JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Carotenoid, Chlorophyll, and Chlorophyll-Derived Compounds in Grapes and Port Wines

MARIA MANUELA MENDES-PINTO,† ANTÓNIO CÉSAR SILVA FERREIRA,† CATHERINE CARIS-VEYRAT,§ AND PAULA GUEDES DE PINHO*,†

Escola Superior de Biotecnologia, Universidade Católica Portuguesa, R. Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal, and INRA, UMR 408, Sécurité et Qualité des Produits d'Origine Végétale, Avignon, F-84914, France

Carotenoids and chlorophyll-derived compounds in grapes and Port wines were investigated by HPLC-DAD and HPLC-DAD-MS (ESP$^+$) analysis. A total of 13 carotenoid and chlorophyll-derived compounds are formally reported in grapes, 3 are identified for the first time, pheophytins *a* and *b* and (13*Z*)-β-carotene, and 3 others remain unknown. In Port wines 19 compounds with carotenoid or chlorophyll-like structures are present, 8 still unidentified. The young wines showed higher total carotenoid content and chlorophyll-like compounds compared to aged Ports, with lutein and β-carotene as major carotenoids. Among samples analyzed of monovarietal *Vitis vinifera* L. cultivar wines produced with the five most important Douro varieties, Tinta Roriz contained the highest levels of carotenoids and Touriga Franca the lowest. The forced-aging study indicated that lutein was more sensitive to temperature than β-carotene. Additionally, aged wines showed higher ratios of β-carotene/lutein concentrations compared to new Ports. Rates of degradation of chlorophyll derivative compounds were higher than those for carotene and lutein.

KEYWORDS: Carotenoids; chlorophylls; chlorophyll derivatives; grapes; Port wines; HPLC-DAD-MS (EPS$^+$); degradation; aging

INTRODUCTION

In grape berries the presence of carotenoids is well recognized. β-Carotene and some xanthophylls (neoxanthin, flavoxanthin, and lutein) are abundant before veraison and subsequently decrease dramatically (*1*−*3*). Three other xanthophylls, namely, violaxanthin, luteoxanthin, and 5,6-epoxylutein, appear after veraison. Cultivar, viticultural region, exposure to sunlight, and ripening stage all affect carotenoid concentrations in grapes (*4*−*7*). It is well-known that carotenoid contents in plants are related to the metabolic processes of plant cells, which are dependent on climatic factors, agricultural practices, and plant variety. Carotenoids are mostly synthesized from the first stage of fruit formation until veraison and then degrade between veraison and maturity to produce C13-norisoprenoid compounds (*2*), which have been reported as odor-active substances responsible for typical aromas of some grape varieties (*2*−*5*). Effects on carotenoid concentrations in grapes due to climatic conditions and sunlight exposure have already been studied (*3*, *6*−*8*). In general, the highest carotenoid levels occurred in grapes produced in hot regions. Nevertheless, at maturity, grapes exposed to sunlight seem to have lower carotenoid concentrations than shaded grapes.

It was reported that norisoprenoids could come from the direct degradation of carotenoid molecules such as β-carotene, lutein, neoxanthin, and violaxanthin (*9*−*12*) and also from the hydrolysis of glycoside molecules (*13*−*15*). Carotenoids and nonaromatic intermediates are known to be precursors of aroma-active norisoprenoids such as α- and β-ionone or β-damascenone, responsible for the typical aroma of some grape varieties (*9*, *14*, *16*). A recent study showed that carotenoids can also be found in Port wines in very small amounts (*17*). The fact that these compounds are present in wines might be important because it is possible that during aging these molecules are degraded into aromatic compounds, norisoprenoids, which can affect wine flavor. Some norisoprenoids have already been identified in Ports: 2,2,6-trimethylcyclohexanone (TCH) (*18*), ionone(s) isomers (*19*), and 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) (*20*) all make contributions to wine flavor. It is at present conjecture but consistent with the post-harvest behavior of carotenoids in other food systems that these compounds might degrade *in situ* to aromatic norisoprenoids. A number of mechanisms for the reaction and decomposition, in foodstuffs, of carotenoids into norisoprenoids with 9−13 carbon atoms are given in the literature. These include enzymatic processes, autoxidation, and thermal decomposition (*10*, *11*).

In the present work, some carotenoid and chlorophyll-derived compounds were identified by HPLC-DAD-MS (ESP$^+$) in grapes and Port wines for the first time. The quantification

* Corresponding author (e-mail pinho@esb.ucp.pt).
† Universidade Católica Portuguesa.
§ INRA, UMR 408.

10.1021/jf0503513 CCC: $30.25
Published on Web 12/01/2005

of carotenoids was performed in grapes and Port wines from the Douro demarcated region of Portugal. As chlorophyll and carotenoid-derivative molecules are present in Port wines, their degradation during aging can be expected. For this purpose chlorophyll and carotenoid profiles in Ports from different vintages were determined. A forced-aging protocol (involving temperature and oxygen) was adopted to evaluate rates of degradation of the major wine carotenoid and chlorophyll derivative molecules.

MATERIALS AND METHODS

Material for Quantitative Analysis. Seventy-nine Port wines were supplied by Ramos Pinto and Barros producers. Samples were collected from different harvesting years: "Colheitas", ranging from 1 to 20 years old, 57 wines from the 2002 vintages and 22 wines more than 10 years old. Wines were made from five different cultivars of *Vitis vinifera* L. cv.: Touriga Nacional (TN), Touriga Franca (TF), Tinta Roriz (TR), Tinto Cão (TC), and Tinta Barroca (TB). Their origins were from two different subregions of the Douro Demarcated Region, Douro Superior (DS) and Cima Corgo (CC). The winemaking processes were "lagar" or foot treading [i] and tank [ii]: [i] lagar capacity = 7000 kg; [ii] classical, red vinification in stainless steel vessels, employing active pumping-over, tank capacity = 12 000 kg filled. All samples were kept at controlled room temperature of 20 °C before analysis.

Material for Identification Analysis. Two Port wines from the 2002 vintage [wine I, from Tinta Roriz (TR) variety obtained from Ramos Pinto producer, and wine II, from Touriga Nacional (TN) from Barros producer]. These two wines were selected from the 79 Port wines analyzed, as they contained higher concentrations of carotenoid compunds.

Tinta Barroca grapes were collected on two different occasions and stored at −4 °C or analyzed immediately.

