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Evaluation of Some Carotenoids in Grapes by Reversed- and Normal-Phase Liquid Chromatography: A Qualitative Analysis

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Carotenoids in grapes of three Port winemaking cultivars were investigated. Extracts were obtained with *n*-hexane/diethyl ether mixtures (0/100; 20/80; 50/50; 100/0) and analyzed by normal and reversed phase HPLC-DAD. Selection and identification of peaks were based on spectroscopic characteristics − λ_{max}, (%III/II) and k′ values, leading to 28 probable carotenoids. Using pure standards, it was possible to identify seven compounds previously described (neochrome, neoxanthin, violaxanthin, flavoxanthin, zeaxanthin, lutein, and β-carotene), one more type of neochrome reported here, for the first time, and in addition, two geometrical isomers of lutein and β-carotene were tentatively described. The remaining 17 need to be further identified. High polarity solvent mixtures lead to qualitatively richer chromatograms. Reversed-phase separations allowed the detection of flavoxanthin and the possible geometrical isomer(s) of β-carotene. Under normal phase, zeaxanthin was detected, and neochromes were better separated from neoxanthin. Extraction with 50/50 *n*-hexane/diethyl ether mixtures and reversed-phase conditions was the best combination for analysis of the carotenoids, known as precursors of compounds with high aroma impact in wines.

KEYWORDS: Port wine grapes; carotenoid identification

INTRODUCTION

Carotenoids are mostly C_{40}-tetraterpenoid compounds with an extensive conjugated polyene backbone (*1*). They are a group of more than 600−700 natural pigments with great structural diversity, separated into two main classes: (i) the carotenes made up with only carbon and hydrogen and (ii) xanthophylls, the enzymatically formed oxidation products of α- and β-carotene.

Widely distributed in nature, they can be found in higher plants, algae, fungi, and bacteria. In grapes, conditions such as soil and climate regulate the maturation stage and therefore the carotenoid profile (*2*−*10*).

Because carotenoids are extremely unstable compounds, they are easily degraded by these similar environmental influences. In fact, high temperatures, oxygen, and light have been described in the literature as being responsible for degradation of carotenoids (*11*−*13*).

Many carotenoid metabolites are involved in the degradation pathways of carotenoids, including C_{13}-norisoprenoids, common plant constituents, which significantly contribute to the flavor of grapes and wines (*2*, *5*, *14*−*19*). Therefore, the carotenoid content in grapes has been studied (*3*−*10*). Although these works report a number of carotenoids considerably lower than those found in other fruits such as red paprika and sweet orange, for instance with 34 and 25 carotenoid compounds, respectively (*20*, *21*), they can play an important role as aroma precursor compounds.

Due to the chemical properties of carotenoides, high-performance liquid chromatography (HPLC) is at present the method of choice for its study. Reversed-phase columns have generally been used as the most suitable tools for separation of these relatively apolar compounds, as reported in several contributions, also with other carotenogenic samples (*20*−*24*). On-line UV−vis spectroscopic data, recorded by a photodiode array detector (DAD) have been extensively applied for carotenoid analysis. The spectroscopic characteristics of carotenoids, position of the absorption maxima, λ_{max} (usually three peaks), and spectral fine structure, defined as the ratio of the peak heights between absorption bands, as a percentage (%III/II), give information about the chromophore (*1*, *25*, and literature cited within). The type of functional groups contained in the molecule can be assessed by various chemical tests, for example acetylation of primary and secondary hydroxy groups or epoxide-furanoid rearrangement, among others (*26*). As a way to reduce misidentification of carotenoids, a minimum identification criteria is described (*27*). It is a combination of absorption spectroscopic data, chromatographic properties, and a mass spectrum (MS). Other methods are also available for carotenoid identification, such as NMR, IR, and CD spectroscopy.

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As carotenoids are present in Port wines (*10*), the aim of this study was to investigate the carotenoid profile in black grapes of three varieties of *Vitis vinifera* L. cv., for example, Tinta Barroca, Touriga Francesa, and Tinta Roriz, typically used in the production of these wines. It was also attempted to establish the most suitable combination between solvent polarity for carotenoid extraction and chromatographic conditions for carotenoid separation, to obtain as much qualitative information as possible concerning these substances, recognized as aroma precursors. The norisoprenoids β-ionone and 2,2,6-trimethylcyclohexanone (TCH) present in some Port wines (*17*, *18*) are also reported as resulting from chemical degradation of β-carotene (*13*), while neoxanthin is suggested as the precursor of β-damascenone, a compound with high aroma impact in wines (*19*). For this purpose, liquid chromatography with a combination of normal and reversed phase separations was used to maximize the separation capacity of carotenoids present in grape material. Additionally, different proportions of hexane and diethyl ether were also applied to differentiate between carotenoids according to their polarity. This study is also potentially useful to gather more information concerning the presence of carotenoid substances present in grape material, which can be precursors of high aroma impact molecules in Port wines.

