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Application of Tristimulus Colorimetry To Estimate the Carotenoids Content in Ultrafrozen Orange Juices

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Tristimulus Colorimetry was applied to characterize the color of Valencia late orange juices. Color measurements were made against white background and black background. The profile of the main carotenoids related to the color of the juices was determined by HPLC. Significant correlations ($p < 0.05$) between b^*, C_{ab}^* and h_{ab} and the content of β-cryptoxanthin, lutein + zeaxanthin and β-carotene were found. The correlations between the color parameters L^*, a^*, b^*, C_{ab}^* and h_{ab} and the carotenoids content were also explored by partial least squares. The results obtained have shown that it is possible to obtain equations, by means of multiple regression models, which allow the determination of the individual carotenoid levels from the CIELAB color parameters, with R^2 values always over 0.9. In this sense, equations have been proposed to calculate the retinol equivalents (1 RE = 1 μg retinol = 12 μg β-carotene = 24 μg α-carotene = 24 μg β-cryptoxanthin) of the orange juice analyzed as a function of the color parameters calculated from measurement made against white and black backgrounds. The average RE per liter of juice obtained by HPLC was 51.07 ± 18.89, whereas employing these equations, average RE values obtained were 51.16 ± 1.36 and 51.21 ± 1.70 for white background and black background, respectively.

KEYWORDS: Carotenoids; CIELAB; color; orange juice; partial least squares (PLS)

INTRODUCTION

Color is one of the most important attributes of orange juice and is mainly due to carotenoid pigments. The importance of color as a quality parameter in citrus products has been demonstrated in several studies (*1*). In certain countries, such as the United States, the color of citrus juices is one of the parameters that is evaluated for the commercial classification of the product in relation to its quality (*2*, *3*). In this sense, Valencia orange juices are worldwide appreciated due to their deep orange color (*4*, *5*).

Although orange juice color is mainly due to carotenoids, in juices from some varieties, such as blood orange juices, anthocyans pigments are also responsible for this attribute. It has been suggested that carotenoids do not contribute equally to the orange juice color (*6*), even though the contribution of each one is unknown yet. However, β-cryptoxanthin is supposed to be the main carotenoid related to the orange juice color. Large quantities of this pigment in orange and mandarin juices produce a highly desirable bright orange color (*7*).

At present, carotenoid determination in foods consists of several stages: extraction, saponification, and analysis by HPLC, in the main. Analysis in triplicate of a sample can last several hours, so there is a considerable risk of degradation, because carotenoids are highly unstable. Objective measurement of color, in contrast, has several advantages, because it is a nondestructive technique that makes it possible to obtain several parameters in a few seconds.

The correlation between some color parameters and pigments content in foods has been evaluated in some studies by simple statistical methods (*8*−*11*), although the relationships between the individual carotenoids and the color parameters have not been studied exhaustively. The aim of this paper was to study, by means of statistical multivariate analysis, the application of the instrumental analysis of orange juice color to estimate the content of the main carotenoids related to the color of orange juice. Furthermore, this work is also aimed at studying the individual contribution of carotenoids to the color of the product. Due to the advantages mentioned above, the objective measurement of orange juice by means of tristimulus colorimetry might be a very useful tool for quality control of carotenoids in the industry. In this sense, it has also been our objective to estimate the retinol equivalents due to the provitamin A carotenoids of the juice analyzed, by means of color parameters.

MATERIALS AND METHODS

Samples. Seventeen ultrafrozen Valencia late orange juices were analyzed. The samples were obtained directly from the industry, which

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was asked to provide representative juices of the whole season. In this sense, samples corresponding to the beginning, the middle and the end of the season were supplied. In the industry, the juices are cooled and immediately frozen using liquid nitrogen in an industrial freezing tunnel. The commercial samples are kept in freezing chambers between −18 and −21 °C until its distribution. The samples analyzed in this study were kept at −21 °C until analysis. Thawing was carried out at room temperature (23 °C) for 24 h.

Sample Preparation. The orange juice bottle was vigorously shaken before taking three 10-mL aliquots. Each aliquot was centrifuged for 5 min at 5000 rpm. The clarified juice was discarded and the pellet containing the carotenoids was successively centrifuged with methanol (5 min at 5000 rpm, 5 mL methanol) until no more color remained. The extractant contained a small proportion of butylated hydroxytoluene (BHT) (0.004%) (*12*−*14*). The colored methanol phases containing the carotenoids were combined and used in the saponification reaction. For the saponification of the extract, 10 mL of methanolic potassium hydroxide (10%, w/v) was added and the mixture allowed to stand in the dark at room temperature for 1 h. The mixture was transferred to a separatory funnel and carotenoids were extracted with 30 mL of methylene chloride containing BHT (0.004%). The potassium hydroxide was removed by washing four times with water (15−20 mL). The yellow extract was evaporated to dryness at room temperature and finally redissolved in 1.5 mL of methanol/acetone (2:1, v/v). Before the chromatographic analysis, the aliquot was filtered using Millipore PVDF Millex filters (13 mm × 0.45μm) (Bedford, MA). Operations were carried out under dim light, and all samples were analyzed in triplicate.