Extraction of Carotenoids from Grape Material. Approximately 50 g of fresh berries, of seeds, were homogenized using a "Turrax" homogenizer at 9500 rpm for 15 min. This procedure provided 40 g of sample (80−90% of homogenate prepared) that was spiked with 200 μL of internal standard, 170 mg/L of β-apo-8′-carotenal, and diluted with 40 mL of water (18.3 MΩ/cm) (EASY pure LF, Barnstead). Extraction was done with 40 mL of hexane/diethyl ether (1:1, v/v) by agitating the mixture for 30 min. The resulting upper layer was separated. The extraction procedure was repeated twice for the lower phase using 20 mL of ether/hexane. The pooled extract was evaporated to dryness using a rotavapor Buchi- RE121. The residue was dissolved in 1 mL of acetone/hexane (1:1, v/v) and used for carotenoid analysis by HPLC. Sample handling, homogenenization, and extraction were carried out on ice under dim yellow light to minimize light-induced isomerization and oxidation of carotenoids.

Extraction of Carotenoids from Port Wines. Wine samples (250 mL) were spiked with 50 μL of internal standard (β-apo-8′-carotenal; 50 mg/L) followed by the addition of 40 mL of hexane/diethyl ether (50:50, v/v). The mixture was stirred mechanically for 30 min. The resulting upper layer was separated. The extraction process was repeated twice for the lower phase using 20 mL of the same solvent. The pooled extract was evaporated to dryness using a rotavapor. The residue was resuspended in 500 μL of acetone/hexane (1:1, v/v) and used for the analysis of carotenoids (*17*).

Preparation of Pheophytins *a* and *b*. Standard chlorophylls *a* and *b*, respectively (0.1 mg each), were dissolved separately in acetone, acidified with 4−5 drops of ethanolic KCl (0.1 M), and analyzed after 2 min with spectrophotometric and chromatographic techniques.

Preparation of Chlorophyllins *a* and *b*. Standard chlorophylls *a* and *b*, respectively (0.1 mg each), were dissolved separately in acetone, alkalinized with 4−5 drops of ethanolic KOH (0.1 M), and after 30 min, components were extracted using diethyl ether. The extract was washed several times using ultrapure water (18.3 MΩ/cm) (EASY pure LF, Barnstead). The organic phase was dried with nitrogen and the residue suspended in 1 mL of acetone for the UV−vis and HPLC analysis.

Reagents and Commercial Standards. HPLC grade solvents hexane, dichloromethane, methanol, ethyl acetate, acetonitrile, and acetone were purchased from Merck. Lutein and β-carotene were purchased from Sigma-Aldrich, whereas (9′*Z*)-neoxanthin and violaxanthin were obtained from CaroteNature. Standards of neochrome a and neochrome b were prepared by acid catalysis of (9′*Z*)-neoxanthin and characterized as reported in a previous study (*21*). β-Apo-8′-carotenal was purchased from Fluka, and chlorophylls *a* and *b* were purchased from Sigma-Aldrich. Ultrapure water (18.3 MΩ/cm) (EASY pure LF, Barnstead) was used for all of the analyses.

HPLC-DAD: Reversed Stationary Phase Conditions: column, Nova-Pak C18 60 Å, 4 μm (3.9 × 300 mm), Waters; eluents, 100% ethyl acetate (solvent A) and 90% acetonitrile in H_2O (v/v) (solvent B); flow rate, 1 mL/min. The following binary gradient system was employed: 0−1 min (0% B); 1−30 min (to 60% B); 30−51 min (60% B); 51−55 min (to 0% B); 55−60 min (0% B). Diode array detection was between 270 and 600 nm. Sample injection was 20 μL, and absorbance was recorded at 447 nm.

HPLC-DAD-MS Apparatus and Conditions. HPLC analyses were carried out with a Hewlett-Packard model 1050 equipped with a quaternary pump solvent delivery and a diode array detector (DAD, model 1100). Positive electrospray mode was used for ionization of molecules. The program used for data analysis was Masslynx version 3.4. Mass spectra parameters, and especially cone voltage, were optimized to avoid fragmentation. Two different reversed stationary phases were employed: column 1, Nova-Pak C18 60 Å, 4 μm (3.9 × 300 mm), Waters. The eluents were 100% ethyl acetate (solvent A) and 90% acetonitrile in H_2O (v/v) (solvent B); flow rate was 1 mL/min. The following binary gradient system was employed: 0−1 min (0% B); 1−30 min (to 60% B); 30−51 min (60% B); 51−55 min (to 0% B); 55−60 min (0% B). Column 2 was a YMC pack C30 (YMC Inc., Wilmington, NC) 5 μm (4.6 × 250 mm) with a pre-column C30 5 μm (4.6 × 20 mm). The following gradient system was used with H_2O (solvent A), methanol (solvent B), and *tert*-butyl methyl ether (solvent C): 0−2 min, %A−%B−%C, 40−60−0; 5 min, %A−%B−%C, 20−80−0; 10 min, %A−%B−%C, 4−81−15; 60 min, %A−%B−%C, 4−11−85; 70 min, %A−%B−%C, 4−11−85; 70.01 min, %A−%B−%C, 0−100−0. The flow was maintained at 1 mL/min. Acquisition of the mass data between *m*/*z* 100 and 700 was performed in the positive electrospray mode.

Qualitative and Quantitative Analysis. Positions of absorption maxima (λ_{max}), the degree of vibrational fine structure (% III/II), and the capacity factor values k', were the parameters used for qualitative analysis. Identification was performed by comparison with standard spectra. Quantification was made by using the calibration curves standards of lutein and β-carotene with $r = 0.9968$ and 0.9979, respectively.

Forced-Aging Experimental Protocol. A volume of 9000 mL of 1-year-old Port with 73 μg/L of lutein and 190 μg/L of β-carotene (pH 3.7) was divided into two portions. One was kept at 2.5 mg/L oxygen content, whereas the other was saturated with oxygen at 4.8 mg/L by stirring at room temperature. Each portion was further divided into three equal parts and submitted to different storage temperatures (20, 40, and 60 °C). Samples were protected from light during the experiment. After the reaction, samples in duplicate were drawn and analyzed for the oxidation products. Control samples that were not exposed to O_2 were also treated in a similar manner.

Statistical Analysis. Principal component analysis (PCA) was carried out using an XLSTAT-Pro version 6.1.9. The PCA method shows similarities between samples projected on a plane and makes it possible to determine which variables determine these similarities and in what way. Analysis of variance (ANOVA) using Excel software from Windows 98 v 7.0 was applied to the experimental data, and the results were considered to be significant if the associated *p* value was <0.05.