MATERIALS AND METHODS

Grape Material. Three varieties of *Vitis vinifera* L. cv., Tinta Barroca (TB), Touriga Francesa (TF), and Tinta Roriz (TR), harvested at the end of August from the same subregion Cima Corgo of the Douro region of northern Portugal, were used. To minimize post-harvest loss and modification of carotenoids, samples were kept at −20 °C prior to analysis. Work was carried out under subdued light, at controlled temperature (20−23 °C) and by avoiding the presence of oxygen to prevent photoisomerization and degradation of carotenoids.

Reagents and Commercial Standards. HPLC grade solvents hexane, dichloromethane, methanol, ethyl acetate, acetonitrile, and acetone, all from Merck (Whitehouse Station, NJ), were used. Lutein and β-carotene were purchased from Sigma-Aldrich (St. Louis, MO) and (9′Z)-neoxanthin and violaxanthin from CaroteNature, Switzerland. β-Apo-8′-carotenal was obtained from Fluka, Switzerland, and chlorophylls a and b were purchased from Biochemika, Switzerland.

Ultra-pure water (18 Ωm) (EASY pure LF, Barnstead) was used.

Organic Solvents Selection. Different organic mixtures of hexane/diethyl ether 0/100, 20/80, 50/50, and 100/0, with corresponding polarities of 2.8, 2.24, 1.4, and 0, were used to extract carotenoids. Other mixtures of hexane/diethyl ether with a wide range of polarity, including 80/20, 60/40, and 40/60 were also tested in a preliminary study.

Carotenoid Extraction. Eight extracts of grapes from each variety were obtained using each of the different solvent proportions. Four extracts were analyzed by reversed-phase HPLC, and the others were analyzed by normal phase HPLC.

An amount of 40 g of grape berries (pulp and skin, without seeds) was homogenized with a mixer Ultra-TURRAX T25 (Janke & Kunker IKA-Labortechnic) for 10 min, at 13500 rpm and 40 mL of ultrapure water was added. Subsequently, each solvent mixture was added in two portions: First, 20 mL were used. After 30 min of stirring, another 20 mL were added. The organic phases were pooled, dried with anhydrous Na_2SO_4, and evaporated to dryness in a rotatory evaporator under 20−23 °C.

HPLC. (*a*) *Equipment.* The chromatographic equipment consisted of a Beckman System Gold, equipped with the 508 autosampler, 126 programmable solvent system. A 168 diode array detector module was set to scan from 270 to 600 nm. Sample injection volume was 20 μL, and absorbance was recorded at 447 nm. A binary gradient elution system was used in both stationary phases.

(*b*) *Reversed Stationary Phase Conditions.* Column: Nova-Pak C18 60 Å 4-μm (3.9 × 300-mm), Waters. The eluents were 100% ethyl acetate (solvent A) and 90% acetonitrile (v/v) in H_2O (solvent B). Flow rate was 1 mL/min with a gradient elution system of 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.

(*c*) *Normal Stationary Phase Conditions.* Column: Spherisorb 5-μm CN (4.6- × 250-mm), Waters. The eluents were 100% methanol (solvent A), 75% hexane, 25% dichloromethane, and 0.5% methanol (v/v/v) (Solvent B). Flow rate was 1 mL/min with a gradient elution of 0−3 min, 0% B; 3−19.5 min, to 22% B; 19.5−20.5 min, 22% B; 20.5−25.5 min, to 0% B; 25.5−26.5 min, 0%B.

The resulting extract from carotenoid isolation was resuspended on 1 mL of mobile phase (50% A, 50% B) according to the HPLC separation to be performed. A known amount of β-apo-8′-carotenal was added to calculate the chromatographic parameter capacity factor (k').

Qualitative Analysis. Spectroscopic characteristics (positions of absorption maxima (λ_{max}) and the degree of vibrational fine structure (%III/II)), and capacity factor values k', were the basis for qualitative analysis of carotenoid compounds. Peaks with the ratio S/N (6 sigma) (parameter of Beckman 32 Karat software) lower than 2 were not selected. Identification of carotenoids in samples was based on on-line spectral data obtained by photodiode array detection, by comparison with standard spectra and reported values (*1*). Spectral behavior of carotenoid compounds in both HPLC eluent systems was also compared. As retention times are difficult to reproduce, even under controlled chromatographic conditions, mean capacity factors were calculated according to ref 28.

