Carotenoids and colour diversity of traditional and emerging Tunisian orange cultivars (*Citrus sinensis* (L.) Osbeck)

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Abstract

Citrus are among the most cultivated fruit crops worldwide and occupy a paramount importance in the Tunisian agricultural sector being one of the strategic products. Despite of that, little is known about the quality parameters of Tunisian sweet oranges and some cultivars have not been characterized yet. This work aims to study the coloration diversity of twenty-five Tunisian grown oranges by analyzing their total and individual carotenoids content and the relationship with CIELab color coordinates. Experimental results proved large diversity in the carotenoid composition of the studied juices. Maltaise demi-sanguine cultivar was distinguished from the rest due to its high content of ζ -carotene, lutein and total carotenoids. An unusual lycopene accumulation was detected in the juice of Meski Ahmer. The Tunisian grown Washington Navel cultivar ranked within the rich-carotenoids juices. In addition, significant negative correlations were found between lightness parameters (L^*) and Hue angle (H) while they were positively related with redness attributes (a^*). Furthermore, (all-*E*)-violaxanthin and ζ -carotene were both significantly correlated to yellow color intensity index (b^*) while zeaxanthin and lutein were positively related to Chroma (C^*) and (b^*) indices. These findings are of interest since the carotenoids content and composition are directly related to color and citrus juice quality. The results obtained could be part in a selection program-guide of Tunisian cultivars and help to understand the physiological and biochemical bases of sweet orange coloration.

Keywords: *Citrus*, diversity, color attributes, oranges, carotenoids content.

1. Introduction

Fruits from the genus *Citrus* (Rutaceae family) represent one of the main crops in the world with 120 million tons produced in 2014 (FAO STAT, 2016). *Citrus* is a large genus and includes several major cultivated species (Xu et al., 2013; Wu et al., 2014). Sweet orange is the most widely grown and consumed *citrus* type, its production reached 68 million tons on 2014 (FAO STAT, 2016). Sweet oranges can be classified in four groups: blond or common oranges, acidless oranges, blood oranges, and Navel oranges (Swingle and Reece, 1967; Hogdson, 1967; Ladaniya, 2008). The global fruit juice industry is dominated by *Citrus* juice as it retains the physicochemical and pleasant organoleptic features of the orange in addition to its convenient packaging and long shelf life compared to other fresh fruits (Aschoff et al., 2015). Orange juice (OJ) is also believed to be an easy way to promote the consumption of bioactive compounds by consumers whose concern about health has greatly increased and whose demands have been oriented to healthier diets. Many studies underlined the balance of vitamins, minerals, sugars and antioxidants OJ gives (Girones-Vilaplana et al., 2014; Rampersaud and Valim, 2015; Stinco et al., 2015). Lately, the interest in carotenoids has been increased since are recognized as health-related and nutritionally important compounds (Fanciullino et al., 2006; Melendez-Martinez et al., 2007; Melendez-Martinez et al., 2008). Carotenoids are isoprenoid derived pigments responsible for the characteristic yellowish and orange color of navel, blond and acidless oranges (Gross, 1987), while the purple-reddish tint of blood oranges is due to the accumulation of anthocyanins (Barrett & Rhodes, 1976; Maccarone et al., 1983; Lo Piero, 2005). Interestingly, a few number of spontaneous sweet orange mutants have been described to develop red pulp, similarly to red grapefruit, due to the unusual accumulation of the carotene lycopene (Liu et al., 2007; Alquézar et al., 2008). Besides the provitamin A activity of specific carotenoids, their intake has been related to numerous health benefits (Takayanagi et al., 2011; Yamaguchi, 2012). Furthermore, carotenoids are believed to have an enormous influence on both external and internal appearance of *Citrus* fruit and consumer acceptance. OJ is believed to have a complex pattern of carotenoids because of the isomerization and epoxy groups generated by the juice acidity and processing

treatments (Melendez et al., 2008). The main carotenoid in ordinary OJ is the (9Z)-violaxanthin isomer. Other compounds such as antheraxanthin, zeaxanthin, lutein, the colorless phytoene and phytofluene, ζ -carotene, α -carotene and β -carotene are also present but in lower proportions (Rodrigo et al., 2015). The lycopene accumulation, a linear carotene imparting red color, is unusual and it has been reported in three sweet orange mutants: Shara, (Monselise and Halevy, 1961), Cara Cara (Liu et al., 2007), and Hong Anliu (Xu et al., 2006). Moreover, a sweet orange mutant selection with dark-orange pulp called Rhode red has been also characterized and color pulp intensity is due to higher β -cryptoxanthin content (Wei et al., 2014). Tunisia has a long tradition in citriculture which began probably in the 7th century (Mzali and Lasram, 2007). Nowadays Tunisian oranges are recognized globally (Tounsi et al., 2011) due to their high organoleptic qualities. They are exported mainly to Europe and also Africa and Asia markets (GIFruits, 2008). Orange production in Tunisia in 2014 reached 371,000 tons (FAO STAT, 2016) with 130,000 tons of Maltese demi-sanguine the main cultivated cultivar (Letaief et al., 2016). Orange production covers 16,500 Ha in the Cap Bon, the main production area with 45 % of the lands devoted to this orange (Snoussi et al., 2012). Nevertheless, some cultivars are threatened by extinction due to their substitution by others more productive, the urbanization, scarcity and low quality of irrigation water, changing in cultural practices and expansion of diseases. Thus, it is urgent to maintain and enhance the local germplasm that allows agriculture to adapt to uncertain future. The current study, focused on Tunisian orange cultivars, aims to evaluate the content of carotenoids in local OJ to better characterize these genetic resources and enhance juice production and quality control programs.

