Vitis **40** (1), 17–22 (2001)

Evolution of proanthocyanidins in bunch stems during berry development (*Vitis vinifera* L.)

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Summary

Proanthocyanidins from bunch stems of two red varieties (Castelão Francês and Touriga Francesa) and one white variety (Viosinho) (*Vitis vinifera* L.) harvested in 1998, were separated into monomers (catechins), oligomers (degree of polymerization ranging from 2 to 12-15) and polymers (degree of polymerization >12-15), and then quantified during grape development (40 d before veraison until ripening). In addition, low molecular weight catechins [(+)-catechin and (-)-epicatechin], dimeric procyanidins (B1, B2, B3, B4), galloylated dimeric procyanidins (B1-3-0-gallate, B2-3-0-gallate, B2-3'-0-gallate), and trimeric procyanidins (C1, T2) from grape stems were quantified by HPLC.

At harvest the polymeric fraction was the most abundant (28.0 - 35.8 mg·g^{-1} stem). For the three grape varieties, the content of catechins, oligomeric and polymeric fractions decreased during berry development mainly after veraison. Grape stems are also an important source of proanthocyanidins in the grape cluster when compared to the flavanol content in seeds. From an industrial point of view, stems seem to be an important source of proanthocyanidins for potential use as nutriceutical, enological products, chemical standards or even in winemaking to regulate flavanol composition in wine.

K e y w o r d s : grapevine cluster stem, maturation, proanthocyanidins, polymerization degree.

Introduction

Proanthocyanidins (flavanols or condensed tannins) are present in solid parts of grape clusters (skins, seeds and stems) and in traces in the pulp. They play a very important role in red wine ageing due to their high reactivity: polymerization, condensation with anthocyanins, reaction with proteins and oxidation reactions (Ricardo da Silva *et al*. 1991 a, b; Cheynier *et al*. 1992). It has been considered that grape and wine proanthocyanidins may play a positive role in human health, in particular due to their effects on arteriosclerosis (Masquellier 1988) and their oxygen radical-scavenging ability (Ricardo da Silva *et al.* 1991c; Frankel *et al*. 1992). According to several authors, these properties largely depend on chemical strutures, their levels, and especially their degree of polymerization (Haslam 1974; Singleton 1992; Rigaud *et al*. 1993).

The flavanol content of grape varieties varies widely and depends on the vintage (Ribéreau-Gayon 1971; Bourzeix *et al*. 1986; Dumon *et al*. 1991; Fernandez de Simon *et al*. 1992; Fuleki and Ricardo-da-Silva 1997). In the case of oligomeric procyanidins, most authors reported procyanidin B1 to be the major oligomer in stems and skins, while procyanidin B2 has its highest concentration in seeds (Bourzeix *et al*. 1986; Ricardo-da-Silva *et al*. 1992 a, b; Santos-Buelga *et al*. 1995; Fuleki and Ricardo-da-Silva 1997; Jordão *et al*. 1998). Galloylated procyanidins are present in considerably lower concentrations than the non-galloylated forms.

The highest levels of proanthocyanidins in grapes were found at the onset of ripening (Dumazert *et al*. 1973; Czochanska *et al*. 1979), thereafter they decreased (Dumarzet *et al*. 1973, Romeyer *et al*. 1986; Fernandéz *et al*. 1992; De Freitas 1995; Jordão *et al*. 1998). For some grape varieties and for some procyanidins, a maximum is sometimes observed at veraison (Romeyer *et al*. 1986; Lee and Jaworski, 1989; Fernandez *et al.* 1992).