Preparation of Neochromes, Flavoxanthin, and Zeaxanthin Standards. Neochromes were prepared by acid catalysis of neoxanthin with a few drops of ethanolic hydrogen chloride (0.1 M). Flavoxanthin and zeaxanthin were isolated from *Ranunuculus acer* and *Lycium halimfolium*, respectively (*29*). The neochromes a and b and flavoxanthin were purified using preparative column chromatography (CC), with $CaCO_3$ as adsorbent and 4−5% acetone in toluene as eluent accordingly to ref 30. Zeaxanthin was purified in the same way, except for the eluent, which was 45−50% toluene/hexane. After crystallization, their purity was controlled by HPLC, according to ref 35. The obtained purity was higher than 90%. The neoxanthin spectrum in ethanol was recorded in a double beam UV−vis spectrophotometer (Shimadzu 1601) within a wavelength range 270−600 nm for comparative analysis, with the resulting product from acidification after 2−3 min. Spectral data in both HPLC reversed-phase and normal phase, were analyzed. Furthermore, the obtained carotenoid compounds were resuspended in ethanol and also analyzed spectrophotometrically by comparison with reference values (*1*, *7*).

Standards were also characterized by FTIR spectroscopy (spectra were recorded on an ATI Mattson/Genesis, Winfirst v.2. 10 Sofware, in KBr pellets).

Study of Carotenoid Stability Under the Applied Experimental Conditions. To determine whether (*Z*)-isomers and furanoid oxides were natural carotenoids from grapes or artifacts due to sample treatments (extraction and separation), a solution of all available standards was added to one grape sample and then extracted. Comparison was made between the resulting chromatogram and the chromatogram of one extracted grape sample to which standards were added after extraction. On the other hand, a standard mixture was also injected individually to determine the extent of coelution of the most polar xanthophylls. Carotenoids were extracted with solvent mixture of hexane/diethyl ether 50/50. Both HPLC separations (reversed and normal phases) were carried out.

Statistical Treatments. An analysis of variance (ANOVA), using the Excel software Windows 98 V. 7.0, was applied to the experimental data. The results were considered significant if the associated *p*-value was <0.05.

RESULTS AND DISCUSSION

Spectral Data of Neochrome, Flavoxanthin, and Zeaxanthin Standards. The analysis of the spectrum in ethanol of the acidified neoxanthin showed a hypsochromic shift of about 20 nm, which indicates that epoxide−furanoid rearrangement had occurred (i.e., the 5,6-epoxide neoxanthin, λ_{max} = 415, 439, 467

Table 1. Chromatographic and Spectroscopic Characteristics for Carotenoid Identification on All Grape Extracts Analyzed

HPLC Reversed Phase Eluents

peak	compound[a]	k′[b]	λ_{max}	2nd D[c]	%(III/II)[b]	grape varities[d] TB	TF	TR
1	neochrome/a	1.881	400; 422; 450	450	93	x	x	x
2	neoxanthin	2.029	415; 438; 466	466	69	–	x	x
3	neochrome/b	2.053	400; 422; 450	450	92	x	x	x
4	violaxanthin	2.203	418; 441; 471	471	90	x	x	x
5	(*13/13′Z*)-or (*15Z/15′*)-unknown	2.480	320; 397; 418; 444	444	69	x	x	x
6	unknown 1RP	3.122	421; 446; 474	476	19	–	x	x
7	flavoxanthin	4.747	398; 422; 448	450	95	x	x	x
8	unknown 2 RP	5.003	(406); 428; 454	456	47	x	x	x
9	*(all-E*)- lutein	6.035	(422); 447; 476	476	53	x	x	x
10	unknown 3 RP	6.315	(423); 446; 472	475	19	x	x	x
11	(*13/13′Z*)-or (*15Z/15′*)-lutein	6.483	333; (420); 442; 468	468	22	x	x	x
12	unknown 4 RP	13.247	415; 435	434	0	x	x	x
13	(all-*E*)-β -carotene	15.801	(428); 454; 482	486	20	x	x	x
14	*(13/13′Z)*- ou *(15/15′Z)*-β - carotene	16.031	338; 449; 478	480	7	x	x	x
IS	β -apo-8′- carotenal	8.694	460	460	0			