2. Materials and methods

2.1. Plant material and sample preparation

Twenty-two Tunisian sweet orange cultivars belonging to navel, acidless, common and blood groups were studied (Table 1). Washington navel, Moro and Double Fine Améliorée were also included in this study for comparative purposes. These cultivars are grown in a traditional Citrus production area located in the Cap-Bon peninsula (North-East of Tunisia, 37° 05' N 11° 02' E). The soil of the orchards were classified as sandy-loam. The Mediterranean climate characterises Cap-

Bon region, with a mean annual rainfall of 500 mm. Trees were irrigated regularly twice per month from March until October. The average maximum and minimum temperature were 31°C and 8°C, respectively. All the oranges were grown conventionally and grafted onto *Citrus aurantium*. Oranges were harvested during the 2014/2015 season between December 1st and March 15th. They exhibit large diversity of fruit shapes and weight, fruit skin color, soluble solids content and acidity (Saddoud *et al.*, 2013). From each of the cultivars selected at least 25 fruits were harvested from at least three different trees at ripen stage (Table 1). Immediately after harvesting, fresh juice was obtained with an electric citrus juicer (Santos Classic N°11, Lyon, France) then filtered through a stainless steel sieve with 1mm mesh, directly frozen in liquid nitrogen and stored at -80 °C until analysis.

2.2. Juice color determination

An aliquot of fresh juice was used for color determination as described in Pérez-Vicente *et al.* (2004) by using a Minolta CM-508i® tristimulus colour spectrophotometer (Osaka, Japan) coupled with a CMA760 transmittance adaptor, illuminant D65 and 10° observer-viewing angle according to the CIELAB 76 convention. Recording and processing CIE color data (L^* , a^* and b^*) used the Minolta Software Chromacontrol S, PC-based colorimetric data system. The psychometric index of lightness L^* takes values from 0 to 100 and measures the luminosity between black and white and equivalent to a member of the grey scale. The a^* index shows positive values for reddish colors and negative values for the greenish colors. The b^* index shows positive values for yellowish colors and negative values for the blue. Chroma (C^*_{ab}) is the quantitative colorfulness attribute as it determines the difference degree in comparison to a grey color with the same lightness for each hue. Hue angle (H^*_{ab}) is a parameter that defines the colors traditionally as pinkish, yellowish and greenish (Melendez *et al.*, 2003). The (H^*) was calculated from $1/\tan(b^*/a^*)$, (C^*) from $(a^{*2} + b^{*2})^{1/2}$. Data were obtained in triplicate.

2.3. Carotenoid analysis

2.3.1. Extraction of carotenoids from juice

Extraction and saponification of carotenoids from orange juice were performed following the protocol described by Melendez *et al.* (2003) with slight modifications. Briefly, 2 mL aliquots of

OJ samples were mixed with 3 mL of extraction mixture (methanol-acetone-dichloromethane, 25:25:50, v/v/v) (all solvents were HPLC grade, Scharlau), vortex for 1 min and sonicated 5 min at room temperature. The samples were then centrifuged for 5 min at 3500 g and 4°C and the lower phase was recovered. The upper phase was re-extracted with 1.5 mL of dichloromethane, sonicated and centrifuged as before until being colorless. Carotenoids extract was saponified with 5ml of KOH methanolic solution (12% p/v) for 2 h in the dark at room temperature with gently shake. The saponified extract was extracted with dichloromethane and washed with a solution of 5% NaCl in MilliQ water until the aqueous phases discarded was neutral. The extract was then concentrated to dryness under a N₂ stream and stored at -20°C until further analysis. At least three replicates of each sample were analyzed and all the operations were carried out in ice under a dim light to avoid photodegradation, isomerization or structural changes of the carotenoids.

2.3.2. High-performance liquid chromatography conditions

Dried extracts were dissolved with a solution of chloroform:methanol:acetone (3:2:1, v/v/v) and centrifuged for 2 min at 10000 g immediately before injection in the HPLC system. The carotenoid composition was analyzed by HPLC on a liquid chromatography system equipped with a 600E pump and 2998 photodiode array detector, and Empower software (Waters, Barcelona, Spain). To separate the pigments by HPLC, a C₃₀ carotenoid column (250 × 4.6 mm, 5 µm) coupled to a C₃₀ guard column (20 × 4.0 mm, 5 µm) (YMC, Teknokroma, Spain) were used. A ternary gradient elution was used for carotenoid separation following the chromatographic conditions (Alquezar et al., 2008). The mobile phase was pumped at 1 mL/min, the column temperature was set to 25 °C, and the injection volume was 20 µL. Chromatograms were monitored from 250 to 540 nm. A Maxplot chromatogram was obtained for each elution where each carotenoid peak was plotted at its corresponding maximum absorbance wavelength. The identification of carotenoids was based on their retention time, absorption and fine spectra (Rodrigo et al., 2015). The carotenoid peaks were integrated at their individual maximal wavelength, and their contents were calculated using the appropriate calibration curves (Alquezar et al., 2008; Carmona et al., 2012). The sum of the individual carotenoids represented the total carotenoids value.

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146 2.4. Statistical analysis

147 Statistical analyses were conducted using a one-way analysis of variance ANOVA and the
 148 significant difference between means was determined by Duncan's multiple range test ($p < 0.05$).

149 The results were presented as mean values $\pm$ SD (standard deviation). Data were analyzed also to
 150 determine correlations between color and carotenoids attributes using Pearson correlation.

151 Statistical analyses were carried out using the SPSS 13.0 software.

152 Table 1: Denominations geographical origins and maturity parameters of sweet orange cultivars
 153 selected for this study