High-Performance Liquid Chromatography. The HPLC apparatus employed consisted of a Hewlett-Packard 1050 system, equipped with an isocratic pump and a UV−vis detector. A 50-μL loop was used for injection. The column was a C18 Kromasil 5 μm (250 × 4.6 mm) with a guard-column of the same material (10 × 4 mm) (Hichrom Ltd., Reading, UK). The mobile phase was a quaternary mixture of methanol/acetonitrile/methylene chloride/water (50:30:15:5, v/v/v/v) to which 0.1% BHT and 0.1% triethylamine was added. All solvents were HPLC grade. The column was kept at room temperature (23 °C) and the flow rate was 2.5 mL/min. The wavelength was adjusted to 486 nm. The chromatograms were registered using a ChemStation software (Hewlett-Packard, Palo Alto, CA). More details regarding the chromatographic method used were given in a previous work (*15*).

Quantitative Analysis. The concentrations of carotenoid pigments were expressed as relative percentage of total peak area. To calculate the absolute concentrations of the provitamin A carotenoids, β-carotene was used as external standard.

Color Measurement. Color measurements were made with a CAS 140 B spectroradiometer (Instrument Systems, Munich, Germany), equipped with a Top 100 telescope optical probe and a Tamron zoom mod. SP 23A. For this purpose, a plastic cuvette for reflectance measurements was used (475 × 350 × 10 mm). Blank measurements were made with the cuvette filled with distilled water against a reference white background ($BaSO_4$ pressed plate). An aliquot of each juice was measured against a white background and a black background, because some studies have shown that sometimes reflectance measurements against a black background (*16*−*19*) are better correlated with visual assessment of color and other parameters. The spectroradiometer was set to take three consecutive measurements of each sample, so color coordinates obtained were averages of three measurements.

The whole visible spectrum (380−770 nm) was recorded ($\Delta\lambda = 1$ nm) and Illuminant D65 and 10° Observer were considered as references. The color parameters corresponding to the uniform color space CIELAB (*20*) were obtained directly from the apparatus. Within the uniform space CIELAB, two color coordinates, a^* and b^*, as well as a psychometric index of lightness, L^*, are defined. a^* takes positive values for reddish colors and negative values for the greenish ones, whereas b^* takes positive values for yellowish colors and negative values for the bluish ones. L^* is an approximate measurement of luminosity, which is the property according to which each color can be considered as equivalent to a member of the gray scale, between black and white, taking values within the range 0−100.

Table 1. Relative Content of Carotenoid Pigments in the Valencia Late Ultrafrozen Orange Juices Analyzed ($n = 17$)

carotenoid	relative content ± SD
neoxanthin	5.73 ± 0.60
violaxanthin	7.26 ± 0.65
antheraxanthin	14.33 ± 0.94
lutein + zeaxanthin	35.65 ± 1.92
lutein-5,6-epoxide	15.69 ± 1.07
α-cryptoxanthin	4.66 ± 0.77
β-cryptoxanthin	11.68 ± 2.45
α-carotene	1.57 ± 0.70
β-carotene	3.40 ± 0.52

From the uniform color spaces, new parameters are defined, such as chroma (C_{ab}^*) and hue (h_{ab})

$$C_{ab}^* = [(a^*)^2 + (b^*)^2]^{1/2} \quad h_{ab} = \arctan(b^*/a^*)$$

Chroma (C_{ab}^*) is the attribute that allows the determination of the degree of difference in comparison to a gray color with the same lightness for each hue, so it is considered the quantitative attribute of colorfullness. Hue (h_{ab}) is the attribute according to which colors have been traditionally defined as reddish, greenish, etc. It is the attribute that allows a color to be distinguished with reference to a gray color with the same lightness. This attribute is related to the differences in absorbance at different wavelengths and is considered the qualitative attribute of color.

Statistical Analysis. Correlations between carotenoids content and color parameters were studied by both simple and multiple regression. Multiple regression analyses were carried out by means of partial least squares (PLS). PLS can be used in situations in which the usage of traditional multivariate statistical methods might be limited, because PLS is a quite robust method.

