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Effect of Genotype and Environment on Citrus Juice Carotenoid Content

Claudie Dhuique-Mayer,*,† Anne-Laure Fanciullino,§ Cecile Dubois,†,‡ and Patrick Ollitrault†,‡

†Département PERSYST UMR Qualisud, TAB-95/16 and ‡Département BIOS UPR 75, Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), 34398 Montpellier Cedex 5, France, and §Institut National de Recherche Agronomique (INRA), UR GEQA Site de San Giuliano, F-20230 San Giuliano, France

A selection of orange and mandarin varieties belonging to the same *Citrus* accession and cultivated in Mediterranean (Corsica), subtropical (New Caledonia), and tropical areas (principally Tahiti) were studied to assess the effect of genotype and environmental conditions on citrus juice carotenoid content. Juices from three sweet orange cultivars, that is, Pera, Sanguinelli, and Valencia (*Citrus sinensis* (L.) Osbeck), and two mandarin species (*Citrus deliciosa* Ten and *Citrus clementina* Hort. ex Tan), were analyzed by HPLC using a C_{30} column. Annual carotenoid content variations in Corsican fruits were evaluated. They were found to be very limited compared to variations due to varietal influences. The statistical analysis (PCA, dissimilarity tree) results based on the different carotenoid compounds showed that citrus juice from Corsica had a higher carotenoid content than citrus juices from tropical origins. The tropical citrus juices were clearly differentiated from citrus juices from Corsica, and close correlations were obtained between β-cryptoxanthin and phytoene (r = 0.931) and β-carotene and phytoene (r = 0.918). More broadly, Mediterranean conditions amplified interspecific differentiation, especially by increasing the β-cryptoxanthin and *cis*-violaxanthin content in oranges and β-carotene and phytoene−phytofluene content in mandarins. Thus, at a quantitative level, environmental conditions also had a major role in determining the levels of carotenoids of nutritional interest, such as the main provitamin A carotenoids in citrus juice (β-cryptoxanthin and β-carotene).

KEYWORDS: *Citrus*; genotype; carotenoid content; geographical origin; environmental factor

INTRODUCTION

Carotenoids are essential components of photosynthetic organisms. In pigment−protein complexes, they act as light sensors for photosynthesis but also prevent photo-oxidation induced by excessively strong light intensities. Some of them (9-*cis*-epoxycarotenoids) also serve as precursors of abscisic acid, a key phytohormone in plant growth and development and involved in various stress responses (*1*, *2*). In horticultural crops, they play a major role in fruit, root, or tuber coloration and in nutritional quality. The nutritional value of citrus juice, owing particularly to their carotenoids, is now well established in the prevention and/or protection against major human diseases (*3*−*5*). In addition to their provitamin A activity, carotenoids have several other biological activities, including antioxidant properties, modulation of the immune function, and regulation of cell differentiation and proliferation (*6*, *7*). Citrus carotenoids have also been identified as the main constituents of the nutritional quality in fresh juices (*8*). In addition, the importance of the peel and pulp color, mainly due to carotenoid accumulation in chromoplasts, depends on several factors including genetic factors and environmental conditions.

Citrus juices have complex carotenoid profiles. The major carotenoids reported in orange [*Citrus sinensis* (L.) Osb.] and mandarin (*Citrus deliciosa* Ten) juices were found to be β,β-carotene type xanthophylls, which are mainly responsible for their characteristic color. Indeed, β-cryptoxanthin was reported to be the main precursor of vitamin A in orange and mandarin (*8*). The authors of a broad range of studies on different cultivated *Citrus* species have agreed on the differentiation of mandarins and oranges from other cultivated species [citron (*Citrus medica* L.), lemon [*Citrus limon* (L.) Burm. f.], pummelo [*Citrus maxima* (Burm.) Merr.], or grapefruit (*Citrus paradisi* Macf.)], with the accumulation of both violaxanthin and β-cryptoxanthin noted only in the former species (*9*−*11*). Melendez-Martinez et al. (*12*) clearly established that violaxanthin [mainly (9-*cis*)-violaxanthin], antheraxanthin [mainly (9-*cis*)-antheraxanthin], zeaxanthin, mutatoxanthin, and β-cryptoxanthin are the major carotenoids in ultrafrozen juices from Valencia oranges. However, qualitative and especially quantitative differences were found among various mandarin or orange varieties (*8*, *10*, *13*). According to Goodner et al. (*13*), mandarins, oranges, and their hybrids could be distinguished by their β-cryptoxanthin contents.

*Author to whom correspondence should be addressed [telephone +33(0)467614482; fax +33(0)467614433; e-mail claudie.dhuique-mayer@cirad.fr].

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During fruit ripening, chlorophyll is degraded and carotenoids accumulate in the flavedo (epicarp) and the pulp of citrus fruit (*9*, *14*−*17*). In contrast with the flavedo, chlorophyll degradation and carotenoid biosynthesis in the pulp were found to occur earlier, resulting in a progressive change in pulp color (*17*). It was established that these metabolic changes during citrus fruit maturation are species- and variety-dependent and are sensitive to environmental conditions, especially temperature and light (*9*, *11*, *18*−*22*).

The process of chlorophyll degradation and carotenoid accumulation in citrus fruit was described by Erickson (*19*). Mild day (20 °C) and cold night (5 °C) air temperatures seem to be required to produce well-colored citrus fruit. Air temperatures below 13 °C caused the disappearance of chlorophyll pigments, revealing carotenoids and a bright orange color (*14*, *19*, *22*). Consequently, the peel of citrus fruit produced in tropical or subtropical conditions remains green or poorly colored. In contrast, the alternation of high daytime and cool night temperatures in Mediterranean zones seems to create an optimum environment for chlorophyll degradation and carotenoid synthesis (*8*, *12*, *15*, *20*). Barry et al. (*22*) confirmed that low-temperature treatment (2 °C for 30 min, 4 °C for 6 h, and 20 °C for 72 h) of mature fruit could enhance flavedo coloration and increase carotenoid contents.