RESULTS AND DISCUSSION

Carotenoid, Chlorophyll, and Chlorophyll Derivatives Profile in Grapes. The analysis of grapes by RP-18-HPLC-DAD-MS (EPS^+) showed that several compounds were detected (**Table 1** and **Figure 1**). The carotenoids neochrome a and b, (9′*Z*)-neoxanthin and violaxanthin were identified. Some

Table 1. HPLC-DAD-MS (EPS$^+$) Characteristics of Carotenoids, Chlorophylls, and Chlorophyll Derivatives in Grapes

peak	compound	*k′*	λ_{max} (nm)	2nd derivative	% (III/II)	identification	M
1	neochrome *a*	1.8811	400; 422; 450	450	93	standard, UV[a]	
2	(9′*Z*)-neoxanthin	2.0292	415; 438; 466	466	69	standard, UV[a]	
3	neochrome *b*	2.0528	400; 422;450	450	92	standard, UV[a]	
4	violaxanthin	2.2032	418; 441; 471	471	90	standard, UV[a]	
5	lutein-5,6-epoxide	4.2357	417; 441; 471	470	89	UV[a]	
6	flavoxanthin	4.7472	398; 422; 448	450	95	UV[a]	
7	unknown	5.0028	(406); 428; 454	456	47		
8	(*all-E*)-lutein	6.0345	(422); 447; 476	476	53	standard, UV[a]	
9	unknown (lutein-like structure)[f]	6.0848	(421); 444; 472	478	52	UV[a]	
10	unknown (lutein-like structure)[g]	6.2420	(420); 443; 472	476	50	UV[a]	
11	(13*Z*)- or (13′*Z*)-lutein	6.4242	333; (420); 442; 468	468	22	UV[a]	
12	chlorophyll *b*	10.900	455; 594; 645			standard	
13	pheophytin *b*	13.410	436; 528; 600; 654			UV;[b] MS[c]	885
14	pheophytin *a*	14.845	410; 506; 536; 666			UV;[b] MS[c]	871
15	(*all-E*)-β-carotene	16.034	(428); 454; 482	486	20	standard[d]	537
16	(*13Z*)-β-carotene	16.254	338; 449; 478	480	7	UV[a]	537
IS	β-apo-8′-carotenal	8.9231	460	460	0	standard[e]	

[a] Identificaton by comparison with UV spectrum of the "parent" compound. [b] Identificaton by comparison with UV spectrum of the "parent" standard obtained by acidification of the respective chlorophyll. [c] Identification by LC-MS is consistent with that of van Breemen et al. (*23*). [d] Pure standard (Sigma-Aldrich, St. Louis, MO) [e] Pure standard (Fluka, Switzerland) [f] (9*Z*)-Lutein. [g] (9′*Z*)-Lutein.

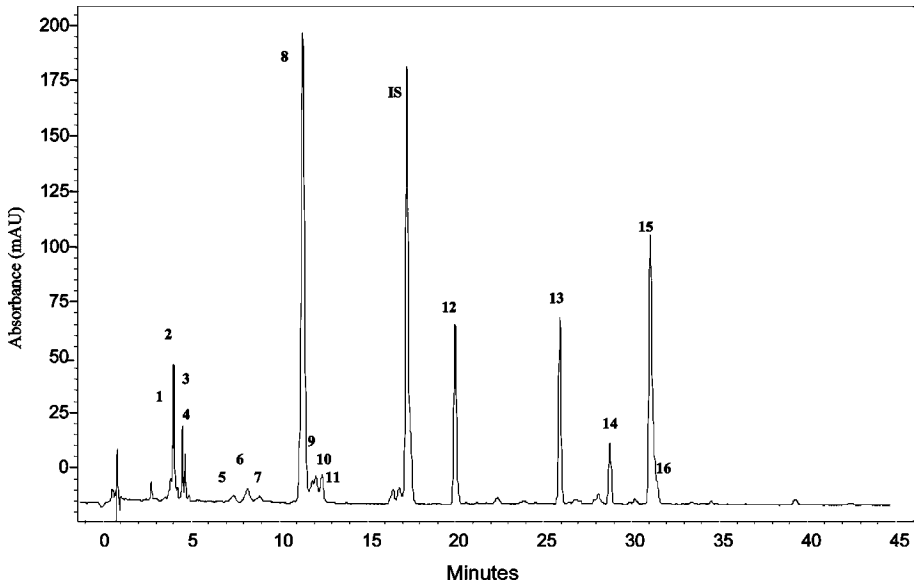

Figure 1. HPLC profile of carotenoids, chlorophyll, and chlorophyll derivatives isolated from grapes. Conditions: column, Nova-Pak C18 60 Å, 4 μm end-capped; detection at 447 nm; flow rate, 1 mL/min; binary gradient elution system of acetonitrile/water (9:1) and ethyl acetate. Peaks: (1) neochrome *a*; (2) (9′*Z*)-neoxanthin; (3) neochrome *b*; (4) violaxanthin; (5) lutein-5,6-epoxide; (6) flavoxanthin; (7) unknown; (8) (*all-E*)-lutein; (9) unknown (lutein-like structure); (10) unknown (lutein-like structure); (11) (13*Z*)- or (13′*Z*)-lutein; (12) chlorophyll *b*; (13) pheophytin *b*; (14) pheophytin *a*; (15) (*all-E*)-β-carotene; (16) (13*Z*)-β-carotene. IS, internal standard, β-apo-8′-carotenal.

compounds with carotenoid-like structure were identified tentatively, although spectral data are available. These include (9*Z*)-lutein (peak 9) and (9′*Z*)-lutein (peak 10). Peaks 12−14 were identified by spectral characteristics, co-injection of standards, and analysis of mass spectra. They correspond to chlorophyll *b*, pheophytin *a*, and pheophytin *b*. The compounds existing in higher amounts in grapes were lutein (peak 8), β-carotene (peak 15), chlorophyll *b* (peak 12), pheophytin *a* (peak 13), and pheophytin *b* (peak 14). Surprisingly, there was no detection of chlorophyll *a* under the RP-HPLC conditions applied for analysis of samples. This is due to the coefficient response of chlorophyll *a*, which is 4 times lower than the coefficient response of chlorophyll *b* (data not shown).

