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A Biochemical Approach to the Evolution of Procyanidins in Grape Seeds During the Ripening of Red Grapes (*Vitis vinifera* L. cv. Merlot Noir)

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ABSTRACT *Seed extracts of one clone of Merlot Noir were studied during maturation using low-pressure and high-performance liquid chromatography to observe changes in composition and to follow the evolution of the main procyanidins. These observations have prompted our laboratory to investigate the polymerisation of procyanidins associated with their extraction with an alcoholic solvent. We report the observed correlations for procyanidin dimers, which are relatively high between procyanidins B_2/C_1/A_2 themselves (linked by C_4—C_8 bond), but which are non-existent between procyanidins linked by C_4—C_8 and by C_4—C_6 bonds. These observations have enabled us to propose a hypothesis regarding procyanidin oligomer biosynthesis.*

Introduction

The condensed tannins of grapes and wines are essentially polymers consisting of (+)-catechin and (−)-epicatechin structural units (procyanidins) (Haslam, 1980). These monomeric compounds have been shown to give rise to a series of condensed procyanidins including the dimers known as B_1—B_8 (Freitas, 1995) and also A_2 (Vivas *et al.*, 1996). Over the past few years, there has been increased interest in studies on the conformation and configuration of procyanidin dimers (Hemingway *et al.*, 1982; Prieur *et al.*, 1994; Self *et al.*, 1986). All these phenolic constituents found in grapes are derived from solid parts (grape seeds and skins).

Grape seeds have been shown to contain much higher levels of these polyphenolic compounds (≈ 500 mg g^{-1}) than the skins (≈ 20 mg g^{-1}) (Bourzeix *et al.*, 1986). In fact, procyanidins from grape seeds are not usually associated with other phenolic constituents. They are in a free state or can be esterified with gallic acid. On the other hand, procyanidins from grape skins tend to assemble with macromolecular cell wall compounds, such as polysaccarides, lignins and proteins. The polyphenolic structure in the

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skins is probably more complex and variable. Since the seeds are the main source of such polyphenols, it is easier to extract phenolic constituents from grape seeds than from skins.

Procyanidins can polymerise and combine with anthocyanins, polysaccharides and proteins. The organoleptic characteristics are undoubtedly the result of the evolution of these condensed forms. Our research therefore examined the problem of procyanidin polymerisation during maturation. Numerous studies have likewise attempted to demonstrate that phenolic constituents are the main components affecting gustative qualities of wine.

This study focuses on procyanidin evolution during the maturation of grapes and involved four stages. First, grape samples were picked at different states of maturity. Second, we suggest a method for extracting phenolic compounds from grape seeds, the main source of procyanidins in grapes. Because of the complexity and variety of these compounds, as well as the presence of impurities, it is impossible to quantify them directly by high-performance liquid chromatography (HPLC). Therefore, the third stage of the research involved the fractionation of the seed extract by low-pressure liquid chromatography (LPLC). The different fractions obtained were analysed by liquid secondary ion mass spectrometry (LSIMS) and the fractions containing monomer and dimer procyanidins were collected. Finally, a quantitative determination of polyphenolics ((+)-catechin, (−)-epicatechin and the dimers) was undertaken by HPLC.

This methodology permitted us to obtain the rate of production of oligomeric procyanidins during grape maturation and to follow their evolution. These results allowed us to establish a correlation between two groups of procyanidin dimers and to suggest that procyanidin evolved according to their structure (C_4—C_6 or C_4—C_8). These final results enable us to propose a hypothesis of procyanidin biosynthesis.

Materials and Methods

Phenolic Constituent Extraction

The method of extraction used was very similar to the procedure described by Darné and Madero Tamargo (1979). This extraction technique to obtain the seed extract was selected because:

- the method closely approximates wine conditions (hydroalcoholic solvent, pH);
- the extraction process is not brutal with phenolic compounds (no alkaline pH, no high temperatures);
- it eliminates some non-phenolic compounds (lipids, carotenoids) which can interfere in the quantity determination of polyphenols.

Grapes were picked in 1995 from a single clone of Merlot Noir in a vineyard near Bordeaux (Gironde, France). Five samples of grapes were collected while they were ripening: M1(21/08); M2(28/08); M3(4/09); M4(7/09); M5(11/09, harvest). All were repeated three times, on a series of four samplings for M1–M5. For each case, average values and standard deviations have been calculated.

