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COMPOSITIONAL CHANGES IN LOWER MOLECULAR WEIGHT FLAVANS DURING GRAPE MATURATION

ZOFIA CZOCHANSKA, LAI YEAP FOO and LAWRENCE J. PORTER
Chemistry Division, Department of Scientific and Industrial Research, Petone, New Zealand

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Key Word Index— *Vitis vinifera*; Vitaceae; grapes; seasonal variation; catechins; proanthocyanidins.

Abstract—Grapes of the Gamay Beaujolais and Siebel varieties have been sampled during the period from just before the onset of colouration to harvest. Extraction has yielded data on the composition and quantities of lower molecular weight and polymeric flavanoids present. The structure of the major flavans of these grapes has been established and the variation in their relative amounts monitored during the growth season. The basic structural unit of the grape proanthocyanidin polymer is a monomer with (−)-epicatechin stereochemistry.

INTRODUCTION

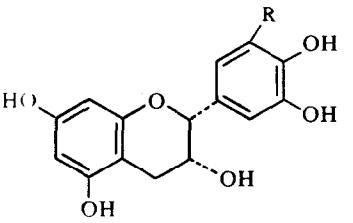
There has been much interest in the composition of the phenolics of grapes because of the direct bearing this has on the final quality of wine [1]. For example, it has been demonstrated that anthocyanins and catechins combine with sulphur dioxide and acetaldehyde which determine the colour and taste of mature red wines [2–5]. It is of interest, therefore, to have some knowledge of the initial composition of phenolics in ripe grapes before they are fermented. Of equal importance, from a plant physiological point of view, is the question of how the phenolic composition of grapes varies throughout the growth season.

There have been many studies made on this topic in terms of total phenolics and tannins [1]. Somers [6] has recently studied pigment development in ripening Shiraz grapes. The current study attempts to follow in some detail the changes in composition of the lower molecular weight flavans and polymeric proanthocyanidins.

RESULTS

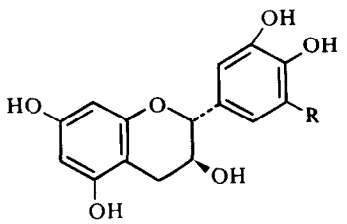
Relatively large samples (0.5—1 kg wet wt) of Gamay Beaujolais and Siebel grapes were sampled at regular intervals through the growth season from just before veraison ('first colour', the onset of fruit ripening) to harvest. The grapes were extracted with aqueous acetone, as described by Jones *et al.* [7] except that an EtOAc extraction was used to isolate the lower MW flavans.

The proportion of flavans in the EtOAc extract and the proanthocyanidin polymer fraction (defined in Experimental) was estimated by a modified [8] vanillin–HCl colorimetric determination. Proanthocyanidin standards were prepared from polymers shown to be homogeneous by ^{13}C NMR [9]. The absorbance per 100 μg of proanthocyanidin polymer was the same as that reported by Broadhurst and Jones [8]. The proanthocyanidin polymer fractions from the first grape samples (2/2) were unpigmented, but as the colour in the grapes developed this fraction became intensely coloured.

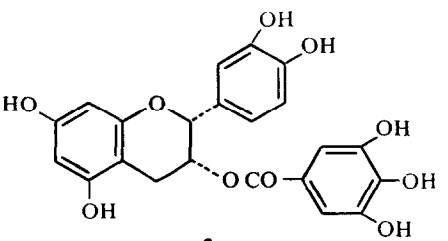


2 R = H
9 R = OH

The major lower MW flavan constituents were isolated by chromatography of combined EtOAc-soluble fractions on Sephadex LH-20 in EtOH [10]. This yielded samples, after derivatization and rechromatography, of the peracetates of gallic acid, (+)-catechin (**1**), (−)-epicatechin (**2**), (−)-epicatechin 3-*O*-gallate (**3**) and procyanidin B-2 (**4**). Their structures were confirmed by ^1H NMR and MS.