Carotenoid and Chlorophyll derivative Profile in Wines. The analysis of wines by RP-18-HPLC-DAD-MS (EPS$^+$) showed that eight carotenoid compounds were identified (**Table 2**). **Figure 2** shows the chromatogram obtained with a 1-year-old Port (wine I). Apart from lutein (peak 8) and β-carotene (peak 18), five other compounds are reported for the first time in wines; neochromes a and b, two of them have spectral characteristics similar to those of lutein (peaks 9 and 10), and peak 11 was identified as (13*Z*)- or (13′*Z*)-lutein, by spectral and mass determinations. Peaks 12 and 13 remain unknown, as there is no coincidence with UV−vis and MS data to suggest possible carotenoid structures. Six new chlorophyll-derived compounds were also identified. Three of them (peaks 7, 14, and 16) were identified with the help of standards and with their mass spectra, which were consistent with those published earlier (*22*, *23*). Three other compounds have the spectral characteristics and even mass spectra consistent with chlorophyll-derived compounds (peaks 6, 15, and 17); nevertheless, a formal identification cannot be made. The identification of the last peak of the chromatogram (peak 19) as the isomer (13*Z*)-β-carotene was done with the help of spectral characteristics and later confirmed by MS. The isomers *Z* are suggested by the small hypsochromic shift (displacement of λ_{max} to shorter

Table 2. HPLC-DAD-MS (EPS+) Characteristics of Carotenoids, Chlorophylls, and Chlorophyll Derivatives in Port Wines

peak	compound	K'	λ_{max} (nm)	2nd derivative	% (III/II)	identification	m/z	(m/z – H_2O)	wine I	wine II
1	unknown	1.5549	430	435	0		nd		x	x
2	neochrome *a*	1.8811	400; 422; 450	450	93	standard, UV[a]				x
3	(9*Z*)-neoxanthin	2.0292	415; 438; 466	466	69	standard, UV[a]				x
4	neochrome *b*	2.0528	400; 422;450	450	92	standard, UV[a]				x
5	violaxanthin	2.2032	418; 441; 471	471	90	standard, UV[a]				x
6	unknown (chlorophyll-derived compound)	1.6968	436; 526; 652	435		UV[a]	635		x	
7	pheophorbide *b*	2.1463	436; 526; 652	435		UV;[b] MS[d]	607		x	x
8	(*all-E*)-lutein	5.8973	(422); 447; 476	476	53	standard[e]		551	x	x
9	unknown (lutein-like structure)[g]	6.0848	(421); 444; 472	474	52	UV[a]	nd		x	x
10	unknown (lutein-like structure)[h]	6.2400	(420); 443; 472	474	50	UV[a]	nd		x	x
11	(13*Z*)- or (13′*Z*)-lutein	6.4242	333; (420); 442; 468	468	22	UV[a]		551	x	x
12	unknown (carotenoid-like structure)	9.4453	(423); 448; 476	478	52	UV[a]	554	536	x	x
13	unknown (carotenoid-like structure)	10.202	(407); 427; 454	456	19	UV[a]	546		x	x
14	pheophytin *b*	13.410	436; 528; 600; 654			UV;[c] MS[d]	885		x	
15	pheophytin *b*-like compound	13.723	436; 528; 600; 654			UV[a]	844			x
16	pheophytin *a*	14.845	410; 506; 536; 666			UV;[c] MS[d]	871		x	x
17	pheophytin *a*-like compound	15.137	410; 506; 536; 666			UV[a]	nd		x	x
18	(*all-E*)-β-carotene	16.0343	(428); 454; 482	486	20	standard[e]	537		x	x
19	(13Z)-β-carotene	16.254	338; 449; 478	480	7	UV[a]	537		x	x
IS	β-apo-8′-carotenal	8.9231	460	460	0	standard[f]				

[a] Identificaton by comparison with UV spectrum of the "parent" compound. [b] Identificaton by UV spectrum is consistent with Canjura and Schwartz (*22*). [c] Identificaton by comparison with UV spectrum of the "parent" standard obtained by acidification of the respective chlorophyll. [d] Identification by LC-MS is consistent with that of van Breemen et al. (*23*). [e] Pure standard (Sigma-Aldrich, St. Louis, MO). [f] Pure standard (Fluka, Switzerland). [g] (9*Z*)-Lutein. [h] (9′*Z*)-Lutein.

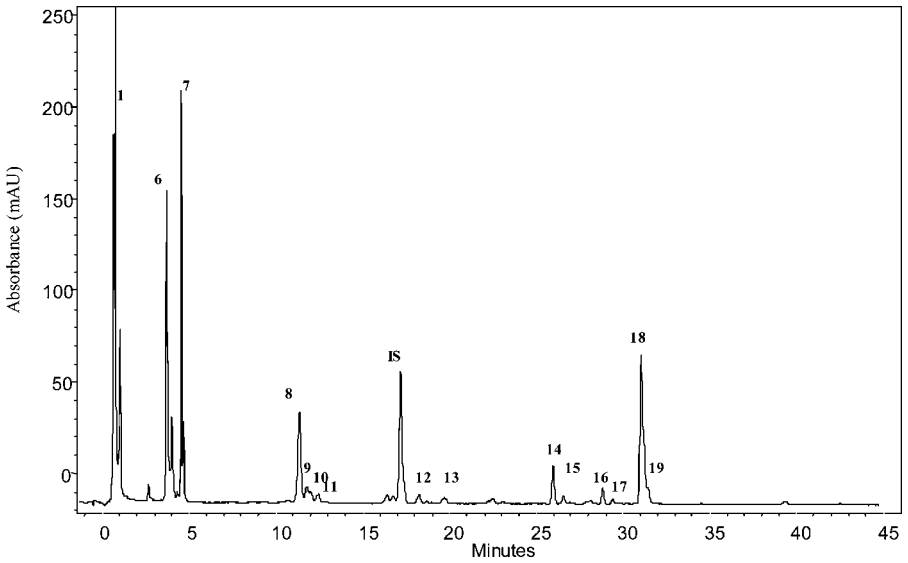

Figure 2. HPLC profile of carotenoids, chlorophyll, and chlorophyll derivatives isolated from Port wine. Conditions: column, Nova-Pak C18 60 Å, 4 μm end-capped; detection at 447 nm; flow rate, 1 mL/min; binary gradient elution system of acetonitrile/water (9:1) and ethyl acetate. Peaks: (1) unknown; (2) neochrome *a*; (3) (9*Z*)-neoxanthin; (4) neochrome *b*; (5) violaxanthin; (6) unknown (chlorophyll-derived compound); (7) pheophorbide *b*; (8) (*all-E*)-lutein; (9) unknown (lutein-like structure); (10) unknown (lutein-like structure); (11) (13*Z*)- or (13′*Z*)-lutein; (12) unknown (carotenoid-like structure); (13) unknown (carotenoid-like structure); (14) pheophytin *b*; (15) pheophytin *b*-like compound; (16) pheophytin *a*; (17) pheophytin *a*-like compound; (18) (*all-E*)-β-carotene; (19) (13*Z*)-β-carotene. IS, internal standard, β-apo-8′-carotenal.

wavelength, 2−6 nm) in the λ_{max} compared to (*all-E*)-β-carotene and the presence of a strong absorption band in the near-UV region (320−380 nm) known as the cis band or cis peak (*24*).

