Z Lebensm Unters Forsch (1995) 200: 221–224

Zeitschrift für
Lebensmittel-
Untersuchung
und-Forschung


*Original paper*

# Proanthocyanidins in skins from different grape varieties

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Received May 20, 1994

**Proanthocyanidine in den Fruchtschalen verschiedener Traubensorten**

**Zusammenfassung.** Die Proanthocyanidine der Schale von Weinbeeren wurden analysiert und mit denen der Kerne verglichen. Innerhalb derselben Sorte wurden qualitative und quantitative Unterschiede zwischen den Gewebearten gefunden. Die Gehalte der Samen lagen generell am höchsten. In den Samen konnten nur Procyanidine nachgewiesen werden, während die Fruchtschalen auch Prodelphinidine enthielten. Insgesamt dominieren jedoch Catechin-Grundeinheiten.

**Abstract.** The proanthocyanidin composition of grape skins was studied and compared with that of grape seeds. Qualitative and quantitative differences in proanthocyanidin contents were observed between skins and seeds from the same variety. Grape seeds were found to have higher amounts of flavan-3-ol than skins; only procyanidins were found in the former, while in the skins prodelphinidins were also present, a predominance of (+)-catechin being found in their elemental flavan-3-ols.

## Introduction

As has been reported by several authors [2, 10, 12], the flavan-3-ols of grapes are formed from a mixture of procyanidin and prodelphinidin units. However, until now, only monomers and oligomers of the procyanidin type have been identified. Among the monomers, there is a clear predominance of (+)-catechin and (−)-epicatechin, although relatively high amounts of (−)-epicatechin-3-*O*-gallate and smaller amounts of (+)-gallocatechin, (−)-epigallocatechin and (+)-catechin-3-*O*-gallate have also been found [2, 15, 16].

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The flavanol composition of grape seeds has been studied by different authors [1, 4, 7, 8, 19]. However, less attention has been directed to grape skins, since the analysis of these is more complex because their flavan-3-ols are accompanied by significant amounts of other phenolic compounds. Different procedures have been used to purify skin extracts and continue specifically with the analysis of flavanols, such as selective elution through Sep-pak C18 cartridges [14, 18], polyamide columns [1, 3] or Sephadex LH-20 [9, 11].

In the studies available some differences have been found between the flavanol composition of the skins and the better known composition of the seeds. Skin flavan-3-ols seem to show a higher degree of condensation and they have been reported to contain a predominance of (+)-catechin [12]; the proportions of (+)-catechin and (−)-epicatechin are however, better balanced in the seeds [1, 20]. Also it has been reported that while B2 [i.e. epicatechin-(4β→8)-epicatechin] is the major dimer of grape seeds, B1 [i.e. epicatechin-(4β→8)-catechin] is the major dimer of the skins; additionally, among the trimers, C1 [i.e., epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin] usually predominates in the seed while epicatechin-(4β→8)-epicatechin-(4β→8)-catechin predominates in the skin [5, 6].

The present work addresses the flavanol composition of grape skins, comparing the results with those found for the seeds of grapes of different varieties; the latter information has been published in an earlier work [20].

## Materials and methods

*Samples.* Grapes from 12 varieties of *Vitis vinifera* were analysed, three of them being white: Verdejo (from the district of Rueda), Malvasía (Toro) and Sauvignon (Toro); and nine being red: Garnacha tinta (Toro), Juan García (Fermoselle), Malbec (Rueda), Mencía (El Bierzo), Merlot (Rueda), Pinot noir (Valladolid), Prieto picudo (Valdevimbre), Tempranillo (Ribera del Duero) and Tinta del País (Ribera del Duero). The grapes were harvested in 1992, when ripe, and were grown in experimental plots situated in different wine-producing

**Table 1.** Retention times and experimental maxima absorption wavelengths ($\gamma_{max}$) of anthocyanidins as recorded by HPLC-DAS

| Anthocyanidin | Parameter | | |
|---|---|---|---|
| | Retention time (min) | γ max | (nm) |
| Delphinidin | 11 | 273 | 531 |
| Cyanidin | 16 | 275 | 526 |
| Petunidin | 18 | 273 | 533 |
| Peonidin | 22 | 276 | 526 |
| Malvidin | 23 | 273 | 536 |

Chromatographic conditions as in [13]

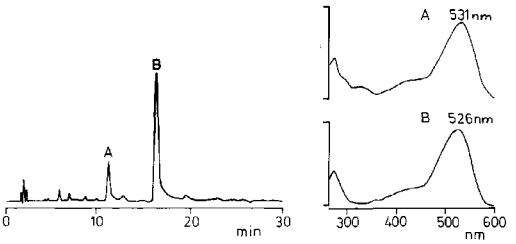


**Fig. 1.** HPLC chromatogram recorded at 520 nm and peak spectra obtained after acid hydrolysis of skins residue from Malvasia variety

districts controlled by the Enological Station of Castilla-Leon (at Rueda, Spain).