However, studies designed to evaluate the effects of environmental conditions on carotenoid biosynthesis and accumulation in citrus fruit pulp are still limited. The influence of environmental conditions on carotenoid accumulation in citrus pulp and juice should be more accurately quantified because of the high nutritional potential. In Valencia oranges, Saunt (*23*) observed that the pulp and juice produced in tropical regions were paler than those of Valencia oranges produced in Mediterranean climates. Mouly et al. (*20*, *21*) differentiated the geographical origins of several processed Valencia orange juices on the basis of carotenoid profiles. Thus, according to Mouly et al. (*20*), pure Valencia juices from oranges grown in Mediterranean regions had a higher total carotenoid content than those from tropical and subtropical regions. In these works, only processed juices of orange varieties were analyzed, and they was different from fresh juices. The difficulty of obtaining fruit produced by *Citrus* varieties from well-identified genotypes and derived from the same germplasm collection could explain why little information is available on the influence of various environmental conditions during the whole fruit maturation on the carotenoid contents of citrus fruit juices.

This study addressed the impact of both environmental and genetic variations on the carotenoid contents of juices from three orange varieties (*C. sinensis* cv. Sanguinelli, Pera, and Valencia), two mandarin varieties (*C. deliciosa* cv. Commune and Hansen), and one clementine variety (*C. clementina*). All of these varieties are of major commercial importance in the citrus industry. We thus collected citrus fruit belonging to the same *Citrus* accessions from the Corsican germplasm collection, which had been cultivated under Mediterranean, subtropical, and tropical climates during the 2003−2006 seasons. Standardized sampling, carotenoid extraction, and analysis were carried out. Taking into account the statistical limits, as the fruit sampling included not only few samples but also fruits sampled in local markets, the aim of this paper was to classify citrus juices using statistical analysis based on carotenoid contents of various *Citrus* species from different geographical origins.

MATERIALS AND METHODS

Citrus Fruits. Different varieties of oranges (*C. sinensis* (L.) Osbeck), mandarins (*Citrus deliciosa* Ten and *Citrus reticulata* Blanco), and their natural hybrid clementine (*Citrus clementina* Hort. ex Tan) were chosen: sweet oranges, cv. Pera; ICVN (International *Citrus* Variety Numbering) 0100399; cv. Valencia ICVN 0100246; cv. Sanguinelli ICVN 0100243; Willow Leaf mandarin ICVN 0100133 (*C. deliciosa* Ten); mandarin Hansen ICVN 0100356 (*Citrus reticulata* Blanco); and SRA 85 Clementine ICVN 010085 (*C. clementina* Hort. ex Tan) in the Tanaka classification. Mediterranean, subtropical, and tropical fruits from the same ICVN genotypes were collected from the INRA-CIRAD collection at the INRA GEQA research unit in Corsica; the Agronomic Institute Neo-Caledonian (IAC) Fruit Research Station at Pocquereux, New Caledonia; the Tahiti collection-Papuakeikaha, Ua Huaka Island, Marquezas Islands. Other citrus fruits from Brazil (Sao Paulo), Costa Rica, and Cuba were obtained from local markets. Citrus fruits, geographical origins, and climatic data are summarized in **Table 1**.

All fruits were harvested during the 2005−2006 seasons and during three seasons for Pera, Sanguinelli, and Valencia oranges from Corsica, 2003−2006. The climatic conditions of Corsica were based on weather observations gathered by the National Meteorological Services (http://france.meteofrance.com/france/climat_france) (*24*)] and are 30-year averages. New Caledonia is situated at the southern limit of the tropical zone and thus benefits from a semitropical climate (*25*). The Marquezas Islands are located in French Polynesia, consisting of 118 islands in the middle of the Pacific Ocean. The climate of the Marquezas Islands is typically humid tropical, with an annual average temperature of 26.4 °C. There are two dry and humid seasons with low temperature variations ranging from 23 °C (minimum) to 31 °C (maximum) (*24*).

Samples (15 fruits/cultivar) were collected from three trees in germplasm plots (Corsica, New Caledonia, Tahiti) or in local markets (other origins). A sample size of 15 fruits per cultivar was chosen on the basis of a previous experiment (*8*). Citrus fruits were harvested at commercial maturity [fruit maturity was estimated using commercial maturity indicators such as soluble solid contents (SSC), titratable acidity (TA), and maturity index (SSC/TA ratio)]. This ratio varies with cultivar and location. However, fruits are marketable when a minimum SSC/TA ratio is attained, and this ratio generally ranges from 7 to 10 for oranges and from 10 to 14 for mandarins (*26*). Fruits were thus collected when the minimum value was attained for this ratio. Fruits were immediately hand-squeezed, and the juice was then filtered through a stainless steel strainer (1 mm mesh). Juices of fruits from the same sample were pooled to obtain an experimental unit. Juices were placed in sealed amber vials (15 mL) under nitrogen and kept frozen (−20 °C) until analyzed. Note that the Pera variety fruits harvested in Corsica in year 2 were atypical fruits with a particularly small size and were thus considered to be not representative. Consequently, the carotenoid content for Pera in year 2 was not included in the variance analysis.

Reagents. Extraction solvents were of RPE grade: hexane, ethanol, and dichloromethane from Carlo-Erba (Val de Reuil, France). Analytic solvents were of HPLC grade: methanol, acetonitrile, and tetrahydrofuran (THF) from Carlo-Erba (Val de Reuil, France); and methyl*tert*-butyl ether (MTBE) from Sigma-Aldrich (Steinheim, Germany). Reagents for analyses were of pure grade: sodium chloride and sodium sulfate from Carlo-Erba (Val de Reuil, France). Standards used were purchased from Extrasynthese (Genay, France): β-carotene, β-cryptoxanthin, zeaxanthin, lutein, and lycopene.

Carotenoid Extraction. Carotenoids were extracted and analyzed according to the methods of Dhuique-Mayer et al. (*8*). Briefly, 20 g of orange juice was homogenized by magnetic stirring for 5 min with 120 mg of $MgCO_3$ and 35 mL of extraction solvent (ethanol/hexane, 4:3 v/v, containing 0.1% of BHT as antioxidant). Lycopene (750 μL of solution equivalent to 90 μg) was added as internal standard. The residue was separated from the liquid phase by filtration through a filter funnel (porosity 2) and re-extracted with 35 mL of ethanol/hexane, as previously described (*8*). Ethanol (30 mL) and hexane (30 mL) were successively used to wash the residue. Organic phases were transferred to a separation funnel and washed with 2 × 50 mL of 10% sodium chloride and 3 × 50 mL of distilled water. The aqueous layer was removed.