Recent investigation (Souquet *et al*. 1996; Sun *et al*. 1996; Cheynier 1997) shows that in grapes proanthocyanidins exist essentially in highly polymerized forms confirming ancient works of Czochanska *et al.* (1979). Prieur *et al*. (1994) reported that mean degrees of polymerization ranged from 2.3 to 16.7 in grape seeds. Recently Sun *et al.* (1998 a) showed that the mean degree of polymerization for the oligomeric and polymeric proanthocyanidin fraction in seed extracts was 9.8 and 31.5, respectively. In grape skins the mean degree of polymerization ranged from 3.4 to 83.3 (Souquet *et al.* 1996). Czochanska *et al.* (1979) reported that polymeric, proanthocyanidins consist exclusively of repeating flavan 3-ol units with predominantly a 2.3-*cis* stereochemistry with the same configuration as (-)-epicatechin (Czochanska *et al.* 1980).

Sun *et al*. (1998 b) studied the proanthocyanidin content of several Portuguese varieties at harvest and evaluated total catechins, oligomeric and polymeric proanthocyanidins in seeds, skins and pulp, showing that these compounds occurred primarily in seeds and, to a smaller degree in skins, very little was found in the pulp. The polymeric forms were the most abundant in all parts. Moreover, Sun *et al*. (1999) studied the transfer of catechins and all proanthocyanidins from the solid parts of the grape cluster into wine and suggested that grape seeds and cluster stems are together with skins important sources of proanthocyanidins in wine.

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Only a few studies considered the analysis of polymeric fractions in the grape cluster. The majority of publications concerning the proanthocyanidin fractions have only mentioned some individual dimeric and trimeric compounds from entire grape berries, skins and seeds, but less attention has been directed to grape stems (CZOCHANSKA *et al*. 1979; BOURZEIX *et al*. 1986; RICARDO-DA-SILVA *et al*. 1992 a, b; DE FREITAS 1995; FULEKI and RICARDO-DA-SILVA 1997; JORDÃO *et al*. 1998).

The main objectives of the present study were to analyse the total levels of flavanols in grape stems (catechins, oligomeric and polymeric structures) during grape berry development and to evaluate the potential utilisation of grape stems as a source of flavanols.

Material and Methods

Harvest of grapes: Clusters (1 kg each) of Castelão Francês, Touriga Francesa (red varieties) and Viosinho (white variety) were harvested in 1998 from a vineyard in the region of Palmela (south of Lisbon). Grapes were kept frozen at -18 ºC until processing. For all grape varieties sampling started 40 d before veraison and lasted until technological maturity (65 d after veraison for red varieties, 40 d after veraison for the white variety).

Physico-chemical analysis of grapes: Grapes were analysed for weight of 200 berries, potential alcohol degree, pH, titratable acidity, total phenols, total anthocyanins, colour density, colour hue, malic acid, tartaric acid and total nitrogen, using the analytical methods recommended by the OIV (1990).

Flavanol extraction: The stems (approximately 40 g) were removed manually from the berries and were placed in 100 ml methanol at -24 ºC in the dark for 16 h; 5 mg of ascorbic acid were added to avoid flavanol oxidation. The extraction followed the procedure of BOURZEIX *et al.* (1986): 100 ml of 80 % methanol was followed by 100 ml of 50 % methanol, each for 4 h and distilled water was added to the samples which were held at -24 ºC during 15 h. Thereafter the residue was extracted with 75 % acetone for 1 h. Finally, all extracts were assembled.

For each sample, 5 ml of each extract were purified and fractionated on a polyamide column (Macherey-Nagel, Düren, Germany) as described by RICARDO-DA-SILVA *et al*. (1990). Elution was carried out with 80 ml of water adjusted to pH 7 to eliminate phenolic acids. Before HPLC analysis, 50 ml of acetonitril (30 % v/v) were used to elute catechins followed by 50 ml of acetone (75 % v/v) to elute oligomeric procyanidins.

Fractionation of proanthocyanidins according their polymerization degree: The stem extracts were separated into three fractions containing catechins (monomers), oligomeric (degree of polymerization ranging from 2 to 12-15) and polymeric proanthocyanidins (degree >12-15), using C_{18} Sep-Pak column as described by SUN *et al*. (1998 a). Thus, each sample was passed through the two preconditioned neutral Sep-Pack cartridges connected in series. To eliminate phenolic acids, 4 ml de-alcoholized medium was adjusted to pH 7.0 and then passed through the two connected Sep-Pak cartridges preconditioned with 10 ml of water adjusted to pH 7.0.

