Particular Behavior of Epoxy Xanthophylls during Veraison and Maturation of Grape

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Seven carotenoids (β-carotene, lutein, flavoxanthin, lutein-5,6-epoxide, luteoxanthin, violaxanthin, and neoxanthin) have been identified and quantitatively determined in three grape cultivars (Muscat of Alexandria, Sauvignon, and Syrah) during the maturation period and in mature grapes. Evidence is presented that lutein-5,6-epoxide, luteoxanthin, and violaxanthin have a particular behavior with consistent rises around the end of veraison. This evolution appears quite different for β-carotene and lutein, the highest grape carotenoids, and for flavoxanthin and neoxanthin, which decrease at the same period. The chief role of veraison in the fate of grape carotenoids is discussed.

Keywords: *Grape; Vitis vinifera L; carotenoids; xanthophylls; maturation*

INTRODUCTION

Carotenoid levels are low in mature grapes (∼0.8−2.5 mg/kg), whatever the variety (Curl, 1964; Gross, 1984; Razungles et al., 1987). However, these compounds can play an important role in the organoleptic quality of wines because they are considered as precursors of flavorants (Sanderson et al., 1971; Enzell, 1981; Belitz and Grosch, 1982). As shown by Enzell (1985), carotenoid degradation can give rise to different C_{13}-norisoprenoid derivatives, each specific to the initial pigment and highly flavorant. Grapes and wines contain several C_{13}-norisoprenoids [e.g., 3-oxo-α-ionol, 3-hydroxy-β-damascone, damascenone, vitispirane, β-ionone, and 1,1,6-trimethyl-1,2-dihydronaphthalene (Schreier et al., 1976; Strauss et al., 1987; Abbott et al., 1990; Winterhalter, 1991; Williams et al., 1992)], so it would be expected that the carotenoid composition of grapes would have a noticeable effect on wine flavor (Razungles et al., 1987, 1993; Marais et al., 1990, 1991).

Carotenoid composition was investigated in the different parts of the berry (Razungles et al., 1988). The skin contains a lot more carotenoids than the pulp. Furthermore, carotenoids are absent in the juice. The respective proportions of these pigments in the berries (Gross, 1984; Razungles et al., 1987) are similar to the proportions observed in the leaf (Moneger, 1968; Varadi et al., 1992). Indeed, green berries are supposed to behave like leaves, with both photosynthetic and photoprotection activities (Goodwin, 1980). These activities are probably higher in skins than in pulps, which would account for the fact that carotenoid levels were highest in skins. Contrary to numerous fruits and as was observed in cherry (Okombi et al., 1975), carotenoid levels decrease during grape maturation (Razungles et al., 1988). The beginning of this metabolism occurs at veraison when chlorophylls disappear.

The first aim of this investigation was to complete the identification and to examine the concentration of the different carotenoids in mature berries. Because these concentrations vary during maturation, our second aim was to investigate the changes in carotenoid concentrations during this period. Finally, because veraison appears to be of major importance for physiological aspects of grape development and the aromatic profile of the future wine will depend on the carotenoid composition in immature berries, the third aim was to establish the fate of carotenoids around the veraison period.

MATERIALS AND METHODS

Plant Material. Grapes from three cultivars (Muscat of Alexandria, Sauvignon, and Syrah) were supplied by the INRA Experiment Station, Domaine du Chapitre, near Montpellier, France.

Samples. For each cultivar, samples of 10 bunches were picked at random from July 27 to September 19. For the assays with mature grapes and with grapes from veraison to maturity, sampling was made at weekly intervals. For the assay with Syrah grapes around veraison (July 27 to August 11), sampling was much more frequent. From the sampled bunches, 100 g of berries were picked, freeze-dried, and stored under reduced pressure in the dark at −20 °C.

