A NOVEL AND ENHANCED APPROACH FOR THE ASSESSMENT OF THE TOTAL CAROTENOID CONTENT OF FOODS BASED ON MULTIPOINT SPECTROSCOPIC MEASUREMENTS

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ABSTRACT

We have devised a more sensible approach to estimate the carotenoid content of orange juices, which can be regarded as a model system of food with intricate carotenoid pattern. For this purpose spectroscopic information at several wavelengths and spectra of the juices and not from their carotenoid extracts were considered, such that more accurate and rapid quantitative assessments can be achieved. The wavelengths proposed on the basis of the characteristic vector method were 420, 455, 515, 545 and 610 nm or 420, 445, 510, 545 and 605, depending on the measurement conditions. The correlations between the carotenoid content and the reflectances at these wavelengths were very good (R = 0.94 and 0.90, respectively). Additionally, it was demonstrated that the colour of the juices could be assessed with very good accuracy considering them. Due to its simplicity and rapidity, this method is intended to facilitate the quality control of the carotenoid content of foodstuffs in the industry and/or in the field.

KEYWORDS: carotenoids; characteristic vector; citrus; colour; foods; orange juice; quality control; tristimulus colorimetry

INTRODUCTION

The carotenoids are isoprenoid compounds biosynthesized by plants, fungi, algae and bacteria that animals have to incorporate through the diet (Fraser & Bramley, 2004). They are natural pigments that provide colour to many structures (flowers, fruits, seeds, tubers, roots, feathers, egg yolk, etc.) and therefore play key roles in the communication between animals, the pollination, the dispersal of seeds, and the acceptability of foods, among others. Pertaining to the involvement of carotenoids in acceptability of foods it is important to consider that some aroma compounds (like safranal, β-ionone, β-damascenone, etc.) are cleavage products of carotenoids (Baldermann, Naim & Fleischmann, 2005; Carmona, Zalacain, Salinas & Alonso, 2006; Silva Ferreira, Monteiro, Oliveira & Guedes de Pinho, 2008; Fleischmann & Zorn, 2008). Nevertheless, they also play essential roles in the photosynthesis, contributing to the harvesting of light and, what it is more important, protecting the photosynthetic organisms from the deleterious effects derived from photooxidation (Telfer, Pascal & Gall, 2008). Additionally they are beneficial for animals and not only because some act as vitamin A precursors. In this regard, it is well-known that some carotenoids exhibit antioxidant activity (Burton, 1989; Demmig-Adams & Adams III, 2002; Astley, Hughes, Wright, Elliott & Southon, 2004) and evidence is accumulating that they can be beneficial for the prevention of serious human disorders (cancer, cardiovascular disease, age-related macular degeneration, etc.) (Krinsky, 1989; Olson, 1999; Giovanucci, 2002).(Voutilainen, Nurmi, Mursu & Rissanen, 2006; Trumbo & Ellwood, 2006; Lidebjer, Leanderson, Ernerudh & Jonasson, 2007).

Due to all the facts enumerated above, the need to assess the carotenoids occurring in foodstuffs is indisputable. Although scientists can choose from a wide choice of HPLC methods to determine them, more rapid methods are needed in some

situations, such as quality control in the industry and/or the field. In this regard, it is important to note that the chromatographic analysis of carotenoids involves typically several steps (extraction, saponification, chromatographic analysis), so they are not appropriate for almost real-time assessments. Even though the estimation of the total carotenoid content of a source by spectrophotometry is still commonplace in some laboratories, such procedure presents drawbacks. The most important one is without any doubt that the accuracy of these measurements is far from desirable, since the extinction coefficients for carotenoids are not as accurate as they should be and sometimes even arbitrary coefficients are used (Britton & Young, 1993). On the other hand, only the spectroscopic information at one point is considered, despite the fact that the visible spectra of most carotenoids have three maxima within an interval of some 40 nm. Additionally, the pigments have to be extracted from the food, which inevitably requires some time and poses a drawback if the levels of carotenoids have to be determined on site.

In this paper we describe a novel approach that simplifies and improves the determination of the total carotenoid content of foods by considering spectroscopic information of the food itself (not from an extract) at several wavelengths. Additionally, this procedure allows to accurately assess the colour of the product, which is one of the main attributes related to its acceptability.

EXPERIMENTAL PROCEDURES

Samples

Eighty-two commercially available orange juices (31 ultrafrozen and 51 thermally-treated juices) were analyzed. The samples were selected such that they covered all the possible colours of orange juice that can be found in the Spanish market.

The thermally-treated samples were purchased from retailers based in Seville (Spain) and the deep-frozen ones were kindly provided by Zumos Vitafresh. The manufacturers' recommendations concerning the storage of the orange juices were followed until their analysis. The deep-frozen orange juices were thawed at ambient temperature.

The samples were considered independently in order to obtain the maximum possible variability and make the correlations between colour or spectroscopic information and pigments more robust since the set of samples varied widely in these parameters.

Carotenoid analysis

The analysis of carotenoids was carried out according to the routine protocol used in our laboratory. This methodology is fully explained elsewhere (Meléndez-Martínez, Vicario & Heredia, 2007a). In brief the carotenoids were extracted with a mixture hexane/methanol/acetone (50:25:25, v/v/v, containing 0.1% butylated hydroxytoluene) and subsequently subjected for 1 hour to an alkaline hydrolysis with ethanolic KOH (10% w/v) under dim light and at room temperature. After washing four times with water the coloured extracts were dried out on a rotary evaporator and re-dissolved in 1 ml of acetone:methanol (1:2, v/v, containing 0.1% butylated hydroxytoluene) for the HPLC analyses.

The HPLC analysis were performed on an Agilent 1100 system fitted with a quaternary pump, a column temperature control module, a PDA detector and an autosampler set to draw 20 μl from the vials (Agilent, Palo Alto, CA, United States). The carotenoids were separated on an YMC C_{30} column (250 × 4.6 mm, 5 μm) (YMC, Wilmington, NC, USA). Water, methanol (MeOH) and methyl-ter-butyl ether (MTBE)

were used in the mobile phase according to a gradient elution. The flow rate was 1ml/min and the peaks were monitored at 430 nm.