Comparison between C-30 and C-18 separations was not possible because there was a very complex system with two families of compounds (carotenoids and chlorophylls and their derivatives), which behave very differently on the two systems. Hence, data obtained with the C-30 column for chromatographic separation of both grape and wine extracts were not considered in the present analysis.

Comparison between Grape and Wine Profiles. The analysis of both chromatograms obtained from grape and wine extracts (**Figure 3**) shows some differences in the correspondent detected compounds. Peak 14 in the chromatogram of wine extracts is comparable with the peak 13 of the Douro grape extracts (**Tables 1** and **2**). Compounds such as (9′*Z*)-neoxanthin, neochromes *a* and *b*, violaxanthin, (*all-E*)-lutein, (13*Z*)- or (13′*Z*)-lutein, pheophytins *a* and *b*, (*all-E*)-β-carotene, and (13*Z*)-β-carotene exist either in grapes or in some Port wines (**Figure 3**). Chlorophylls were not detected in Port wines. Winterhalter and Rouseff (*28*) reported that during the process of fermentation, chlorophylls present in the grape were degraded, and the products were identified as pheophytins, pyropheophytins, and other pheophytin and chlorophyll-derived compounds. These derivatives are yet to be identified.

Quantitative Analysis. The maximum levels of carotenoids found in young Port was 720 μg/L, generally showing higher total carotenoids content compared to aged Ports. Among 79

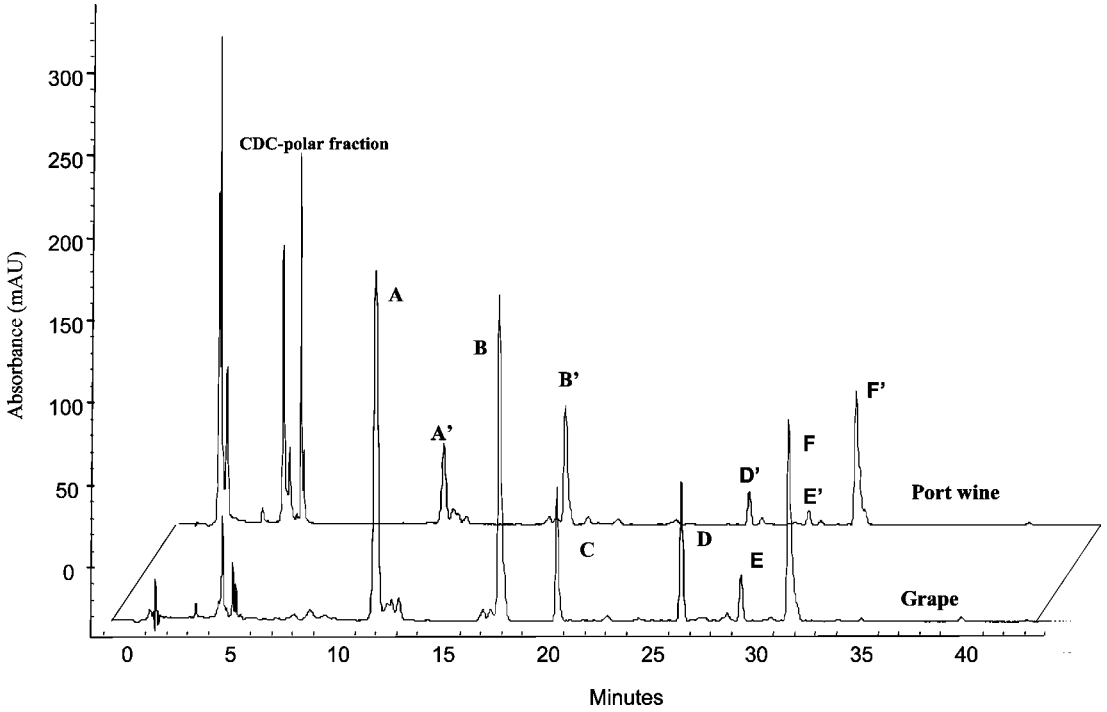

Figure 3. Comparison of HPLC profile of carotenoids, chlorophylls, and chlorophyll derivatives isolated from grape and Port wine. Conditions: column, Nova-Pak C18 60 Å, 4 μm end-capped; detection at 447 nm; flow rate, 1 mL/min; binary gradient elution system of acetonitrile/water (9:1) and ethyl acetate. CDC-polar fraction, chlorophyll derivative compounds; A, A′, lutein polar fraction; B, B′, internal standard (IS), β-apo-8′-carotenal; C, chlorophyll *b*; D, D′, pheopythin *b*; E, E′, pheophythin *a*; F, F′, β-carotene.

Table 3. Results Obtained from the Analysis of 20 DS Port Wines and 20 CC Port Wines[a]

	polar fraction	lutein	pheo-phytin *b*	β-carotene	sum of Car, Chl-DC
wines from DS					
av	129	67	85	102	383
SD	61	32	51	57	100
wines from CC					
av	67	22	25	24	138
SD	32	6	19	5	38

[a] Polar fraction corresponds to polar compounds that have a low retention with the stationary phase C18, compounds such as neoxanthin, neochrome, violaxanthin, and chlorophyll-derivative compounds (the only one identified is the pheophorbide *b*).

Port wines analyzed, the highest values found for lutein and β-carotene were 106 and 358 μg/L, respectively. The analysis of the sum of carotenoid and chlorophyll-derived compounds (Car,Chl-DC) shows that young Ports had a total of Car,Chl-DC ranging between 28 and 720 μg/L. Conversely, old Port wines had a Car,Chl-DC content ranging between traces and 24 μg/L. However, 24.5% of the young Ports had no β-carotene and lutein, and only chlorophyll-derived compounds were present.

Carotenoid and Chlorophyll-Derivative Compounds, Douro Subregions. The analysis of 40 Port wines produced by the two more important subregions of the Douro, Cima Corgo (CC) and Douro Superior (DS), shows that there is a clear differentiation between the two classes of wines (**Table 3**). Considering that the DS region is a hot climatic subregion, grapes produced there are richer in Car,Chl-DC, which is in agreement with results published by other authors (*3*, *6*−*8*). The profile existing in grapes is maintained in the respective wines.