No.	Group*	Cultivar	Code	Collection site	TSS (°Brix)	MI
1	Navel	Bourouhin	BRH	Gobba Kbira	10.9 $\pm$ 0	8.31 $\pm$ 0.1
2	Navel	Washington Navel**	WNN	Gobba Kbira	12.70 $\pm$ 0	11.38 $\pm$ 0.14
3	Acidless	Meski Malti	MEM	Bouargoub	10.57 $\pm$ 0	14.61 $\pm$ 0.15
4	Acidless	Meski Ahmer	MEH	Bouargoub	11.63 $\pm$ 0.5	19.61 $\pm$ 0.09
5	Acidless	Meski Ansli	MEA	Khlidia	10.10 $\pm$ 0.1	14.57 $\pm$ 0.14
6	Acidless	Meski Arbi	MES	Khlidia	9.63 $\pm$ 0	12.85 $\pm$ 0.45
7	Acidless	Meski Boujnab	MBM	Bouargoub	12.93 $\pm$ 0	10.54 $\pm$ 0.16
8	Acidless	Meski Sifi	MEI	Gobba Kbira	10.33 $\pm$ 0	14.56 $\pm$ 0.02
9	Common	Malti Trabelsi	MTR	Gobba Kbira	10.23 $\pm$ 0	7.49 $\pm$ 0.01
10	Common	Malti Twil	MTW	Gobba Kbira	10.47 $\pm$ 0.1	8.72 $\pm$ 0.21
11	Common	Malti Abiadh	MSW	Gobba Kbira	11.63 $\pm$ 0	11.09 $\pm$ 0.26
12	Common	Malti Abiadh Boujnab	MBB	Morneg	12.5 $\pm$ 0.1	11.57 $\pm$ 0.36
13	Common	Malti Mdawer	MDW	Menzel Bouzalfa	11.2 $\pm$ 0	10 $\pm$ 0.2
14	Common	Ballerin	BAL	Gobba Kbira	11 $\pm$ 0.1	9.54 $\pm$ 0.05
15	Common	Beldi Abiadh	BAB	Bouargoub	10 $\pm$ 0	8 $\pm$ 0.19
16	Blood O	Maltaise Petit-Pierre	MPP	Gobba Kbira	12.3 $\pm$ 0.1	11.25 $\pm$ 0.13
17	Blood O	Double fine améliorée**	DFM	Takelsa	12.4 $\pm$ 0.1	10.42 $\pm$ 0.6
18	Blood O	Maltaise demi-sanguine	MDM	Gobba Kbira	13.5 $\pm$ 0.5	11.19 $\pm$ 0.15
19	Blood O	Malti Lsen Asfour	MLS	Hammamet	13.2 $\pm$ 0.2	10.64 $\pm$ 0.05
20	Blood O	Malti Ahmer	MSS	Gobba Kbira	11.6 $\pm$ 0.1	10.3 $\pm$ 0.04
21	Blood O	Chemi	CHE	Gobba Kbira	11.7 $\pm$ 0.1	8.12 $\pm$ 0.26
22	Blood O	Moro**	MRO	Bouargoub	11.8 $\pm$ 0.1	11.48 $\pm$ 0.3
23	Blood O	Boukhobza	BKH	Gobba Kbira	13.6 $\pm$ 0.1	11.86 $\pm$ 0.14
24	Blood O	Beldi Ahmer	BAH	Bouargoub	11.3 $\pm$ 0.1	8.63 $\pm$ 0.04
25	Blood O	Sakasli	SAK	Gobba Kbira	13.53 $\pm$ 0	11.97 $\pm$ 0.14

154 TSS: total soluble solids content; MI : Maturity index

155 *This was based on the classification proposed by Hodgson (1967) and Ladaniya (2008)

156 ** Non-tunisian varieties included for comparison

157 3. Results

158 3.1. Diversity of coloration and total carotenoid content in Tunisian sweet orange juices

Each orange cultivar has its characteristic juice color that varied visibly from red pigmented to pale yellow. In fact, all juice color parameters varied significantly among cultivars when they were evaluated in the CIE L*a*b* system (Table 2). Based on lightness parameter L*, most of juices exhibited light color. Meski Arbi (MES) and Moro (MRO) cultivars showed, respectively, the highest and the lowest values. Redness and yellowness of the OJ are indicated by (a*) and (b*) indices, respectively. Cultivar MRO had the highest redness color and MES, Meski Sifi (MEI) and Meski Abiadh (MSW) the lowest ones. For yellowness (b*), MES recorded the highest value followed by Meski Malti (MEM), while MSW exhibited the lowest one. Concerning the colorfulness attribute represented by Chroma (C) parameter, MRO juice showed the highest color intensity whereas MSW juice had the lowest Chroma values. Regarding H or hue angle, the obtained values matched for most of them with orange colors. Some exceptions were found: MEM and MES exhibited yellowish juices, while Boukhobza (BKH), Sakasli (SAK), Chemi (CHE), Malti Ahmer (MSS) and Moro (MRO) showed red-tinted juices.

A comparative study was held to check whether the genotype affected the total and individual carotenoid contents. Eleven carotenoids were identified in the OJ and their chromatographic and spectral characteristics are reported in Table 3. This study identified the carotenoids (all-*E*)-violaxanthin, (9*Z*)-violaxanthin, lutein, lycopene, ζ -carotene, β -carotene, phytofluene, antheraxanthin, zeaxanthin, β -cryptoxanthin and phytoene. Qualitative and quantitative carotenoid composition was significantly different ($P<0.05\%$) among the different OJ samples (Table 4). The types of carotenoids and the concentrations of each specific carotenoid varied among the studied oranges. Total carotenoid content, as the sum of individual carotenoids, was determined in the juice of the selected sweet orange fruits (Figure 1). Maltaise demi-sanguine (MDM) was the richest cultivar concerning the total carotenoid content with an average of 13.320 mg/L. Washington navel (WNN), Boukhoza (BKH), Malti Isen asfour (MLS), Meski Arbi (MES) and Ballerin (BAL) were also among the richest juices in total carotenoids with, respectively, 10.401 mg/L, 9.920 mg/L, 9.740 mg/L, 9.220 mg/L, 8.710 mg/L. The blond orange Malti Abiadh (MSW) seemed to contain the lowest total carotenoid content (1.570 mg/L) and the simplest pattern.

