Determination of Carotenoid Profiles in Grapes, Musts, and Fortified Wines from Douro Varieties of *Vitis vinifera*

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β-Carotene and six xanthophylls (lutein, neoxanthin, violaxanthin, luteoxanthin, cryptoxanthin, and echinenone) have been identified and semiquantitatively or quantitatively determined in musts and port wines for the first time. An HPLC method was developed and compared with that of one based on thin layer cromatography with scanning densitometry. The most abundant carotenoids present in red grape varieties are *β*-carotene and lutein. In wines, significant quantities of violaxanthin, luteoxanthin, and neoxanthin were found. This study was done with berries (skin and pulp), musts, and fortified wines. Some experiments were performed to follow carotenoid content from grapes to wines. Although the levels of *β*-carotene and lutein found in fortified wines were lower than those found in musts, other xanthophylls, such as neoxanthin, violaxanthin, and luteoxanthin, exist in appreciable amounts in young ports.

Keywords: *Carotenoids; wine composition; winemaking; aroma precursors*

INTRODUCTION

The most abundant species of carotenoids present in grapes are *β*-carotene and lutein, although violaxanthin and neoxanthin are also present at detectable levels (*1*). Carotenoids and nonaromatic intermediates are known to be precursors of aroma-active nor-isoprenoids, such as α- and *β*-ionone or *β*-damascenone, which are responsible for the typical aroma of some grape varieties (*2*). Studies of the evolution of the carotenoid profile of grapes during maturation showed that *β*-carotene and several xanthophylls (neochrome, neoxanthin, flavoxanthin, and lutein) are abundant before veraison and the levels decrease during ripening (*3*, *4*). There is, however, no reference in the literature to levels of carotenoids in musts and wines. The fact that these compounds are present in wines might be important since it is possible that during aging these molecules are degraded into aromatic compounds, nor-isoprenoids, which can impact wine flavor. Some nor-isoprenoids have already been identified in ports: 2,2,6-trimethylcyclohexanone (*5*), ionone(s)-isomers (*6*), and 1,1,6-trimethyl-1,2-dihydronaphthalene (*7*) all make contributions to wine flavor. Although it is at present conjecture, it is consistent with the post-harvest behavior of carotenoids in other food systems that these might degrade in situ to aromatic nor-isoprenoids. A number of mechanisms for the reaction and decomposition in foodstuffs of carotenoids into nor-isoprenoids with nine to 13 carbon atoms are given in the literature. These include enzymatic processes, auto-oxidation, and thermal decomposition (*8*, *9*). In this present work, an HPLC method for the quantification of carotenoids in wine and grape material was developed and applied to samples from the Douro demarcated region of Portugal. Levels of carotenoids are determined in finished wines (ports) and their evolution from grapes through must to wine is followed.

MATERIAL AND METHODS

Grapes, musts, and fortified wines used were obtained from five cultivars (Touriga Nacional, TN; Touriga Francesa, TF; Tinta Roriz, TR; Tinto Cão, TC; and Tinta Barroca, TB) harvested at two different subregions (Cima Corgo, CC; and Douro Superior, DS) of the Douro region of northern Portugal. Grapes and musts were obtained in October 1999 (harvesting date) and immediately frozen at −20 °C. Ports were available in February 2000. The evolution of carotenoids in musts was followed in laboratory scales microvinification.

Reagents and Materials. Reference samples used were obtained from commercial sources: lutein (Sigma-Aldrich, USA), chlorophylls (Fluka, Switzerland), and *β*-carotene (Merck, Germany). The internal standard employed for HPLC study was *β*-apo-8-carotenal (Fluka, Switzerland) and HPLC grade solvents ethyl acetate, acetonitrile, and hexane (Merck, USA).

Extraction of Carotenoids from Grapes, Musts, and Fortified Wines. (a) Grapes. A total of 200 g of grapes were separated into pulp and skin, both materials being freeze-dried, and 50 *μ*L of the internal standard being added to the powder obtained. Extraction was performed by stirring overnight in 20 mL of hexane. The organic layer was dried with anhydrous Na_2SO_4 and concentrated to dryness using a rotavapor at 20 °C.

