonzalo, 1988; Pascual-Teresa et al., 2000; Takeota et al., 1997; Yoshida et al., 1996) coincide in indicating a process of purification of the extracts, previous to both quantitative and qualitative analyses, as absolutely necessary.

In our case, different cleaning processes were assayed, giving variable results. Initially, a simple purification was tested, consisting of retaining the anthocyanins of the sample in a $C_{18}$ cartridge which was submitted to a clean up process with ultra-pure water and a later elution of the anthocyanins with 0.01% MeOH:HCl. This process supposed a loss of 73% of the anthocyanins with regard to the non-purified extract (A1), due in great part to the high turbidity of the solution obtained.

It was then decided to vary the clean-up process, once the extract was retained in the $C_{18}$ cartridge, opting to use ethyl acetate instead of water. This solvent had already been used with this objective by Takeota et al. (1997). The results improved considerably, although it was found that an important loss of anthocyanins still occurred, due to the partial solubility of these compounds in the solvent. This loss of compounds supposes a great obstacle for the correct identification and quantification of the extracts.

Bearing in mind that the results were not very satisfactory, it was decided to employ Amberlita XAD-7 (non-ionic resin) instead of the $C_{18}$ cartridge, following the protocol cited by Yoshida et al. (1996). Thus, fractions of great purity were obtained, but the avoidance of the loss of compounds was not achieved. Furthermore, this process was very long and increased the cost of the technique.

On the basis of the results obtained, we proceeded to a modification of the method proposed by Takeota et al. (1997). First, a liquid–liquid extraction was performed, using hexane as solvent, to obtain a purified aqueous phase (A1). It was observed chromatographically that this process, not only did not produce the loss of compounds of interest, but also improved the resolution of the chromatographic peaks. This aqueous phase was then submitted to a second process of purification, testing different $C_{18}$ cartridges, the best results being obtained with less porous cartridges.

With this process of extraction and purification optimised, an aqueous extract was obtained (A2), free of interferences and 2.7 times richer in anthocyanins than that obtained by the methods described in the bibliography (Takeota et al., 1997; Yoshida et al., 1996).

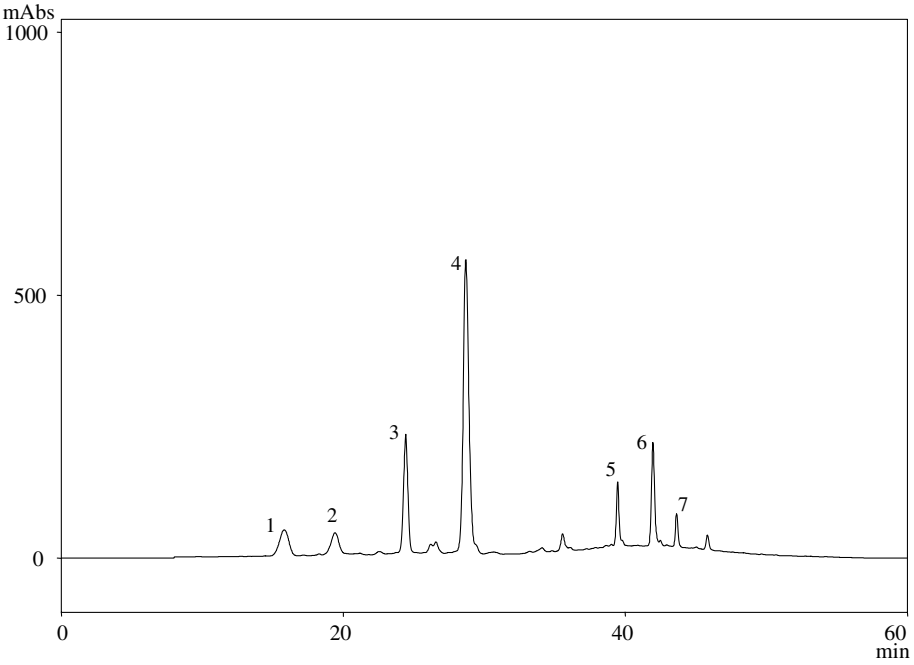


Fig. 2. Chromatogram profile at 520 nm, corresponding to an extract of seed coat of *P. vulgaris* L. var. Tolosa with MeOH (0.5% HCl).