For all statistical analyses, Statistica v. 5.5 software (*21*) was used.

RESULTS AND DISCUSSION

Chromatographic Determination of Carotenoids. Table 1 shows the relative percentage of the carotenoids of interest in the orange juice analyzed. Carotenoids are usually monitored at 450 nm; however, in this study, the wavelength 486 nm has been chosen because it is more selective for the meaningful orange juice carotenoids as far as color is concerned (*22*), because the absorption in the red-orange region of the visible spectrum is maximum at this wavelength. This more selective wavelength allows the determination by means of an isocratic elution of those carotenoids more related to the color of the orange juice, which is very suitable for the industry, because analysis time is shorter in comparison to gradient methods. The major carotenoids in the ultrafrozen juices analyzed were lutein + zeaxanthin, lutein-5,6-epoxide, antheraxanthin, and β-cryptoxanthin. The last one is considered the main source of provitamin A in orange juice. The other provitamin A carotenoids, α-carotene and β-carotene, occurred in very low proportions.

Color Characterization. The color characteristics of the orange juice are shown in **Table 2**. One of the peculiarities of the orange juice analyzed in this study is its deep orange color, because the juice is subjected neither to high temperatures nor to concentration process during the production.

For both backgrounds, samples were located within the first quadrant, showing a^* and b^* positive values. As it can be observed, a^* and b^* values were lower when the black background was used. L^* is a relative measurement between the light reflected and absorbed by the samples, so its values were lower when the black background was used. Chroma (C_{ab}^*) showed higher values for the white background. How-

Table 2. Color Parameters of the Orange Juices

	L^*	a^*	b^*	C_{ab}^*	h_{ab}
			white background		
mean ± SD	71.61 ± 0.87	25.04 ± 1.29	78.02 ± 3.73	81.95 ± 3.75	72.19 ± 0.85
range	69.85–72.80	20.94–26.42	69.47–88.66	72.56–91.93	71.34–74.69
			black background		
mean ± SD	63.23 ± 1.27	16.18 ± 0.56	64.64 ± 3.78	66.64 ± 3.66	75.90 ± 0.91
range	61.39–66.29	15.02–17.05	60.38–76.73	62.48–78.27	74.78–78.63

Table 3. Correlation Matrices between Chromatic Parameters and Relative Contents of Carotenoids

	L^*	a^*	b^*	C_{ab}^*	h_{ab}
		white background			
neoxanthin	0.009	0.180	0.160	0.171	–0.056
violaxanthin	0.077	0.072	–0.304	–0.282	–0.336
antheraxanthin	–0.092	–0.009	–0.524	–0.500	–0.442
lutein + zeaxanthin	–0.188	–0.107	–0.640	–0.621	–0.434
lutein–5,6–epoxide	–0.448	0.432	–0.308	–0.249	–0.685
α–cryptoxanthin	0.235	–0.177	0.475	0.434	0.580
β–cryptoxanthin	0.206	–0.054	0.603	0.569	0.569
α–carotene	–0.014	–0.089	0.023	0.013	0.104
β–carotene	0.314	–0.067	0.585	0.550	0.563
		black background			
neoxanthin	0.087	–0.164	0.131	0.126	0.193
violaxanthin	–0.050	0.166	–0.346	–0.343	–0.347
antheraxanthin	–0.354	0.113	–0.591	–0.591	–0.506
lutein + zeaxanthin	–0.502	0.210	–0.655	–0.652	–0.606
lutein–5,6–epoxide	–0.473	0.500	–0.518	–0.504	–0.636
α-cryptoxanthin	0.438	–0.252	0.605	0.600	0.587
β–cryptoxanthin	0.466	–0.278	0.691	0.686	0.665
α–carotene	0.079	–0.025	0.055	0.055	0.044
β–carotene	0.485	–0.244	0.643	0.640	0.602

ever, hue (h_{ab}) showed higher values for the black background. For any of the background used, chroma and hue values agreed with vivid orange colors, which are typical in Valencia late juices.

The colorimetric analysis of the orange juices analyzed in this study indicated that the color of the product is very constant during the season. **Table 2** shows that standard deviations of the color parameters were quite low in any of the background used, although the variability of L^* values was a bit higher for the black background. Variability for b^* values was higher than that for a^* values, independent of the background used in the measurements, and a^* showed higher standard deviation for white background.