This dried extract was dissolved in 1 mL of a solution of 50% of ethyl acetate and 50% of acetonitrile/water (9/1, v/v). To prevent photoisomerization, all the manipulations were performed in almost complete absence of light. A sample volume of 20 *μ*L was injected onto the HPLC.

(b) Musts. Crushed grape material, 100 mL volume of sample, and 100 *μ*L of internal standard (*β*-apo-8-carotenal; 108 mg/L) were placed in a 250-mL volumetric flask, protected from light. This slurry was stirred (1000 rpm) for 12 h with 50 mL of hexane. The organic layer was separated, and the extraction was repeated with 20 mL of the same solvent during 30 min. The organic layers were combined and dried with

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Table 1. Data Used for Carotenoid Identification in Grape Berries, Musts, and Wines

	tr (HPLC)	rf (TLC)	HPLC eluent spectral data λ max (nm)			source of pigment
neoxanthin	8.6	0.29	417	440	417	yellow nettle
violaxanthin	10.9	0.31	419	440	469	yellow nettle
luteoxanthin	12.9	0.33	381	401	427	yellow nettle
lutein	14.6	0.35	419	444	471	sigma/aldrich
zeaxanthin	14.7	0.35	419	444	471	green algae
b-apo-8'-carotenal (IS)	16.4					fluka
chlorophyll b	17.2	0.34				fluka (spinach)
chlorophyll b'	18.2	0.34				fluka (spinach)
cryptoxanthin	19.9	0.46	340	454	480	green algae
echinenon	20.3	0.64	356	457	484	green algae
xantophyll monoesters	21.5					green algae
xantophyll monoesters	22					green algae
chlorophyll a	23.2	0.37		408		fluka (spinach)
chlorophyll a'	13.7	0.38		400		fluka (spinach)
β-carotene	24.8	0.9		452	469	merck

anhydrous Na_2SO_4. The following steps of the analytical procedure until injection are the same those described above.

(c) Wines. A 350 mL volume of wine was spiked with 100 μL of internal standard and placed in a 500 mL flask; 20 mL of hexane was added and the mixture was stirred (1000 rpm) for 30 min. The organic layer was separated, and the extraction was repeated using 20 mL and 10 mL of the same solvent. The three organic layers were dried with anhydrous Na_2SO_4. The following steps of the analytical procedure until injection are the same to those described above.

Chromatographic Procedures. (a) Thin Layer Chromatography was performed in precoated silica gel 60 F254 aluminum sheets 20 × 20 cm (Merck, Germany). The solvent system employed was acetone/*n*-hexane, 3/7 (*v*/*v*) (*10*) and a prerun in 2.5% (w/v) solution of citric acid in methanol (*11*). After the prerun, the TLC plates were dried under air flow, at 80 °C for 30 min. Qualitative analysis of TLC plates was performed using an imaging densitometer (model Q5-700, Bio-Rad, USA).

(b) HPLC Quantification. The quantification of carotenoids was performed by HPLC, using an Beckman System Gold equipped with the 502 autosampler, 126 programmable solvent system, and the 168 diode array detector modules. The data were stored and processed using the System Gold software (v6.0). The absorption spectra were recorded between λ = 270−550 nm, with an spectral scan rate of 1 (Hz). The wavelength selected for the system monitoring and chromatogram integration was 447 nm.

(c) Stationary Phase. Spherisorb ODS 2 (25 cm × 4.6 mm × 5 μm diameter of particles) (Merck, Germany).

Mobile-phase: Solvent A, ethyl acetate; solvent B, acetonitrile/water (9:1 v/v), flow rate of 1 mL/min. The following gradient was used: 0−16 min (0−60% A); 16−36 (100% A); 36−46 min (100% A); 46−56 min (0% A).