The seeds were recovered, freeze dried and reduced to powder. To prepare the LPLC samples, 4 g of powder were ground for 2 min in a blender with 10 ml of ethanol 95% (v/v) and 10 ml of aqueous solution containing 1 g l^{-1} of $NaHSO_3$ (antioxidizing agent). Chloroform (20 ml) was then added and the mixing was continued for 1 min. The resulting mixture was centrifuged for 10 min ($4000 \times g$). Two phases were separated out by an interface made up of the solid matter. The green lower phase (containing chloroform, lipids, pigments, etc.) was eliminated. The yellow superior phase

Table 1. HPLC gradient elution

Time (min)	0	5	90	95	100	105
A (%):	93	93	80	0	0	93
B (%):	7	7	20	100	100	7

(hydroalcoholic solution) containing the phenolic constituents was recovered. This extraction was repeated six times on the powder remaining in the centrifuge tube. All the hydroalcoholic extracts were collected and evaporated to remove ethanol (temperature $\leq 30°C$). The aqueous solution obtained was filtered. This solution (20 ml) was extracted with ethyl acetate (6×20 ml). The organic phases were collected and the solvent was evaporated (temperature $\leq 30°C$). The extracts obtained were dissolved in 5 ml of methanol before being injected into a low-pressure column.

Low-pressure Liquid Chromatography

The samples were injected into a low-pressure column (1.6×35 cm) of gel TSK Toyopearl HW-40(S). They were eluted with methanol and the flow rate was 0.8 ml min^{-1}. Each fraction was recovered and analysed by LSIMS. The fractions containing the oligomer procyanidins were then collected. The solvent was completely evaporated (temperature $\leq 30°C$). The extracts were dissolved in 0.5 ml of methanol before being analysed by HPLC.

Usually, gel TSK Toyopearl HW-40(S) is used for size exclusion chromatography (SEC): molecules are eluted according to their decreasing molecular mass. However, polyphenols have the particularity to adsorb themselves on this gel by hydrophobic linkages, so their separation is based on the principle of affinity chromatography, not SEC. In this case, the more condensed forms have an important affinity for this gel and are eluted more slowly, so phenolic compound elution is realised according to their increasing molecular mass (Biau, 1996).

LSIMS Analysis

LSIMS spectra were recorded using a VG-Autospec EQ instrument, equipped with a caesium ion gun in negative mode: caesium ion beam energy, 35 keV (2 μA); temperature $\leq 40°C$. Calibration was performed with caesium iodide salt (200–1500 Da). Thioglycerol was used as matrix. Data acquisition was performed using Vax Station 3100 Digital equipment (OPUS system). For each fraction collected by LPLC, solvent was evaporated, then samples were dissolved in the minimum quantities of anhydrous methanol required to solubilise the polyphenols.

HPLC Analysis

Extract (20 μl) was injected into two BECKMAN ultrasphere ODS C_{18} (250×46 mm; 5 μm) columns in series at 20°C ($\pm$ 1°C), eluted with a flow rate at 1 ml min^{-1}. Compositions of the two solvents were: solvent A was formic acid:water (2.5:97.5 v/v); solvent B was solvent A:acetonitrile (20:80 v/v).

The analytical method used was very similar to the procedure described by Ricardo da Silva *et al.* (1991). The gradient conditions are given in Table 1. Detection was

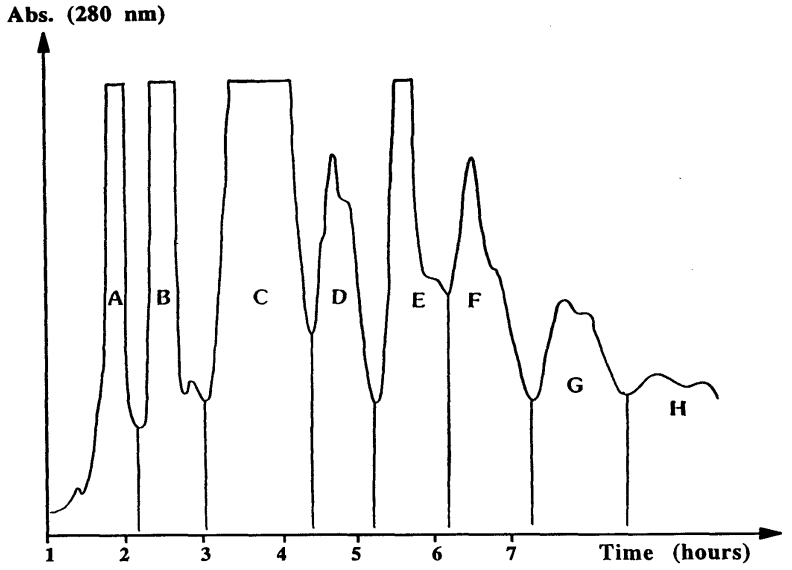

Figure 1. Low-pressure chromatogram of a seed extract (Merlot Noir, 1995).