1 R = H
8 R = OH



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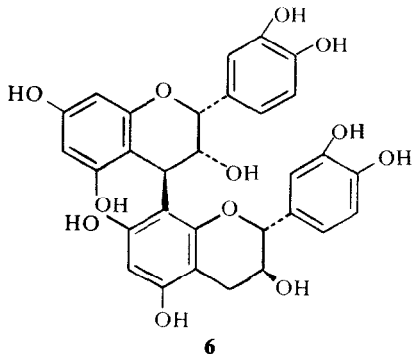

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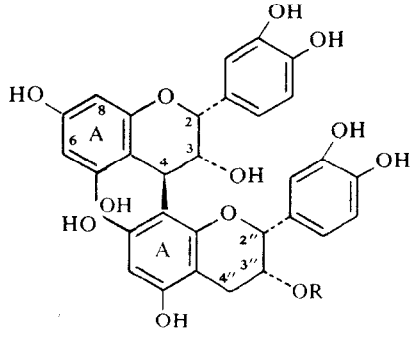
The predominant monomer unit of the proanthocyanidin polymer of grapes was determined by degradation of a combined sample of polymer fractions with phloroglucinol and HCl in dioxan–H_2O [11]. This yielded the phloroglucinol adduct of the (−)-epicatechin carbocation. The structure of the adduct (**5**) was confirmed by comparison of the ^1H NMR spectrum of the octaacetate with that of authentic material.

The detailed analysis of the lower MW flavans was performed by GLC analysis of the TMSi ethers of the phenolics. The method used was essentially that pioneered by Collier and Mallows [12] for the analysis of tea flavans. However, JXR methyl silicone was found to be a more satisfactory liquid phase than OV-1 and a short column was used so that reliable quantitative analyses of the procyanidin dimers could be obtained [13]. (The procyanidin pairs B-1 and B-2; B-3 and B-4 were inseparable by GLC on this liquid phase however.) The retention indices for the individual flavan constituents were obtained by independent injections of appropriate substances obtained from other plant sources. The GLC data for the major flavan constituents are summarized in Table 1.

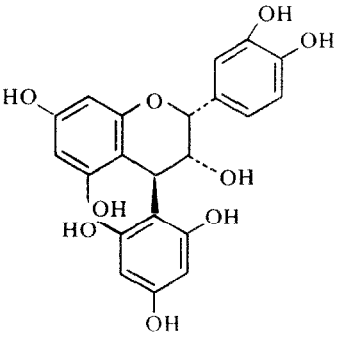


4 R = H
7 R = gallate

DISCUSSION

Structure of the lower MW flavans

The major components of both Beaujolais and Siebel grapes are (+)-catechin (**1**), (−)-epicatechin (**2**), (−)-epicatechin 3-*O*-gallate (**3**) and procyanidin B-2 (**4**). Weinges and Piretti [14] isolated a similar series of compounds from *Vitis vinifera* cv Albana grapes, but



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found, however, that procyanidin B-1 (**6**) rather than B-2 (**4**) was the major procyanidin dimer. This difference may be readily explained by the fact that Albana contained a much higher proportion of (+)-catechin (**1**, the lower flavan unit of B-1) than we found in either Beaujolais or Siebel. Thus the ratio of (+)-catechin (**1**) to (−)-epicatechin (**2**) in Albana was 8:1 [14] whereas it was approximately 1:1 in both grape varieties in this study (see Table 2).

Procyanidin B-1 (**6**) was a minor constituent of both grape varieties and was identified by co-chromatography with authentic material on cellulose TLC. Thus both the major procyanidin dimers possess an upper flavan unit with (−)-epicatechin stereochemistry, which is consistent with the observation that the procyanidin polymer also possesses units predominantly of this type.