HPLC Normal Phase Eluents

peak	compound[e]	k′[a]	λ max	2nd D	%(III/II)[b]	grape varities TB	TF	TR
1	(*all -E*)-β -carotene	0.074	(428); 454; 482	486	14	x	x	x
2	unknown 1 NP	0.164	(405); 428; 453	456	25	x	x	x
3	unknown 2 NP	0.213	404; 427; 453	455	0	–	x	x
4	unknown 3 NP	0.604	422; 448; 475	477	31	–	x	x
5	unknown 4 NP	0.788	434	432	0	x	–	–
6	unknown 5 NP	1.188	415; 435	434	0	x	x	x
7	*(all-E*)- lutein	3.630	(422); 447; 476	476	56	x	x	x
8	zeaxanthin	3.649	(426); 452; 483	484	18	x	x	x
9	unknown 6 NP	3.687	(420); 442; 470	472	47	x	x	x
10	unknown 7 NP	3.773	(422); 447; 470	472	15	x	x	x
11	unknown 8 NP	3.634	400; 424; 450	450	112	–	–	x
12	(*13/13′Z*)-or (*15Z/15′*)-lutein	3.695	333; (420); 442; 468	468	21	–	x	x
13	unknown 9 NP	3.907	420; 442; 468	471	14	–	x	x
14	unknown 10 NP	3.926	402; 423; 448	450	33	x	x	x
15	unknown 11 NP	4.024	419; 442; 472	472	44	x	x	x
16	unknown 12 NP	4.057	422; 447; (471)	471	3	x	x	x
17	unknown 13 NP	4.214	400; 424; 450	450	64	x	x	x
18	unknown 14 NP	4.283	419; 442; 472	472	63	x	x	x
19	unknown 15 NP	4.707	422; 447; 478	478	40	–	x	x
20	neochrome/b	4.691	400; 422; 450	450	84	x	x	x
21	neochrome/a	4.757	400; 422; 450	450	81	x	x	x
22	neoxanthin	4.848	415; 438; 466	466	84	x	x	x
IS	β -apo-8′- carotenal	0.423	460	460	0			

[a] RP- reversed phase. [b] Average values. [c] 2nd D, second derivative. [d] TB, Tinta Barroca; TF, Touriga Francesa; TR, Tinta Roriz; x, detected; –, not detected. [e] NP, normal phase.

was converted to the corresponding 5,8-epoxide neochrome, λ_{max} = 400, 420, 450). The other isolated compounds showed the following absorption maxima (nm): flavoxanthin, λ_{max} = 398, 422, 448; zeaxanthin, λ_{max} = (426), 450, 481. Data were consistent with reference values (*1*, *7*). The spectral fine structure (%III/II) values found were neochrome 92, flavoxanthin 95, and zeaxanthin 25, which were similar to values reported in the literature (*1*). HPLC analyses of the resulting product from neoxanthin acidification showed two peaks with identical spectra, which were denominated as neochrome a and neochrome b.

The FTIR spectra of these compounds, compared to reported data (*29*) allowed their characterization and the confirmation that no other major compounds were present. Neochrome a IR γ_{max} (cm^{-1}): 3427, 2956, 2922, 2852, 1925, 1701, 1643, 1541, 1454, 1371, 1259, 1151, 1097, 1032, 959. Neochrome b IR γ_{max} (cm^{-1}): 3431, 2958 (shoulder), 2922, 2852, 1918, 1736, 1645, 1541, 1458, 1381, 1259, 1153, 1097, 1028, 960. Flavoxanthin IR γ_{max} (cm^{-1}): 3410, 2956, 2920, 2858, 1712, 1653, 1454, 1365, 1257, 1159, 1126, 1093, 1057, 1020, 966. Zeaxanthin IR γ_{max} (cm^{-1}): 3340, 2953, 2918, 2852, 1707, 1650, 1456, 1365, 1259, 1099, 1038, 962.

Carotenoid Profile. The pool of carotenoid products found in all grape extracts from all varieties analyzed is given in **Table 1** for reversed phase separations (RP) and normal phase separations (NP). It should be noted that not all carotenoids were detected in all samples (**Table 1**). Neoxanthin, violaxanthin, (all-*E*)-lutein and (all-*E*)-β carotene, flavoxanthin, zeaxanthin, and two neochromes, with identical spectral data and different *k′* values, denominated as neochrome a and neochrome b, were identified by comparison with standards and reported values. The presence of these compounds is in agreement with previous works (*3*−*10*), but only one neochrome (without specification) has been reported. The xanthophylls, luteoxanthin, lutein 5,6-epoxide, echinenone, and cryptoxanthin were not detected. As α-carotene was present in trace amounts, as also reported in a former study (*7*), it was not considered since the ratio S/N (6 sigma) was lower than 2. Together with (all-*E*)-lutein and (all-*E*)-β-carotene, the most abundant carotenoids in grapes (*3*−*10*), an unknown compound with absorption maxima