*Extraction of flavan-3-ol from grape skins.* Approximately 20 g of frozen grape skins were ground and homogenized at –30° C in methanol containing 0.5 g/l of ascorbic acid, in a similar fashion to that described for the extraction of flavan-3-ol from grape seeds [8]; water was added and the methanol was removed under vacuum. The aqueous extract was washed with *n*-hexane to eliminate liposoluble substances and the remaining solvent was eliminated by evaporation.

A similar extraction technique was also performed using 70% acetone instead of methanol with a view to establishing which of the two solvents would be more efficient for the extraction of flavan-3-ol from the grape skins.

*Purification of extracts.* The aqueous extracts were passed through a Sep-pak C18 cartridge. A wash with water adjusted to a pH of 7 was performed to eliminate phenolic acids, and the flavan-3-ols were eluted with ethyl acetate; a small volume of water was added to this eluate and the solvent was evaporated under vacuum. The aqueous extracts were then placed in a small column (5 × 1 cm) of Sephadex LH-20, thereafter washing first with 30% ethanol containing 1% 1N HCl, then with distilled water, and finally eluting with 70% acetone. The acetone was removed by evaporation under vacuum and the filtered aqueous extracts were analysed by HPLC.

*Acid hydrolysis.* Aliquots of the extracts were dried in a vacuum and the residue was redissolved in *n*-butanol/HCl (95:5 vol.:vol.) solution. The mixture was heated to 100° C for 20 min and the hydrolysate was dried in a rotary evaporator and then redissolved in wine-like synthetic solution containing 5 g/l tartaric acid buffer solution and 10 vol.% ethanol (pH = 3.2). The anthocyanin pigments formed were analysed in this latter solution by HPLC, under the conditions reported by Hebrero et al. [13]. Acid hydrolysis of the solid residues remaining after extraction from the skins and seeds was also performed; in this case, after the hydrolysis the mixtures were centrifuged and the supernatant fluid containing the pigments was analysed by HPLC as indicated above. In the grapes from the red varieties where some anthocyanins remained in the residue, these were removed before performing hydrolysis by repeated extraction with methanol: 1N HCl (95:5) until the skins were completely discoloured. This was done to ensure that the anthocyanidins formed were indeed exclusively derived from the proanthocyanidins. The pigments formed by hydrolysis were identified by comparison of their chromatographic characteristics and visible-UV spectra with those of the anthocyanidins obtained by acid hydrolysis of anthocyanins extracted previoulsy from grape skins as described previously [13]. Table 1 shows the chromatographic and spectral characteristics of these anthocyanidin standards obtained with a diode array detector.

*Analysis of flavan-3-ol by HPLC.* The conditions used for this were the same as reported by Escribano-Bailón et al. [8].

## Results and discussion

In all the varieties of grape studied, acid hydrolysis of the extracts and the residues of the seeds produced only cyanidin; this means that the proanthocyanidins present were of the procyanidin type, that is, derivatives of (+)-catechin and (–)-epicatechin. The amounts of cyanidin formed were significant, both in the residues and in the extracts of seeds, indicating that the procyanidins present are in both alcohol-soluble and alcohol-insoluble forms.

Hydrolysis of the skin extracts afforded very low amounts of pigment owing to the existence of low amounts of soluble proanthocyanidins in the skins, as was later observed in the analysis of the proanthocyanidin composition by HPLC. This low yield of cyanidin was observed both in the extracts obtained with methanol, and those obtained with acetone, even though the latter is considered to be able to permit the partial solubilization of some condensed proanthocyanidins or proanthocyanidins bound to proteins [11, 12]. However, in the hydrolysis of the residues remaining after extraction of the skins, significant amounts of delphinidin and cyanidin were formed. As an example, Fig. 1 shows the chromatogram obtained from the hydrolysis performed on the skin residue from the Malvasia variety. One important conclusion of these results is the observation that the condensed tannins of the skins are formed from procyanidins and prodelphinidins while only procyanidins are found in the seeds, at least in the varieties analysed here.