Carotenoid, Chlorophyll, and Chlorophyll-Derivative Compounds/Monovariety, V. vinifera L. Cv. The analysis of Car,-Chl-DC from 29 monovarietal wines, produced with the principal *V. vinifera* L. cv. varieties TN, TB, TR, TC, and TF, shows a significant difference among them (**Figure 4**). For the year of study (2002), the ANOVA of the data showed differences between cultivars and between the different compounds, $p = 0.0265$ and 1.11E−08, respectively, at the 95% level. PCA showed that wines from TR variety were the richest in Car,-Chl-DC. For the year of study TF wines had the lowest content of these compounds and TN wines were richer only in the polar fraction compounds.

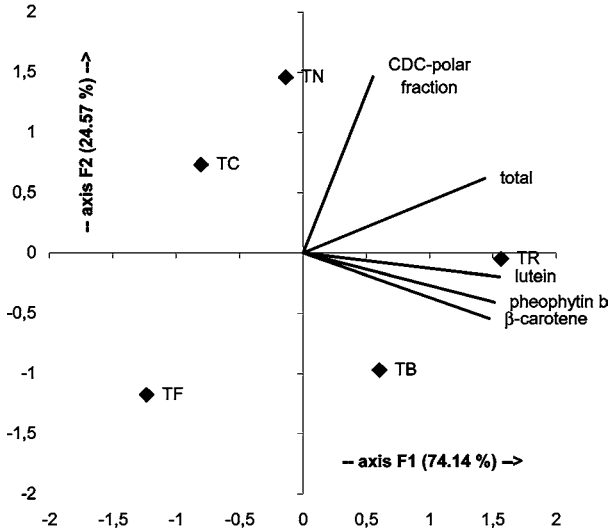

Figure 4. Principal component diagram of the Car,Chl-DC contents and 29 Port wines produced from 5 different varieties. TF, Touriga Franca (5 wines); TN, Touriga Nacional (7 wines); TR, Tinta Roriz (5 wines); TC, Tinto Cão (1 wine); TB, Tinta Barroca (6 wines). Factor score plot 1–2: axis 1 and 2 account for 98.71% of the total variance.

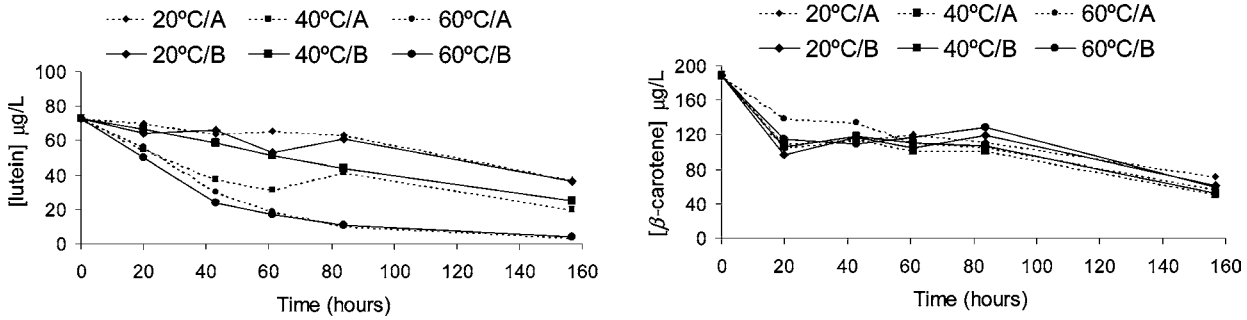

Figure 5. Lutein and β-carotene degradation during the forced-aged protocol, using different storage temperatures (20, 40, and 60 °C) and different dissolved oxygen levels [(A) 2.5 mg/L; (B) 4.8 mg/L]. Values of lutein and β-carotene are expressed in micrograms per liter of wine.

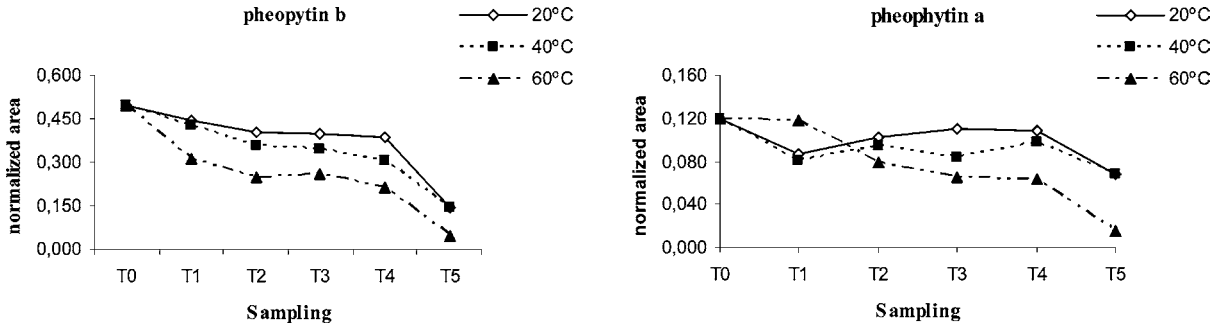

Figure 6. Pheophytin *a* and *b* degradation during the forced-aged protocol, using different storage temperatures (20, 40, and 60 °C). Values are expressed in normalized area. To simplify the demonstration of the obtained results, only results for condition A are reported.

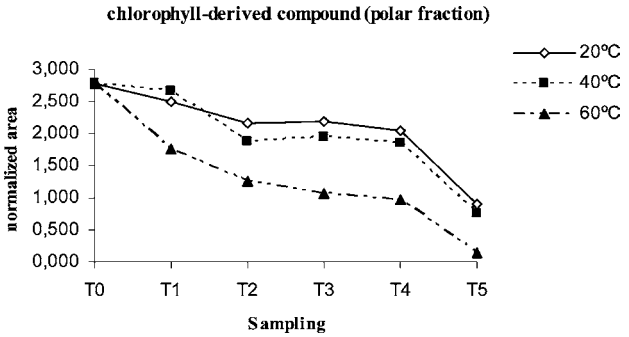

Figure 7. Chlorophyll-derived compounds (polar fraction) degradation during the forced-aged protocol, using different storage temperatures (20, 40, and 60 °C). Values are expressed in normalized area. To simplify the demonstration of the obtained results, only results for condition A are reported.