### 3.3. Qualitative analysis by HPLC/MS

The chromatographic analysis of the purified extract, registered at 520 nm, shows the existence of seven principal anthocyanins (Fig. 2). Based on the retention time and the UV–Vis spectrum (Table 1), it was possible to identify, by comparison with previously analysed patterns, the major peaks 3 and 4 that correspond to the monoglucosides of cyanidin and pelargonidin, respectively. These data were later confirmed by mass analysis (Table 1) in which, for peak 3, an [$M^+$] ion at *m*/*z* 449, which in the $MS^2$ analysis produced a fragment at *m*/*z* 287 (loss of a hexose). Peak 4 presented an [$M^+$] ion at *m*/*z* 433 and a major fragment in $MS^2$ at *m*/*z* 271. These data are concordant with those indicated by Yoshida et al. (1996), but different from those reported by Takeota et al. (1997), who, analysing different samples of *P. vulgaris* L., indicated delphinidin as the major anthocyanin, followed by petunidin and malvidin.

The MS analysis of peak 1 showed an [$M^+$] ion at *m*/*z* 611 and two fragments at *m*/*z* 449 and 287, which would correspond to the losses of one and two hexoses, respectively. The same occurred for peak 2 with an [$M^+$] ion at *m*/*z* 595 and two fragments at *m*/*z* 433 and 271. Taking into account the data of masses and the order of elution and the spectral characteristics, it was possible to identify peaks 1 and 2 as the corresponding diglucosides of cyanidin and pelargonidin, respectively. The presence of these compounds in *P. vulgaris* L. had not been previously described by any author, although Yoshida et al. (1996) found variable quantities of petunidin and malvidin 3,5-diglucoside in a sample of *P. vulgaris* L. from North America.

Peaks 5, 6 and 7 appear in a very late zone of the chromatogram, which indicates the lesser polarities. Initially, it was thought that they could be anthocyanins joined to a hydroxycinnamic type of acid, which would explain their longer retention time. Nevertheless, when their UV–Vis spectra are observed (Fig. 3), they present a lack of shoulders, characteristic of this type of compound, at wavelengths close to 300–330 nm, which discounts the possibility that they are this type of acylated derivative. The MS data analysis for these three peaks shows molecular ions at *m*/*z* 287, 301 and 271, which would be in accordance with the molecular masses of the anthocyanidins cyanidin, peonidin and pelargonidin, respectively. Moreover, these compounds had retention times and spectral characteristics (Fig. 3) equal to those of the aglycones resulting from the acid hydrolysis of the sample.

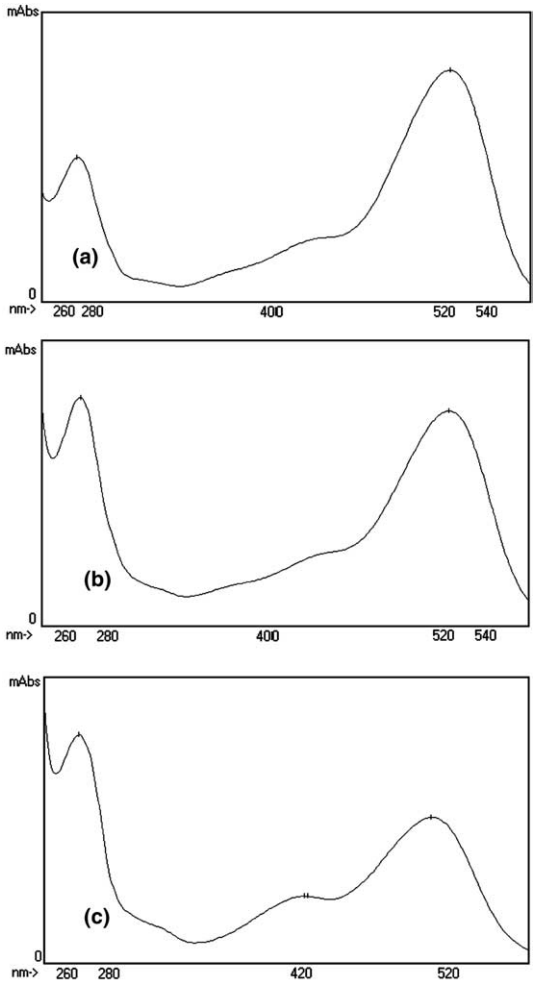


Fig. 3. UV–Vis spectra corresponding to compounds 5, 6 and 7 of the anthocyanic extract from *P. vulgaris* L. var. Tolosa: (a) cyanidin, (b) peonidin, (c) pelargonidin.