Table 2. Phenolic constituents in different seed fractions (Freitas, 1995)

Seed fractions	Molecular ion [M—H]	Constituents
A	153/169/179	*p*-coumaric acid, gallic acid and cafeic acid
B	289	(+)-catechin and (−)-epicatechin
C	577	procyanidins B_1, B_2, B_3, B_4, B_5, B_7
	441	(−)-epigallocatechin
D	577	procyanidins B_6, B_8
	865	trimer C_1
E	865	procyanidin trimers
	729	galloylated procyanidins B_1, B_2, B_3, B_4, B_5
F	865	procyanidin trimers
	729	galloylated procyanidin B_6
G	865	procyanidin trimers
	1017	galloylated procyanidin trimers
	1153	procyanidin tetramers
H	881	digalloylated procyanidins B_1, B_2, B_3, B_7
	1153	procyanidin tetramers

monitored at 280 nm. The levels of dimer procyanidins were quantified using standard curves developed from reference standards given by Freitas (University of Porto).

Results

Separation and Identification of Different Fractions in Seed Extract

Figure 1 represents a typical low-pressure chromatogram of seed extract of Merlot Noir grapes. These seed samples all contained eight different fractions (A–H). Each fraction

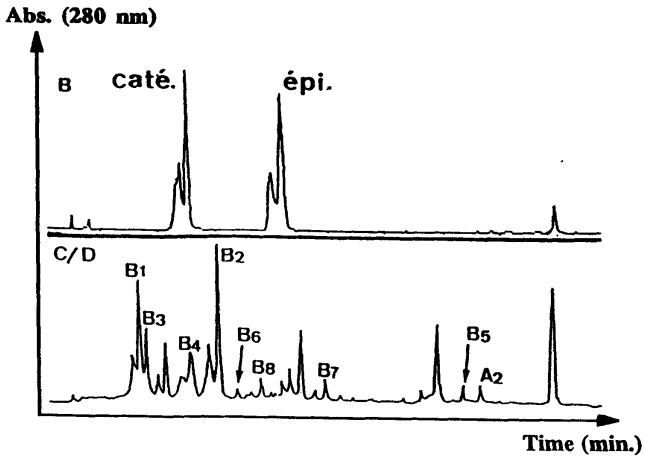

Figure 2. HPLC chromatogram of two fractions collected (fraction B and fraction C/D).

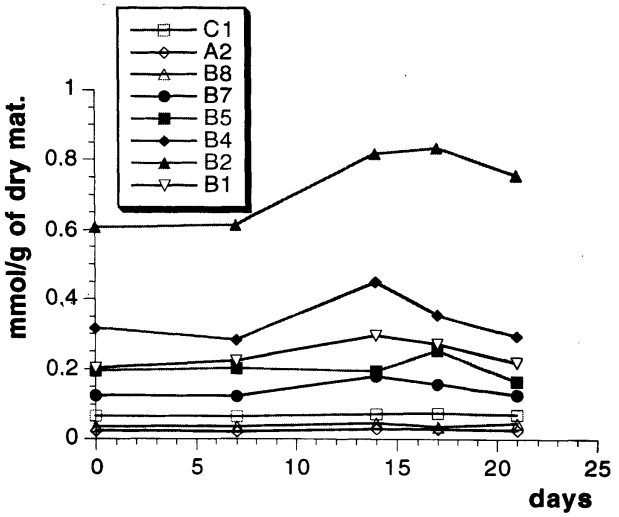

Figure 3. Procyanidin evolution during maturation.

was recovered and directly analysed by LSIMS. Procyanidins were separated according to their degree of polymerisation (Table 2). The following observations can be made: oligomer procyanidins (molecular weight ≤ 1170) and catechins represent 70% of the seed extract; (+)-catechin, (−)-epicatechin and the dimer procyanidins represent 30%; the remaining oligomers, 40%; and the more polymerised forms, 30%.

Subsequently, only the fractions associated with (+)-catechin, (−)-epicatechin (fraction B), the dimers (B_1–B_8 and A_2) and the trimer C_1 (fraction C/D) were collected and quantified by HPLC (Figure 2).