Retention values: neoxanthin (8.7 min); violaxanthin (9.6 min); luteoxanthin (12.8 min); lutein (14.4 min); zeaxanthin (14.5 min); chlorophyll b (17.3 min); cryptoxanthin (19.7 min); echinenone (20.2 min); astaxanthin esters (21.2 min); α-carotene (21.7 min); chlorophyll a and a′ (22.9 and 23.4 min); β-carotene (24.8 min).

(d) Identification. The carotenoids in the samples were identified by comparison of the retardation factor values (R_f) in TLC, retention time in HPLC, and UV spectra obtained either from commercially available standards (β-carotene; lutein) or extracts of nettles and yellow pepper (zeaxanthin, cryptoxanthin, echinenone, neoxanthin, violaxanthin, and luteoxanthin) or published data (*12*) (Table 1).

RESULTS AND DISCUSSION

Quantitative Determination of Carotenoids in Musts and Wines (Validation of the HPLC Method). β-carotene and lutein were assayed by comparison with the commercial compounds mentioned above. The other xantophylls were quantified as lutein equivalents.

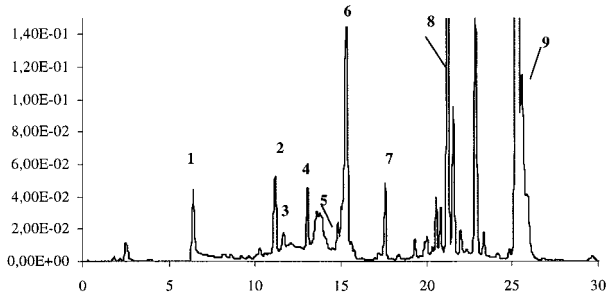

Figure 1. HPLC chromatogram of carotenoids of Tinta Roriz must. DO = 447 nm. (1) Neoxanthin, (2) violaxanthin, (3) luteoxanthin, (4) unknown (lutein 5,6-epoxy?), (5) zeaxanthin, (6) lutein, (7) internal standard, (8) xantophylls esters, and (9) β-carotene.

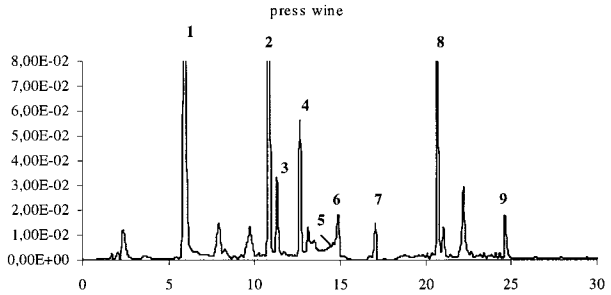

Figure 2. HPLC chromatogram of carotenoids of Tinta Roriz press wine. DO = 447 nm. (1) Neoxanthin, (2) violaxanthin, (3) luteoxanthin, (4) unknown (lutein 5,6-epoxy?), (5) zeaxanthin, (6) lutein, (7) internal standard, (8) xantophylls esters, and (9) β-carotene.

(a) Repeatability and Linearity. The repeatability of the method was assessed with extracts of port for seven replications, and the relative error was 12.4% for β-carotene, 11.7% for lutein, 7.8% for violaxanthin, 2.7% for neoxanthin, 3.3% for luteoxanthin, and 17.1% for xantophyll monoesters. Using the HPLC method, β-carotene and lutein were determined with a linear response factor calculated from reference solutions. Violaxanthin, neoxanthin, and luteoxanthin concentrations were expressed as lutein equivalents. Each determination was performed in duplicate. The concentration given is the mean values. Relative error (SD/mean) calculates from six replicates of musts was less than 10% for β-carotene and lutein. A linearity study performed for β-carotene in musts and wines showed r^2 = 0.9983 and 0.9805, respectively. Figures 1 and 2 show a HPLC separation of carotenoids of a Tinta Roriz must and wine, monitored at 447 nm.