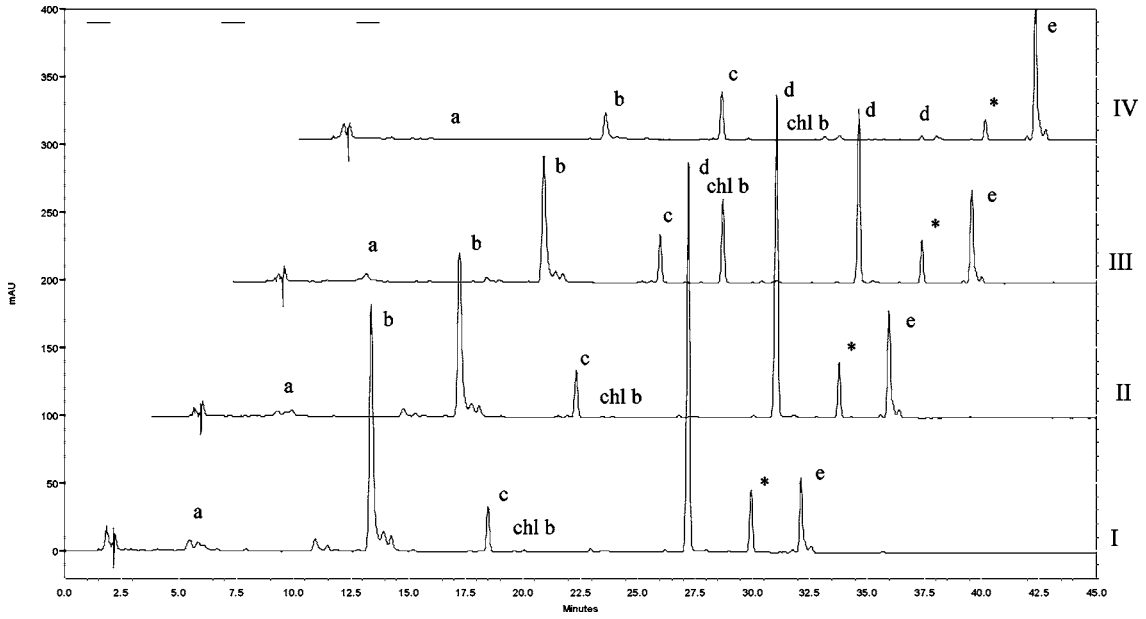

Figure 1. HPLC reversed-phase separations using the four different solvent mixtures. I, 0h/100de; II, 20h/80de; III, 50h/50de; IV, 100h/0de; (h, hexane; de, diethyl ether). a, neoxanthin polar fraction; b, lutein polar fraction; c, internal standard; d, unknown (415; 435 nm); e, β-carotene. chl b, chlorophyll b (λ_{max} 435; 458 nm; 2nd D 458); *, chlorophyll? (λ_{max} 325; 409; 506; 536 nm; 2nd D 412).