3.2. Concentration of individual carotenoids in Tunisian sweet orange juices

A total of eleven carotenoids were identified by liquid chromatography-photodiode array detector. In the studied OJ samples, concentrations of (9Z)-violaxanthin were the highest ranging from 0.821 to 4.405 mg/L for the cultivars Malti Abiadh (MSW) and Meski Arbi (MES), respectively. (all-*E*)-violaxanthin was detected in all samples and varied unequally among them. The highest amount (1.823 mg/L) was recorded in the cultivar Malti Ahmer (MSS). The concentration of β -cryptoxanthin varied widely among the juice samples (Table 4). Blood oranges Malti Lsen Asfour (MLS), Skasali (SAK) Chemi (CHE) and double-fine améliorée (DFM) held important amounts of this xanthophyll, respectively, 0.853, 0.523, 0.579 and 0.539 mg/L and the navel cultivar WNN showed the most important concentration reaching 1.328 mg/L. Besides, among the studied sweet orange juices, β -carotene is not detected or only as low levels: 0.255, 0.055 and 0.051 mg/L in the juices of Malti Ahmer (MSS), Double Fine Améliorée (DFM) and Maltaise Demi-Sanguine (MDM), respectively. Concerning phytoene, a colorless carotene, Ballerin (BAL) cultivar exhibited the highest amount (3.072 mg/L). The two others upstream carotenes of the pathway, phytofluene and ζ -carotene, were present in the OJ. The juices of all pigmented oranges except those of Maltaise Petit-Pierre (MPP), Moro (MRO) and Meski Ahmer (MSS) contained significant amounts of both compounds, and Boukhobza (BKH) and Malti Demi-Sanguine (MDM) had the highest amounts, respectively, 1.232 and 1.229 mg/L of phytofluene and 0.876 and 0.331 mg/L of ζ -carotene. In Malti Twil (MTW) and Meski Sifi (MEI) juices none of these carotenes were detected. Other carotenoids such as zeaxanthin and lutein, were found in detectable levels for some samples with a range of 0-0.786 and 0-3.976 mg/L respectively. Antheraxanthin was also detected and varied significantly among samples. Its concentration was less than 1.372 mg/L. It is worth to mention that the red carotene lycopene was detected exclusively in the Meski Ahmer (MEH) juice in a concentration of 0.409 mg/L.

3.3. Correlation analysis

In order to evaluate the relationship between the individual and total carotenoids with the juice color, a correlation analysis among juice attributes was performed (Table 5). The total carotenoids

were highly and negatively correlated with lightness parameters (L^*) and Hue angle (H), and positively and highly related with redness attributes (a^*). It was also noticed that yellow intensity index (b^*) was better correlated to individual carotenoids (Table 5). (all-*E*)-violaxanthin as well as ζ -carotene were significantly correlated to yellow color intensity index (b^*) while zeaxanthin and lutein were positively related to Chroma (C^*) and (b^*) coordinates.

Table 2. Juice color parameters (CIE Lab) of the twenty-five studied orange cultivars

Cultivar	CIE Lab ^a				
	L^*	a^*	b^*	C^*_{ab}	H^*_{ab}
BRH	70.49 ± 5.3 ^{hi}	2.80 ± 1.2 ^g	17.25 ± 1.3 ^{ef}	17.12 ± 3.4 ^{gh}	80.68 ± 1.9 ^{cd}
WNN	71.3 ± 3.9 ^h	6.50 ± 0.9 ^f	28.70 ± 0.3 ^b	30.60 ± 0.3 ^{de}	76.31 ± 3.4 ^{ab}
MEM	81.17 ± 10.6 ^{ef}	0.50 ± 0.4 ^{hi}	34.25 ± 1.1 ^a	35.50 ± 0.2 ^c	89.59 ± 0.8 ^a
MEH	80.75 ± 2.4 ^{ef}	25.5 ± 2.1 ^c	25.5 ± 0.7 ^c	19.87 ± 1.7 ^f	79.25 ± 1.5 ^{cde}
MEA	85.96 ± 6.7 ^{bc}	1.05 ± 0.4 ^{gh}	9.85 ± 0.25 ^{ijk}	9.88 ± 2.9 ^j	83.70 ± 0.1 ^c
MES	95.21 ± 17.3 ^a	0.15 ± 0.2 ^{hij}	35.45 ± 2.9 ^a	41.95 ± 4.9 ^b	89.89 ± 1.2 ^a
MBM	83.1 ± 2.7 ^{bcd}	0.55 ± 0.1 ^{hi}	8.96 ± 1.4 ^{jk}	8.72 ± 1.6 ^{jk}	80.56 ± 3.1 ^{cd}
MEI	86.15 ± 14.7 ^{bc}	-0.10 ± 0.1 ^k	6.75 ± 0.25 ^k	6.80 ± 0.8 ^{jkl}	89.15 ± 0.25 ^a
MTR	90.02 ± 11.9 ^{bc}	0.74 ± 0.5 ^{hi}	19.07 ± 2.9 ^{de}	18.97 ± 8.5 ^{fg}	87.90 ± 2.0 ^b
MTW	87.01 ± 6.6 ^{bc}	0.57 ± 0.23 ^{hi}	6.50 ± 0.1 ^{ijk}	6.52 ± 2.5 ^{jkl}	83.75 ± 0.4 ^c
MSW	88.76 ± 0.4 ^b	-0.21 ± 0.1 ^k	2.16 ± 0.9 ^m	1.67 ± 0.1 ^h	80.96 ± 2.9 ^{cd}
MBB	77.51 ± 19.2 ^f	1.5 ± 0.3 ^g	15.42 ± 3.9 ^{efg}	13.77 ± 2 ^{hi}	81.67 ± 2.1 ^{cd}
MDW	86.15 ± 8.4 ^{bc}	0.81 ± 0.45 ^h	7.90 ± 1.2 ^k	7.92 ± 1.4 ^{jk}	83.60 ± 0.3 ^{ab}
BAL	80.24 ± 13.4 ^{ef}	0.02 ± 0.1 ^j	4.50 ± 0.4 ^l	4.50 ± 0.4 ^m	88.93 ± 1.0 ^b
BAB	81.57 ± 3.6 ^{def}	0.68 ± 0.3 ^{hi}	7.56 ± 1 ^{kl}	7.48 ± 0.5 ^{jk}	84.77 ± 0.3 ^c
MPP	68.4 ± 2.7 ^{gh}	4.99 ± 0.25 ^{ef}	13.96 ± 1.4 ^{ghi}	14.83 ± 1.6 ^{hi}	70.33 ± 0.3 ^{ef}
DFM	63.38 ± 2.9 ^h	6.19 ± 1.3 ^f	14.50 ± 2.3 ^{ghi}	15.43 ± 2.4 ^{ghi}	66.17 ± 5.1 ^f
MDM	66.81 ± 0.3 ^h	7.5 ± 0.49 ^d	18.5 ± 0.6 ^{de}	19.71 ± 12.3 ^f	73.96 ± 5.3 ^e
MLS	63.38 ± 2.9 ^h	6.59 ± 1.3 ^f	19 ± 2.3 ^{de}	17.75 ± 2.4 ^{gh}	69.24 ± 5.1 ^{ef}
MSS	54.21 ± 14.3 ^{ij}	14.03 ± 0.5 ^e	16.50 ± 0.9 ^{efg}	21.64 ± 2.9 ^{def}	49.38 ± 5.1 ^{gh}
CHE	52.75 ± 1.1 ^j	16.21 ± 0.1 ^c	16.00 ± 0.2 ^{efg}	28.54 ± 0.4 ^c	46.39 ± 1.4 ⁱ
MRO	47.16 ± 0.8 ^k	56.66 ± 2.9 ^a	17.00 ± 1.1 ^{ef}	59.18 ± 10.2 ^a	13.94 ± 9.1 ^k
BKH	50.09 ± 1.4 ^h	29.57 ± 4.54 ^c	13.12 ± 0.4 ^{hi}	32.70 ± 8.5 ^{de}	24.05 ± 5.7 ^j
BAH	62.25 ± 5.2 ^h	8.13 ± 2.4 ^d	25.75 ± 0.7 ^c	36.19 ± 6.7 ^c	51.22 ± 1.8 ^g
SAK	48.93 ± 13.5 ^j	32.12 ± 7.6 ^b	12.77 ± 0.8 ^{hij}	34.74 ± 1.7 ^c	21.66 ± 6.4 ^j