Extraction of Carotenoids. Extraction was carried out as previously discussed (Razungles et al., 1987, 1988). Grape berries (100 g) placed in liquid nitrogen were crushed in a ballgrinder. The powder obtained was mixed with cold acetone and magnesium hydroxy carbonate (3 g for mature berries, 6 g for green berries), the mixture was filtered, and the filtrate containing carotenoids was recovered. Acetone was evaporated, and the residue was submitted to saponification with an ethanol:potassium hydroxide mixture (50 mL of ethanol + 5 mL of KOH, 60% in water). Carotenoids were extracted with diethyl ether (previously purified on aluminum oxide act.1), concentrated to dryness, redissolved in acetone containing β-apo-8′-carotenal (13 mg/L; Fluka, Buchs, Switzerland) as internal standard, and analyzed by HPLC techniques.

Fractionation. The carotenoids were fractionated and determined by HPLC techniques (Razungles et al., 1987, 1988). The equipment used was a Varian 5500 system fitted with a 250 × 4.6 mm column packed with bonded silica phase, C18 5 μm (Brownlee Labs, Santa Clara, CA). The following gradient was used with an acetone:water mixture: 0 to 20 min, acetone: water at 70:30 (v/v) to 100% acetone; 20 to 30 min, constant 100% acetone. The flow rate was 1 mL/min. The effluent was monitored with a photodiode array detector (λ: 350 → 600 nm; Waters 990).

Identification. Carotenoids were identified by comparison either with commercially available standards [β-carotene (Merck, Darmstadt, Germany); lutein (Extrasynthese, Genay,

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Table 1. Chromatographic and Spectroscopic Characteristics Used for Pigment Identification in Grape Berries

pigment identified	R_t value[a]	R_f value[b]	spectral data, λmax, nm			hypsochromic shift, nm in ethanol	acetylation	source of pigment
			HPLC eluent	chloroform	ethanol			
neoxanthin	7.4	0.19	415, 438, 468	419, 443, 472	413, 437, 465	17	+	nettle
neochrome	7.8	0.27	402, 422, 451	405, 428, 456	398, 421, 448	0	+	green grape
violaxanthin	9.1	0.31	415, 441, 471	422, 447, 477	416, 440, 467	39	+	mango-nettle
luteoxanthin	9.6	0.34	400, 422, 449	405, 427, 454	398, 419, 446	20	+	nettle
lutein 5,6-epoxide	11.1	0.37	415, 441, 471	429, 451, 478	418, 443, 469	20	+	mango
flavoxanthin	11.8	0.40	402, 424, 451	407, 430, 459	399, 421, 448	0	+	chrysanthemum
zeaxanthin	12.8	0.41	(429), 452, 479	(434), 459, 488	(427), 450, 477	0	+	Hoffmann-La Roche
lutein	13.2	0.42	(427), 449, 477	430, 452, 482	421, 444, 472	0	+	Extrasynthèse
β-carotene	21.2	0.96	(425), 455, 482	(435), 461, 487	(427), 449, 475	0	−	Merck

[a] HPLC conditions: column, C18 5 μm; solvent gradient used: 0 to 20 min, acetone:water at 70:30 (v/v) to 100% acetone; 20 to 30 min, constant 100% acetone. [b] Chromatographic support for TLC: silica gel G 60; solvent system: light petroleum ether:acetone:diethylamine (10:4:1).

Table 2. Carotenoid Content in Three Varieties of *V. vinifera* at Maturity

carotenoids	Muscat of Alexandria		Sauvignon		Syrah	
	μg/kg	% of total carotenoids	μg/kg	% of total carotenoids	μg/kg	% of total carotenoids
β-carotene	285	30.4	305	31.9	396	35.4
lutein	516	55.1	485	50.7	556	49.7
flavoxanthin[a]	45	4.8	35	3.7	25	2.2
lutein 5,6-epoxide[a]	16	1.7	29	3.0	21	1.9
luteoxanthin[a]	10	1.1	12	1.2	16	1.4
violaxanthin	18	1.9	34	3.6	24	2.1
neoxanthin	47	5.0	56	5.9	82	7.3
total carotenoids	937		956		1120	

[a] Expressed as lutein equivalent.