Statistical Analysis. As a preliminary stage, the relation between the color parameters and the carotenoids content was explored by means of simple correlations (**Table 3**). The usage of a black background led to the highest correlations. L^* and a^* were the color parameters less correlated with the carotenoid content, mainly when the white background was used. This fact indicates that the yellow component (b^*) is the most important in relation to the definition of the juice color. Significant correlations ($p < 0.05$) were found between b^*, C_{ab}^* and h_{ab} and the content of β-cryptoxanthin (0.691, 0.686, and 0.665, respectively), lutein + zeaxanthin (−0.655, −0.652, and −0.606, respectively) and β-carotene (0.643, 0.640, and 0.602, respectively) for measurements made against a black background. Independently of the background used, the content of α-carotene was the least correlated with those color parameters.

However, to define the color of any object completely by means of Tristimulus Colorimetry, the scalar coordinates L^*, a^*, b^*, or L^* and the angular coordinates (h_{ab} and C_{ab}^*) have to be considered together, due to the tridimensional nature of color. Hence, to achieve a more accurate evaluation of the correlation between color and carotenoid pigments, multiple regression studies by means of partial least squares (PLS) were explored. The content of each carotenoid was considered as dependent variable and the parameters L^*, a^*, b^* and L^*, C_{ab}^*, h_{ab} were considered as predictor or independent variables. R^2 values obtained are shown in **Table 4**. As it can be observed, there were large differences in these values neither as a function of the background used nor as a function of the set of predictor parameters considered. The highest R^2 values were obtained for the contents of lutein + zeaxantin, lutein-5,6-epoxide, antheraxanthin, violaxanthin, and neoxanthin, in this order (**Table 4**). For these carotenoids, it can also be observed that the higher relative content the higher R^2 value, although differences in these R^2 values are minimum. This appreciation was not applicable to α- and β-cryptoxanthin and α- and β-carotene. In the case of α-cryptoxanthin and β-carotene, correlations were higher than those corresponding to β-cryptoxanthin, despite their lower levels (4.66 and 3.40%, respectively, in comparison to 11.68%). In this sense, R^2 values for α-carotene might also indicate that this carotenoid has a remarkable qualitative importance on the color of orange juice, because its content in the orange juice analyzed is nearly insignificant (1.57%). This might suggest that β-cryptoxanthin is not the main carotenoid related to the color of orange juices, as it was supposed (*7*).

Table 4. R^2 Values for the Correlations between Carotenoid Levels and Color Parameters Explored by Multiple Regression by Means of PLS

dependent variables	predictor variables: white background		black background	
	L^*, a^*, b^*	L^*, C_{ab}^*, h_{ab}	L^*, a^*, b^*	L^*, C_{ab}^*, h_{ab}
neoxanthin	0.990	0.990	0.990	0.990
violaxanthin	0.991	0.991	0.990	0.990
antheraxanthin	0.994	0.994	0.992	0.994
lutein + zeaxanthin	0.995	0.995	0.994	0.995
lutein-5,6-epoxide	0.994	0.994	0.992	0.993
α-cryptoxanthin	0.978	0.978	0.980	0.979
β-cryptoxanthin	0.966	0.965	0.968	0.966
α-carotene	0.844	0.844	0.845	0.845
β-carotene	0.982	0.981	0.984	0.982

In relation to the contribution of each predictor variable to the regression models mentioned above, there were qualitative differences depending on the background employed in the measurements. For white background, when the scalar parameters (L^*, a^*, b^*) were considered, yellowness (b^*) was the variable with the highest weight in the models, followed by L^*, with a similar weight, and a^* with very low weight. When the psychometric parameters (L^*, C_{ab}^* and h_{ab}) were considered, C_{ab}^* was the color parameter with the highest weight, followed by L^* and h_{ab}, both with a similar contribution. On the other hand, when the black background was used, b^* was also the scalar parameter with the highest weight in the models, but the contribution of L^* in the regression models increased and the

Table 5. Regression Equations for the Calculation of Carotenoid Levels as a Function of Scalar Color Parameters