After drying the column with N_2 elutions were carried out first with 25 ml ethyl acetate to elute catechins and oligomeric proanthocyanidins and then the polymeric fraction was eluted with 10 ml methanol. To separate the monomeric from oligomeric fraction, both fractions were evaporated to dryness under vacuum at 25 ºC, dissolved in distilled water and then redeposited onto the same connected cartridges preconditioned with distilled water. After drying the cartridges with N_2, catechins and oligomeric proanthocyanidins were eluted sequentially with 25 ml diethyl ether (catechins fraction) and finally with 10 ml methanol (oligomeric fraction).

Vanillin assay: For each fraction obtained previously, the quantification of flavanols was performed by the modified vanillin assay described by SUN *et al.* (1998 c). Quantification was done in duplicate.

HPLC analysis of individual catechins and oligomeric proanthocyanidins: For analytical HPLC a Merck Model L-200A pump (Merck-Hitachi, Darmstadt, Germany) connected to a Waters 717 plus autoinjector (Milford, MA, USA) was used. Detection: Konic UV-vis detector (UVIS 200) coupled to a Konichrom data treatment system at 280 nm. The column (250x4.6 mm, 5 μm particle size) was a reverse-phase C18 Lichrosphere® 100 (Merck, Darmstadt, Germany) protected by a guard column of the same material.

A modification of the method described by RICARDO-DA-SILVA *et al*. (1990) and DALLAS *et al*. (1995), was used for the HPLC separation of individual catechins and procyanidins. The mobile phases for catechins were (A) acetic acid/bidistilled water (2.5:97.5, v/v) and (B) acetronitrile/A (80:20, v/v). The linear gradient was run from 93 % of A and 7 % of B during 26.1 min, followed by 88 % of A and 22 % of B, during 90 s. For 15 min the column was washed with methanol/water (50:50, v/v). The flow rate was 0.9 ml·min^{-1}.

The mobile phases for procyanidins were (A) acetic acid/bidistilled water (10:90, v/v) and (B) bidistilled water. A linear gradient was run from 10 % of A and 90 % of B to 70 % of A and 30 % of B, during 45 min, followed by another gradient from 90 % of A and 10 % of B during 25 min and a constancy for more than 12 min. The flow rate was 1 ml·min^{-1}.

Between two injections, the column was washed with methanol/water (50:50, v/v) during 15 min. All analyses were done in duplicate.

Results and Discussion

In Tab. 1, the physico-chemical composition of cvs Touriga Francesa, Castelão Francês and Viosinho grapes at harvest is presented. The results show that total phenols, total anthocyanins and colour density in cv. Touriga Francesa grapes were much higher than in cv. Castelão Francês.

Fig. 1 shows the evolution of monomers (catechins) and oligomers (degree of polymerization ranging from 2 to 12-15) from stems during cluster development. For the three cultivars the contents of the two flavanol fractions decreased throughout grape development, but not in a uniform way.

Table 1

Physico-chemical composition of Touriga Francesa, Castelão Francês and Viosinho grape varieties at techinological maturity

	Touriga Francesa	Castelão Francês	Viosinho
Weight of 200 berries (g)	343.8	385.6	226.1
Estimated degree of alcohol (% v/v)	11.0	10.9	12.9
Titratable acidity ($g \cdot l^{-1}$ tart. acid)	2.9	3.3	2.9
pH	4.3	4.0	3.6
Total anthocyanins ($mg \cdot g^{-1}$ berry)	1.25	0.95	---
Colour density	11.5	3.7	0.9
Colour hue	0.62	0.70	---
Total phenols (o.d. x 100)	46.1	19.0	52.0
Malic acid ($g \cdot l^{-1}$)	1.6	3.3	1.5
Tartaric acid ($g \cdot l^{-1}$)	4.4	4.6	4.1
Total nitrogen ($mg \cdot l^{-1}$)	1064	843	1246