France); zeaxanthin (Hoffmann-La Roche, Basel, Switzerland)] or with reference xanthophylls extracted from mango fruits (violaxanthin, lutein 5,6-epoxide), nettles (neoxanthin, violaxanthin, luteoxanthin), and chrysanthemum flowers (flavoxanthin). Neochrome was isolated from our green grape extracts. These extracts from different sources were separated by thin layer chromatography (Minguez-Mosquera et al., 1992), and the identifications were carried out using retardation factor (R_f) values and UV−vis spectral data in several solvents (Table 1); (Davies, 1976; Minguez-Mosquera and Garrido-Fernandez, 1989; Cano, 1991; Cano and De Ancos, 1994); 5,6-epoxide groups were investigated by addition of 2% HCl in ethanol, and hydroxyl groups were studied by acetylation with acetic anhydride in pyridine (Davies, 1976; Minguez-Mosquera and Hornero-Mendez, 1994). Identifications were confirmed by cochromatography of reference compounds with a grape carotenoid extract.

Quantitative Determination. β-Carotene, lutein, violaxanthin, and neoxanthin were determined with linear response factor calculated from reference solutions. Flavoxanthin, lutein 5,6-epoxide, and luteoxanthin concentrations were expressed as lutein equivalents because no sufficient amounts were isolated for calibration and authentic commercial specimens were not available. Zeaxanthin, detected in several extracts, was not determined because its corresponding peak co-eluted with the large peak of lutein. Determinations were made in duplicate. The concentrations given are the mean values. Relative error calculated from five replicates was <5% for β-carotene and lutein and ∼10% for the other xanthophylls in relation to their low concentrations.

Color Measurement of Berries. Twenty five grape berries were placed in a Petri dish to form a homogeneous surface, and a glass plate was placed upon the berries. External color was measured with a Minolta CR 200 tristimulus color analyzer (Bakker et al., 1986). For this study, only the *b** scale corresponding to yellow/blue chromaticity was used. Determinations were made with six repetitions.

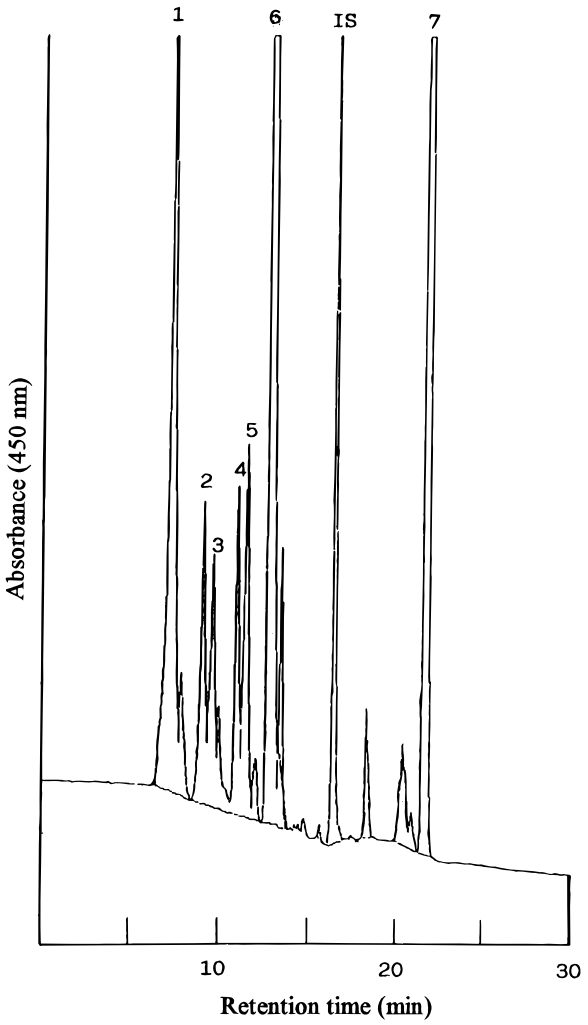

Figure 1. HPLC separation of carotenoids of Syrah mature berries, monitored at 450 nm: (1) neoxanthin; (2) violaxanthin; (3) luteoxanthin; (4) lutein 5,6-epoxide; (5) flavoxanthin; (6) lutein; (7) β-carotene; (IS) internal standard (β-apo-8′-carotenal).