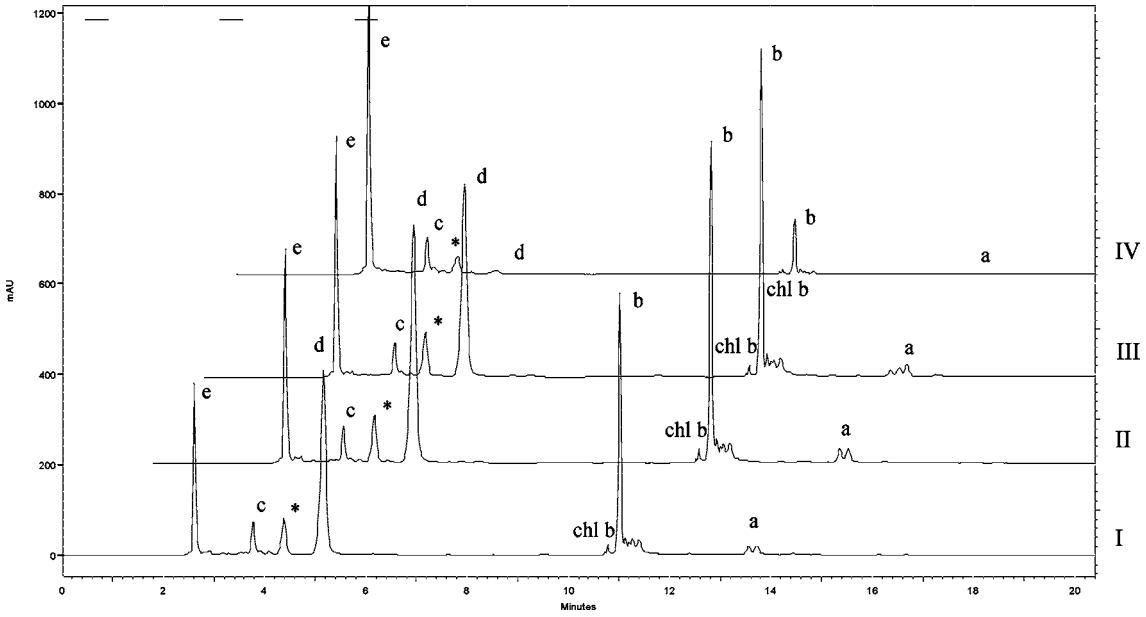

Figure 2. HPLC normal-phase separations using the four different solvent mixtures. I, 0h/100de; II, 20h/80de; III, 50h/50de; IV, 100h/0e; (h, hexane; de, diethyl ether). a, neoxanthin polar fraction; b, lutein polar fraction; c, internal standard; d, unknown (415, 435 nm); e, β -carotene; chl b, chlorophyll b (λ_{max} 435; 458 nm; 2nd D 458); *, chlorophyll? (λ_{max} 325; 409; 506; 536 nm; 2nd D 412).

of 415 and 435 nm, and a major predominance when extracted with the high polarity solvent mixtures, with a k' value near to that of β-carotene, was found. These compounds, unknown 4 RP and unknown 5 NP (**Table 1**), were present in high amounts in all samples extracted.

Additionally, two (*Z*)-isomers were tentatively described in grape material, namely (13/13′Z)- or (15/15′Z)-isomers of lutein and β-carotene. Such observation is supported by the small hypsochromic shift (displacement of λ_{max} to shorter wavelength, 4−6 nm) in the λ_{max} compared to (all-*E*)-isomers and by the presence of a strong absorption band in the near UV region (320−380 nm) known as the cis-band or cis-peak (*1*, *22*, *25*). The high intensity of this band indicates the presence of isomers such as 13,13′ and 15,15′, in which the cis double is near the center of the compound (*1*). One compound with an absorption band in 320 nm is reported as (13/13′Z)- or (15/15′Z)-unknown.

Slight differences seem to exist relative to the presence of some xanthophylls within the three grape varieties analyzed. This is the case of compounds unknown 8 NP, (13/13′Z)-or (15/15′Z)-lutein, unknown 9 NP, and unknown 15 NP but also for other more apolar compounds such as unknown 2 NP and unknown 3 NP, which were only present in Touriga Francesa and Tinta Roriz grape samples analyzed. The compound unknown 4 NP was only detected in grape samples of Tinta Barroca (see **Table 1**). However, it should be emphasized that to clarify if these differences were the result of a varietal effect, which was not the purpose of this work, further experiments should be carried out.