Another difference encountered between both parts of the grape was that for the same weight of sample, the amounts of anthocyanin pigments formed were much higher in the seeds than in the skins, confirming that the former are a much richer source of condensed tannins. Additionally, the proanthocyanidins of the skins were mainly in unextractable form, possibly because they exist in the form of large polymers, or because they are bound to matrix substances, as indicated previously [9, 11, 12]. However, in the seeds a significant part of the condensed tannins occurs in soluble form, possibly as oligomers containing between two and six elemental flavan-3-ol units [8].

The existence of procyanidins and prodelphinidins in the polymer chains of the condensed tannins of grape skins has been reported by Haslam [12], who indicated an approximate ratio of 4:1 in the proportions of cyanidin and delphinidin formed during hydrolysis. In our case, the

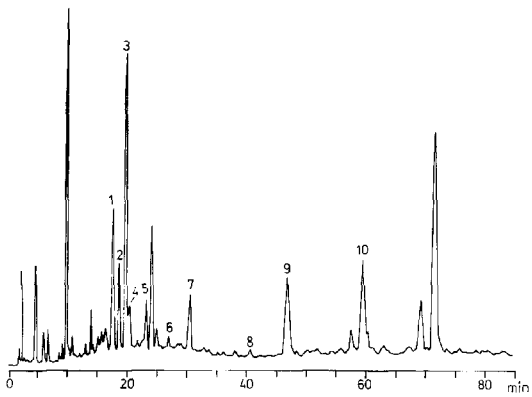


**Fig. 2.** HPLC chromatogram recorded at 280 nm of a grape skin purified extract from the Tempranillo variety. Only peaks corresponding to flavan-3-ol are numbered. Numbering of peaks as in Table 2

ratio between these two pigments ranged between 2:1, found in the Tinta del País variety, and 5:1 in the Sauvignon variety. Apart from these, no other type of pigment was formed, showing that there are no type-A double bonds among the elemental flavan-3-ol units of grape proanthocyanidins, since had they been present the formation of other pigments resulting from the alternative breakage of interflavan bonds would be observed.

Analysis of soluble flavan-3-ols in grape skins is more difficult than in seeds since they are present in lower concentrations and are accompanied by other phenolic compounds such as flavonols, anthocyanins or hydroxycynammic derivatives. Accordingly, previous assays were carried out to achieve the separation of the flavan-3-ols from other compounds present in the skin extracts that might interfere with their determination by HPLC. Purification in polyamide and Sephadex LH-20 columns and through Sep-pak C218 cartridges was attempted. However, it was not possible to obtain a satisfactory separation of the flavan-3-ols with any of these methods individually, so a combination of the techniques was chosen. Initially, the extracts were passed through a Sep-pak C18 cartridge, where the phenolic acids and hydroxycynammic derivatives were eliminated by washing and part of the anthocyanins and flavonols were retained in the cartridge. Later, additional purification was carried out in a Sephadex LH-20 column, largely eliminating the anthocyanins and another part of the flavonols. Figure 2 shows the chromatogram of a grape skin extract of the Tempranillo variety after the complete purification process. As may be seen, some compounds do remain in the extract, although they do not interfere with the observation of the peaks corresponding to the flavan-3-ols.

Only 10 peaks corresponding to flavan-3-ol compounds were detected in the skin extracts of the different varieties of grape analysed; these compounds appear in Table 2. In these same varieties, the flavanol composition of the seeds is more diverse, it being possible to identify 27 different compounds, as reported elsewhere [20]. No spectra corresponding to prodelphinidin-type compounds could be detected in the chromatograms, either in the skins or the seeds, confirming the results obtained previously with acid hydrolysis.

In assays performed with seed extracts of known flavanol composition and with standards of catechins and procyanidins, it was observed that the purification process, to which the skin extracts were subjected, involved a loss of flavan-3-ol ranging between 7% and 20%. Despite this loss, purification proved to be necessary since in the chromatograms previously obtained, the flavanols could not be located well since their peaks were masked by those of other phenolic compounds present in much higher concentrations. The degree of loss of flavanols occurring during the purification process is random and it was not possible to establish any relationship between the type of compound and the percentage of loss, and hence the results obtained on the flavanol composition of the skins were not considered from the quantitative point of view.