^a Data are mean ± SD.

L^* : color lightness; a^* : color redness; b^* : color yellowness; C^*_{ab} : Chroma; H^*_{ab} : hue angle

Different lowercase letters on the same column represent significant differences ($p < 0.05$) among samples

Table 3. Chromatographic and spectroscopic characteristics of carotenoids found in the studied orange juice samples.

Peak	Retention time (min)	Carotenoid	UV-Vis absorption maxima (nm)	D _B /D _{II} ^c
1	11.0	NI	sh, 441,469	0.20
2	14.7	NI	328(Z), sh, 431,455	
3	16.3	^a (all- <i>E</i>)-violaxanthin	415 438,469	
4	17.3	NI	sh, 441	
5	17.6	NI	328(Z), sh, 425	
6	18.4	NI	sh, 429, 456	0.07
7	18.7	NI	397, 421,448	
8	19.9	Mix NI	336(Z), sh, 401, 424, 447	
9	21.9	^a (9Z)-violaxanthin	327(Z), 412, 435, 462	
10	22.3	^b Luteoxanthin	310, 416,443	
11	23.7	^b Mutatoxanthin	sh, 426, 452	0.08
12	24	^a Lutein	sh, 447, 472	
13	26.4	^a Zeaxanthin	429, 450, 477	
14	27.4	^b Z-Antheraxanthin	329(Z), 420, 441, 469	
15	30.3	^a (15Z)-Phytoene	285	
16	31.2	^b (9Z)-zeaxanthin	338(Z), sh, 444, 473	0.08
17	32.1	^a Phytofluene	331, 347, 364	
18	34.4	^a β-cryptoxanthin	423, 450, 477	
19	37.1	^a ζ-carotene	376, 398, 424	
20	43	^a β-carotene	426, 451, 473	
21	64.2	^a (all- <i>E</i>)-Lycopene	446,472, 503	

^a identified using authentic standards; ^b Tentatively identified (Melendez-Martínez et al., 2008 and 2015); ^c Intensity of Z peak as ratio D_B/D_{II}. NI, non identified

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249 **Table 4:** Concentration of carotenoids (mg /L) in orange juices from the twenty-five studied cultivars^a

		Phytoene	Phytofluene	ζ-Carotene	Lycopene	Lutein	β-Carotene	β-Cryptoxanthin	Zeaxanthin	Antheraxanthin	(all- <i>E</i>)violaxanthin	(9 <i>Z</i>)-violaxanthin
BRH	Navel	0.353 ^b	0.062 ^b	0.022 ± 0.031 ^g	N.D ^b	0.467 ± 0.07 ^c	N.D ^c	0.431 ± 0.056 ^{cdefg}	0.284 ± 0.043 ^c	0.695 ± 0.015 ^{abcde}	0.813 ± 0.123 ^{def}	2.844 ± 0.057 ^{cde}
WNN	Navel	0.794 ^b	0.170 ± 0.017 ^b	0.235 ± 0.015 ^c	N.D ^b	0.027 ± 0.038 ^j	N.D ^c	1.328 ± 0.055 ^a	0.259 ^c	1.338 ± 0.141 ^a	1.625 ± 0.013 ^{ab}	3.521 ± 0.058 ^{bc}
MEM	Acidless	0.309 ± 64 ^b	0.085 ± 21 ^b	N.D ^g	N.D ^b	0.183 ± 38 ^g	N.D ^c	0.424 ± 0.08 ^{cdefg}	0.154 ± 0.09 ^d	1.210 ± 0.09 ^{ab}	1.204 ± 0.24 ^{bc}	4.030 ± 0.06 ^{bcd}
MEH	Acidless	0.677 ± 0.275 ^b	0.237 ± 0.057 ^b	0.127 ± 0.025 ^{def}	0.409 ± 0.038 ^a	0.417 ± 0.020 ^c	N.D ^c	0.112 ± 0.037 ^{gh}	0.113 ± 0.041 ^{de}	0.439 ± 0.021 ^{bcd}	0.809 ± 0.135 ^{def}	2.697 ± 0.092 ^{def}
MEA	Acidless	2.019 ± 0.025 ^a	0.092 ± 1 ^b	N.D ^g	N.D ^b	0.171 ± 63 ^g	N.D ^c	0.408 ± 0.010 ^{cdefg}	0.185 ± 0.089 ^d	0.552 ± 0.039 ^{bcd}	0.996 ± 0.063 ^{bcd}	2.010 ± 0.062 ^{ghij}
MES	Acidless	1.626 ± 0.154 ^a	0.174 ± 0.075 ^b	0.134 ± 0.058 ^{de}	N.D ^b	0.231 ± 0.041 ^f	N.D ^c	0.264 ± 0.017 ^{cdefgh}	0.071 ± 0.031 ^{def}	0.765 ± 0.029 ^{abcde}	1.303 ± 0.052 ^{abc}	4.406 ± 0.692 ^a
MBM	Acidless	0.337 ± 0.025 ^b	0.099 ± 0.014 ^b	0.119 ^{def}	N.D ^b	N.D ^k	N.D ^c	0.701 ± 0.27 ^{bc}	0.786 ± 0.023 ^a	0.841 ± 0.236 ^{abcde}	1.192 ± 0.401 ^e	3.192 ± 0.092 ^{bcd}
MEI	Acidless	0.758 ± 0.097 ^b	N.D ^b	N.D ^g	N.D ^b	0.134 ± 0.019 ^{gh}	N.D ^c	0.123 ± 0.018 ^{fgh}	0.030 ^{ef}	0.269 ± 0.057 ^{de}	0.513 ± 0.107 ^{ef}	1.135 ± 0.078 ^{ij}
MTR	Common	0.477 ± 0.037 ^b	0.136 ± 0.014 ^b	N.D ^g	N.D ^b	N.D ^k	N.D ^c	0.755 ± 0.063 ^{bc}	0.194 ^c	0.970 ± 0.063 ^{abcd}	0.501 ± 0.031 ^{ef}	3.396 ± 0.087 ^{bc}
MTW	Common	N.D ^c	N.D ^b	N.D ^g	N.D ^b	0.063 ± 0.089 ⁱ	Tr. ^c	0.245 ^{cdefgh}	0.047 ^{ef}	0.329 ± 0.070 ^{de}	0.462 ± 0.086 ^f	1.454 ± 0.011 ^{ghij}
MSW	Common	N.D ^c	N.D ^b	N.D ^g	N.D ^b	0.014 ^j	N.D ^c	0.079 ^{gh}	0.123 ± 0.146 ^d	0.089 ± 0.026 ^{ef}	0.361 ± 0.050 ^f	0.821 ± 0.136 ^{ijk}
MBB	Common	0.249 ± 0.014 ^b	0.028	0.016 ^{fg}	N.D ^b	0.114 ± 0.038 ^h	N.D ^c	0.798 ± 0.017 ^{bc}	0.261 ± 0.038 ^c	0.728 ± 0.171 ^{abcde}	1.050 ± 0.013 ^{bcd}	2.485 ^{def}