Saponification. The hexanic extract was evaporated to dryness with a rotary evaporator, redissolved with 20 mL of hexane, and placed in a 50 mL amber vial to which 20 mL of 10% methanolic KOH was added. Saponification was carried out overnight in the dark at room temperature. The sample was shaken under nitrogen in the sealed vial. The sample was then transferred to a separation funnel to which 50 mL of distilled water was added to separate the layers. The hexanic layer was rinsed until free

Table 1. Citrus Species, Geographical Origins, and Climatic Data

	geographical origin					
Citrus	Corsica	New Caledonia	Tahiti	Brazil	Cuba	Costa Rica
geographical situation[a]	I 42° 18′ N L 9° 29′ E	I 22° 17′ S L 166° 27′ E	I 9° 48′ S L 139° 2′ W	I 23° 30′ S L 46° 37′ W	I 21° 30′ N L 80° 8′ W	I 9° 56′ N L 84° 5′ W
temperature (°C)[b]	11 < 16 < 21	20−26	23−31	15 < 20 < 35	25−28	17−30
rainfall (mm)[c]	840	1070	1420	1390	1120	1800
sweet oranges						
Pera	X X X			X		
Sanguinelli	X X X X X X	X				
Valencia late	X	X	X		X	X
mandarins						
Clementine	X	X				
Willow Leaf	X	X				
Hansen	X	X	X			

[a] I, latitude; L, longitude. [b] < Average < or min−max. [c] Average mm per year.

Table 2. Chromatographic and Spectral Data Obtained by HPLC-DAD Detection of Carotenoids Analyzed in Citrus Juices

				λ_{max} (nm) observed					
no.	RT (min)[a]	tentative identification		peak I	peak II	peak III	% III/II	% D_B/DII[c]	ref[d]
1	21.37	9-*cis*-violaxanthin	cis326	412	436	464	81	0.109	*32*, *33*
2	22.08	lutein[b]		422	444	472	48		
4	23.65	zeaxanthin[b]		426	450	476	17		
5	27.81	phytoene		276	286	298			*27*
6	29.95	β-cryptoxanthin[b]		427	450	477	20		
7	29.72	phytofluene		331	348	368	68		*27*
8	37.14	β-carotene[b]			452	477	12		

[a] RT, retention time. [b] identified using authentic standards. [c] Ratio of the absorbance of the *cis* peak in the UV/vis spectrum to that of the second absorption band in the visible region. [d] References confirming identification.

of alkali. The methanolic KOH layer was extracted with 3 × 15 mL of dichloromethane. The extracts were pooled and washed to remove alkali. Aqueous traces from organic extracts were removed with anhydrous sodium sulfate; then the extracts were filtered and evaporated to dryness under vacuum. Carotenoid extracts were dissolved as described above. The analyses were conducted under red light to avoid carotenoid degradation during extraction and saponification. The coefficient of variation for extraction−saponification was < 5%. The hexanic phase was dried using anhydrous sodium sulfate and filtered before evaporation to dryness under vacuum at 40 °C. Carotenoid extracts were dissolved in 500 μL of dichloromethane and 500 μL of a MTBE/methanol 80:20 mixture. This solution was diluted 6-fold in MTBE/methanol (80:20; v/v) and stored in an amber vial before HPLC analysis.

HPLC Analysis of Carotenoids. Carotenoids were analyzed by reverse-phase high-performance liquid chromatography using an Agilent 1100 system (Massy, France) Carotenoids were separated along a C_{30} column (250 × 4.6 mm i.d., 5 μm YMC (EUROP GMBH, Germany), and the mobile phases were H_2O as eluent A, methanol as eluent B, and MTBE as eluent C. The flow rate was set at 1 mL min^{-1}, the column temperature was set at 25 °C, and the injection volume was 20 μL. A gradient program was performed: the initial condition was 40% A/60% B; 0−5 min, 20% A/80% B; 5−10 min, 4% A/81% B/15% C; 10−60 min, 4% A/11% B/85% C; 60−71 min, 100% B; 71−72 min, back to the initial condition for re-equilibration. Absorbance was monitored at 290, 350, 450, and 470 nm using an Agilent 1100 photodiode array detector. Chromatographic data and UV−visible spectra were collected, stored, and integrated using Agilent ChemStation Plus software.

Identification was carried out by HPLC-DAD through the combined use of the retention time, UV−visible spectral data, and co-injection with authentic standards. In addition, the spectral fine structure value III/II and the ratio (D_B/DII) of the absorbance of the cis peak in the UV−vis spectrum to that of the second absorption band in the visible region were compared with those reported in the literature to confirm identification of the geometrical isomer of violaxanthin (see **Table 2**). Some chemical structures among the carotenoids identified are illustrated in **Figure 1**.

Quantification of carotenoids was achieved using calibration curves with β-carotene, β-cryptoxanthin, lutein, and lycopene at five concentration levels. The correlation coefficients ranged from 0.994 to 0.998. Recoveries were completed by the addition of internal standard (lycopene) before extraction of each sample analyzed and used to correct the carotenoid level after HPLC analysis. The mean recovery was 84% for β-cryptoxanthin.

Preparation of Standards. Concentrations of external standard solutions were determined using a molar extinction coefficient (ε_{mol}) in appropriate solvent checked by spectrophotometry according to the method of Britton et al. (*27*). Internal standards were diluted in dichloromethane.

Statistical Analyses. Seven types of carotenoids were chosen for their nutritional relevance and their weight in the different carotenoid patterns. The data analyzed were means of four determinations performed for each experimental unit of these carotenoid concentrations (mg/L) (**Tables 3** and **5**). To evaluate the significance of variety and year effects, a two-way analysis of variance was performed without replicates, and then years were considered as a block factor and interaction as a random effect (GML procedure of SAS 9.1 for Windows, SAS Institute Inc., Cary, NC). To classify Valencia orange juices as a function of their geographical origins, a dissimilarity analysis using a tree representation (weighted neighbor-joining tree construction method on a Euclidean distance matrix) was performed with @DARwin 5.0 software (Montpellier, France) (*28*). Normalized principal component analysis (PCA) findings were used to visualize the carotenoid correlations and compare the sample contents (XLSTAT-Pro 7.0 Addinsoft, France).