Table 1. Compositional changes in the flavan constituents of grapes during berry development

		Siebel Sampling dates									Gamay Beaujolais Sampling dates						
		2/2	17/2	1/3	8/3	15/3	22/3	29/3	6/4	14/4	2/2	17/2	1/3	8/3	15/3	22/3	29/3
Proportion of flavans in:																	
EtOAc fraction (% w/w)	Relative retention	4.8	8.6	1.8	3.1	2.1	2.5	2.7	2.8	5.0	8.0	6.6	5.3	4.9	2.8	4.8	3.7
Polymer fraction (% w/w)	time, V_R	10.1	5.8	1.4	2.2	2.5	3.8	2.9	2.4	2.5	12.5	6.4	7.0	9.8	3.5	5.9	4.6
Gallic acid*	0.35	0.5	2.8	2.6	3.2	3.9	4.3	3.6	4.2	4.8	1.3	0.8	0.9	1.3	1.8	1.7	1.4
(−)-Epicatechin*	1.0	27.0	38.5	41.4	38.8	34.9	39.6	41.7	40.5	40.7	32.3	22.5	38.7	31.8	39.0	32.7	40.1
(+)-Catechin*	1.01	31.5	40.9	40.5	43.3	39.5	39.6	42.5	41.0	35.0	35.5	58.5	56.7	60.5	48.8	57.7	53.4
(−)-Epigallocatechin*	1.06	0.4	1.7	3.4	2.4	1.1	1.5	1.1	0.9	0.8	1.7	10.1	tr	tr	3.4	1.6	2.7
(+)-Gallocatechin*	1.14	0.5	tr	0.2	tr	0.4	0.4	0.4	0.4	0.6	1.1	0.5	tr	tr	0.2	0.1	0.6
(−)-Epicatechin* gallate	1.71	33.0	13.1	10.7	10.0	19.1	13.5	8.8	10.6	11.9	20.7	4.4	2.6	5.5	4.6	2.5	0.9
Procyanidin* B-1 + B-2	2.30	6.6	2.3	0.7	2.1	0.9	1.0	1.7	2.1	4.9	6.7	1.9	0.9	0.7	2.2	2.5	0.8
Procyanidin B-2* 3″-*O*-gallate	2.87	0.5	0.6	0.4	0.3	0.1	0.1	0.1	0.4	1.3	0.8	1.3	0.2	0.1	0.1	1.2	tr

* Percentage of each constituent has been calculated as an area %, uncorrected for response factors. The values are the relative proportion of each constituent in the EtOAc fractions.

The GLC studies showed that a minor component, with a retention time similar to the TMSi ethers of procyanidin B-3 or B-4, $V_R = 2.87$ (see Table 1), was also present. However, the fact that neither procyanidin B-3 nor B-4 were detected in comparative PC against standards, nor their peracetates isolated, implied that the likely identity of the $V_R = 2.87$ component is a 3″-*O*-gallate ester of procyanidin B-2, which was isolated as its peracetate derivative during the course of isolation of procyanidin B-2 (**4**) decaacetate. The MS of the peracetate confirmed its dimeric nature and treatment with *n*-BuOH–HCl produced cyanidin.* The ^{1}H NMR spectrum was consistent with structure **7**, procyanidin B-2 3″-*O*-gallate, and was assigned by consideration of the spectra of (−)-epicatechin (**2**) pentaacetate, (−)-epicatechin 3-*O*-gallate (**3**) heptaacetate and procyanidin B-2 (**4**) decaacetate. Pertinent features of the ^{1}H NMR spectrum of the acetate of **7** are the occurrence of a sharp singlet at δ 7.61, due to the gallate ring aromatic protons, and the appearance of the H-4″ signal of the lower epicatechin moiety as a narrow doublet, $J = 3.4$ Hz and δ 3.02. This resonance is at lower field than the normal position for the H-4″ signal, δ 2.90, as observed in (−)-epicatechin (**2**) pentaacetate and procyanidin B-2 (**4**) decaacetate. The positions of the upper flavan unit A-ring protons, δ 6.12 and 6.25, imply the presence of a strong paramagnetic shielding effect due to overlap of the catechol ring of the lower flavan unit [11]. This in turn implies that the lower flavan unit is bonded axially to the upper flavan unit at C-4, as shown in **7**.