These results would seem to demonstrate, for the first time, the presence, in natural form, of aglycones in vegetable samples. Until now, the bibliographical data have indicated that, in nature, this type of compound is only

Table 1
Retention time, UV–Vis and Mass spectral characteristics for the anthocyanic composition of *P. vulgaris* L. var. Tolosa

| Peak | Compound | $R_t$ | $\lambda$ | [$M^+$] | $MS^2$ | $MS^3$ |
|---|---|---|---|---|---|---|
| 1 | Cy 3,5-digluc | 16.0 | 279, 513 | 611 | 449, 287 | 287 |
| 2 | Pg 3,5-digluc | 19.5 | 280, 484, 498 | 595 | 433, 271 | 271 |
| 3 | Cy 3-gluc | 24.6 | 280, 515 | 449 | 287 | 287 |
| 4 | Pg 3-gluc | 28.7 | 275, 429, 501 | 433 | 271 | 271 |
| 5 | Cyanidin | 39.6 | 275, 525 | 287 | 287 | 287 |
| 6 | Peonidin | 42.4 | 275, 524 | 301 | 287/259 | 259 |
| 7 | Pelargonidin | 43.0 | 274, 426, 513 | 271 | 271 | 271 |

found in heteroside forms. Therefore, the possibility that these aglycones were the consequence of a hydrolysis produced during the extraction stage was considered; however, it is true that other authors who use the same type of solvent do not describe the appearance of anthocyanidins. Some authors (Dao et al., 1998; Merken et al., 2001) describe processes of hydrolysis, principally of acylated anthocyanins, but always using concentrations of HCl above 1.2 M whereas, in our case, the molarity of the acid was 0.06. Merken et al. (2001), utilising pure extracts of anthocyanins, assayed different concentrations of HCl (0.6–2.4 M) in 50% aqueous methanol, proving that, for the hydrolysis to be effective, it was necessary to use concentrations above 1.8 M.

It was decided to test the possible hydrolysis by modifying the conditions of extraction, decreasing the concentration of acid and using a less aggressive acid than HCl (Yoshida et al., 1996). Assays were carried out with 0.2% and 0.1% MeOH:HCl, instead of 0.5% and finally using 3% and 2% MeOH:TFA. In the first case (Fig. 4(a)), in spite of the concentration of acid being lower, surprisingly, the peaks of the three aglycones continued to be important. In the other three cases (Fig. 4(b)–(d)), some of the possible aglycones always appear, although in concentrations below the quantification limit.

In view of these results, it appears that, effectively, with concentrations greater than 0.1% of HCl, a significant hydrolysis of the anthocyanins is produced. Nonetheless, when Table 2 is carefully observed, it is found that the efficacy of the extraction is proportional to the concentration of acid, which must, undoubtedly, equally influence the extraction of the corresponding anthocyanidin. When 0.5% MeOH:HCl was used 24.1 mg/100 g were extracted from the sample whereas, when MeOH:TFA or 0.1% MeOH:HCl were used, this quantity decreased to 8 mg/100 g in the best of cases.

What is more, the presence of peonidin monoglucoside, or its acylated derivative, was not detected in any of the assays, which would justify the appearance of the corresponding aglycone as a consequence of a possible hydrolysis. Moreover, the sample was submitted to acid hydrolysis, to affirm the hypothesis that peaks 5, 6 and 7 corresponded to the cited aglycones. To carry out this confirmation, a new extraction of the anthocyanins was performed, using the original conditions (0.5% MeOH:HCl) and continuing to a later purification. The aqueous extract obtained had a pH of 1.88, which, according to the bibliographical data (Coutalte, 1986; Dao et al., 1998; Rivas-Gonzalo, 2003), is not sufficient to produce drastic damage in the original anthocyanin structure.

This aqueous extract was submitted to the process of hydrolysis, as indicated in Section 2 and later analysed by liquid chromatography. In the case of the acid hydro-