RESULTS AND DISCUSSION

Carotenoid Determinations in Mature Grapes. As shown in Table 2, the levels of neoxanthin, violaxanthin, lutein 5,6-epoxide, lutein, and β-carotene are in agreement with those from a previous work (Razungles et al., 1987). Lutein and β-carotene had contents ∼5−10-fold higher than those of other compounds.

In addition to these five carotenoids, our results confirmed the presence of luteoxanthin. Although this compound was previously identified in Thompson Seedless (Curl, 1964) and in Dabouki and Riesling (Gross,

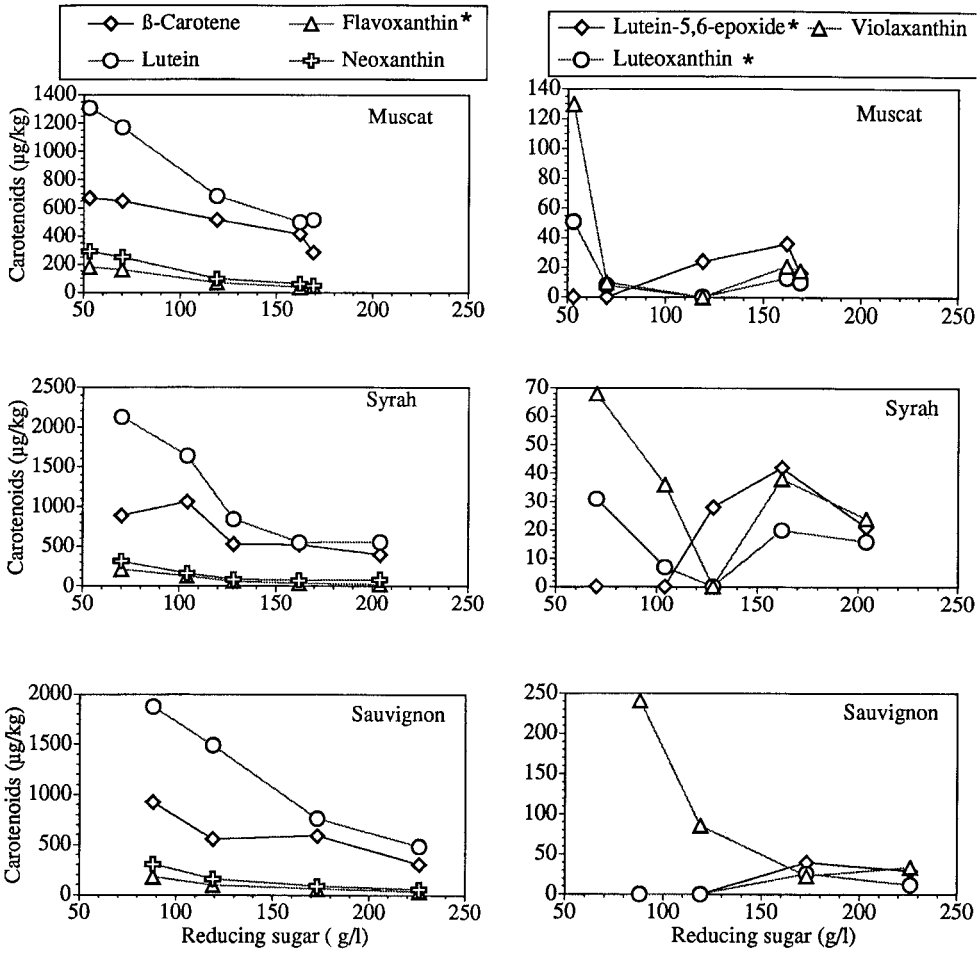

Figure 2. Change in several carotenoid contents during grape maturation of three varieties of grapes (July 27 to September 4; *expressed as lutein equivalent).