Most of the carotenoids detected were identified by comparison of their UV/Vis spectroscopic and chromatographic characteristics with those of reference standards isolated from appropriate sources or semi-synthesized. These standards were stereomutated to help identify geometrical isomers. More detailed information concerning the identification of the carotenoids occurring in orange juices is provided in previous papers.

The absolute concentrations of the pigments were worked out by external calibration and the total contents of carotenoids as the sum of the content of the individual pigments.

Colour measurement

A more detailed description of the methodology followed for the objective colour measurements can be found anywhere else (Meléndez-Martínez, Vicario & Heredia, 2004). In brief, visible reflectance measurements (380-770 nm) were recorded at 1 nm intervals on a CAS 140 B spectroradiometer (Instrument Systems, Munich, Germany) equipped with a Tamron zoom mod. SP 23A (Tamron USA, Inc., Commack, NY, USA), a Top 100 telescope optical probe (Instrument Systems, Munich, Germany) and an external incandescent lamp. The apparatus takes three consecutive measurements at 1 second intervals in order to compensate for possible variations in the conditions of the measurements, such that the colour parameters obtained are averages of that triplicate.

The within-laboratory repeatability (within-day precision) was developed according to ISO-5725-2:1994 (ISO, 1994). It was ascertained by analyzing ten times within the same day the colour of an orange juice. The repeatability is expressed as the

coefficient of variation (CV) or relative standard deviation (percentage of SD), being lower than 3.6 % for all the CIELAB colorimetric variables (0.66% for L*, 3.57% for a*, 2.40% for b*, 2.41% for C^*_{ab}, and 0.29% for h_{ab}).

The Illuminant D_{65} and the 10° Observer were taken as references and the colour coordinates of the uniform colour space CIELAB were considered (CIE, 1978).

A transparent plastic cuvette was used for the measurements. The blank measurements were made with the cuvette filled with distilled water and placed against a reference $BaSO_4$ pressed plate (USRS-99-010, Labsphere Inc. North Sutton, NH, USA). The samples were measured against a white and a black background because it has been reported that the colour parameters obtained by using one or another are differently correlated with the visual perception of the colour and other parameters (Meléndez-Martínez et al., 2004).

Mathematical method of the characteristic vector method

The analysis of the characteristic vectors has been carried out following the adaptation of the method made by Simonds (Simonds, 1963), using software developed by Laboratorio de Color de la Rioja.

The fundamentals of the characteristic vector analysis (Lebart, Morineau & Fenelon, 1985) can be summarized as follows: the response data ρ_λ (visible reflectance spectrum) are available for r = 301 levels of the variable λ (wavelength) and can be plotted as a curve. Consequently, for each sample the r values of ρ_λ make up a one-row r-column vector of response data. For *n* samples the vectors can be laid out as a matrix of r columns and n rows. In this regard, a set of p characteristic vectors ($p < r$), can be found such that, when they are properly added to the mean response vector, they will approximate to any of the primary response vectors. The outcome is that the components of the generic vectors, that is, the reflectance spectra, can be expressed as:

$$\rho_\lambda = \overline{\rho}_\lambda + M_1 V_{1,\lambda} + M_2 V_{2,\lambda} + ... + M_p V_{p,\lambda} \qquad \text{Eq. [1]}$$

In this equation, ρ_λ refers to the spectral reflectance of the sample, $\overline{\rho}_\lambda$ to the component of the mean vector, $V_{i,\lambda}$ to the components of the characteristic i^{th} vector and M_i to the specific coefficients, named scalar multiples, of every reconstructed vector.

This analysis is to be applied to *n* experimental curves. The model computes the M_i coefficients necessary for the reconstruction of each of them, as well as the percentage of the variability among the family of homologous response curves explained for each characteristic vector. The formulae to work out the tristimulus values, in accordance to the Commission Internationale de l'Eclairage (CIE) recommendations, would be:

$$X_{10} = k \sum_{\lambda=380}^{\lambda=780} \rho_\lambda L_\lambda \bar{x}_\lambda \Delta\lambda \qquad Y_{10} = k \sum_{\lambda=380}^{\lambda=780} \rho_\lambda L_\lambda \bar{y}_\lambda \Delta\lambda \qquad Z_{10} = k \sum_{\lambda=380}^{\lambda=780} \rho_\lambda L_\lambda \bar{z}_\lambda \Delta\lambda \qquad \text{Eq.[2]}$$

where *k* is a normalizing factor, L_λ the spectral emission of the reference illuminant, $\bar{x}_\lambda$, $\bar{y}_\lambda$, $\bar{z}_\lambda$, the colour-matching functions corresponding to the observer consider as reference, and $\Delta\lambda$ the interval of the spectral measurements.

If Eq. [1] is substituted into Eq. [2], the following expressions would be obtained for the tristimulus values (in this case X, although analogous expressions are obtained for Y and Z):

$$X_{10} = k \sum_{\lambda=380}^{\lambda=780} \left(\overline{\rho}_\lambda + M_1 V_{1,\lambda} + M_2 V_{2,\lambda} + \cdots + M_p V_{p,\lambda}\right) L_\lambda \bar{x}_\lambda \Delta\lambda$$

Expressed otherwise:

$$X_{10} = k \sum_{\lambda=380}^{\lambda=780} \overline{\rho}_\lambda L_\lambda \bar{x}_\lambda \Delta\lambda + M_1 k \sum_{\lambda=380}^{\lambda=780} V_{1,\lambda} L_\lambda \bar{x}_\lambda \Delta\lambda + \cdots + M_p k \sum_{\lambda=380}^{\lambda=780} V_{p,\lambda} L_\lambda \bar{x}_\lambda \Delta\lambda \qquad \text{Eq.[3]}$$

as the M_i coefficients are not dependent on the wavelength for any single reconstructed curve.

Calling

$$X_i = k \sum_{\lambda=380}^{\lambda=780} V_{i,\lambda} L_\lambda \bar{x}_\lambda \Delta\lambda$$

where (i=1,...,*p*), Eq. [3] can be expressed in this way:

$$X_{10} = X_{0,10} + M_1 X_{1,10} + M_2 X_{2,10} + ... + M_p X_{p,10} \qquad \text{Eq. [4]}$$

being $X_{0,10}$ the tristimulus value corresponding to the mean reflectance spectrum and the $X_{i,10}$ could be considered the theoretical tristimulus values of each characteristic vector.