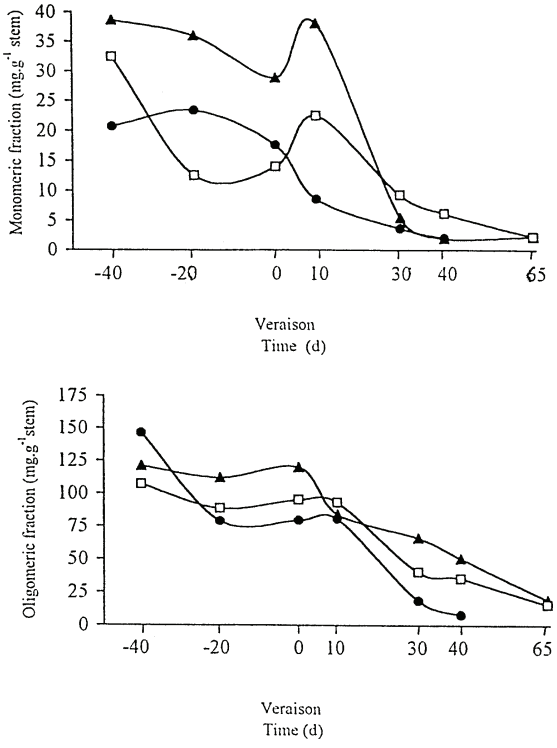

Fig. 1: Evolution of the monomeric and oligomeric fraction in cluster stems of cvs Castelão Francês (□), Touriga Francesa (▲) and Viosinho (●).

For the two red varieties the concentrations of the two fractions were high 40 d before veraison and decreased until veraison (Fig. 1). At veraison the concentration of the monomeric and oligomeric fractions increased sharply and 10 d after veraison decreased continuously. In contrast, in the white cv. Viosinho, a continuous decrease was observed during grape development. The results obtained for the oligomeric and monomeric fractions indicate that differences between cultivars were significantly greater at the early stages of development. However, grape stems of Touriga Francesa exhibited higher values than Castelão Francês and Viosinho in all stages of development.

For Viosinho, the values of monomeric and oligomeric fractions were less abundant than in red varieties.

Fig. 2 shows that the polymeric fraction for the three varieties continuously decreased. On the other hand, it becomes evident that the polymeric fraction is the most abundant proanthocyanidin fraction from stems during grape development (Figs 1 and 2). Values of the polymeric fraction at maturity are presented in Fig. 3: in contrast, the concentration of the monomeric forms exhibited smaller values in all varieties. These results confirm recent investigations of PRIEUR *et al.* (1994), SUN *et al.* (1996, 1998 a) analyzing seeds and skins at harvest.

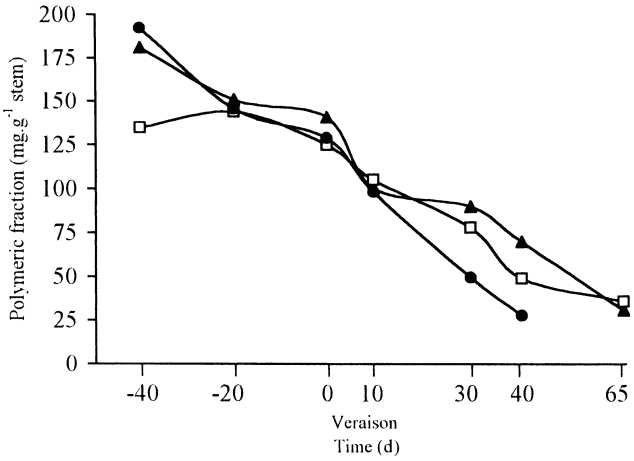

Fig. 2: Evolution of the polymeric fraction in cluster stems of cvs Castelão Francês (□), Touriga Francesa (▲) and Viosinho (●).