Table 3. Repartition of soluble and insoluble procyanidins in two seed extracts (average values)

	M1[a]	M5[a]
Dimer procyanidins (eluted by H_2O)	1804.3 (± 1.4)	1451.8 (± 0.9)
Polymerised procyanidins[b] (eluted by CH_3CN)	633.1 (± 1.6)	987.9 (± 1.0)
Sum of the two types of procyanidins	2437.4 (± 2.3)	2439.7 (± 2.7)

[a]Results obtained by peak area integration.
[b]The presence of tannins polymerised in this peak has been shown by a reaction of Bate–Smith (1954).

Table 4. Correlation: procyanidin dimers C_4—C_8 themselves; procyanidins C_4—C_8 with procyanidins C_4—C_6

	Linear regression	Correlation coefficient r ($r_{0.01} = 0.917$)
$[A_2]/[B_2]$	$y = 0.0010995 + 0.033407$	0.961: correlation > 99%
$[A_2]/[C_1]$	$y = -0.029644 + 0.79087$	0.937: correlation > 99%
$[B_2]/[B_5]$	$y = 0.12946 + 0.10083$	0.352: no correlation
$[A_2]/[B_5]$	$y = 0.019818 + 0.027526$	0.226: no correlation

Procyanidin Evolution During Maturation

This method permitted the evolution of procyanidins to be followed during grape maturation. Figure 3 shows the various transformations during maturation leading to the different procyanidins made up of (−)-epicatechin units. The evolution of these procyanidins through time was similar: accumulation then decreased at maturity, although some of the procyanidins ($B_1/B_4/B_7/B_8$) made up of (+)-catechin and (−)-epicatechin units decreased more rapidly than procyanidins made up of only (−)-epicatechin units ($B_2/B_5/A_2/C_1$).

On the other hand, to understand the polymerisation process, we studied the procyanidin repartition according to the degree of polymerisation during the grape ripening. Table 3 shows the distribution of soluble and non-soluble procyanidins in an aqueous solvent for seed extracts M1 (minimum tannin concentration) and M5 (tannin concentration at maturity). The levels of procyanidins which are feebly polymerised ($n \leq 3$) and thus extractable by a hydroalcoholic solvent decrease during maturation, whereas the more polymerised procyanidins, extractable by an organic solvent (CH_3CN), increase. The sum of the two types of procyanidins remains similar in both cases.

In the same way, we have tried to correlate the concentration evolution of procyanidin between them. The results (linear regression and correlation coefficient ($r_{0.01}$)) of these correlations between $[A_2]/[B_2]$, $[A_2]/[C_1]$, $[B_2]/[B_5]$ and $[A_2]/[B_5]$ are reported in Table 4. We can observe the following. The quantity determination of the procyanidins, B_2: epicat (4β—8) epicat, C_1: epicat (4β—8) epicat (4β—8) epicat and A_2: epicat (4β—8; C_2—O—C_7) epicat, only made up of (−)-epicatechin units linked by a C_4—C_8 bond, shows a significant correlation between these three molecules. On the other hand, quantity determination of the procyanidins made up of (−)-epicatechin units linked by a C_4—C_6 bond (procyanidin B_5) is no longer correlated with the dimer procyanidins C_4—C_8.

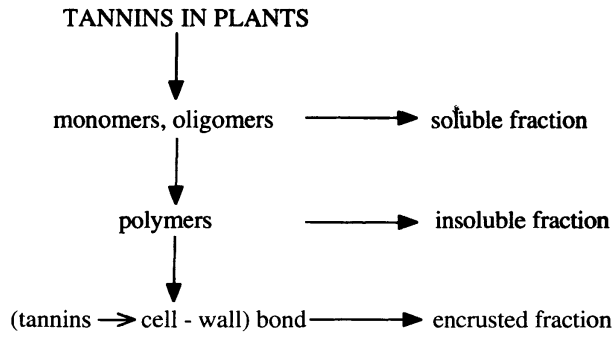

Figure 4. Scheme of reticulation proposed by Monties (1992).