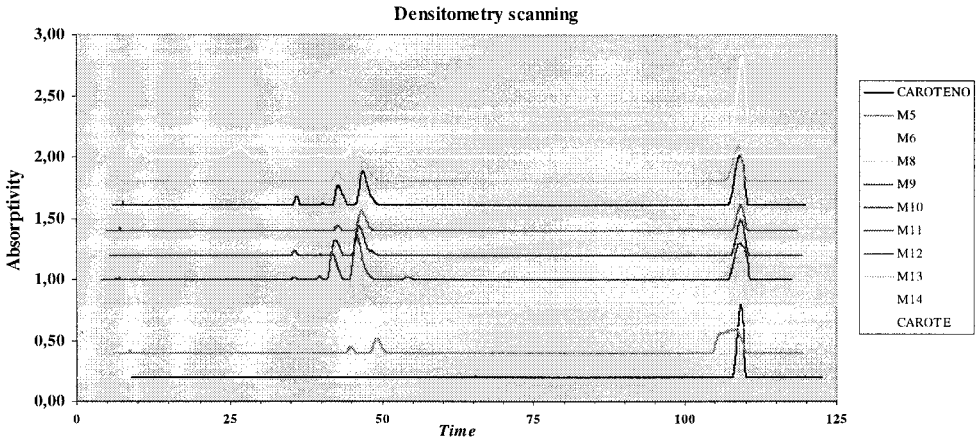

Figure 3. TLC/scanning densitometry of nine musts.

Table 2. Levels of Carotenoids in Pulp and Skin. Sum of Xantophylls, Neoxanthin, Violaxanthin, Luteoxanthin, Cryptoxanthin, and Echinenone

μg/kg	skin	pulp
sum of xantophylls	131	112
lutein	666	603
sum of monoesters	160	169
β-carotene	1.692	519

(b) Levels of Carotenoids in Grapes, Musts, and Wines (Ports). The study of carotenoids in grapes shows a very different profile between skin and pulp (Table 2). Skin contributes to aproximately 65% of carotenoides (lutein, monoesters of xantophylls, and β-carotene), while the contribution of pulp is only 35%; these results are in according to those obtained by Razungles et al. (1998). In skin and pulp, we noticed the presence of neoxanthin. There was 3 times as much in skin than in pulp. Levels of lutein and monoesters of xantophylls are approximately the same in skin and pulp. The proportion of β-carotene in skin is 3 times higher than in pulp.

The determination of carotenoids in musts shows the presence of β-carotene and some xanthophylls. The identification of these molecules was done by TLC and HPLC methods. The determination of a carotenoid profile by TLC/densitometry (Figure 3) shows some qualitative differences among varieties. These results were confirmed by HPLC quantification (Table 3). Among the carotenoids studied, β-carotene was present in musts in higher levels, followed by xanthophylls (Table 3).

Although in dry red wine carotenoids are absent, we have found these molecules in fortified wine extracts. In this type of fortified wine, the fermentation of the grape mash, including the skins, is stopped after 3−4 days by the addition of neutral grape spirit (77% alcohol). The wine obtained has approximately 20% (v/v) alcohol content. The short fermentation period, and the high level of ethanol could account for the persistence of these highly liposoluble molecules in this type of wine.

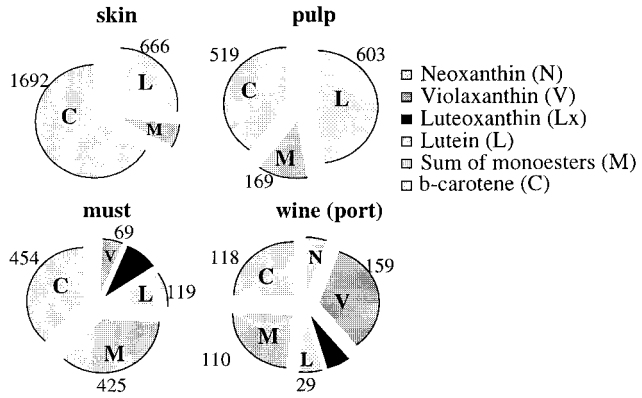

Figure 4. Profile of the most abundant carotenoids in skin, pulp, must, and fortified wine. Results are expressed in % and in μg/L (for pulp and skin) and μg/L (for must and wine).