To apply the results to spectra from any given sample, the corresponding M_i coefficients must be calculated. For this purpose, the $\rho_{\lambda i}$ reflectances at as many wavelengths as characteristic vectors appear in Eq. [1] must be measured, and the following algorithms must be solved, being the values of $\bar{\rho}_{\lambda i}$ and $V_{j,\lambda i}$ results previously obtained:

$$\begin{aligned}
\rho_{\lambda 1} &= \bar{\rho}_{\lambda 1} + M_1 V_{1,\lambda 1} + M_2 V_{2,\lambda 1} + ... + M_p V_{p,\lambda 1} \\
\rho_{\lambda 2} &= \bar{\rho}_{\lambda 2} + M_1 V_{1,\lambda 2} + M_2 V_{2,\lambda 2} + ... + M_p V_{p,\lambda 2} \\
&..... \\
\rho_{\lambda p} &= \bar{\rho}_{\lambda p} + M_1 V_{1,\lambda p} + M_2 V_{2,\lambda p} + ... + M_p V_{p,\lambda p}
\end{aligned} \qquad \text{Eq. [5]}$$

Due to the fact that the M_i coefficients obtained after the application of Eq. [5] are functions of the measured reflectances, $\rho_{\lambda i}$, they can be substituted in Eq. [4]. As a result, Eq. [6] is obtained, where the coefficients of $\rho_{\lambda i}$ are expressed as C_{iX} :

$$X_{10} = C_{0X_{10}} + C_{1X_{10}} \rho_{\lambda 1} + C_{2X_{10}} \rho_{\lambda 2} + ... + C_{pX_{10}} \rho_{\lambda p} \qquad \text{Eq. [6]}$$

Analogously Y and Z could be worked out by applying the following formulae, which express the tristimulus values as a function of the measured reflectances:

$$Y_{10} = C_{0Y_{10}} + C_{1Y_{10}} \rho_{\lambda 1} + C_{2Y_{10}} \rho_{\lambda 2} + ... + C_{pY_{10}} \rho_{\lambda p}$$

$$Z_{10} = C_{0Z_{10}} + C_{1Z_{10}} \rho_{\lambda 1} + C_{2Z_{10}} \rho_{\lambda 2} + ... + C_{pZ_{10}} \rho_{\lambda p}$$

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. $C_{\frac{1}{0}} \quad {}_{2} + \quad + \quad {}_{pZ} \ \rho_{\lambda p}$

Upon the computation of the first six characteristic vectors, and, taking into account the high percentage of the variability of data explained by them (specifically 0.9976 for white backing and 0.9804 for black backing), six reconstructions of each spectrum were performed using the mathematical method. This method starts with the mean and the first characteristic vectors and then adds the corresponding characteristic vector to each following reconstruction, subsequently multiplying each vector V_i by its M_i coefficient. In theory, any set of p wavelengths could be used to obtain the reflectances $\rho_{\lambda i}$ in Eq. [5], even though it has been found that it is necessary to select, using a scanning process, those which give the best results for calculating the tristimulus values. From the tristimulus values, the colour coordinates in whatever CIE system can be calculated. In this study, the colour coordinates of the uniform colour space CIELAB were computed (CIE, 1978), from both the experimental and the reconstructed spectra, considering the CIE 1964 standard observer and the D_{65} standard illuminant.

Colour differences have been worked out using the coordinates obtained after applying the CIE method to the experimental spectra as reference coordinates and the ones obtained after applying the characteristic vector method as "recalculated coordinates".

Multivariate statistical analysis

The Statistica v.6.0 software (Statsoft, 2001) was used for the statistical analyses of the data. Multiple regressions analyses were performed in order to evaluate the correlations existing between the colour of the orange juices and the total content in carotenoids.

RESULTS AND DISCUSSION

Orange juice is one of the most intricate sources of carotenoids among foods. Whilst carotenoid-rich products like for instance carrots and tomatoes for instance

contain mainly carotenes (Marx, Schieber & Carle, 2000; Seybold, Fröhlich, Bitsch, Otto & Böhm, 2004), orange juice contains carotenes, monohydroxyxanthophylls, dihydroxyxanthophylls, 5,6-epoxycarotenoids and 5,8-epoxycarotenoids. More specifically, the major carotenoids by far in carrots and tomatoes are β-carotene (β,β-carotene) and lycopene (ψ,ψ-carotene), whilst, in the case of orange juice, there are several carotenoids which are important quantitatively, such as violaxanthin (5,6:5',6'-diepoxy-5,6,5',6'-tetrahydro-β,β-carotene-3,3'-diol) or its 5,8-epoxycarotenoid isomer auroxanthin (5,8:5',8'-diepoxy-5,8,5',8'-tetrahydro-β,β-carotene-3,3'-diol), antheraxanthin (5,6-epoxy-5,6-dihydro-β,β-carotene-3,3′-diol) and its 5,8-epoxycarotenoid isomer mutatoxanthin (5,8-epoxy-5,8-dihydro-β,β-carotene-3,3′-diol), β-cryptoxanthin (β,β-caroten-3-ol) and zeaxanthin (β,β-carotene-3,3'-diol) (Melendez-Martinez, Britton, Vicario & Heredia, 2008). These facts have important implications in relation to the visible spectra of the carotenoid extracts of these foods. Thus, those corresponding to carrots and tomatoes bear a certain resemblance to the ones corresponding to their major carotenoids. This signifies that, for certain purposes, a rapid rough estimation of the total carotenoid content in these sources by spectrophotometric measurements may be carried out considering the wavelength of maximum absorption and the extinction coefficients corresponding to those major carotenoids. In the case of orange juices, since the carotenoid pattern is much more intricate, the visible spectrum does not resemble clearly that of a given carotenoid. Consequently, the inherent inaccuracy of the spectrophotometric determination of the total carotenoid content is much higher in orange juices than in carrots and tomatoes. In this regard, we have reported recently that the assessment of the carotenoid content of orange juices considering the absorption of the extract at only one wavelength was very inaccurate (Melendez-Martinez et al., 2008).