1984) grapes, this is the first time it has really been quantified. In these previous works, data were only given in percentage of the total carotenoids. Our results are in agreement with those of Gross (1984) and are different from those of Curl (1964) who reported much lower levels of violaxanthin in mature Thompson Seedless; this difference probably indicates a varietal effect.

A peak (no. 5) between lutein 5,6-epoxide and lutein is evident in Figure 1. Comparison of the spectral data of this compound in three solvents with those in the literature (Davies, 1976; Khachik et al., 1986) shows the absence of the hypsochromic shift in acidic medium and that the retention time of this peak (11.8 min) is after the retention time of lutein 5,6-epoxide and just before the retention time of lutein (Khachik et al., 1986; Taylor et al., 1992). These retention times are consistent with those of flavoxanthin. Its content is about the same as that of violaxanthin.

Carotenoid Changes during Grape Maturation. The results of this study both confirmed previous results about the major carotenoids and revealed original evolutions of certain xanthophylls during the maturation period.

β-Carotene, Lutein, Flavoxanthin, and Neoxanthin. As shown in Figure 2, carotenoid changes in the three cultivars studied were similar to those in Carignan, Grenache, and Syrah grapes (Razungles et al., 1988). Concentrations of these compounds fall dramatically after veraison, slow down at maturity, with a leveling off for lutein and neoxanthin. This leveling off probably results from the fact that, in grape berries after veraison, chloroplasts are not replaced by chromoplasts (Branas, 1974) that are able to synthetise carotenoids (Camara and Moneger, 1978). The carotenoids are liberated into the cell and can be submitted to chemical or enzymatic degradations [low pH, presence of lipoxygenase implicated in the catabolism of carotenoids (Belitz and Grosch, 1982)]. Moreover, the structure of these pigments can be modified, as indicated in the next paragraph.

Lutein 5,6-Epoxide, Luteoxanthin, and Violaxanthin. At the beginning of maturation (two first samplings), whatever the cultivar, there was no lutein 5,6-epoxide in grapes (Figure 2). This compound increases only afterwards, with the highest level noted when the sugar concentration is ~165 g/L. The recent improvement of our equipment (using a photodiode array detector) gave evidence of a rise in this epoxy lutein, whereas its isomer flavoxanthin as well as other major carotenoids were degraded. Changes of luteoxanthin and violaxanthin were quite similar for Muscat of Alexandria and Syrah cultivars; that is, a drastic decrease to a sugar level of 120−130 g/L, followed by an increase to 160−170 g sugar/L and, ultimately, a renewed decrease down to maturity. The general fate of xanthophylls was quite similar for Sauvignon compared with Syrah and Muscat grapes; that is, a decrease followed by a slight increase towards maturity and overmaturity for violaxanthin and a rise for lutein 5,6-epoxide and luteoxanthin, with a maximum at ~170 g sugar/L (this maximum occurs earlier for the two other cultivars).