The HPLC determinations show a different profile from skin, pulp, musts, and wines (Figure 4).

Figure 4 shows that in skin β-carotene exists in much higher levels, and in pulp β-carotene and lutein exist in similar amounts. The other xanthophylls are almost absent. Musts and wines have lower contents of lutein and β-carotene. Nevertheless, they are richer in oxygenated xantophylls such as neoxanthin, violaxanthin, and luteoxanthin. This is due essentially to a precipitation of more highly lipophilic molecules such as β-carotene (which as no hydroxylic group) and the much easier solubilization of hydroxy-xanthophylls, such as neoxanthin and violaxanthin. In wines, these xanthophylls are the most important group; β-carotene represents only 25% of the total carotenoid content. Violaxanthin, neoxanthin, luteoxanthin, and lutein represent in wine 52% of total carotenoid content, while for instance, in skin they represent only 17%. There are typical profiles of carotenoids in grapes, musts, and wines. In grapes

Table 3. Carotenoid Results Obtained by HPLC/Photodiode Array for Musts from Different Varieties and Different Subregions

	M5 (TNCC)	M6 (TNDS)	M8 (TBDS)	M9 (TFCC)	M10 (TFCC)	M11 (TRCC)	M12 (TRDS)	M13 (TCCC)	M14 (TCDS)
lutein	69.0	31.5	134.2	68.8	24.5	49.0	25.3	108.9	119.1
violaxanthin	11.9	39.9	87.4	46.5	75.3	10.0	41.9	36.2	69.4
neoxanthin	2.0	9.4	7.4	10.7	9.5	1.8	5.8	1.7	9.9
luteoxanthin	4.0	52.8	170.5	70.5	102.0	12.0	55.1	58.1	108.7
sum of xantophylls	86.9	133.5	399.4	196.5	211.2	72.8	128.0	204.9	307.1
β-carotene	94.0	148.0	425.0	416.0	465.0	174.0	469.0	328.0	454.0

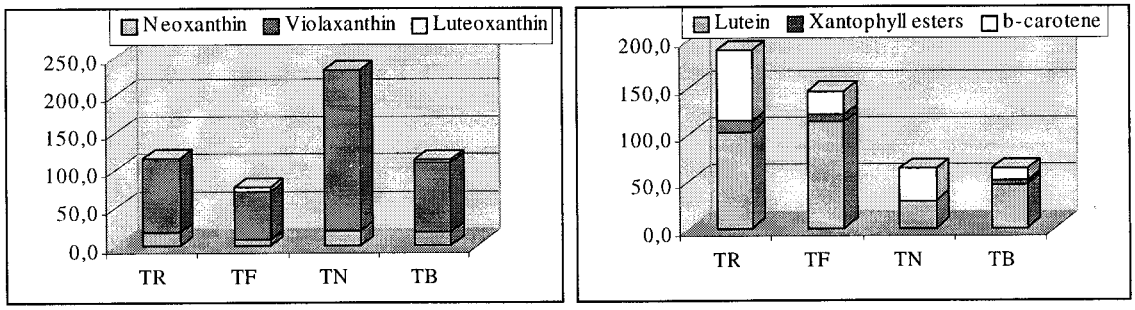

Figure 5. Carotenoid profile in four ports. Results are expressed in μg/L. Neoxanthin, violaxanthin, and luteoxanthin results are expressed as lutein equivalent.