A typical absorption spectrum in the visible region of the spectra (380-770 nm) of an orange juice carotenoid extract is depicted in Figure 1a. The comparison between this figure and Figure 1b (visible reflection spectra of an orange juice) is very useful to infer that the colour of the orange juice is mainly owed to its carotenoid fraction. Thus, regardless of the background used for the measurements, it can be clearly observed that the reflectance of the juice is lowest in the region of the visible spectrum where the carotenoids typically absorb maximally and that the reflectance is maximum where the carotenoids do not absorb. In any case, when comparing Figure 1a and 1b several things have to be considered, which may explain the differences between them. Firstly, the absorption spectra in Figure 1a correspond to carotenoid extracts in organic solvents, whilst the spectra in Figure 1b correspond to an orange juice, that is, an aqueous liquid with carotenoid-containing pulp particles suspended in it. In this regard, it is well-known that the absorption maxima of carotenoids in a lipid and/or protein milieu appear at longer wavelengths relative to those found in solvents like ethanol or hexane (Britton, 1983). Secondly, the pulp particles are very important not only because they contain the pigments, but also because they produce turbidity and scattering, and these phenomena do play an important role in the reflection of light by the juices (Meléndez-Martínez, Vicario & Heredia, 2005). Additionally, some carotenoids under certain circumstances can aggregate in chromoplasts, and these associations do lead to noticeable changes in the absorption of light (Britton, 1995; Kön et al., 2008).

Taking all these facts together we hypothesized that a better estimation of the carotenoid contents of foods with complex carotenoid patterns, like orange juice, should be done by considering more than one wavelength. In addition, since carotenoids account for the colour of orange juice and many other foods, we hypothesized that this assessment may be done considering a visible spectra of the food itself.

277277

278 Characteristic vector method

279 The application of the characteristic vector method leads to a simplification of
280 the measurement of colour with the added advantage that the measurement is very
281 accurate. This methodology has already been successfully applied to assess the colour
282 of several products, like red wines (Ayala, Echávarri, Juárez & Negueruela, 1993),
283 vinegars (García-Parrilla et al., 1998) and virgin olive oils (Moyano et al., 2001).
284 Basically, the mathematical model leads to the selection of a few wavelengths (normally
285 between 3 and 6) from which the spectrum can be reconstructed in a way that this
286 reconstructed spectrum matches the original one. Regarding the tristimulus values
287 (X,Y,Z), they are computed from the reflectance values at the selected wavelengths.

288 When the mathematical model was applied to the dataset corresponding to the
289 measurements made with white background, the following expressions for the
2902 calculation of the tristimulus values were obtained:
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$$X_{10} = -0.573753 + 4.64532\,\rho_{420} + 17.2818\,\rho_{455} - 0.419262\,\rho_{515} + 21.2178\,\rho_{545} + 55.6781\,\rho_{610} + 32.6615\,\rho_{\quad} + 1.75831\,\rho_{\quad} + 14.5068\,\rho_{\quad} + 17.6706\,\rho_{\quad} + 38.9295\,\rho_{\quad} \quad \text{Eq. [7]}$$

$$Y_{10} = -0.455165 - 0.74817\,\rho_{420} + 26.1632\,\rho_{455} - 79.4236\,\rho_{515} + 1.71666\,\rho_{545} + \rho_{610} \quad \text{Eq. [8]}$$

$$Z_{10} = -\rho_{420} + 5.53728\,\rho_{455} - \rho_{515} + 2.06994\,\rho_{545} \quad \text{Eq. [9]}$$

300

Likewise, the following equations were obtained for the measurements made with black background:

$$X_{10} = 0.125891 + 6.09084\ \rho_{420} + 12.8171\ \rho_{445} + 2.57889\ \rho_{510} + 15.1968\ \rho_{545} + 58.9423\ \rho_{605} \quad \text{Eq. [10]}$$

$$Y_{10} = -0.141477 + 2.36885\ \rho_{420} + 4.44265\ \rho_{445} + 22.2363\ \rho_{510} + 33.9817\ \rho_{545} + 37.1951\ \rho_{605} \quad \text{Eq. [11]}$$

$$Z_{10} = 0.423821 + 22.995\ \rho_{420} + 74.9983\ \rho_{445} + 10.288\ \rho_{510} - 1.51727\ \rho_{545} + 0.0107494\ \rho_{605} \quad \text{Eq. [12]}$$

In the light of the results obtained and considering Figure 1b it can be claimed that the reflectance spectra of the orange juices studied can be reconstructed by considering wavelengths at which the absorption of light is very high (420, 445 and 455 nm), intermediate (510, 515 and 545 nm) and low (605 and 610 nm). Since the original reflectance spectra obtained for the juices were clearly different as a function of the background used for the measurements, some of the wavelengths obtained were different depending on the background used for the colour measurements.

The CIELAB colour coordinates corresponding to the 82 orange juice samples were calculated from the tristimulus values obtained considering the equations 7-12. To compare the colour coordinates computed from the original spectra (summarized in Table 1) with the ones calculated from the reconstructed spectra their colour differences were calculated. Notwithstanding that the CIEDE2000 colour difference formula is recommended for industrial applications for small colour differences under reference

conditions (CIE, 2004), we opted for using the CIELAB colour difference formula (CIE, 2004) because visual colour differences were not involved.

Considering the measurements made with white background, the highest differences between the original and the "reconstructed" colour coordinates were 0.99 CIELAB units for a*, 1.69 units for b* and 0.17 units for L*. Regarding the measurements made with black background, the highest differences were 0.6, 1.65, and 0.23 CIELAB units for a*, b* and L*, respectively. In this sense it was observed that the highest colour differences (ΔE_{ab}) were 1.70 and 1.60 CIELAB units for white and black background, respectively. In any case, it was observed that the values of ΔE_{ab} were lower than 0.5 CIELAB units in most of the cases (81% of the cases considering the white background and 74% of the cases considering the black background) (Figure 2). These results indicated that the reflectance spectra of diverse orange juices can be reconstructed very accurately using as few as 5 wavelengths.