RESULTS

Annual Carotenoid Content Variations in Juices from Three Sweet Orange Cultivars. To investigate the variety versus annual effects on carotenoid contents, Pera, Valencia, and Sanguinelli *Citrus* varieties were analyzed during three seasons. The results are reported in **Table 3**. The main carotenoids were identified by

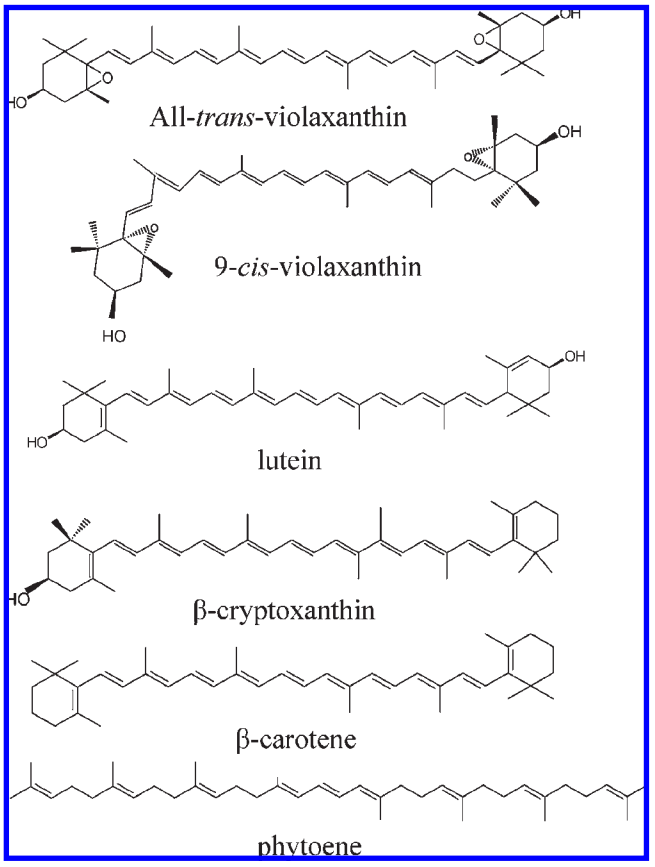

Figure 1. Some chemical structures of the carotenoids analyzed.

comparison with authentic standards, online spectral data, and chromatographic retention patterns and were quantified as discussed in previous studies (*6*, *19*). In the carotenoids analyzed, the main nutritional compounds were β-cryptoxanthin, lutein, zeaxanthin, and β-carotene. *cis*-Violaxanthin was the major compound in these sweet oranges, with concentrations ranging from 4.48 ± 0.23 to 7.23 ± 0.22 mg L^{-1} followed by β-cryptoxanthin, with contents ranging from 1.67 ± 0.06 to 4.18 ± 0.05 mg L^{-1}. The *cis*-violaxanthin/β-cryptoxanthin ratio was over 1 (1.17−3.12) regardless of the variety and season. Phytoene and phytofluene are two colorless carotenoids, which absorb at 290 and 350 nm, and they are produced in the two first steps of the carotenoid biosynthetic pathway. Statistical analyses (**Table 4**) revealed significantly higher phytofluene and β-cryptoxanthin contents in Sanguinelli than in Valencia oranges. The Pera cultivar showed intermediate contents of these carotenoids. A significant difference between varieties was also revealed for *cis*-violaxanthin and, to a lesser extent, for phytoene. For all carotenoids showing significant variability, except for lutein, varietal effects were more marked than annual effects. This was particularly true for β-cryptoxanthin, the major pro-vitamin A carotenoid. A lower level of significance for the year factor indicated that the between-year variability was less than that between varieties. The genetic variability among sweet orange cultivars was associated with significant variations in the contents of four carotenoids (*cis*-violaxanthin, β-cryptoxanthin, and phytoene−phytofluene), regardless of the season under Mediterranean conditions.

Variations in Valencia Oranges from Five Geographical Origins. In this paragraph, we focus on the impact of environmental conditions on carotenoids from orange juices by assessing oranges produced under highly contrasting conditions, that is, comparing Valencia oranges grown in the Mediterranean area (Corsica) with Valencia oranges grown in subtropical (New Caledonia) and tropical areas (Tahiti, Costa Rica, and Cuba). The tree (**Figure 2**) was drawn up with the contents of seven carotenoids (β-cryptoxanthin, lutein, *cis*-violaxanthin, β-carotene, zeaxanthin, phytofluene, and phytoene). This representation underscored that the quantitative compositions were more geographical origin-dependent than annual variation-dependent. Moreover, the subtropical origin (New Caledonia) is located in an intermediary position between the tropical and Mediterranean origins. **Table 5** shows the quantitative carotenoid compositions of the Valencia juices from the five origins. We found that the total carotenoid contents (calculated as the sum of the seven carotenoid contents) were lower in fruits from semitropical (New Caledonia) and tropical areas (Costa Rica, Tahiti, and Cuba) when compared to oranges from the Mediterranean area. There were 1.5- and 2.3-fold decreases in total carotenoid contents for Valencia oranges from New Caledonia and Costa Rica, respectively, reaching 4.7- and 9.7-fold for Valencia oranges from Tahiti and Cuba, respectively. For β-cryptoxanthin, that is, the main provitamin A carotenoid, the highest amount was found in Valencia oranges from Corsica, with 2.15 mg L^{-1} followed by Valencia oranges from New Caledonia (0.92 mg L^{-1}), Costa Rica (0.56 mg L^{-1}), Tahiti (0.11 mg L^{-1}), and Cuba (0.10 mg L^{-1}). Consequently, the vitamin A level was substantially lower in oranges from subtropical and tropical areas (see **Table 5**). It is interesting to note that *cis*-violaxanthin was still the most abundant carotenoid in all juices analyzed.