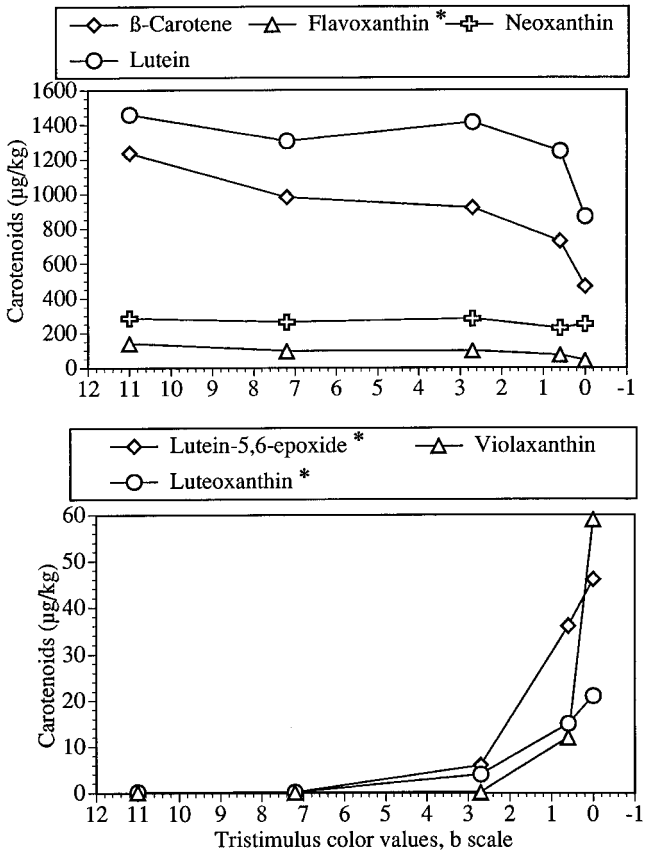

Figure 3. Change in several carotenoid contents of Syrah during veraison period (July 27 to August 11; *expressed as lutein equivalent).

The rise during the end of veraison and maturation of violaxanthin and lutein 5,6-epoxide possibly comes from bioconversions from β-carotene to violaxanthin and from α-carotene to lutein 5,6-epoxide (Matus et al., 1991). Evidence has already been presented that α-carotene, present only in trace amounts in the mature berry (Curl, 1964; Gross, 1984; Razungles et al., 1987), could possibly follow the same pathway as its isomer β-carotene (Deli et al., 1992). Because of the difficulties in determining α-carotene in green berries before veraison, it is not possible to check this hypothesis.

The simultaneous presence of 5,6- and 5,8-epoxy xanthophylls in the green berries extracts should confirm that the latter are not artifact compounds. Our results indicated a decrease of the ratio between furanoid epoxides (flavoxanthin and, only in the green berries, neochrome) and the corresponding 5,6-epoxides (lutein 5,6-epoxide and neoxanthin). For violaxanthin and luteoxanthin, the evolution was different. Luteoxanthin decreased consistently before and during the first part of veraison then increased slightly. This increase could be related to an epoxidation of zeaxanthin (Matus et al., 1991); such a particular behavior of luteoxanthin has already been observed during the maturation of dancy tangerine (Gross, 1982) and of black paprika (Deli et al., 1992). Concerning grapes, we confirm here our previous works that suggested the importance of veraison as a chief physiological step (Razungles et al., 1988); therefore, it was deemed worthwhile to focus our interest on veraison.

Carotenoid Changes around Veraison. To more accurately investigate the phenomenon of carotenoid changes around veraison, six samplings of Syrah were made between July 27 and August 11. The first four samplings took place at the same moment of the day. Berries were picked and separated according to their color. As pointed out in the previous assay, there was a decrease of β-carotene, lutein, neoxanthin, and flavoxanthin during veraison (Figure 3). However, the decrease begins only when the color of the berries turned entirely pink (b^* value = 0.7). From this moment, the negative slope of the different changes increases up to the end of veraison (b^* value = 0), when the color of the berries turned to red. The fate of lutein 5,6-epoxide, luteoxanthin, and violaxanthin was quite different. Their concentration rose consistently at the end of veraison, and after, as previously shown, during maturation. This evolution, which is concomitant and opposite to that of the other carotenoids, is consistent with changes in structure of the chief compounds. This change in structure could arise from an epoxidation related to the drastic decrease of the berry acidity during veraison. The formation of anthocyanins changing the lighting of cells of the berry or the increase in the shadow of the leaves could also lead to these modifications in structure (Hager, 1969; Wilkinson, 1977; Yamamoto, 1979).

CONCLUSION

This study of grape carotenoids during the maturation period has led to the following results:

(1) new epoxy xanthophylls have been identified and three of them (flavoxanthin, luteoxanthin, and violaxanthin) have been quantitatively determined.

(2) lutein-5,6-epoxide, violaxanthin, and luteoxanthin have a particuliar behavior during the maturation period and at maturity, and their evolutions don't follow those of the major carotenoids previously described.

(3) the end of veraison (and not the beginning as we thought) appears to be a key moment for the changes in the carotenoid ratios.

These data are important for the understanding of grape carotenoid metabolism and to promote the synthesis of these compounds, which are considered as interesting flavor precursors.

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