Relationships spectroscopic measurements–carotenoid content

Even though the instrumental colour measurements by Tristimulus Colorimetry cannot provide detailed information regarding the carotenoid profile of complex sources of carotenoids they are very useful for other purposes. These measurements have inherent advantages (ultra-rapidity, versatility, non-destructiveness, possibility of automatization, portability, etc.) that make them very appropriate to obtain almost real-time information and therefore amenable for quality control purposes. Thus, the applicability of objective colour assessments to estimate the carotenoid content of diverse foods has been the goal of several studies conducted in the last years (Mínguez-Mosquera, Rejano-Navarro, Gandul-Rojas, Sánchez-Gómez & Garrido-Fernández,

1991; Meléndez-Martínez, Vicario & Heredia, 2003; Konopka, Kozirok & Rotkiewicz,

2004; Ruiz, Reich, Bureau, Renard & Audergon, 2008; Ornelas-Paz, Yahia & Gardea, 2008; Moyano, Meléndez-Martínez, Alba & Heredia, 2008a; Moyano, Meléndez-Martínez, Alba & Heredia, 2008b). Likewise, they have proved to be useful for the estimation of the vitamin A activity of diverse orange juices (Meléndez-Martínez, Vicario & Heredia, 2007b). In any case, some of these methods required expensive apparatus which are not always affordable for the quality control departments of the industry or for producers. Likewise, the interpretation of the data involves being familiar with the objective measurement of colour by tristimulus colorimetry, which is not as intuitive for technicians as just taking absorbance/reflectance readings at only a few wavelengths. In this sense we have proposed as few as five wavelengths from which the whole visible spectra (380-770 nm) of orange juices can be reconstructed. These wavelengths are expected to be appropriate for the rapid estimation of the total carotenoid content of any orange juice, as the miscellaneous set of samples analyzed in this study differed greatly in colour and in their carotenoid content (Table 1). Thus, considering quantitative data, it was observed that the total carotenoid content of the orange juices analyzed ranged roughly from 2 to 35 mg/l. Important qualitative changes in the carotenoid profiles of the samples were also noticed. In fact, in the light of the data we have reported in previous surveys (Melendez-Martinez et al., 2008) it can be inferred that clear qualitative changes take place in the carotenoid pattern of orange juices as a result of the treatments they undergo in the industry and the length and conditions of storage. At this point it is also important to note that the carotenoid content of oranges (and of all vegetable products) depends on other factors, like genotype, stage of ripeness, the climatic conditions of the area of production, etc. (Mouly, Gaydou, Lapierre & Corsetti, 1999; Lee & Castle, 2001), which adds further variability to our set of samples.

To ascertain whether the wavelengths selected by the mathematical model could be really used to estimate the carotenoid content of the diverse orange juice surveyed, multiple regression analyses were carried out. For this purpose, the total contents of carotenoids were considered as dependent variable and the reflectances at the selected wavelengths as independent variables. The results are summarized in Table 2. The regression coefficients were high regardless of the background used for the measurements, although they were higher when the reflectances obtained for the white background were considered ($R = 0.94$ and 0.90 for white and black measurements, respectively). Likewise, high significance ($p<0.01$) correlations between the total content of carotenoids and the reflectances at 515, 545, and 610 nm (for white background) and at 420, 510, and 545 nm (for black background). These results indicated that it is possible to obtain a good rapid quantitative assessment of the carotenoid fraction of orange juices from the reflectances values obtained at 5 wavelengths. In this regard, the B coefficients that enable to work out the total carotenoid content from the reflectances at those wavelengths were computed (Table 2).

When the correlations colour coordinates-carotenoid content were considered, some interesting information was obtained. Taking into account the colour coordinates computed from the original spectra it was observed that a* was the only coordinate having high significance ($p<0.001$), for measurements made with the white and the black backgrounds. When the set of scalar colour coordinates (L*, a*, b*) was included in the multiple regression model to consider complete colorimetric information (Meléndez-Martínez et al., 2007b), a* was also the only coordinate having high significance. Analogous simple and multiple regression analyses were carried out considering the L*, a* and b* coordinates obtained from the reconstructed spectra. The results of these regression analyses were in agreement with those obtained taking into

account the colour parameters obtained from the original spectra (Table 3), which also confirms the goodness of the 5 wavelength selected by the mathematical model. With regard to the regression coefficients obtained when the total carotenoid content was considered as dependent variable and the set of scalar coordinates as independent variables, several interesting remarks can be made. On one hand, it was seen that the coefficients obtained for the colour coordinates computed from the original spectra (R=0.82 and R=0.81 for the white and the black backgrounds, respectively) were almost identical to those obtained from the coordinates computed from the reconstructed spectra (R=0.81 and R=0.81 for the white and the black backgrounds, respectively). This observation is and additional proof of the goodness of the characteristic vector method to simplify the colour measurement without losing much accuracy. On the other hand, it is important to note that these coefficients are clearly lower than those obtained when the correlations between the reflectances at the wavelengths selected and the carotenoid content were evaluated (Table 2). These data seem to indicate that the consideration of reflectance values at a number of key wavelengths may be more useful to assess the pigment content of carotenoid-containing foods than their colour coordinates. In any case, the frequency histograms of the differences between the total carotenoid content values obtained by HPLC and those calculated from colour coordinates and the selected wavelengths (Figure 3) indicate that both types of parameters can be used to estimate with good accuracy the total pigment content.

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REFERENCES

Astley, S. B., Hughes, D.A., Wright, A.J.A., Elliott, R.M., & Southon, S. (2004). DNA damage and susceptibility to oxidative damage in lymphocytes: Effects of carotenoids in vitro and in vivo. *British Journal of Nutrition, 91*(1), 53-61.

Ayala, F., Echávarri, J.F., Juárez, M.C., & Negueruela, A.I. (1993). Propuesta de un nuevo método de medida del color de vinos tintos y rosados. *Óptica Pura y Aplicada, 26(2),* 533-541.

Baldermann, S., Naim, M., & Fleischmann, P. (2005). Enzymatic carotenoid degradation and aroma formation in nectarines (*Prunus persica*). *Food Research International, 38,* 833-836.