	white background
neoxanthin	$0.034605L^* + 0.012112a^* + 0.037730b^* + 0.000512$
violaxanthin	$0.043844L^* + 0.015336a^* + 0.047706b^* + 0.000648$
anteraxanthin	$0.086568L^* + 0.030273a^* + 0.094175b^* + 0.001280$
lutein + zeaxanthin	$0.215388L^* + 0.075307a^* + 0.234326b^* + 0.003185$
lutein-5,6-epoxide	$0.094744L^* + 0.033190a^* + 0.103161b^* + 0.001401$
α-cryptoxanthin	$0.028146L^* + 0.009824a^* + 0.030760b^* + 0.000416$
β-cryptoxanthin	$0.070598L^* + 0.024662a^* + 0.077316b^* + 0.001043$
α-carotene	$0.009468\ L^* + 0.003305a^* + 0.010321b^* + 0.000140$
β-carotene	$0.020557L^* + 0.007181a^* + 0.022475b^* + 0.000304$
	black background
neoxanthin	$0.042873L^* + 0.010964a^* + 0.043853b^* + 0.000718$
violaxanthin	$0.054302L^* + 0.013905a^* + 0.055420b^* + 0.000909$
antheraxanthin	$0.107193L^* + 0.027452a^* + 0.109392b^* + 0.001796$
lutein + zeaxanthin	$0.266699L^* + 0.068314a^* + 0.272242b^* + 0.004468$
lutein-5,6-epoxide	$0.117330L^* + 0.030079a^* + 0.119781b^* + 0.001966$
α-cryptoxanthin	$0.034897L^* + 0.008907a^* + 0.035822b^* + 0.000584$
β-cryptoxanthin	$0.087569L^* + 0.022328a^* + 0.090066b^* + 0.001464$
α-carotene	$0.011737L^* + 0.003001a^* + 0.012006b^* + 0.000196$
β-carotene	$0.025486L^* + 0.006505a^* + 0.026159b^* + 0.000426$

weight of a^* was even lower than in the white background. For the psychometric parameters, hue was the variable with the highest weight and the contribution of chroma and L^* to the regression models was similar.

Equations that allow to calculate the relative percentages of carotenoids as a function of color parameters were calculated (**Table 5**) based on scalar parameters.

Taken into account that, for measurements made against the white background, b^* and C_{ab}^* are the parameters with the highest weights in the models, the contribution of the carotenoids considered jointly to those parameters has been calculated. For this purpose, b^* and C_{ab}^*, were considered as dependent variables separately, and the relative contents of carotenoids were considered as predictor variables. R^2 values obtained for b^* and C_{ab}^* were 0.995 and 0.996, respectively. In both cases, it was observed that the higher relative content, the higher contribution to the color parameter considered. In the case of the measurements made against the black background, b^* and h_{ab} were considered as dependent variables, and R^2 values obtained were 0.994 and 0.999, respectively. Regarding the contribution of carotenoids to those color parameters, it was also observed that the higher relative content, the higher weight in the model.

For quality control purposes in the industry, it might be very interesting to have almost on-line information regarding the contents of provitamin A carotenoids during the production, due to their nutritional importance. In this sense, objective measurement of color by Tristimulus Colorimetry could be a quite important tool. The average levels of provitamin A carotenoids in the juice are shown in **Table 6**. Retinol equivalents, taken into account the new recommendations (*23*), were calculated as follows:

$$\text{RE} = (\mu\text{g } \beta\text{-carotene}/12) + (\mu\text{g } \alpha\text{-carotene} + \mu\text{g } \beta\text{-cryptoxanthin}/24)$$

The correlations between RE and the color parameters were explored by PLS. For color measurements made against the white background, R^2 values obtained were 0.890 and 0.889, when L^*, a^*, b^* and L^*, C^*_{ab}, h_{ab} were considered as predictor variables, respectively. When the measurements were made against the black background, R^2 values obtained were slightly higher, 0.892 and 0.890, for L^*, a^*, b^* and L^*, C^*_{ab}, h_{ab} as predictor variables, respectively. Equations that allow calculations of the retinol equivalents (RE) in the orange juice as a function of color parameters have been calculated, considering the scalar coordinates:

Table 6. Levels of Provitamin A Carotenoids in Valencia Late Ultrafrozen Orange Juices by HPLC ($n = 17$)

carotenoid	avg content (mg/l)	range
β-cryptoxanthin	0.69 ± 0.27	0.33–1.21
α-carotene	0.11 ± 0.05	n.d.–0.23
β-carotene	0.21 ± 0.07	0.11–0.36

for white background

$$\text{RE} = 0.308382L^* + 0.108054a^* + 0.337946b^* + 0.004563$$

for black background

$$\text{RE} = 0.382527L^* + 0.097892a^* + 0.393434b^* + 0.006401$$

Average RE values per liter of juice, obtained by means of these formulas were 51.16 ± 1.36 and 51.21 ± 1.70, respectively, whereas average RE value considering provitamin A content determined by HPLC was 51.07 ± 18.89.

The results obtained in this study indicate that it seems feasible to estimate the provitamin A content in orange juice by means of color parameters, applying Tristimulus Colorimetry and multivariate statistics. For the best results, black background should be used.

These equations could be used, as a first step, in the quality control of juices showing quite constant color during the whole season, although further studies should be carried out to extend the applicability of tristimulus colorimetry to estimate the carotenoids content in juices differing greatly in color characteristics.

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