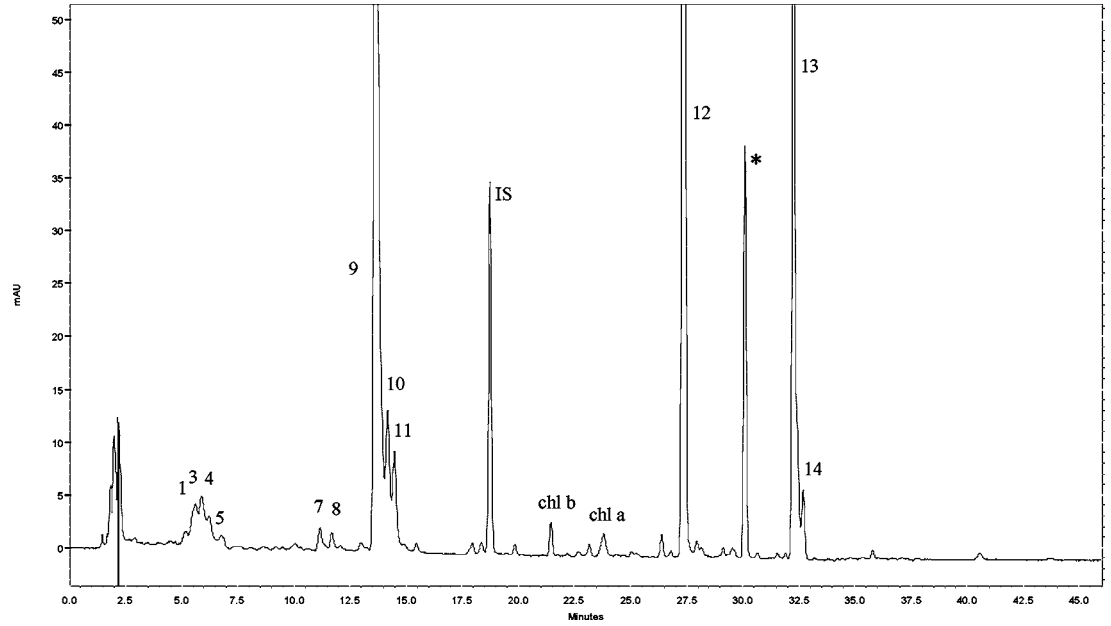

Figure 3. HPLC reversed-phase separation of a grape sample extracted with solvent mixture 50h/50de (h, hexane; de, diethyl ether). Peak identification described in **Table 1**. chl b, chlorophyll b (λ_{max} 435; 458 nm; 2nd D 458); chl a, chlorophyll a (λ_{max} 333; 380; 411; 430 nm; 2nd D 411); *, chlorophyll? (λ_{max} 325; 409; 506; 536 nm; 2nd D 412).

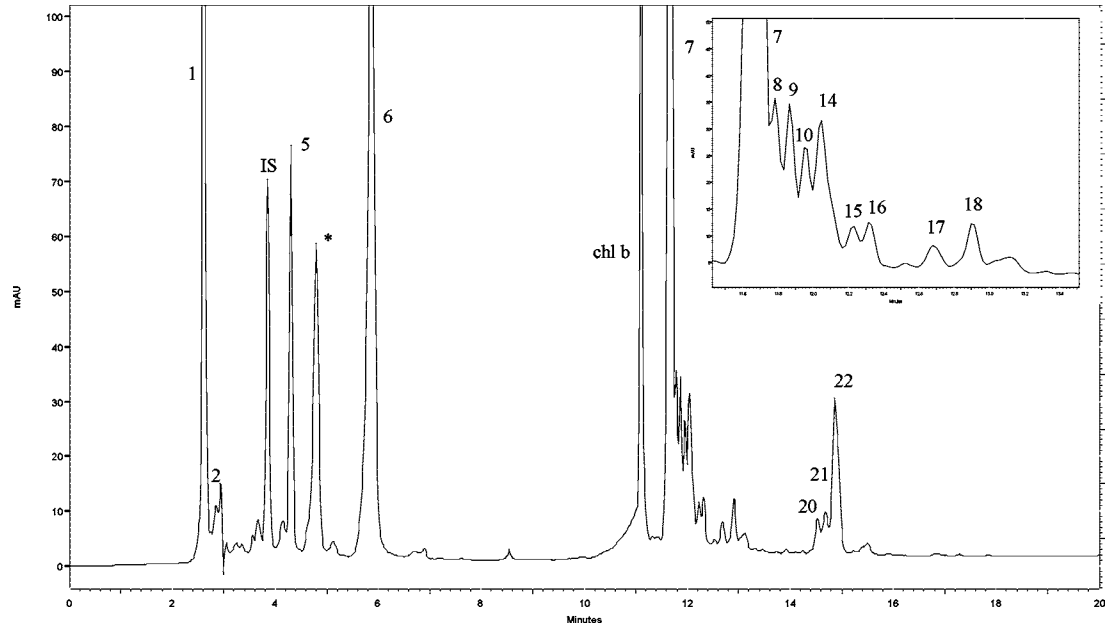

Figure 4. (**a**) HPLC normal-phase separation of grape sample extracted with solvent mixture 50h/50de (h, hexane; de, diethyl ether). Peak identification described in **Table 1**. Upper trace: (**b**) lutein polar fraction. chl b, chlorophyll b (λ_{max} 435; 458 nm; 2nd D 458); *, chlorophyll? (λ_{max} 325; 409; 506; 536 nm; 2nd D 412).

According to the given qualitative analysis, it was possible to detect 28 compounds with probable carotenoid structure in all analyses performed, with 17 still unidentified.