Influence of Subtropical and Tropical versus Mediterranean Conditions on Several Sweet Orange, Clementine, and Mandarin Cultivars. PCA was used to analyze correlations between the concentrations of seven carotenoids from three sweet oranges grown in six different areas (principally Corsica and New Caledonia with those from four other additional origins, Tahiti, Costa Rica, Cuba, and Brazil). The results are reported in **Figure 3**. Sweet oranges seemed to be separated into two groups according to labels on the graph. The first axis takes 79% of the data matrix total inertia into account. Mediterranean sweet oranges, with high carotenoid contents, are clearly differentiated from those of all other origins. Indeed, the correlation circles highlight that the Mediterranean sweet orange group, which is on the right of the graph, differed from oranges from all other countries by the higher carotenoid contents. All carotenoids were positively correlated, but particularly close correlations were obtained between β-cryptoxanthin, phytofluene, and phytoene ($r = 95$). A second PCA was performed to highlight the origin differentiation by adding three other varieties, namely, Willow leaf and Hansen mandarins (*C. deliciosa*) and clementine (*C. clementina*). The results of this analysis are shown in **Figure 4**. The correlation circles revealed two correlated vector groups. The first factorial axis (horizontal) could be interpreted as including the species with the highest levels of provitamin A carotenoids (β-carotene and β-cryptoxanthin) and precursors, whereas the second axis (vertical) represented species with the highest xanthophyll contents (violaxanthin, lutein, and zeaxanthin). Samples are ordered along the first axis according to species, but also according to origins. First, the mandarin group shows generally higher levels of provitamin A carotenoids and precursors than the orange group. Interestingly, we noted the intermediate position of clementine on this axis, corresponding to the hybrid status between mandarin and sweet orange. Second, within each species, juices from Corsica had higher contents than their tropical homologues. This origin effect on this first group of carotenoids was clearly more marked for mandarins than for oranges and clementines. On the other hand, the second axis reveals a sensitivity of oranges toward origin on xanthophyll contents

Table 3. Carotenoid Content (Milligrams per Liter)[a] in Three Varieties of Corsican Orange Juices over 3 Years

orange variety	phytoene	phytofluene	β-carotene	β-cryptoxanthin	zeaxanthin	*cis*-violaxanthin	lutein
Sanguinelli 1	0.85	0.95	0.38	3.98	2.02	6.98	1.63
SD	0.08	0.13	0.03	0.07	0.07	0.60	0.14
Sanguinelli 2	0.97	1.05	0.54	4.18	1.85	4.89	1.44
SD	0.03	0.07	0.02	0.05	0.05	0.90	0.08
Sanguinelli 3	0.99	1.03	0.38	4.15	1.70	7.23	2.30
SD	0.01	0.06	0.02	0.05	0.02	0.22	0.10
Pera 1	0.68	0.59	0.45	3.67	2.43	4.67	0.87
SD	0.06	0.05	0.04	0.28	0.23	0.33	0.10
Pera 2	0.51	0.48	0.36	2.20	1.73	6.06	1.32
SD	0.06	0.33	0.03	0.15	0.15	0.91	0.19
Pera 3	0.97	0.75	0.69	3.63	3.12	5.04	2.43
SD	0.07	0.04	0.07	0.15	0.150	0.24	0.07
Valencia 1	0.33	0.35	0.30	1.67	1.99	5.18	1.09
SD	0.03	0.01	0.03	0.06	0.15	0.62	0.11
Valencia 2	0.76	0.48	0.53	2.47	1.59	4.48	1.07
SD	0.02	0.03	0.03	0.05	0.11	0.23	0.15
Valencia 3	0.36	0.55	0.49	2.16	1.73	5.39	1.94
SD	0.02	0.03	0.09	0.03	0.05	0.38	0.15

[a] Values are means of four independent determinations. SD, standard deviation; 1, 2, 3 represent years 1, 2, and 3.

Table 4. Intervarietal Differentiation for Carotenoid Contents in Sweet Orange Juice[a]

	phytoene	phytofluene	β-carotene	β-cryptoxanthin	zeaxanthin	*cis*-violaxanthin	lutein
means	0.74	0.72	0.47	3.24	2.05	5.48	1.60
residual CV%	19	5.2	16	6.6	16	8.6	17
test variety[b]	0.0539 +	0.0009 ***	0.1384 NS	0.0029 **	0.1123 NS	0.0393 *	>0.2 NS
variety means[c]							
1 = Sanguin	0.94	1.01 a	0.43	4.10 a	1.86	6.37 a	1.79
2 = Pera	0.90	0.69 b	0.62	3.76 a	2.73	4.36 b	1.49
3 = Valencia	0.48	0.46 c	0.44	2.10 b	1.77	5.02 ab	1.37
test for year [b]	>0.2 NS	0.0380 *	0.1072 NS	>0.2 NS	>0.8 NS	0.0734 +	0.0342 *

[a] Statistical results of a two-way analysis of variance (ANOVA) without interaction (from data in **Table 2**). [b] *F*-test probability: NS, not significant at the 10% level; +, *, **, ***, respectively significant at the 10, 5, 1, and 1‰ levels. [c] Tukey−Kramer multiple-comparison procedure at the 5% level: means are predicted values for a balanced design.

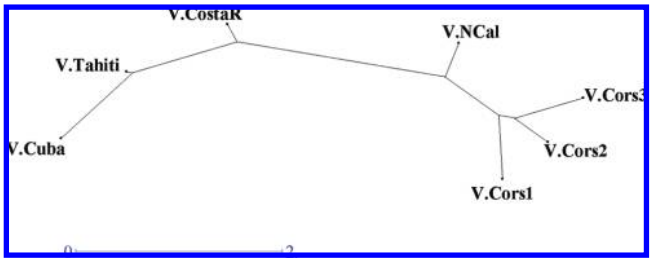

Figure 2. Classification of Valencia orange juices from five different origins. The tree was constructed according to the neighbor-joining method using Euclidean distances (VCors1, Valencia Corsica year 1; VCors2, Valencia Corsica year 2; VCors3, Valencia Corsica year 3; V.NCal:, Valencia New Caledonia; V.CostaR, Valencia Costa Rica; V.Tahiti, ValenciaTahiti; VCuba, Valencia Cuba).