The GLC study also shows that, in addition to the above flavans, small amounts of gallic acid, (+)-gallocatechin (**8**) and (−)-epigallacatechin (**9**) are also present. The latter compound has been reported in grapes by Siashvili *et al.* [16]. Further evidence for the presence of flavans with a trihydroxylated B-ring is that degradation of the proanthocyanidin fraction from the first sample (2/2) with *n*-BuOH–HCl [17] yielded delphinidin as well as cyanidin in a ratio of 1:4.4 for Beaujolais and 1:3.4 for Siebel.

Variation of flavan constituents with fruit development

The data in Table 1 show that the relative proportions of the major components, (+)-catechin (**1**) and (−)-epicatechin (**2**), undergo comparatively little fluctuation with time. Gamay Beaujolais contains a higher proportion of (−)-epicatechin (**2**) than Siebel. The relative amount of (−)-epicatechin 3-*O*-gallate (**3**) undergoes a marked decrease in the berries of both grape varieties as ripening progresses. This trend is most marked in Beaujolais where the relative amount of (−)-epicatechin 3-*O*-gallate (**3**) in ripe grapes is very similar to that reported by Weinges and Piretti [14] for Albana grapes. This is probably of some significance as both Beaujolais and Albana are *Vitis vinifera* varieties whereas Siebel is a hybrid grape.

The results in Table 1 show that the highest concentrations of flavans are present in the grapes in the early stages of development. The level then drops quite quickly to a more or less steady state concentration throughout maturation. The tendency for higher concentrations of flavans to occur in the early stages of berry development is apparently typical and concurs with other work based on the estimation of total phenolics [18].

It may also be seen from Table 1 that the relative amounts of flavans in the EtOAc and polymer fraction undergo marked fluctuations, probably implying that these polyphenols are being actively metabolized throughout the growth season.

Proanthocyanidin polymer fraction

The anthocyanin pigments in the grapes are co-isolated with the proanthocyanidin polymers in the procedure used to isolate the proanthocyanidin polymer fraction. The contribution of the anthocyanins to the absorbance at 500 nm in the vanillin–HCl determination is readily corrected [8] to produce the proanthocyanidin analyses in Table 1. As pointed out earlier the proanthocyanidin fraction becomes increasingly pigmented by anthocyanins as the berry matures, so that although 100% of the weight of the polymer fraction is proanthocyanidins in the 2/2 samples of Beaujolais and Siebel, this falls to about 75% for Beaujolais and 50% for the highly pigmented Siebel grapes in the later samples. This effect may imply a partial diversion of flavonoid biosynthesis from flavan to anthocyanin production as the berry matures.

EXPERIMENTAL

Extraction of grapes. The grapes were separated from the stems and extracted (Waring-Blendor) ×3 with Me_2CO–H_2O, 7:3, containing ascorbic acid 0.1% w/v. The combined Me_2CO–H_2O solns were vigorously stirred and NaCl added until an upper Me_2CO-rich phase separated. The Me_2CO-rich layer was separated from the lower phase and the Me_2CO evapd at 30° *in vacuo* to yield an aq. residue which was diluted with an equal vol. of H_2O and extracted sequentially with $CHCl_3$ (3×) and EtOAc (8×). The resulting aq. soln, containing the polymeric proanthocyanidins, was dialysed, freeze-dried [7] and weighed to obtain the yield of the proanthocyanidin polymer fraction. The weight of the EtOAc fraction was obtained after evapn of the solvent *in vacuo* at 40°. The extracted fruit was oven-dried and weighed to estimate the residue dried weight. This weight was combined with that of the EtOAc and proanthocyanidin polymer fractions to estimate the total dry wt of extracted berries, in order to calculate the % w/w flavan values in Table 1.