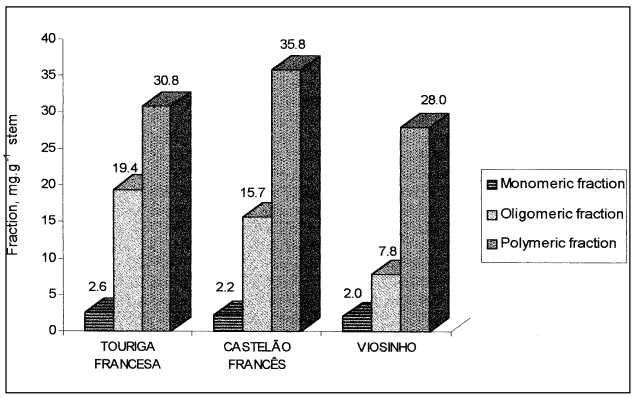

Fig. 3: The flavanol fractions in cluster stems ($mg \cdot g^{-1}$) of cvs Touriga Francesa, Castelão Francês and Viosinho at harvest.

The tendency for higher concentrations of flavanols to occur in the early stages of berry development is probably implying that these polyphenols are metabolized throughout the growing season. For SINGLETON *et al.* (1969), the metabolic process of proanthocyanidin flavanols is similar to that of anthocyanins, sugars and other grape components. VALERO *et al.* (1989) considered that the decrease during grape maturation is a consequence of increasing weight of berries or/and seeds. Tabs 2 and 3 show the content of some oligomeric procyanidins and monomers from

Table 2

Flavanol content (mg·g^{-1}) in cluster stems of the red cvs Castelão Francês and Touriga Francesa during development

	Cluster stem development													
	-40 d		-20 d		Veraison		+10 d		+30 d		+40 d		+65 d	
Flavanols	Castelão Francês	Touriga Francesa	Castelão Francês	Touriga Francesa	Castelão Francês	Touriga Francesa	Castelão Francês	Touriga Francesa	Castelão Francês	Touriga Francesa	Castelão Francês	Touriga Francesa	Castelão Francês	Touriga Francesa
B1	44.8	50.1	24.5	40.2	5.5	30.6	23.7	37.3	10.1	30.6	7.1	20.6	3.5	5.8
B2	0.9	0.4	1.5	0.2	0.4	0.5	0.3	1.2	0.3	1.5	0.3	1.2	0.4	1.2
B3	15.9	19.6	8.8	8.0	2.0	5.9	3.3	6.5	2.0	7.0	0.6	7.3	0.2	1.2
B4	1.2	2.1	0.5	1.1	0.2	0.3	1.0	0.3	1.8	0.3	1.4	0.4	1.0	0.2
B1-3-0-gallate	2.5	0.2	1.3	0.4	0.3	0.4	0.6	2.7	1.0	1.5	0.3	1.1	0.2	0.1
B2-3-0-gallate	1.6	2.4	0.6	3.0	0.2	2.1	0.5	2.6	1.1	1.7	0.8	0.6	0.5	0.4
B2-3'-0-gallate	2.3	3.1	0.5	0.5	0.4	2.3	0.9	2.4	0.7	3.0	0.5	2.8	0.2	0.9
C1	2.9	3.8	2.3	6.5	0.3	4.2	0.9	9.7	0.6	3.8	0.5	1.2	0.6	0.5
T2	15.4	19.9	18.3	32.8	2.0	6.4	4.7	5.8	5.7	5.0	4.4	5.0	1.5	2.3
(+)-Catechin	20.5	36.0	10.6	34.9	10.5	25.6	22.0	35.9	8.5	4.2	5.9	0.9	1.3	2.0
(-)-Epicatechin	1.1	1.1	1.0	0.4	2.2	3.0	1.2	1.1	1.1	0.8	0.8	0.6	0.7	0.5

Each value represents the mean from duplicate samples.