250 ^a Data are mean ± SD. Tr., Traces, values below 0.010 mg. N.D., not detected. Different lowercase letters on the same column represent significant differences (p < 0.05) for a carotenoid among samples.

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		Phytoene	Phytofluene	ζ-Carotene	Lycopene	Lutein	β-Carotene	β-Cryptoxanthin	Zeaxanthin	Antheraxanthin	(all- <i>E</i>)-violaxanthin	(9 <i>Z</i>)-violaxanthin
MDW	Common	0.299 ± 0.050 ^b	N.D ^b	0.011 ^{fg}	N.D ^b	0.435 ± 39 ^c	N.D ^c	0.716 ± 14 ^{bc}	0.201 ± 8 ^c	0.497 ± 80 ^{bcde}	0.779 ± 79 ^{def}	2.565± 48 ^{defg}
BAL	Common	3.072 ± 422 ^a	0.183 ± 59 ^b	N.D ^g	N.D ^b	N.D ^k	N.D ^c	0.581±0.194 ^{bcdef}	0.206 ± 0.072 ^c	1.372 ± 0.126 ^a	0.473 ± 0.140 ^{ef}	3.072 ± 0.422 ^a
BAB	Common	0.400 ± 1 ^b	N.D ^b	0.035 ^{fg}	N.D ^b	N.D ^k	N.D ^c	0.013 ⁱ	0.021 ^{cfig}	0.251±0.01 ^{de}	0.469 ±0.045 ^{ef}	0.400 ^{jk}
MPP	Blood O	0.146 ±0.037 ^b	N.D ^b	N.D ^g	N.D ^b	0.142 ^f	N.D ^c	0.077 ^{gh}	0.039 ^{ef}	0.368±0.04 ^{bcde}	0.548 ±0.02 ^{def}	1.469 ±0.079 ^{ghij}
DFM	Blood O	1.566 ± 0.267 ^{ab}	0.093 ± 5 ^b	0.115 ± 29 ^{def}	N.D ^b	N.D ^k	0.024 ^{ab}	0.539 ±0.0236 ^{bcdef}	0.639 ± 0.063 ^{ab}	1.166 ± 0.219 ^{ab}	0.523 ± 0.062 ^{def}	2.380 ± 0.012 ^{fghi}
MDM	Blood O	1.122 ± 0.223a ^b	1.229 ± 0.235 ^a	0.876 ± 0.088 ^a	N.D ^b	3.976 ± 0.611 ^a	0.051 ± 0.017 ^b	0.089 ± 0.013 ^{gh}	0.405 ± 0.061 ^{bc}	0.515 ± 0.024 ^{bcde}	1.059 ± 0.394 ^{bc}	1.122 ± 0.223 ^{ij}
MLS	Blood O	0.251 ± 0.024 ^b	0.064 ± 0.096 ^b	0.166 ^d	N.D ^b	N.D ^k	N.D ^c	0.853 ± 0.013 ^b	0.412 ± 0.040 ^{bc}	1.122± 0.126 ^{ab}	0.826 ± 0.091 ^{def}	3.205 ± 0.099 ^{bc}
MSS	Blood O	0.200 ± 0.021 ^b	N.D ^b	N.D ^g	N.D ^b	0.376 ± 0.040 ^d	0.255 ± 0.077 ^a	0.090 ^{gh}	0.095 ^{de}	0.522 ±0.075 ^{bcde}	1.823 ± 0.149 ^a	0.200 ± 0.021 ^{kl}
CHE	Blood O	0.647 ± 0.167 ^b	0.079 ^b	0.066 ^{fg}	N.D ^b	0.357 ± 0.056 ^d	N.D ^c	0.579 ± 0.029 ^{bcdef}	0.174 ± 0.037 ^c	0.426 ± 0.072 ^{bcde}	0.407 ± 0.067 ^{ef}	2.521 ± 0.081 ^{defg}
MRO	Blood O	0.340 ± 0.092 ^b	N.D ^b	N.D ^g	N.D ^b	2.865 ± 0.657 ^b	N.D ^c	0.284 ^{defgh}	0.161 ^c	0.503 ± 0.185 ^{bcde}	0.333 ± 0.122 ^g	1.289 ± 0.086 ^{ij}
BKH	Blood O	1.083 ±0.155a ^b	1.232 ±0.201 ^a	0.331 ± 0.060 ^b	N.D ^b	0.153 ±0.038 ^g	N.D ^c	0.166 ± 0.057 ^{defgh}	0.175 ±0.017 ^c	0.681 ± 0.150 ^{bcde}	1.362 ± 0.081 ^{abc}	1.083 ±0.155 ^{ij}
BAH	Blood O	0.575 ± 0.017 ^b	0.100 ± 0.035 ^b	0.054 ± 0.017 ^{fg}	N.D ^b	0.310 ± 0.51 ^e	Tr. ^c	0.092 ± 0.013 ^{gh}	0.138 ±0.018 ^d	0.497 ± 0.058 ^{bcde}	0.336 ± 0.036 ^f	0.575 ± 17 ^{jk}
SAK	Blood O	0.461 ± 0.077 ^b	0.156 ± 0.014 ^b	0.107± 0.024 ^{def}	N.D ^b	N.D ^k	Tr. ^c	0.523 ± 0.022 ^{bcdef}	0.192 ± 0.045 ^c	0.531 ± 0.173 ^{bcde}	0.248 ± 0.080 ^g	2.766 ± 0.142 ^{def}