(higher in Corsica samples), whereas variations within the mandarin−clementine group for this second group of carotenoids appeared to be linked to various cultivars (Mand Hansen > Mand commune > Clem). Likewise, we noted that the difference between Hansen mandarin harvested from Corsica and New Caledonia/Tahiti was due to the β-carotene content and not the β-cryptoxanthin content (see **Table 5**). To complete these PCAs and highlight the differences between mandarins and oranges, he graph in **Figure 5** shows the interspecific variation through a comparison of two geographical origins (New Caledonia/Corsica). **Figure 5A** shows that the orange carotenoid profiles varied as a function of the two principal carotenoids (β-cryptoxanthin and*cis*-violaxanthin) with respect to origins (New Caledonia, Corsica). The carotenoid contents of sweet oranges from New Caledonia were about 4.5-fold lower in β-cryptoxanthin and 2-fold lower in *cis*-violaxanthin than in sweet oranges from Corsica. Concerning the mandarin/clementine carotenoid profiles (**Figure 5B**), the difference between the two origins (New Caledonia versus Corsica) could be explained by the contents of three carotenoids (β-carotene, phytoene, and phytofluene). With regard to vitamin A, the mandarin/clementine group appeared to be the best *Citrus* species, regardless of the geographical origin. The sweet orange Pera and Sanguinelli varieties from Corsica displayed the highest retinol activity equivalent (RAE) contents.

Table 5. Carotenoid Contents (Milligrams per Liter)[a] in Juices of the Same *Citrus* Varieties from Different Geographical Origins

orange variety[b]	phytoene	phytofluene	β-carotene	β-cryptoxanthin	zeaxanthin	*cis* -violaxanthin	lutein	vitamin A[c]
Valencia CO	0.36	0.55	0.48	2.16	1.73	5.39	1.9	130
SD	0.02	0.04	0.06	0.02	0.05	0.1	0.15	
Valencia NC	0.1	0.1	0.14	0.92	1.17	4.6	1.3	50
SD	0.01	0.01	0.01	0.03	0.04	0.04	0.06	
Valencia CR	0.05	0.01	0.19	0.56	1.25	2.04	1.34	39
SD	0.005	0.002	0.04	0.02	0.09	0.12	0.05	
Valencia TA	0	0	0.08	0.11	0.41	1.46	0.62	11
SD	0	0	0.01	0.01	0.01	0.03	0.02	
Valencia CU	0.04	0	0.12	0.1	0.22	0.56	0.25	10
SD	0.003	0	0.05	0.01	0.02	0.01	0.01	
Sanguin CO	1	1.1	0.37	4.18	1.71	7.2	2.3	205
SD	0.06	0.1	0.02	0.01	0.07	0.5	0.2	
Sanguin NC	0.12	0.15	0.1	0.47	0.89	1.3	1.75	28
SD	0.01	0.01	0.02	0.01	0.07	0.15	0.08	
Pera CO	0.5	0.75	0.68	3.8	3.1	5.05	2.4	214
SD	0.07	0.03	0.08	0.06	0.1	0.24	0.07	
Pera B	0.1	0.06	0.26	0.46	1.09	3	1.34	41
SD	0.02	0.01	0.04	0.03	0.08	0.2	0.08	
Mand CO	2.3	3.01	1.98	14	1.06	2.67	0.91	748
SD	0.1	0.22	0.02	1	0.06	0.06	0.04	
Mand NC	0.09	0.74	0.4	10.51	1.1	1.79	1.15	471
SD	0.005	0.01	0.03	0.2	0.09	0.36	0.01	
Mand H CO	4.46	5	1.97	15	1.28	4.67	1.63	789
SD	0.1	0.1	0.02	0.4	0.02	0.1	0.19	
Mand H NC	1	1.1	0.7	16.2	1.43	4.45	2.3	733
SD	0.03	±0.1	0.03	0.54	0.14	0.15	0.2	
Mand H TA	0.24	0.92	0.76	9.16	1.31	5.6	2.1	445
SD	0.006	0.02	0.03	0.19	0.02	0.01	0.03	
Clem CO	0.65	1.22	0.68	8.49	0.63	2.06	0.61	410
SD	0.03	0.04	0.01	0.14	0.02	0.07	0.02	
Clem NC	0.11	0.62	0.17	8.93	1.06	2.15	0.8	386
SD	0.01	0.01	0.01	0.2	0.06	0.2	0.02	

[a] Values are means of four independent determinations. SD, standard deviation. [b] Origins: CO, Corsica; NC, New Caledonia; TA, Tahiti; CR, Costa Rica; CU, Cuba; B, Brazil. Varieties: Sanguin, Sanguinelli; Mand, commune mandarin; Mand H, mandarin Hansen; Clem, clementine. [c] Expressed as retinol activity equivalent L^{-1} (conversion factor 1 μg RAE = 12 μg of β-carotene + 24 μg of β-cryptoxanthin).

DISCUSSION

This study was carried out to determine how environmental conditions affected carotenoid compositions in citrus fruit. We focused on juices of oranges (*C. sinensis*), mandarins (*C. deliciosa*), and clementine (*C. clementina*). The qualitative and quantitative carotenoid compositions of six *Citrus* varieties from six different areas and during three seasons were analyzed by HPLC. The effects of both low variations (several seasons for one area) and high variations (Mediterranean, subtropical, and tropical areas) in environmental conditions on carotenoid contents were characterized.

Influence of Environmental Conditions on Carotenoid Contents Was Variable with Species and Was Particularly High for Sweet Oranges. In this work, we analyzed the responses of six *Citrus* varieties to changes in environmental conditions. As the effects of annual environmental changes on carotenoid contents in three sweet oranges were limited, we investigated more contrasting conditions. Three sweet oranges were also harvested in subtropical (New Caledonia) and tropical (Brazil, Costa Rica, Cuba, and Tahiti) areas. Subtropical and tropical climatic conditions were also found to have marked effects on carotenoid contents. All sweet oranges had lower total carotenoid contents in both of these former conditions as compared to oranges grown in a Mediterranean environment. For Valencia oranges, almost a 10-fold decrease in total carotenoid content was noted in oranges of Cuban origin. The results of Mouly et al. (*20*) had already revealed a marked reduction in total carotenoid contents for Valencia juices of tropical origin. In addition, our results showed that the intercultivar variations observed in Mediterranean conditions differed in the two other areas. For example, contrary to