Britton, G. (1983). *The biochemistry of natural pigments*. Cambridge, United Kingdom: Cambridge University Press.

Britton, G. (1995). Structure and properties of carotenoids in relation to function. *The FASEB Journal, 9,* 1551-1558.

Britton, G., & Young, A. (1993). Methods for isolation and analysis of carotenoids. In A. Young & G. Britton, *Carotenoids in photosynthesis* (pp. 409-458). London: Chapman & Hall.

Burton, G. W. (1989). Antioxidant action of carotenoids. *Journal of Nutrition, 119,* 109-111.

Carmona, M., Zalacain, A., Salinas, M.R., & Alonso, G.L. (2006). Generation of saffron volatiles by thermal carotenoid degradation. *Journal of Agricultural and Food Chemistry, 54,* 6825-6834.

449 CIE (1978). *Recommendations on Uniform Color Spaces, Color-Difference Equations,*
450 *Psychometric Color Terms, CIE Publication No. 15 (E-1.3.1) 1971, Supplement*
451 *2*. Vienna: Bureau Central de la CIE.

452 CIE (2004). *Technical Report Colorimetry*. Vienna, Austria: Commission Internationale
453 de l'Eclairage Central Bureau.

454 Demmig-Adams, B., & Adams III, W.W. (2002). Antioxidants in photosynthesis and
455 human nutrition. *Science*, *298*, 2149-2154.

456 Fleischmann, P., & Zorn, H. (2008). Enzymic pathways for formation of carotenoid
457 cleavage products. In G. Britton, S. Liaaen-Jensen, & H. Pfander, *Carotenoids.*
458 *Volume 4: Natural Functions* (pp. 341-366). Basel, Switzerland: Birkhäuser.

459 Fraser, P. D., & Bramley, P.M. (2004). The biosynthesis and nutritional uses of
460 carotenoids. *Progress in Lipid Research*, *43*, 228-265.

461 García-Parrilla, M. C., Ayala, F., Echávarri, J.F., Troncoso, A.Mª., Negueruela, A.I., &
462 Heredia, F.J. (1998). Measurement of wine vinegars' color: application of the
463 characteristic vector method. *Journal of Agricultural and Food Chemistry*, *46*,
464 4238-4241.

465 Giovanucci, E. (2002). Lycopene and prostate cancer risk. Methodological
466 considerations in the epidemiologic literature. *Pure and Applied Chemistry*,
467 *74*(8), 1427-1434.

468 ISO (1994) Accuracy (trueness and precision) of measurement methods and results Part
469 2: Basic method for the determination of repeatability and reproducibility of a
470 standard measurement method. ISO-5725-2:1994

Kön, S., Kolbe, H., Korger, M., Köpsel, C., Mayer, B., Auweter, H., Lüddecke, E., Bettermann, H., & Martin, H.-D. (2008). Aggregation and interface behaviour of carotenoids. In G. Britton, S. Liaaen-Jensen, & H. Pfander, *Carotenoids. Volume 4: Natural functions* (pp. 53-98). Basel, Switzerland: Birkhäuser.

Konopka, I., Kozirok, W., & Rotkiewicz, D. (2004). Lipids and carotenoids of wheat grain and flour and attempt of correlating them with digital image analysis of kernel surface and cross-sections. *Food Research International*, *37*, 429-438.

Krinsky, N. I. (1989). Carotenoids and cancer in animal models. *Journal of Nutrition*, *119*, 123-126.

Lebart, L. A., Morineau, A., & Fenelon, J.P. (1985). *Tratamiento estadístico*. Barcelona, Spain: Marcombo.

Lee, H. S., & Castle, W.S. (2001). Seasonal changes of carotenoid pigments and color in Hamlin, Earlygold, and Budd Blood orange juices. *Journal of Agricultural and Food Chemistry*, *49*, 877-882.

Lidebjer, C., Leanderson, P., Ernerudh, J., & Jonasson, L. (2007). Low plasma levels of oxygenated carotenoids in patients with coronary artery disease. *Nutrition, Metabolism and Cardiovascular Diseases*, *17*(6), 448-456.

Marx, M., Schieber, A., & Carle, R. (2000). Quantitative determination of carotene stereoisomers in carrot juices and vitamin supplemented (ATBC) drinks. *Food Chemistry*, *70*, 403-408.

491 Meléndez-Martínez, A. J., Vicario, I.M., & Heredia, F.J. (2003). Application of
492 Tristimulus Colorimetry to estimate the carotenoids content in ultrafrozen
493 orange juices. *Journal of Agricultural and Food Chemistry*, *51*(25), 7266-7270.

494 Meléndez-Martínez, A. J., Vicario, I.M., & Heredia, F.J. (2004). Correlation between
495 visual and instrumental colour measurements of orange juice dilutions. Effect of
496 the background. *Food Quality and Preference*, *16*, 471-478.

497 Meléndez-Martínez, A. J., Vicario, I.M., & Heredia, F.J. (2005). Instrumental
498 measurement of orange juice colour: A review. *Journal of the Science of Food*
499 *and Agriculture*, *85*, 894-901.

500 Meléndez-Martínez, A. J., Vicario, I.M., & Heredia, F.J. (2007a). Carotenoids, color
501 and ascorbic acid content of a novel frozen-marketed orange juice. *Journal of*
502 *Agricultural and Food Chemistry*, *55*(4), 1347-1355.

503 Meléndez-Martínez, A. J., Vicario, I.M., & Heredia, F.J. (2007b). Rapid assessment of
504 vitamin A activity through objective color measurements for the quality control
505 of orange juices with diverse carotenoid profiles. *Journal of Agricultural and*
506 *Food Chemistry*, *55*(8), 2808-2815.

507 Melendez-Martinez, A. J., Britton, G., Vicario, I.M., & Heredia, F.J. (2008). The
508 complex carotenoid pattern of orange juices from concentrate. *Food Chemistry*,
509 *109*(3), 546-553.

510 Mínguez-Mosquera, M. I., Rejano-Navarro, L., Gandul-Rojas, B., Sánchez-Gómez,
511 A.H., & Garrido-Fernández, J. (1991). Color-pigment correlation in virgin olive
512 oil. *Journal of the American Oil Chemists Society*, *68*(5), 332-336.