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263 ^aData are mean ± SD. Tr., Traces, values below 0.010 mg. N.D., not detected. Different lowercase letters on the same column represent significant differences (p < 0.05) for a carotenoid among samples.

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4. Discussion

Color is an important quality attribute of OJ influencing consumer behavior by visual appeal (Melendez et al., 2005) and allows the evaluation for commercial classification in countries such USA (Tepper, 1993). Previous studies showed that oranges with reddish colored pulp are very appealing to consumers and becoming particularly prized (Lee, 2001 and 2002). Thus, juices derived from Boukhobza (BKH), Sakasli (SAK), Chemi (CHE), Malti Ahmer (MSS), Meski Ahmer (MEH), Malti Lsen Asfour (LSN), Maltaise demi-sanguine (MDM), Maltaise Petit-Pierre (MPP), and Beldi Ahmer (BAH) cultivars may gain interest in juice industry. In particular, MDM cultivar showed the highest concentration of total carotenoids and that, in addition to the anthocyanin presence (Letaief et al., 2016), may contribute to its juice deep color. In fact, the juice obtained from MDM fruit is highly appreciated thanks to the deep orange color in comparison with other juices. This is a relevant characteristic since this is the most exported Tunisian orange cultivar. Regarding the correlation analysis between the individual and total carotenoids with the juice color attributes, it can be indicated that the darker and redder the juices are the richer in carotenoids. This result is in agreement with Melendez-Martinez et al. (2003) that analyzed mature Valencia orange juices and confirmed that (b^*) is the best parameter defining the juice color.

Additional studies also determined that zeaxanthin and lutein were positively related to Chroma (C^*) coordinates (Melendez-Martinez et al., 2010) corroborating then with our findings (Table 5). According to these results, the impact of the chemical structure on the color of carotenoids is well established and depending on the number of conjugated double bonds (c.d.b.) can be categorized in: 7 c.d.b. (auroxanthin, ζ -carotene), 8 c.d.b. (luteoxanthin), 9 c.d.b. (mutatoxanthin, violaxanthin), 10 c.d.b. (antheraxanthin, lutein, α -carotene) and 11 c.d.b. (zeaxanthin, β -cryptoxanthin, β -carotene). Meléndez Martínez et al. (2007b) grouped these compounds in a^*b^* plane according to their number of conjugated double bonds showing that the reddish carotenoids had positive values of a^* and b^* whereas the yellowish ones were located near the b^* axis. Moreover, a^* values decreased from those with 11 c.d.b. to those with 9. However, the hue increased when the ring of the molecule was opened and the conjugation increased as follow: all

(E)-lycopene < β -carotene < β -cryptoxanthin < α -carotene < lutein. In our case, MSS juice recorded the highest β -carotene content (0.255 mg/L) and had a relatively low hue (49.39 CIELab units). The lowest a^* values (-0.10 and -0.21 CIELab units, respectively) and higher H^*ab values (89.15 and 80.93 CIELab units, respectively) correspond to MEI and MSW juices. This shows that those samples were yellower and less red than the rest of the orange juices. Accordingly, they also had the lowest carotenoid content (3.4 mg/L and 1.57 mg/L, respectively).

Figure 1. Total carotenoids content in the juices of studied orange cultivars. Means with different letters indicate significant differences at $P \leq 0.01$ according to Duncan range test.

It is generally accepted that environmental and agronomical practices can have a deep effect on carotenoid content in citrus fruits. When cultivated in Corsica (France), Maltaise cultivar exhibited a slightly lower concentration in carotenoids compared to Tunisia (11 mg/ml in French conditions vs 13.31 mg/ml in Tunisian ones) (Dhuique-Mayer et al., 2005). Interestingly, in the Tunisian conditions Washington Navel juice shows 2 times more total carotenoid content compared to juices from the same cultivar grown in other geographical locations. (Xu et al., 2006; Fanciullino et al., 2008; Rodrigo et al., 2015) The cultivar Moro, a blood-pigmented orange, showed also a higher content of total carotenoids (7.17 mg/L) than the same cultivar cultivated in California and Florida; (5.94 mg L⁻¹ for California and 3.71 or 2.86 mg/L for Florida) (Lee et al., 1990; Choia et al., 2002). The same cultivar exhibited a total carotenoid content of 12.2 mg/L under Algerian