**Table 2.** Flavan-3-ols detected in grape skin

| Peak | Compound |
|---|---|
| 1 | Catechin-(4α→8)-catechin (Dimer B3) |
| 2 | Epicatechin-(4β→8)-catechin (Dimer B1) |
| 3 | (+)-Catechin |
| 4 | Epicatechin-(4β→8)-epicatechin-(4β→8)-catechin |
| 5 | Catechin-(4α→8)-epicatechin (Dimer B4) |
| 6 | Epicatechin-(4β→8)-epicatechin (Dimer B2) |
| 7 | (−)-Epicatechin |
| 8 | Epicatechin-(4β→8)-epicatechin-3-*O*-gallate (B2-3′-*O*-gallate) |
| 9 | Epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin (Trimer C1) |
| 10 | (−)-Epicatechin-3-*O*-gallate |

**Table 3.** Distribution of flavan-3-ols in skins from different grape varieties

| Variety[b] | Compound[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| **Whites** | | | | | | | | | | |
| Verdejo (Rueda) | + | ++ | +++ | ++ | + | + | + | − | − | − |
| Malvasía (Toro) | + | + | +++ | ++ | + | ++ | ++ | + | ++ | + |
| Sauvignon (Toro) | − | +++ | +++ | − | − | − | ++ | − | − | − |
| **Reds** | | | | | | | | | | |
| Tempranillo (Ribera de Duero) | ++ | ++ | +++ | ++ | + | + | ++ | + | ++ | ++ |
| Tinta del País (Ribera de Duero) | + | ++ | +++ | +++ | + | + | + | ++ | + | +++ |
| Garnacha tinta (Toro) | − | − | +++ | + | − | − | − | − | − | − |
| Juan García (Fermoselle) | + | + | +++ | + | + | + | + | − | − | − |
| Malbec (Rueda) | + | + | +++ | ++ | + | − | + | − | − | − |
| Prieto Picudo (Valdevimbre) | + | +++ | +++ | +++ | + | − | ++ | − | − | − |
| Mencía (Bierzo) | + | ++ | ++ | ++ | − | − | + | − | − | − |
| Pinot noir (Valladolid) | − | + | +++ | + | − | − | − | − | − | − |
| Merlot (Rueda) | + | ++ | +++ | +++ | + | − | + | − | − | − |

[a] Numbering of compounds as in Table 2

[b] The district of origin is indicated in parentheses after the variety. Analysis of each sample was performed in triplicate

Table 3 shows the distribution of soluble flavan-3-ols found in the skin extracts of the different varieties of grape analysed. Comparison of these results with those obtained for the flavanol composition of seeds reported elsewhere [20] shows that, in general, there is no correlation between the flavanol composition of the skins and seeds of grapes, both having different distributions of the compounds. However, certain aspects of interest deserve consideration. In this sense, (+)-catechin is the most abundant flavan-3-ol, and in the skin it is clearly the major monomer, however, in seeds (−)-epicatechin is also well represented, some varieties displaying similar amounts of both monomers or even higher levels of (−)-epicatechin. Among the procyanidins of the skins the dimer B1 [i.e. epicatechin-(4β→8)-catechin] predominates together with the trimer epicatechin-(4β→8)-epicatechin-(4β→8)-catechin, both substances having (+)-catechin in their initiation unit, and resulting from successive extensions with epicatechin units. In the skins there are few procyanidins with (−)-epicatechin in the initiation unit, whereas they are well represented in the seeds. Thus, the B2 dimer [i.e. epicatechin-(4β→8)-epicatechin] is the most abundant procyanidin in seeds and there are also relatively high proportions of the trimer C1 [i.e. epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin], resulting from the extension of B2 with epicatechin. The presence of flavanols esterified with gallic acid is one characteristic of grapes. All the varieties analysed here exhibited relatively significant amounts of galloyl derivatives in the seeds. By contrast, the almost complete absence of gallic esters in the skin is striking. Only epicatechin-3-*O*-gallate and B2-3′-*O*-gallate could be detected in some varieties (Malvasia, Tempranillo and Tinta del País).

As is known, flavanols play a significant role in the evolution of the colour of red wines during maturation and ageing [12]. The facts that the amount of flavanols is much lower in skins, and that these substances are present in a relatively insoluble form suggest that it is the soluble flavan-3-ols present in the seeds that are basically carried through into the must during the wine-making process when fermentation is carried out in the presence of the solid parts of the grape (red wine manufacture).

*Acknowledgements.* The authors are grateful to Junta de Castilla y Léon (Grant No. 0907/90) and Comisión Interministerial de Ciencia y Tecnologia (ref. ALI90-0983) for financial support of this work. Author Escribano-Bailón is indebted to Dirección General de Investigación Científica y Tecnica for a fellowship (ref. AP90). Thanks are also due to Nick Skinner for translation of this paper.

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