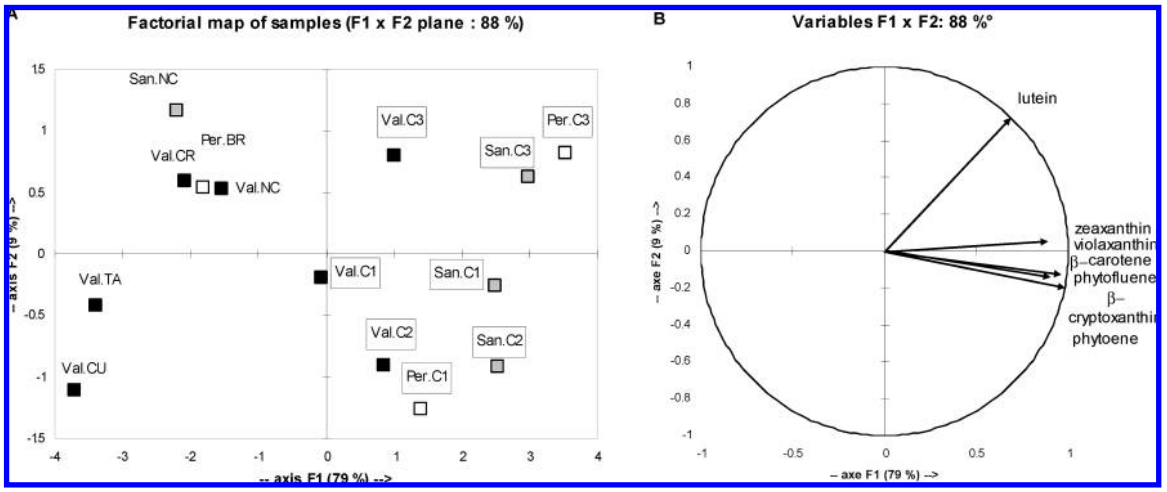

Figure 3. Orange juice origin differentiation according to PCA of carotenoid content: (**A**) differentiation of geographical origins (surrounded labels are drawn to highlight Mediterranean origins); (**B**) correlation circle based on seven carotenoids as variables. Per, Pera oranges; San, Sanguinelli oranges; Val, Valencia oranges; BR, Brazil; C1, Corsica year 1; C2, Corsica year 2; C3, Corsica year 3; CR, Costa Rica; CU, Cuba; NC, New Caledonia; TA, Tahiti.

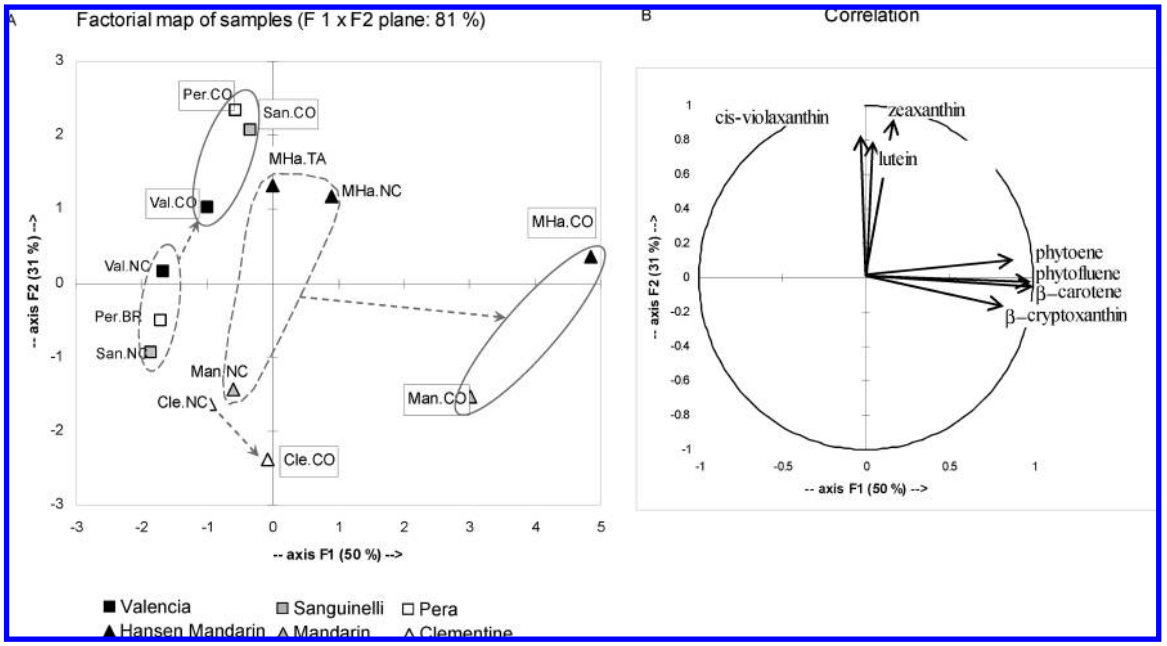

Figure 4. Orange and mandarin juice origin differentiation according to PCA of carotenoid content: (**A**) differentiation of geographical origins; (**B**) correlation circle based on seven carotenoids as variables. CO, Corsica; TA, Tahiti; NC, New Caledonia; BR, Brazil; Val, Valencia oranges; San, Sanguinelli oranges; Per, Pera oranges; Man, mandarin; MHa, mandarin Hansen; Cle, clementine.

what occurred in Mediterranean conditions, under the New Caledonian climate, Valencia juices concentrated higher amounts of β-cryptoxanthin and *cis*-violaxanthin than juices from Sanguinelli oranges. However, when Mediterranean versus tropical/subtropical production areas were compared, for sweet orange juices the environmental factor was stronger than the varietal factor with respect to determining the carotenoid and total vitamin A contents. Analyses of mandarins and clementines revealed that the effect of environmental conditions was dependent on the *Citrus* species. Indeed, for these species, the reduction in total carotenoid contents was less marked in subtropical and tropical areas and only involved carotenes (phytoene, phytofluene, and β-carotene). Moreover, Star Ruby grapefruit had higher total carotenoid contents and increased lycopene contents when grown in subtropical and tropical areas than Mediterranean areas, thus boosting the red color of its flesh. For example, New Caledonian Star Ruby had a higher lycopene content (17 mg L^{-1}) than Corsican Star Ruby (10 mg L^{-1}; our unpublished data). Our results suggested that there are strong interactions between *Citrus* species and environmental conditions for carotenoid production. It seems that Mediterranean conditions enhance β-cryptoxanthin and *cis*-violaxanthin production and accumulation in sweet oranges and that of phytoene, phytofluene, and β-carotene in mandarins and clementines. For these varietal groups, there was a higher interspecific differentiation with respect to carotenoid contents under Mediterranean conditions than under tropical/subtropical conditions. Variations in temperature, humidity, and light intensity were likely involved. These factors, their interaction, and effects on carotenoid biosynthetic pathway regulation should be more accurately quantified.