Flavan determination. The % w/w of flavans in the EtOAc and proanthocyanidin fractions was estimated by the modified vanillin–HCl method of ref. [8]. Proanthocyanidin polymers isolated from *Pinus radiata* phloem and *Ribes sanguineum* leaves (purified by adsorption chromatography on Sephadex LH-20 [7] and shown to be homogeneous by microanalysis and ^{13}C NMR [9]) were used as standards. Our work confirms that the absorbance at 500 nm for 100 μg of proanthocyanidin polymer is 0.55 [8].

Determination of flavans by GLC. The EtOAc-soluble flavans were derivatized with *N,O*-bis(trimethylsilyl)acetamide (BSA) in Py using the optimum conditions determined in ref. [12]. The resulting TMSi ethers were analysed by GLC on a 1 m × 4 mm i.d. glass column with a 3% JXR methyl silicone (Supelco Inc.) liquid phase on 120 mesh Chromosorb W (AW, DMS) using programmed temp. 10°/min from 180 to 310°. Relative retention times (V_R) were estimated by co-injection of appropriate samples of the TMSi ethers of (+)-catechin, (−)-epicatechin (both Fluka), (+)-gallocatechin, (−)-epigallocatechin

* Earlier work [15] has shown that anthocyanidin pigments may be generated with equal facility from proanthocyanidins, or their peracetate derivatives.

(isolated from *Ribes sanguineum* leaves [15]) and procyanidine B-1. B-3 and B-6 (isolated from *Pinus radiata* phloem [19]) and (−)-epicatechin 3-*O*-gallate and procyanidin B-2 (isolated in the current study from grapes).

Isolation of individual flavans. A sample (10 g) of the combined EtOAc-soluble fractions was chromatographed on Sephadex LH-20 with EtOH to yield 3 fractions containing the separated major flavans. These were acetylated and the major flavans isolated as their peracetate derivatives. The first fraction yielded (−)-epicatechin (**2**) pentaacetate and (+)-catechin (**1**) pentaacetate together with gallic acid triacetate. The second fraction yielded (−)-epicatechin 3-*O*-gallate (**3**) heptaacetate. The third fraction yielded procyanidin B-2 (**4**) decaacetate. All compounds were recognized by their specific rotations, mp and ^{1}H NMR spectra, which were consistent with published data [10, 14].

Procyanidin B-2 3″-O-gallate dodecaacetate. Separated from procyanidin B-2 decaacetate by PLC (R_f 0.4; Si gel, C_6H_6–Me_2CO, 4:1) which yielded 3.2 mg of the dodecaacetate, $[\alpha]_D^{20}$ − 47° (*c* 0.07, $CHCl_3$); ^{1}H NMR data (80 MHz; $CDCl_3$; asterisked chemical shifts are for the corresponding protons in (−)-epicatechin 3-*O*-gallate heptaacetate for comparison): δ 5.28 (*s*, H-2), 5.28 (*m*, H-3), 4.99 (H-4), 4.72 (*s*, H-2″), 5.59 (*m*, H-3″), 3.03, 3.02* (*d*, H-4″), 6.12, 6.25; 6.60* (AB_q; H-6, H-8), 6.68 (H-6″), 7.67, 7.61* (*s*, gallate ring aromatic protons), 7.02–7.21 (B-ring protons), 1.25–2.23 (acetate protons).

Polymer degradation. The polymer fraction from either Beaujolais or Siebel was stirred with phloroglucinol in dioxan–0.1M HCl (1:1) for 12 hr [11]. The soln (10% w/v) was diluted with H_2O and extracted with EtOAc (8×) and the resulting extract was chromatographed on Sephadex LH-20 to yield (2*R*,3*R*,4*R*)-4-(2,4,6-trihydroxyphenyl)flavan-3,3′,4′,5,7-pentaol (**9**), which was identified by preparation of the octaacetate and comparison of the ^{1}H NMR spectrum, MS, specific rotation and mp with data obtained from authentic material isolated from crown vetch polymer [15].

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