Table 4. Results of Carotenoids Expressed in %[a]

	samples during alcoholic fermentation/carotenoids (%)						
	T0	T3	T4	T5	T6	T7	press wine
sugar	174	119	*b*	*b*	*b*	<2	<2
neoxanthin	12	5	8	3	14	21	14
violaxanthin	0	3	8	4	4	10	33
luteoxanthin	1	0	2	1	1	3	6
sum of xantophylls	13	8	18	8	19	34	53
lutein	10	16	12	19	17	18	11
xantophyll esters	14	24	20	28	24	17	21
carotene	63	52	50	45	40	31	15

[a] Sugar in g/L. Must: T0; samples during fermentation: T3, T4, T5, and T6; wine: T7; and press wine. [b] Results not available.

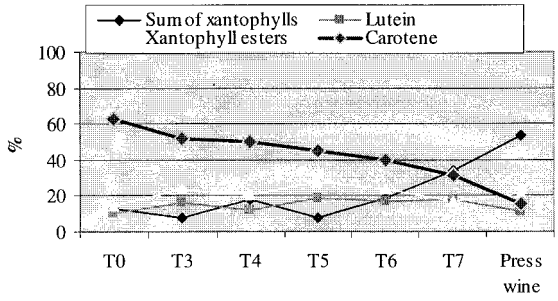

Figure 6. Evolution of carotenoids during alcoholic fermentation. Sum of xantophylls (neoxanthin, xiolaxanthin, luteoxanthin).

(skins and pulp), β-carotene and lutein are dominant. In musts, xanthophylls esters and β-carotene exist in higher levels than lutein and other epoxy-xanthophylls.

In fortified wines, the quantity of carotenoids is less than in grapes and musts; however, lutein, neoxanthin, and violaxanthin were found in appreciable amounts in musts and wines. It is important to notice that this is the first time that these molecules were found in musts and wines.

Four ports were analyzed just after brandy addition. The results show that amounts in xantophylls as neoxanthin and violaxanthin can be greater than lutein and β-carotene, as can be seen for Touriga Nacional wine (Figure 5).

The carotenoid profiles among studied varieties are very different. Touriga Nacional wines are richer in violaxanthin, and Tinta Roriz wines and Touriga Francesa wines are richer in lutein content. The higher levels in β-carotene is found in Tinta Roriz wines.

(c) Evolution of Carotenoids during Alcoholic Fermentation. To understand the role of the fermentation process in the degradation of carotenoids and how this process can change the carotenoid profile, we followed the carotenoid contents throughout the alcoholic fermentation. Results are shown in Table 4. In the six first days, sugars are almost completely consumed. T0 is the must, and T3, T4, T5, T6, and T7 correspond to the first day (T1) until the seventh day (T7). Samples are taken from the bottom of the tank, with no previous treatment before analysis.

As expected, the results showed that during alcoholic fermentation there is a decrease of β-carotene content and a increase in neoxanthin, violaxanthin, and luteoxanthin (Figure 6).

Ongoing work is aimed at trying to determine if the increase in hydroxy and epoxy xanthophylls is only related to their solubility or if enzymatic mechanisms involving lypo-oxygenases enzymes play a role in the transformation of carotene into epoxy or hydroxy xanthophylls. As these molecules exist in ports, we can speculate, and studies are ongoing to prove that they can be converted directly into nor-isoprenoids during aging, with a consequent impact on wine flavor. It is very important to determine the mechanism of degradation of these nonaromatic molecules to volatile compounds.

SUMMARY

The study of carotenoids in grapes, musts, and fortified wines led to the following results:

There are typical profiles of carotenoids in grapes, musts, and wines. In grapes (skins and pulp), β-carotene and lutein are dominant. In musts, xanthophylls esters and β-carotene exist in higher levels than lutein and other epoxy-xanthophylls.

In fortified wines, the quantity of carotenoids is less than in grapes and musts; however, lutein, neoxanthin, and violaxanthin were found in appreciable amounts. It is important to notice that this study is the first time that these molecules have been found in musts and wines (port).

These data could suggest that in young port wines the presence of lutein and other xantophylls could be converted directly into nor-isoprenoids during aging.

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