513 Mouly, P. P., Gaydou, E.M., Lapierre, L., & Corsetti, J. (1999). Differentiation of
514 several geographical origins in single-strength Valencia orange juices using
515 quantitative comparison of carotenoid profiles. *Journal of Agricultural and*
516 *Food Chemistry*, *47*, 4038-4045.

517 Moyano, M. J., Ayala, F., Echávarri, J.F., Alba, J., Negueruela, A.I., & Heredia, F.J.
518 (2001). Simplified measurement of virgin olive oil color by application of the
519 characteristic vector method. *Journal of the American Oil Chemists Society*,
520 *78*(12), -.

521 Moyano, M. J., Meléndez-Martínez, A.J., Alba, J., & Heredia, F.J. (2008a). A
522 comprehensive study on the color of virgin olive oils and its relationship with
523 their chlorophylls and carotenoids indexes (I). CIEXYZ non-uniform color
524 space. *Food Research International*, *41*(505), 512-.

525 Moyano, M. J., Meléndez-Martínez, A.J., Alba, J., & Heredia, F.J. (2008b). A
526 comprehensive study on the color of virgin olive oils and its relationship with
527 their chlorophylls and carotenoids indexes (II). CIELUV and CIELAB uniform
528 color spaces. *Food Research International*, *41*, 513-521.

529 Olson, J. A. (1999). Carotenoids and human health. *Archivos Latinoamericanos de*
530 *Nutrición*, *49*(1-S), 7-11.

531 Ornelas-Paz, J. d. J. s., Yahia, E.M., & Gardea, A.A. (2008). Changes in external and
532 internal color during postharvest ripening of [`]Manila' and [`]Ataulfo' mango
533 fruit and relationship with carotenoid content determined by liquid
534 chromatography-APcI+-time-of-flight mass spectrometry. *Postharvest Biology*
535 *and Technology*, *50*(2-3), 145-152.

536 Ruiz, D., Reich, M., Bureau, S., Renard, C.M.G.C., & Audergon, J.M. (2008).
537 Application of Reflectance Colorimeter Measurements and Infrared
538 Spectroscopy Methods to Rapid and Nondestructive Evaluation of Carotenoids
539 Content in Apricot (Prunus armeniaca L.). *Journal of Agricultural and Food*
540 *Chemistry*, *56*(13), 4916-4922.

541 Seybold, C., Fröhlich, K., Bitsch, R., Otto, K., & Böhm, V. (2004). Changes in contents
542 of carotenoids and vitamin E during tomato processing. *Journal of Agricultural*
543 *and Food Chemistry*, *52*(23), 7005-7010.

544 Silva Ferreira, A. C., Monteiro, J., Oliveira, C., & Guedes de Pinho, P. (2008). Study of
545 major aromatic compounds in port wines from carotenoid degradation. *Food*
546 *Chemistry*, *110*(1), 83-87.

547 Simonds, J. L. (1963). Application of characteristic vector analysis to photographic and
548 optical response data. *Journal of the Optical Society of America*, *53*, 968-.

549 Statsoft (2001). Statistica. (6.0), -.

550 Telfer, A., Pascal, A., & Gall, A. (2008). Carotenoids in photosynthesis. In G. Britton,
551 S. Liaaen-Jensen, & H. Pfander, *Carotenoids. Volume 4: Natural functions* (pp.
552 265-308). Basel, Switzerland: Birkhäuser.

553 Trumbo, P. R., & Ellwood, K.C. (2006). Lutein and zeaxanthin intakes and risk of age-
554 related macular degeneration and cataracts: an evaluation using the Food and
555 Drug Administration's evidence-based review system for health claims.
556 *American Journal of Clinical Nutrition*, *84*, 971-974.

Voutilainen, S., Nurmi, T., Mursu, J., & Rissanen, T.H. (2006). Carotenoids and cardiovascular health. *American Journal of Clinical Nutrition, 83*, 1265-1271.

Table 1

Table 1. Summary of the carotenoid content and CIELAB colour coordinates corresponding to the 82 samples surveyed

		White background					Black background				
	c† (mg/l)	**L***	**a***	**b***	**C^*_{ab}**	**h_{ab}**	**L***	**a***	**b***	**C^*_{ab}**	**h_{ab}**
Mean	13.14	75.30	10.79	66.48	67.43	80.96	63.15	5.49	53.61	53.97	84.38
SD	9.62	2.83	4.05	7.95	8.34	2.70	3.61	3.35	8.68	8.87	3.00

† Total carotenoid content

Table 2

Table 2. Summary of the multiple regression analyses performed to assess the correlations between the reflectances at the wavelengths selected (independent variables) and the carotenoid content (dependent variable).

Background	Reflectance (nm)	B coefficient	Std. error of estimate
White (R=0.94080)	420	32.615	3.3675
	455	-13.856	
	515	-145.571 **	
	545	122.985**	
	610	-65.832*	
	Intercept	29.573**	
Black (R=0.90259)	420	157.465*	4.2770
	445	-176.853	
	510	-162.228**	
	545	111.682*	
	605	-31.638	
	Intercept	13.659*	

* Significant at $p<0.01$; ** significant at $p<0.001$

Table 3

Table 3. Summary of the multiple regression analyses carried out to evaluate the correlations between the total carotenoid content and the colour coordinates obtained from the original and the reconstructed spectra