Forced Aging. Results indicate that lutein degraded more quickly than β-carotene independent of temperature (20, 40, or 60 °C) and oxygen content (A, 2.5 mg/L; B, 4.8 mg/L) (**Figure 5**). At 60 °C, there was a loss of ~95% of lutein after 160 h, at both oxygen levels. β-Carotene degraded with time, with a similar behavior under the different experimental conditions. It seems that the combined effect of acidic conditions of wine with high temperatures is responsible for the higher degradation of lutein compared to β-carotene, which might be related to the presence of hydroxyl groups of lutein. The degradation reactions apparently follow zero-order kinetics. These results support the higher ratio of β-carotene/lutein concentrations (average values for each year group) observed in aged wines compared to new Ports (2002 wines, 1.3; 1996 wines, 3.8), which might suggest that lutein degrades more quickly than β-carotene during wine aging.

Degradation rates of chlorophyll-derived compounds were also determined (**Figures 6** and **7**). For this purpose the pheophytin *b* degradation and chlorophyll-derived compound polar fraction (Chl-DC compound existing in higher concentration) were followed over time. Identical behaviors were observed under conditions of different dissolved oxygen levels (data not shown).

It is interesting to note that at 60 °C both pheophytin *b* and polar fraction chlorophyll-derived compound, including pheophorbide *b*, were degraded more quickly than the former compounds, lutein and β-carotene. After 160 h, these compounds were practically nonexistent. These results explain the slow degradation of lutein and β-carotene and the faster degradation of Chl-DC in Ports. In fact, in old Port wines very low levels of β-carotene can be found but no chlorophyll-derived compounds are present; conversely, old Port wines have a higher aromatic complexity than young Port wines, which might be related to a higher contents of volatile compounds.

The fact that carotenoid and chlorophyll-derived molecules exist in Port wines (*24*) and are nonexistent in red and white table wines is probably related to the winemaking process. Port is a naturally sweet wine produced by interrupting alcoholic fermentation by the addition of grape spirit. As the great part of the Port wine matrix is must (grapes not fermented), the major parts of sugars, amino acids, polyphenols, and aroma precursors remain intact in the respective wines. This is probably the main reason that carotenoid and chlorophyll-derived molecules remain in Port wines. Moreover, the addition of brandy (up to 20% v/v ethanol) may facilitate the solubilization of these molecules. However, levels of carotenoid and chlorophyll molecules found in young Port wines are very low and, probably, they do not have a sensorial impact in wine. Nevertheless, it is important to note that Port wine undergoes long periods of aging (>4 years), both for bottle-aged ("vintage category") and barrel-aged ("tawny" category) wines; consequently, many chemical reactions may occur during the aging process, which can involve the degradation of these molecules with concomitant formation of others.

In fact, some volatile compounds are directly or indirectly related with carotenoid molecules (*9*, *15*, *26*, *27*); these volatiles can be found in old Port wines. On the other hand, young Port

wines are richer in carotenoid and chlorophyll-derived molecules than old Port wines, where these molecules are practically nonexistent. All of these observations imply that during aging carotenoid molecules and probably chlorophyll-derived molecules can be transformed or degraded into smaller volatile molecules and may have a sensorial impact in wine aroma.

The degradation of chlorophyll pigments involves a number of reactions. It has been demonstrated that chlorophyll pigments can be broken down into pheophytins. It has also been demonstrated that not only the formation of pheophytins is involved but also the formation of pyropheophytins (*29*). Some previous work has shown chemical degradation of chlorophyll, and some kinetic studies of the formation of small molecules have been described (*30*−*32*). However, in these works only molecules detected by HPLC methodologies were identified; for this reason no attempts were considered in the formation of volatiles. The future work will be to determine if these molecules can be degraded into compounds that can have a sensorial impact in wines.

This work reports the presence of carotenoids and chlorophyll-derived (Car,Chl-DC) compounds in grapes and Port wines. Using HPLC-DAD-MS analysis it was possible to detect two more carotenoids in grapes and five new carotenoid compounds in wines, being possible the identification of (13,13*Z*′)-lutein and β-carotene isomers both in grapes and in Ports. Furthermore, the chlorophyll-derived compounds, namely, pheophytins *a* and *b*, were detected for the first time in grapes and Port wines. Rates of degradation of Car,Chl-DC were determined, and results were consistent with the observed decreased of these compound in old Port wines compared to young Ports.

Because carotenoids are potential percursors of aroma compounds identified in Port wines such as TCH, β-ionone, TDN, and β-damascenone, it can also be considered that the degradation of chlorophyll-derived compounds in Ports may result in the formation of volatile compounds and, therefore, have a role in the aroma evolution during the wine aging process.

Further research should be done to assess the possible relationship between the presence of carotenoids and chlorophyll-derived compounds in Ports and their conversion into aroma compounds that can have sensorial impact in wines.

ACKNOWLEDGMENT

We acknowledge Michel Carail from INRA Avignon, France, for his assistance in running the HPLC-DAD-MS analysis.

LITERATURE CITED

(1) Razungles, A.; Bayonove, C.; Cordonnier, R.; Baumes, R. Etude des caroténoides du raisin à maturité. *Vitis* **1987**, *26*, 183−191.

(2) Razungles, A.; Bayonove, C.; Cordonnier, R.; Sapis, J. Grape carotenoids: changes during the maturation period and localization in mature berries. *Am. J. Enol. Vitic.* **1988**, *39* (1), 44−48.

(3) Razungles, A.; Babic, I.; Sapis, J.; Bayonove, C. Particular behavior of epoxy xanthophylls during veraison and maturation of grape. *J. Agric. Food Chem.* **1996**, *44*, 3821−3825.

(4) Marais, J.; van Wyk, C.; Rapp, A. Carotenoid levels in maturing grapes as affected by climatic regions, sunlight and shade. *S. Afr. J. Enol. Vitic.* **1991**, *12* (2), 64−69.

(5) Bureau, S.; Razungles, A.; Baumes, R.; Bayonove, C. Effect of vine or bunch shading on the carotenoid composition in *Vitis Vinifera* L. berries. I. Syrah grapes. *Vitic. Enol. Sci.* **1998**, *53* (2), 64−71.

(6) Oliveira, C.; Silva Ferreira, A. C.; Mendes Pinto, M. M.; Hogg, T.; Alves, F.; Guedes de Pinho, P. Carotenoid compounds found in grapes and their relationship to plant water status. *J. Agric. Food Chem*. **2003**, *51*, 5967−5971.

(7) Oliveira, C.; Silva Ferreira, A. C.; Costa, P.; Guerra, J.; Guedes de Pinho, P. Effect of some viticultural parameters on the grape carotenoid profile. *J. Agric. Food Chem.* **2004**, *52*, 4178−4184.