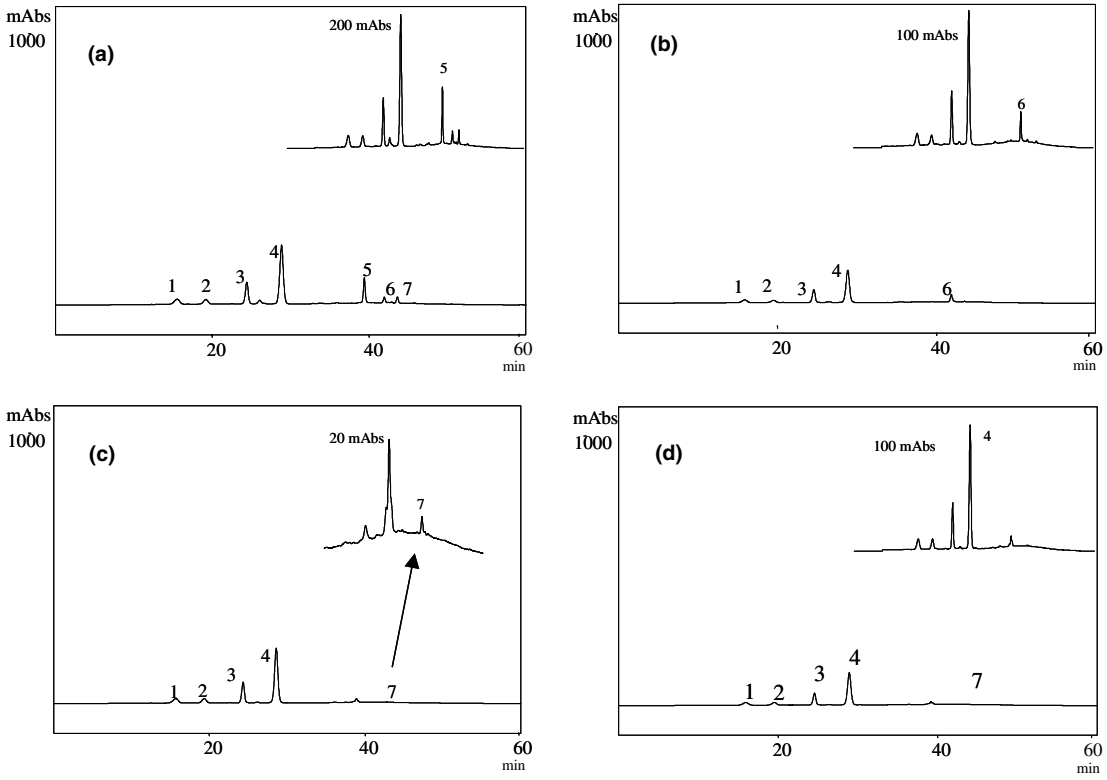


Fig. 4. Chromatograms, registered at 520 nm, corresponding to a methanolic extract of the seed coat of *Phaseolus vulgaris* L. var. Tolosa, with: (a) 0.2% HCl, (b) 0.1% HCl, (c) 3% TFA, (d) 2% TFA.

Table 2
Quantification in mg/100g of sample (%) in seeds of *P vulgaris* L. var. Tolosa determined in the diverse assays performed

| Peak | Compound | HCl 0.5% | HCl 0.2% | HCl 0.1% | TFA 3% | TFA 2% |
|---|---|---|---|---|---|---|
| 1 | Cy 3,5-digluc | 1.98 (8.2) | 0.85 (7.2) | 0.41 (10.0) | 0.76 (9.5) | 0.49 (9.9) |
| 2 | Pg 3,5-digluc | 1.54 (6.4) | 0.82 (7.0) | 0.43 (10.5) | 0.75 (9.4) | 0.56 (11.3) |
| 3 | Cy 3-gluc | 3.99 (16.6) | 1.70 (14.4) | 0.84 (20.5) | 1.61 (20.2) | 0.96 (19.3) |
| 4 | Pg 3-gluc | 12.6 (52.3) | 5.37 (45.6) | 2.41 (58.9) | 4.87 (61.0) | 2.96 (59.6) |
| 5 | Cy[a] | 1.63 (6.8) | 1.36 (11.5) | | | |
| 6 | Pn[a] | 1.36 (5.7) | 1.09 (9.3) | | | |
| 7 | Pg[a] | 0.95 (4.0) | 0.59 (5.0) | | | |
| Total | 24.1 | 11.8 | 4.09 | 7.99 | 4.97 | |

[a] Quantified as its respective monoglucoside.

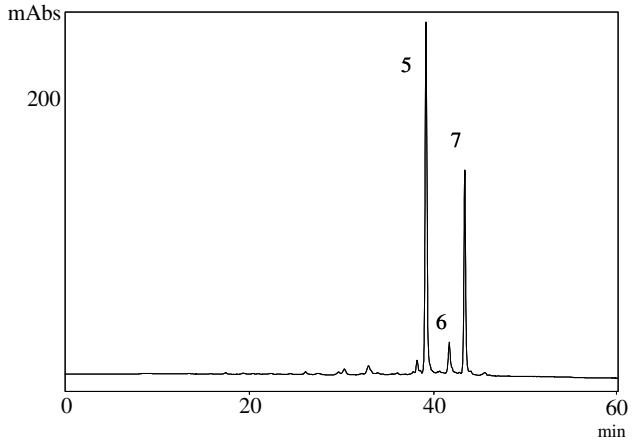


Fig. 5. Chromatogram, registered at 520 nm, corresponding to a methanolic extract of the seed coat of *P. vulgaris* L. var. Tolosa, submitted to acid hydrolysis.

lysis (Fig. 5), only the presence of peaks 5, 6 and 7 is observed. The aglycones of cyanidin (peak 5) and pelargonidin (peak 7) increased considerably as a consequence of the hydrolysis of their mono and diglucoside derivatives, whereas peak 6, corresponding to peonidin, appeared in similar concentration to that of the original extract.