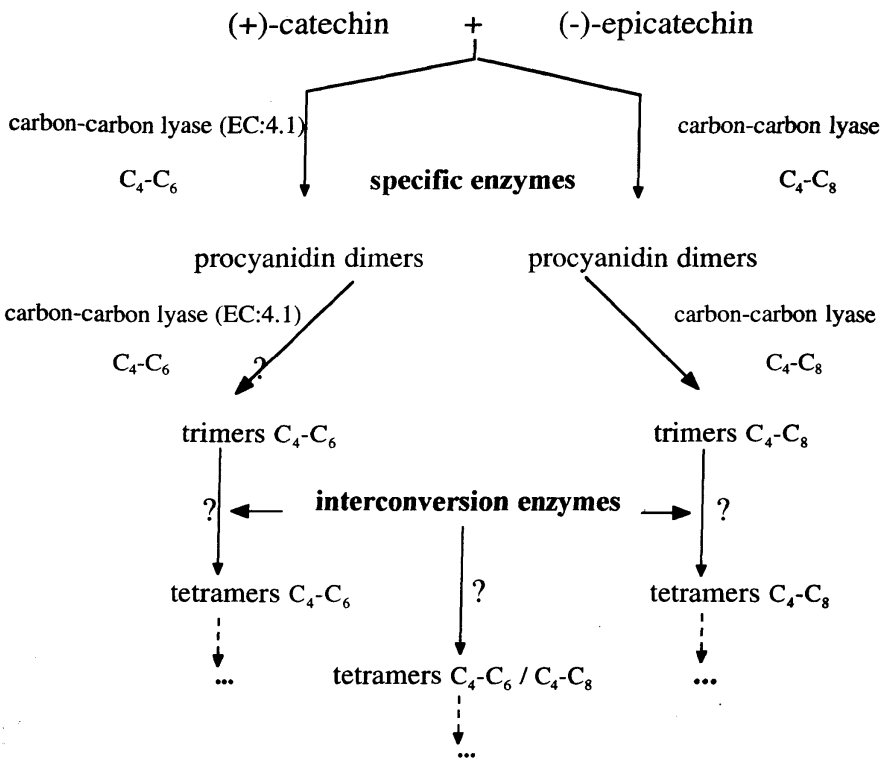

Figure 5. Hypothesis of biosynthesis.

Discussion

A Comparative Evolution of Procyanidin Dimers and Polymers During Maturation

Several research groups have already demonstrated that tannins decrease at maturity (Darné, 1991). It has been shown that seed tannins decrease when the phenolic constituents in the skins increase. The authors explain this result by tannin migration from seeds to skins. Nevertheless, there is no gradient of tannin concentration in pulp to justify their hypothesis: Amrani-Joutei (1993), Amrani-Joutei *et al.* (1994) and Park *et al.* (1995) have shown that there are no tannins in either the pulp or pulp vessels carrying

unrefined or refined juice. It has also been shown that the phenolic constituents are unable to circulate in both unrefined and refined juice, which is not physiologically possible.

A second hypothesis might account for this observation: the decrease of tannins at maturity should be equivalent to the decrease of 'extractive tannins'. In fact, procyanidins continue to polymerise and become decreasingly soluble in an aqueous solvent. This insoluble fraction of tannins cannot be determined quantitatively by an extraction with hydroalcoholic solvent, so the concentration of total tannins decreases without their real rate decreasing. This insoluble fraction of tannins is about 20% (Vaneron, unpublished results). It is thought that there is some bonding between parental compounds and tannins: some of the tannins are found in oligomeric form ($n \leqslant 3$) and some in polymerised insoluble form. Injections by HPLC of seed extracts M1 and M5 seem to confirm this second assumption (Table 3). Tannins are unlikely to migrate at maturity from seeds to skins, but they become less and less extractive during polymerisation. This hypothesis agrees perfectly with the general theory of Monties (1992) (Figure 4).

Correlation Between Concentration Evolution of Procyanidins C_4—C_8 and C_4—C_6

Based on this assumption, we tried to correlate the procyanidins made up of only (−)-epicatechin units (Table 4). This experience enabled us to suggest a hypothesis for the biochemical evolution of these procyanidins. As shown in Table 4, the procyanidins made up of only (−)-epicatechin units linked by a C_4—C_8 bond (B_2; C_1; A_2) are closely interrelated ($r_{0.01} = 0.917$). These three molecules are thus theoretically involved in the same biosynthesis process. On the other hand, the lack of correlation between procyanidins C_4—C_8 and procyanidins C_4—C_6 supposes that there are two different biochemical evolutions for the formation of these two types of dimers. This proposed biochemical evolution hypothesis is summarised in the general scheme shown in Figure 5.

Conclusions

We have demonstrated that seed tannin levels did not decrease at maturity and that procyanidins just continued the polymerisation process. During polymerisation, procyanidins become less and less extractive by a hydroalcoholic solvent, which explains why the quantity determination of these compounds decreases at maturity.

The present study clearly points to a correlation between the C_4—C_8 procyanidins themselves. We can therefore assume that C_4—C_8 procyanidin formation requires a specific enzyme. The same applies for the other dimers C_4—C_6 which also require another specific enzyme.

We intend to continue our investigations in the near future to verify the present findings and to determine the specific enzymatic activity that causes procyanidin polymerisation.

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