Table 3

Flavanol content ($mg \cdot g^{-1}$) in cluster stems of the white cv. Viosinho during development

Flavanols	Cluster stem development					
	-40 d	-20 d	Veraison	+10 d	+30 d	+40 d
B1	50.7	20.6	7.5	16.0	8.1	1.2
B2	0	0.3	0.8	1.0	0.4	0.4
B3	23.1	10.5	5.3	7.2	1.3	0.1
B4	4.3	2.9	0.4	1.2	0.2	0.1
B1-3-0-gallate	4.3	1.9	0.6	3.4	0.3	0.1
B2-3-0-gallate	3.2	2.9	1.1	2.0	0.2	0.6
B2-3'-0-gallate	3.2	3.0	0.8	2.2	0.4	0.2
C1	2.5	1.9	0.4	1.4	0.6	0.4
T2	35.0	25.9	5.6	12.4	1.3	0.9
(+)-Catechin	19.5	21.4	16.8	7.4	2.9	1.5
(-)-Epicatechin	0.8	0.6	0.8	0.5	0.5	0.6

Each value represents the mean from duplicate samples.

stems during cluster development. 40 d before veraison the concentrations of oligomeric procyanidins were high and than steadly decreased until veraison. However, concentrations of dimeric pro-cyanidins (B1, B2, B3, B4), galloylated dimeric (B1-3-0-gallate, B2-3-0-gallate, B2-3'-0-gallate) and trimeric procyanidins (C1, T2) showed a sharp increase at veraison and then decreased continuously to low concentrations at harvest.

For cv. Castelão Francês (Tab. 2), the seasonal variation in oligomeric procyanidins was much less intense than for other varieties. The values of oligomeric procyanidins from cluster stems of the white variety were less abundant than those of the red varieties. These results are similar to those of Ricardo-da-Silva *et al*. (1991 a; 1992 a, b).

The evolution of the individual oligomeric procyanidins was in general similar to that found for the oligomeric fraction during grape maturation (Fig. 1). However, at veraison the magnitude of variation for the monomeric fraction was much lower than for the oligomeric procyanidins. This evolution confirms the studies made in seeds and skins of other grape varieties (Romeyer *et al*. 1986; Lee and Jaworski 1989; Jordão *et al*. 1998).

For the three cultivars studied, (-)-epicatechin decreased until veraison (Tabs 2 and 3). However, red varieties show a sharp increase of (+)-catechin within 10 d after veraison and then decreased continuously.

During maturation the major oligomeric procyanidin in the three varieties was B1 (Tabs 2 and 3). The second most abundant procyanidin in all grape varieties was the trimeric procyanidin trimeric 2; this is similar to previous studies of Ricardo-da-Silva *et al*. (1991; 1992 a, b) and Jordão *et al*. (1998).

From the galloylated dimeric procyanidins (Tabs 2 and 3), B2-3'-O-gallate was the most abundant form in Touriga Francesa, while for Castelão Francês and Viosinho B2-3-O-gallate was the most abundant form at technical maturation. From all oligomeric forms, the less abundant forms were B1-3-O-gallate in Touriga Francesa and procyanidin B3 for Castelão Francês and Viosinho.

The oligomeric procyanidins (dimers and trimers) which until now were usually quantified individually by HPLC, constituted only a very small part of the stem flavanols, as has already been mentioned in literature for seeds and skins.

Conclusions

Grape cluster stems are shown to be important source of catechins and proanthocyanidins. The maximum concentration of these flavanols was observed in the early stages of development. During cluster maturation, the polymeric fraction was the most abundant, followed by the oligomeric fraction.

To our knowledge, this is the first report on a quantitative analysis of monomeric, oligomeric and polymeric flavanol compounds during cluster stem maturation. As this part of the grape cluster is a by-product of winemaking, its extraction might probably be of interest from an industrial point of view to obtain nutriceutical, oenological or chemical products. In addition, if stems are used in maceration during red winemaking this study may help to estimate the contribution of stems to the wine flavanol content.

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Received September 9, 2000