Another possibility, which would justify the appearance of these aglycones would be their presence as a consequence of an enzymatic hydrolysis by the action of β-glucosidase. These enzymes are capable of breaking the *O*-glycoside bonds of the anthocyanins, releasing the aglycones, which are rapidly destroyed. Whightman and Wrostad (1996) indicated that the action of this type of enzyme produces a degradation of the anthocyanins without forming aglycone and Arnaldos, Muñoz, Ferrer, and Calderon (2001) proved, in strawberry, that, upon increasing the polyphenol oxidase activity, the concentration of soluble phenols decreases, although without detecting any corresponding aglycones. Various authors (Mateo & Di Stefano, 1997; Piffaut, Kader, Girardin, & Metche, 1994) indicate that pH below 1 retains the enzymatic activity and that this activity is greater at pH close to neutrality. In our case, as has been indicated previously, we worked at sufficiently acid pH for the activity of the enzymes to be minimal, which is why it does not seem probable that this is the origin of the anthocyanidins found in the sample.

It is important to note, that although several papers about *P. vulgaris* L. have been published, their authors indicate different results. This question can be explained because the composition of the bean is influenced by different factors, such as geographical origin, variety and ripeness. In this paper, the results are only referred to a *P. vulgaris* L. var. Tolosa and maybe other varieties have different anthocyanin content.

### 3.4. Quantitative analysis by HPLC

For each anthocyanin, the practical quantitation limit (PQL), equivalent to 5 times the method detection level (LDM) (Glaser, Foerst, McKee, Quave, & Budde, 1981), was established as a parameter of quantification. In Table 3 the limits for each anthocyanin are presented, expressed in mg/100 g of grain. The repeatability of the method was checked, carrying out the process of extraction and analysis of the sample 10 times and determining the mean and the coefficient of variation (Table 4).

The total anthocyanic content of the sample was 24.1 mg/100 g of grain, bearing in mind that the coat represents 8.6% of the weight of the grain. In Table 2 the distribution of the compounds quantified in the different assays is shown. In the data analysed, it is noteworthy that 83.5% correspond to glycosylated compounds. The major compound is pelargonidin 3-glucoside

Table 3
Limits of detection of the method and of practical quantification established for each anthocyanin, expressed as mg/100 g

| Anthocyanin | PQL | MDL |
|---|---|---|
| Dp 3-gluc | 0.44 | 0.09 |
| Cy 3-gluc | 0.43 | 0.09 |
| Pt 3-gluc | 0.40 | 0.08 |
| Pg 3-gluc | 0.45 | 0.09 |
| Pn 3-gluc | 0.55 | 0.11 |
| Mv 3-gluc | 0.35 | 0.07 |

Table 4
Repeatability of the method performed with the sample of *P. vulgaris* L. var. Tolosa expressed as mg/100 g of sample

| Compound | Mean | CV |
|---|---|---|
| Cy 3,5-digluc | 2.14 | 4.23 |
| Pg 3,5-digluc | 1.98 | 0.72 |
| Cy 3-gluc | 3.49 | 4.16 |
| Pg 3-gluc | 11.9 | 5.24 |
| Cy | 1.18 | 2.97 |
| Pn | 1.72 | 4.32 |
| Pg | 1.56 | 0.84 |
| Total | 20.4 | 8.82 |

(52.3%), whereas the minor one is its corresponding aglycone (4%). The cyanidin 3-glucoside (16.6%) is also important. In any case, the group of compounds of pelargonidin (62.7%) and cyanidin (31.6%) predominates. The presence of peonidin, which is only detected in aglycone form, at 5.7% is noteworthy.

## 4. Conclusions

The improvement in the process of extraction and purification and the later application of an LC-MS method has allowed the identification of the anthocyanins, cyanidin and pelargonidin monoglucosides, as major compounds, and cyanidin and pelargonidin diglucosides which have not been previously detected in *P. vulgaris* L.

The natural presence of aglycones of anthocyanins, and particularly of cyanidin, peonidin and pelargonidin, has been detected. This appears to be a novel, since, to our knowledge, the anthocyanidins are present in nature in a glycosylated form.

## Acknowledgements

This work was supported by the grant AGL-2002-00167 from the Ministerio de Ciencia y Tecnología, Spain and FEDER (UE Programme). We thank Mr. G. H. Jenkins for his help with the English version of the ms.

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