conditions (Achir et al., 2015) and 15.72 mg/ L when cultivated in Spain (Stinco et al., 2016). On the other hand, carotenoid level of Valencia orange in Brazil was around 12 mg/L (Gama and Sylos, 2005), whereas when grown in Sevilla (Spain) its concentration was almost the double (23.17 mg/L) (Stinco et al., 2016). In comparative studies including sweet orange juices from different cultivars it has been concluded that citrus fruits cultivated in tropical or semitropical areas show lower total carotenoid contents as compared to those grown in Mediterranean regions (Mouly et al., 1999; Dhuique-Mayer et al., 2009; Stinco et al., 2016). Specifically, Mediterranean conditions seem to promote accumulation of enhance β -cryptoxanthin and (9Z)-violaxanthin in sweet orange fruits compared to other locations (Dhuique-Mayer et al., 2009). A recent study performed in five Chinese regions also points that temperature and other environmental conditions such as lighting time or rainfall are correlated with total and individual carotenoids content in the sweet orange Cara Cara (Lu et al., 2017). In other citrus species it has been also proposed that light exposure and intensity can also affect carotenoid accumulation in the peel but the effect on carotenoids in the juice remains to be investigated (Cronje et al., 2013; Lado et al., 2015). The effect of water limitation on the synthesis and accumulation of carotenoids remains controversial; the positive or negative impact depends on the intensity of the stress, its duration or its time of application during ontogeny (Poiroux-Gonord et al., 2010). Other studies showed that cultivation practices could be effective to modify the carotenoid contents of fruits and organic farming caused a positive effect on the total content of carotenoids in mandarin juices (Beltran-Gonzalez et al., 2008).

Table 5: Correlation coefficients of color parameters, total and individual carotenoids of orange juices

	CIE L^*	CIE a^*	CIE b^*	Chroma	Hue
Phytoene	0.131	-0.118	0.047	-0.037	0.154
Phytofluene	-0.224	0.201	0.336	0.235	-0.240
ζ -Carotene	-0.165	0.099	0.482*	0.223	-0.126
Lycopene	0.114	-0.1	0.33	-0.096	0.126
Lutein	-0.217	0.156	0.398*	0.435*	-0.271
β -Carotene	-0.231	0.083	0.097	0.029	-0.176
β -Cryptoxanthin	0.042	-0.116	0.120	0.008	0.145
Zeaxanthin	-0.69	-0.045	0.401*	0.456*	-0.048
Antheraxanthin	0.031	-0.126	0.354	0.145	0.149
(all-E)-violaxanthin	0.108	-0.165	0.457*	0.145	0.139
(9Z)-violaxanthin	0.27	-0.287	0.363	0.135	0.347
Total carotenoids	-0.709**	0.627**	-0.198	0.246	-0.743**

* Correlations are significant at $P \leq 0.05$ according to Pearson correlation.

** Correlations are significant at $P \leq 0.01$ according to Pearson correlation

In this study the intercultivar variation in carotenoids observed in Tunisian oranges is qualitative and the genotype contributes highly to such differences since all the studied OJ come from mature fruits with the same growing conditions and similar geographic origins. Major carotenoids in the twenty-five studied juices were similar to those reported by other authors and twenty-one different carotenoid peaks were separated by HPLC-DAD (Table 3) which corroborates the complex carotenoid pattern described for sweet orange juices (Melendez-Martinez et al., 2008; Aschoff et al., 2015; Rodrigo et al., 2015; Lu et al., 2017). The β,β -xanthophylls were predominately found than carotenes, corroborating the results reported by Fanciullino et al. (2006), Melendez-Martinez et al. (2008), Dhuique-Mayer et al. (2009) and Rodrigo et al. (2015). Violaxanthin is formed from epoxidation of zeaxanthin via antheraxanthin and is a precursor of neoxanthin and luteoxanthin (Gross, 1987). Its isomers were predominant in the analyzed OJ and the peak labeled as (9Z)-violaxanthin, which is the major carotenoid in the peel and pulp of orange-colored mature fruit, represents the main form occurring in the analyzed samples. The accumulation of specific β,β -xanthophylls, such as (9Z)-violaxanthin is orchestrated by a decline in lutein content characterized by a massive synthesis of chromoplastic carotenoid pigments (Rodrigo et al., 2004; Kato et al., 2004). The high concentration of (9Z)-violaxanthin in MES cultivar may justify its deep yellow juice. Since violaxanthin pigment renders a yellowish coloration, it has been suggested that it is not the major carotenoid responsible for the intense orange coloration of some varieties (Oberholster et al., 2001; Melendez-Martinez et al., 2007a, Carmona et al., 2012) and other β,β -xanthophylls, in particular the mono-hydroxycarotenoid β -cryptoxanthin give a dark orange tint to citrus fruits (Melendez-Martinez et al., 2007a, Fanciullino et al., 2008, Carmona et al., 2012). Moreover, β -cryptoxanthin is a precursor of vitamin A and citrus fruits are the main source for this carotenoid in a regular european diet (Estevez-Santiago et al., 2015). Breithaupt and Bamedi (2001) found out that Tunisian oranges contain higher values of free β -cryptoxanthin amounts in the edible portion of the fruit compared to those grown in Italy and Spain. Thereafter, Dhuique-Mayer et al. (2009) proved that Mediterranean conditions increase the differentiation between genotypes by enhancing β -cryptoxanthin and 9-Z-violaxanthin content in sweet oranges and the β -carotene, phytoene, and phytofluene content in mandarins. The unusual lycopene accumulation in MEH cultivar may explain

its pleasant unique red-pinkish hue. Lycopene is a highly apolar carotene and is the major carotenoid in red tomatoes and watermelons. In citrus, it has been identified in red cultivars of grapefruit (*Citrus paradisi*) and pumelo (*Citrus grandis*). However, its accumulation in other species of the genus *Citrus* is very unusual and has only been reported in a few spontaneous sweet orange fruits mutants such as Sarah, Hong Anliu and CaraCara (Lee et al., 2001; Xu and Deng, 2002; Alquezar et al., 2008a) and therefore, this new Tunisian cultivar merits further biochemical and agronomical characterization.

5. Conclusion

The data presented in this work highlights the potential of some of the sweet orange Tunisian cultivars evaluated and these results might help in further genomic research as well as in breeding programs. The individual and total carotenoid contents as well as color attributes varied significantly among the studied OJ. The Maltaise demi-sanguine might be considered as carotenoid-rich cultivar, which combines an attractive color with the benefits associated to carotenoid intake, making this orange desirable for juice processing. Moreover, the Meski Ahmer cultivar has been identified as the first sweet orange from the acidless group which accumulates lycopene. Due to the health benefits associated to this carotene, this cultivar deserves additional investigation and may help to understand the process governing of lycopene biosynthesis in orange fruits.

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