Extractability. Among the solvent mixtures used for carotenoid isolation, it was possible to observe that different polarities resulted in chromatograms with different carotenoid elution profiles, in both stationary phases. **Figures 1** and **2** show the four chromatograms obtained from the different extractions of grape material of Tinta Roriz. ANOVA procedure showed significant differences for carotenoid compounds among the four solvent mixtures used ($p < 0.05$), for both reversed- and normal-phase separations. The same results were obtained with Tinta Barroca and Touriga Francesa. The selection of these mixtures was made according to the results obtained in a preliminary study in which no significant differences were observed in the carotenoid pattern, using more different proportions of hexane/diethyl ether, such as 80/20, 60/40, and 40/60, respectively. As expected, the extraction of β-carotene was improved with high levels of hexane (solvent mixture 100/0), while increasing the amount of diethyl ether (solvent mixture 0/100) the extraction of a higher number of polar compounds (the xanthophylls) was generally achieved.

For the purpose of this work, a mixture of 50/50 hexane/diethyl ether was the best choice, as it extracted both neoxanthin and β-carotene (**Figures 1** and **2**, chromatogram III). These carotenoids are suggested as precursors of the aroma compounds β-damascenone (*19*) and β-ionone and 2,2,6-trimethylcyclohexanone (TCH) (*13*), respectively.

Chromatographic Separations−Comparison between Reversed and Normal Phase. Figure 3 shows the reversed-phase separation of carotenoids in grape sample of Tinta Barroca extracted with 50/50 mixture of hexane/diethyl ether. **Figure 4**, parts **a** and **b** show the normal phase separation of the same extract.

The analysis of both chromatograms shows that compounds were detected differently according to the different chromatographic procedures (see **Table 1**). Under reversed-phase conditions, flavoxanthin and the possible geometrical isomer(s) of β-carotene were detected, while normal-phase conditions allowed the detection of zeaxanthin, as well as a significantly higher number of xanthopylls (**Figure 4b**). Under reversed phase conditions, this group of compounds was coeluted with the large peak of lutein. In addition, separation of both neochromes a and b from neoxanthin under normal phase was enhanced, as can be seen by the given capacity values.

It should be noted that no shift occurred in the absorbance maximums of all identified carotenoids in both HPLC systems.

Carotenoid Stability Study. Although maximum care was taken during sample manipulations, it was important to consider the fact that carotenoid compounds could be exposed to some modification, such as (*E*−*Z*) photoisomerization and/or epoxide-furanoid rearrangements. To determine if these chemical modifications occurred, the spectrum of each standard was compared before and after extraction. As the maxima of absorbance for each carotenoid standard were maintained during the experimental procedures (i.e., before and after the extraction) it can be assumed that there was no interconversion of 5,6- to 5,8-epoxides. If this interconversion had occurred, a hypsochromic shift of approximately 20 nm would be expected. Thus, neither 5,6−5,8-epoxide interconversion happened during isolation, nor (*E*−*Z*) isomerization. Thus, flavoxanthin, a 5,8-epoxy xanthophylls, is naturally occurring in grape material (not being an artifact), which corroborates some previous studies (*11*−*14*) but does not confirm some other studies made on yellow pepper that mentioned the presence of 5,8-epoxy xanthophylls as "post-mortem artifacts" (*32*). Furthermore, this test helped to confirm the identification of carotenoids in the sample by an enhancement of the peak height that corresponded to the compound in analysis with identical spectral characteristics and with the identical capacity factor of that in the standard mixture. Besides, as standards showed the same characteristic spectrum under both chromatographic conditions, normal and reversed stationary phases, it can be assumed that there was no degradation during chromatographic separations.

Conclusions. This study reports a considerably high number of compounds with carotenoid structure on grape material compared to those previously reported in the literature. A total of 28 compounds were found in all extracts of the three cultivars Tinta Barroca, Touriga Francesa, and Tinta Roriz, based on online spectral data obtained by HPLC-DAD. This results from the application of different solvent mixtures with different polarities for carotenoid extraction, which clearly enhanced the range of substances that could be extracted. On the other hand, the use of both normal- and reversed-phase chromatographic conditions for their separation maximize information about the detected compounds. The combined effect of solvent extractability versus reversed and normal stationary phases (HPLC) can be an important analytical tool for carotenoid detection not only on grape material.

To improve the knowledge of carotenoid composition of Port winemaking cultivars, ongoing research is under development. This is crucial to identify several unknown carotenoid compounds and also to relate the presence of grape carotenoids with the aroma of Port wines, as carotenoids are suggested as precursors of aroma compounds. Some of these compounds with high aroma impact were identified in some Ports, such as β-ionone, 2,2,6-trimethylcyclohexanone, and β-damascenone.

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