(8) Bureau, S.; Baumes, R.; Razungles, A. Effects of vine bunch shading on the glycosylated flavor precursors in grapes of *Vitis vinifera* L. Cv. Syrah. *J. Agric. Food Chem.* **2000**, *48*, 1290−1297.

(9) Sefton, M. A.; Skouroumounis, G. K.; Massy-Westropp, R. A.; Williams, P. A. Norisoprenoids in *Vitis vinifera* White wine grapes and the identification of a precursor of damascenone in these fruit. *Aust. J. Chem.* **1989**, *42*, 2071−2084.

(10) Mordi, C. R.; Walton, J. C.; Burton, G. W.; Hughes, L.; Ingold, K. U.; Lindsay, D. A. Exploratory study of β-carotene autoxidation. *Tetrahedron Lett*. **1991**, *32*, 4203−4206.

(11) Kanasawud, P.; Crouzet, J. C. Mechanism of formation of volatile compounds by thermal degradation of carotenoids in aqueous medium. 1. β-Carotene degradation. *J. Agric. Food Chem.* **1990**, *38*, 237−243.

(12) Teranishi, W. R., Takeoka, G., Guntert, M., Eds. *Flavour Precursors—Thermal and Enzymatic Conversion*; ACS Series 490; American Chemical Society: Washington, DC, 1993; p 98.

(13) Gunata, Y. Z.; Bayonove, C. L.; Baumes, R. L.; Cordonnier, R. E. The aroma of grapes. I. Extraction and determination of free and glycosidically bound fraction of some grape aroma components. *J. Chromatogr*. **1985**, *331*, 83−90.

(14) Skouroumounis, G. K.; Massy-Westropp, R. A.; Sefton, M. A.; Williams, P. A. Precursors of damascenone in fruit juices. *Tetrahedron Lett*. **1992**, *33*, 3533−3536.

(15) Isoe, S.; Katsumura, S.; Sakan, T. The synthesis of damascenone and β-damascone and the possible mechanism of their formation from carotenoids. *Helv. Chem. Acta* **1973**, *56*, 1514−1516.

(16) Silva Ferreira, A. C.; Guedes de Pinho, P. Nor-isoprenoids profile during port wine ageing influence of some technological parameters. *Anal. Chim. Acta* **2004**, *513*, 169−176.

(17) Guedes de Pinho, P.; Silva Ferreira, A. C.; Mendes-Pinto, M.; Benitez, J. G.; Hogg, T. A. Determination of carotenoid profile in grapes, must and fortified wines from Douro varieties of *Vitis vinifera*. *J. Agric. Food Chem.* **2001**, *49*, 5484−5488.

(18) Freitas, V.; Ramalho, P.; Azevedo, Z.; Macedo, A. Identification of some volatile descriptors of the rock-rose like aroma of fortified red wines from Douro Demarcated Region. *J. Agric. Food Chem.* **1999**, *47*, 4327−4331.

(19) Kotseridis, Y. Etude de l'arôme des vins de Merlot et Cabernet-Sauvignon de la Région Bordelaise. Thèse de Doctorat de L'Université Victor Segalen, Bordeaux II, 1999; 652 pp.

(20) Simpson, R. F. Aroma and compositional changes in wine with oxidation, storage end ageing. *Vitis* **1978**, *17*, 274−287.

(21) Mendes-Pinto, M. M.; Silva Ferreira, A. C.; Oliveira, M. B. P. P.; Guedes de Pinho, P. Evaluation of major carotenoids in grapes by liquid chromatography with reversed and normal phase—a qualitative analysis. *J. Agric. Food Chem*. **2004**, *52*, 3182−3188.

(22) Canjura, F. L.; Schwartz, S. J. Separation of chlorophyll compounds and their polar derivatives by high performance liquid chromatography. *J. Agric. Food Chem*. **1991**, *39*, 1102−1105.

(23) Van Breemen, R. B.; Canjura, F. L.; Schwartz, S. J. Identification of chlorophyll derivatives by mass spectrometry. *J. Agric. Food Chem*. **1991**, *39*, 1452−56.

(24) Mendes-Pinto, M. M.; Silva Ferreira, A C.; Guedes de Pinho, P. Carotenoids profile during port wine ageing. In *3rd International Congress, Pigments in Food: More than Colors*, Quimper, France, June 14−17, 2004; pp 364−367.

(25) Britton, G. UV/Visible spectroscopy. In *Carotenoids*; Britton, G., Liaasen-Jensen, J., Pfander, H., Eds.; Birkäuser Verlag: Basel, Switzerland, 1995; Vol. 1B, Spectroscopy, Chapter 2, pp 13−62 (ISBN 3-7643-2909-2; 0-8176-2902-2).

(26) Skouroumounis, G. K.; Sefton, M. A The formation of β-damascenone in wine. In *Chemistry of Wine Flavour*; Waterhouse,

A. L., Ebeler, S. E., Eds.; American Chemical Society: Washington, DC, 1998; pp 241−254.
(27) Ravichandran, R. Carotenoid composition, distribution and degradation to flavour volatiles during black tea manufacture and the effect of carotenoid supplementation on tea quality and aroma. *Food Chem.* **2002**, *78*, 23−28.
(28) Winterhalter, P.; Rouseff, R. *Carotenoid Derived Aroma Compounds*; ACS Symposium Series 802; American Chemical Society: Washington, DC, 2000; pp 1−19.
(29) Schwartz, S. J.; von Elbe, J. H. Kinetics of chlorophyll degradation to pyropheophytin in vegetables. *J. Food Sci.* **1983**, *48*, 1303−1306.
(30) Minguez Mosquera, M. I.; Gandul-Rojas, B.; Minguez Mosquera, J. Mechanism and kinetics of the degradation of chlorophylls during processing of green table olives. *J. Agric. Food Chem.* **1994**, *42*, 1089−1095.
(31) Suzuki, Y.; Shioi, Y. Detection of chlorophyll breakdown products in the senescent leaves of higher plants. *Plant Cell Physiol.* **1999**, *40*, 909−915.
(32) Rontani, J. F.; Cuny, P.; Gossi, V. Photodegradation of chlorophyll phytyl chain in senescent leaves of higher plants. *Phytochemistry* **1996**, *2*, 347−351.

Received for review February 15, 2005. Revised manuscript received August 24, 2005. Accepted September 27, 2005. This work was supported by the Portuguese Ministry of Agriculture through the AGRO program (Project 820). M.M.M.P. is in receipt of a Research Grant from the Ministry of Agriculture (AGRO 820). We thank Project MCT/FCT/POCTI/AGR/42598/2001 for financial support.

JF0503513