Effects of Environmental Conditions and Regulation of the Carotenoid Biosynthetic Pathway. We focused on quantifying the effects of environmental conditions and detecting potential relationships between changes in environmental conditions and

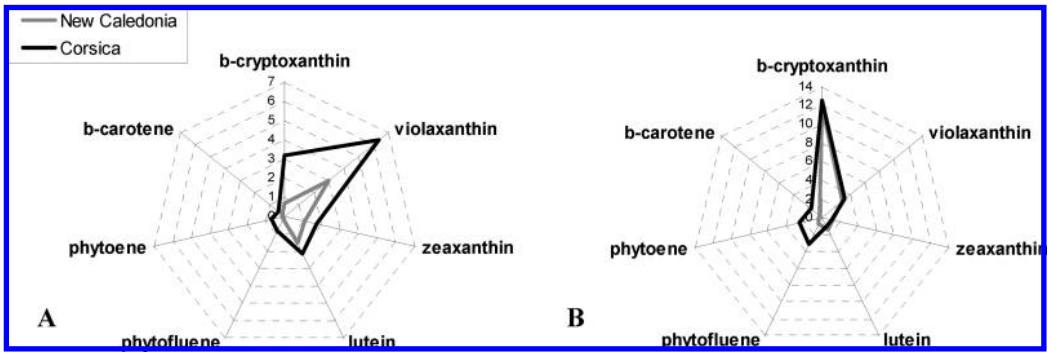

Figure 5. "Radar" chart showing variations in carotenoid profiles of orange (**A**) and mandarin (**B**) with respect to origins (New Caledonia, Corsica 2005).

regulation of specific steps in the carotenoid biosynthetic pathway. As already emphasized, Mediterranean conditions seemed to enhance β-cryptoxanthin and *cis*-violaxanthin production and accumulation in the juice sacs of sweet oranges. We also noted that although the levels of these two compounds were markedly lower under subtropical and tropical climates, *cis*-violaxanthin was still the major carotenoid in juices of all sweet oranges (except for Sanguinelli from New Caledonia for which lutein was the major compound, followed by *cis*-violaxanthin). These results suggest that carotene production and flux into the carotenoid biosynthetic pathway may be limited under tropical conditions. The analyses of carotenoids from mandarin and clementine juices seemed to confirm this hypothesis. Environmental conditions seemed to influence the first step of the carotenoid biosynthesis catalyzed by phytoene synthase (PSY) or the synthesis of carotenoid precursors (via the methylerythritol phosphate pathway). The same regulation mechanism was suggested by Gautier et al. in tomato (*29*). Indeed, these authors found that increasing the temperature from 21 to 26 °C reduced the total carotene content but not the lycopene content, which seems to imply that the first step of the carotenoid biosynthetic pathway is down-regulated. The effect of environmental conditions on citrus fruit pulp is poorly documented. Most studies concern the flavedo color break. Rodrigo et al. (*15*) showed that up-regulation of *Psy* genes at the onset of fruit flavedo coloration enhanced the production of linear carotenes and flux into the pathway, thus confirming the role of *Psy* in the global regulation of carotenoid production. It was demonstrated that environmental conditions, particularly low temperatures, are essential in the citrus peel color change (*19*). In the flavedo of oranges, the findings of Alos et al. (*14*) suggested that, during natural ripening, the flux through the carotenoid biosynthetic pathway increased, whereas the metabolic flux leading to chlorophyll synthesis decreased. The promotion of flavedo coloration by water stress was also described in relation with the higher regulation of NCED genes that code for 9-*cis*-epoxycarotenoid dioxygenase, which catalyzes a limiting step in abscisic acid biosynthesis (*2*). It was also shown that NCED expression was correlated with the regulation of carotenoid accumulation in citrus fruit pulp (*30*). However, flavedo and juice sac sections may present differences in carotenoid biosynthetic pathway regulation mechanisms—chlorophyll degradation and carotenoid biosynthesis occur earlier in pulp than in peel (*17*)—and Oberholster et al. (*31*) suggested that cold stress may be the stimulus for the synthesis of β-citraurin, a peel-specific apocarotenoid, and a degradation product of zeaxanthin or β-cryptoxanthin.

These two regulatory steps (*14*, *18*), that is, DXS (step of the methylerythritol phosphate pathway) and PSY (first step of the carotenoid biosynthetic pathway), may have an important role in the modulation of carotenoid production in both flavedo and juice sacs in relation with variations in environmental conditions. Further analyses are needed to understand the process by which changes in environmental conditions could modify the regulation of these steps in citrus juice sacs and their potential relationship with NCED expression.

In conclusion, our results revealed significant differences in the carotenoid contents of citrus juices from fruits grown in Mediterranean, subtropical, and tropical countries. Using multivariate statistical analyses (PCA), we demonstrated that Mediterranean conditions amplify interspecific differentiation, particularly by increasing β-cryptoxanthin and *cis*-violaxanthin contents in oranges and β-carotene and phytoene−phytofluene in mandarins. Our results underscored the importance of environmental factors in carotenoid synthesis and accumulation in citrus fruits. Moreover, for the first time to our knowledge, our results highlighted that interactions between *Citrus* accessions and environmental conditions may occur with respect to carotenoid biosynthesis in juice sacs. Further analyses are needed to understand how these interactions could control carotenoid biosynthetic pathway regulation mechanisms. Further studies must be conducted to complete the quantification of variations in carotenoid contents of citrus juices from different geographical origins, particularly from Mediterranean production areas such as Spain, Morocco, and Tunisia relative to juices from tropical production areas (Florida and Brazil). The nutritional quality of citrus fruits and juices is a major issue for growers and needs to be taken into account, whereas accurate acquired information on what determines the variability will pave the way for targeted genetic improvements tailored to different production zones.

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