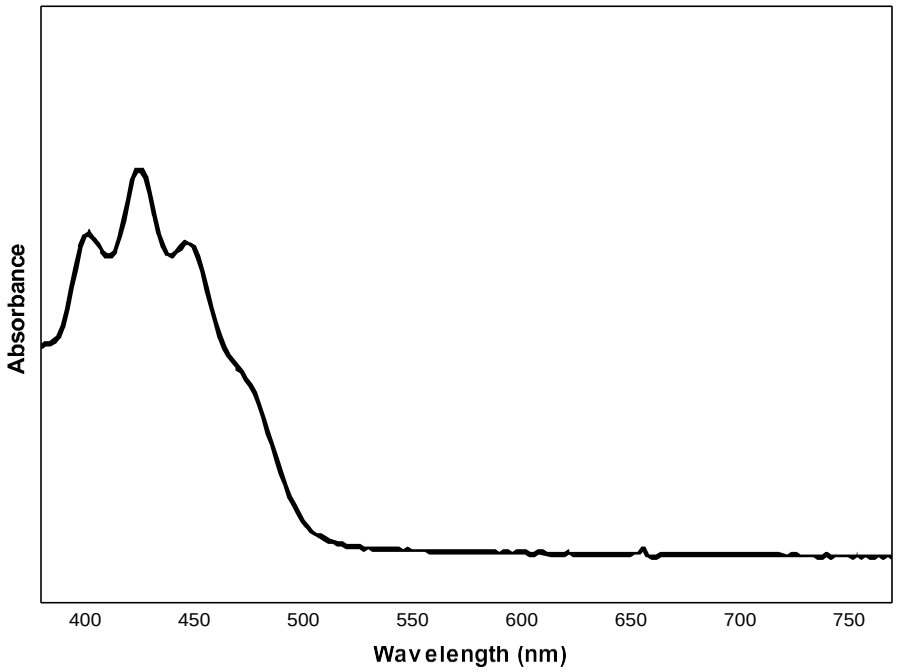
Background	Variables	From original spectra		From reconstructed spectra	
		B coefficient	Std. error of estimate	B coefficient	Std. error of estimate
White	L*	0.1271	5.6257	0.07059	5.6932
	a*	2.1603**		2.07541**	
	b*	-0.1072		-0.08703*	
	Intercept	-12.6542		-8.81539	
Black	L*	-0.021504	5.7294	-0.05097	5.8057
	a*	2.524167**		2.48255**	
	b*	-0.161832*		-0.14881	
	Intercept	9.030370		10.44026	

* Significant at $p<0.01$; ** significant at $p<0.001$

Figure 1

Figure 1. Typical visible spectra:

a. Absorption spectrum of an orange juice carotenoid extract



b. Reflection spectra of an orange juice obtained with white and black backgrounds

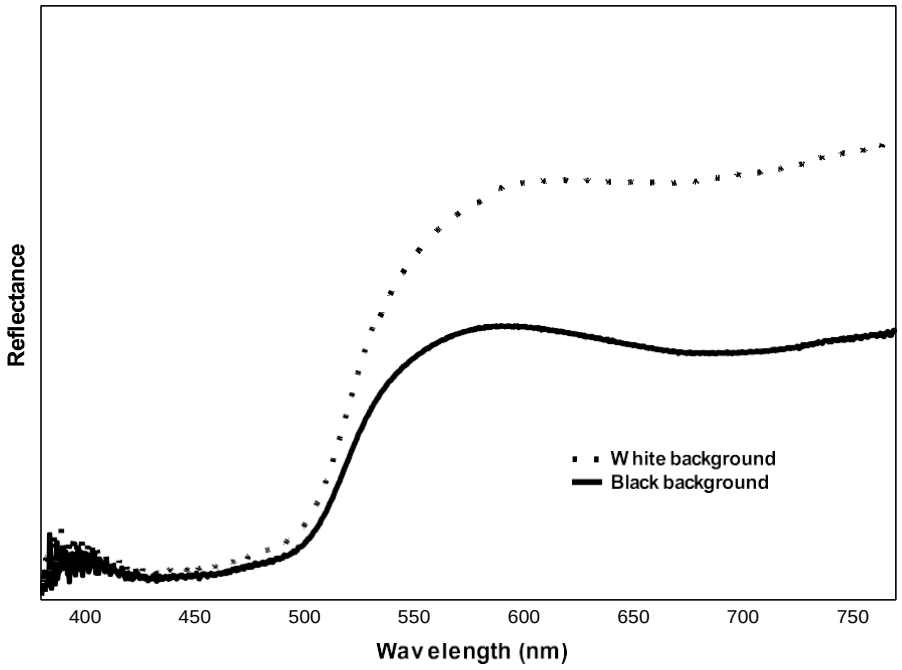
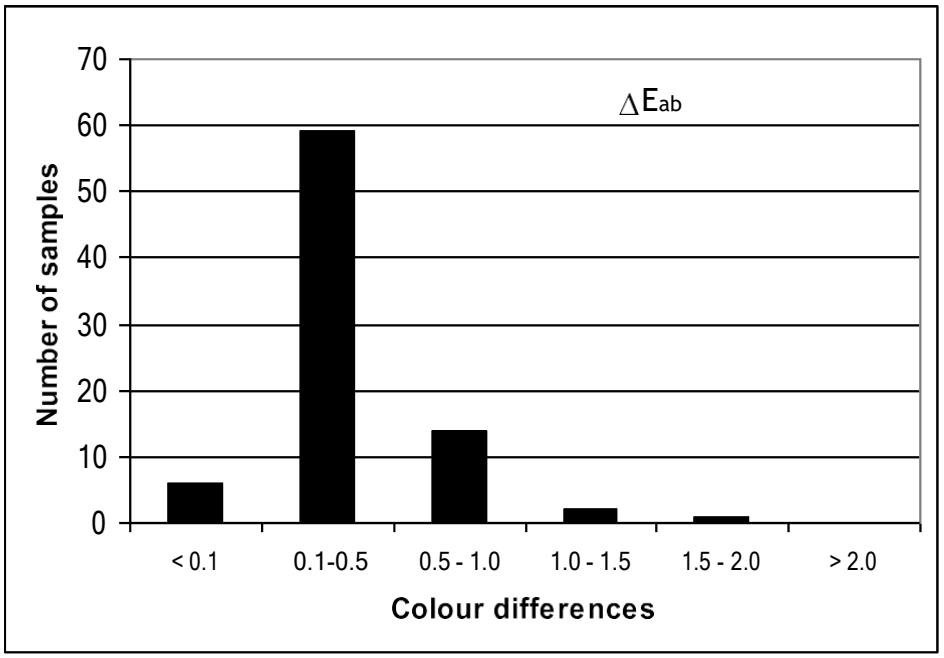


Figure 2

Figure 2. Frequency histogram of colour differences corresponding to original and reconstructed spectra obtained for white (upper figure) and black background (lower figure)



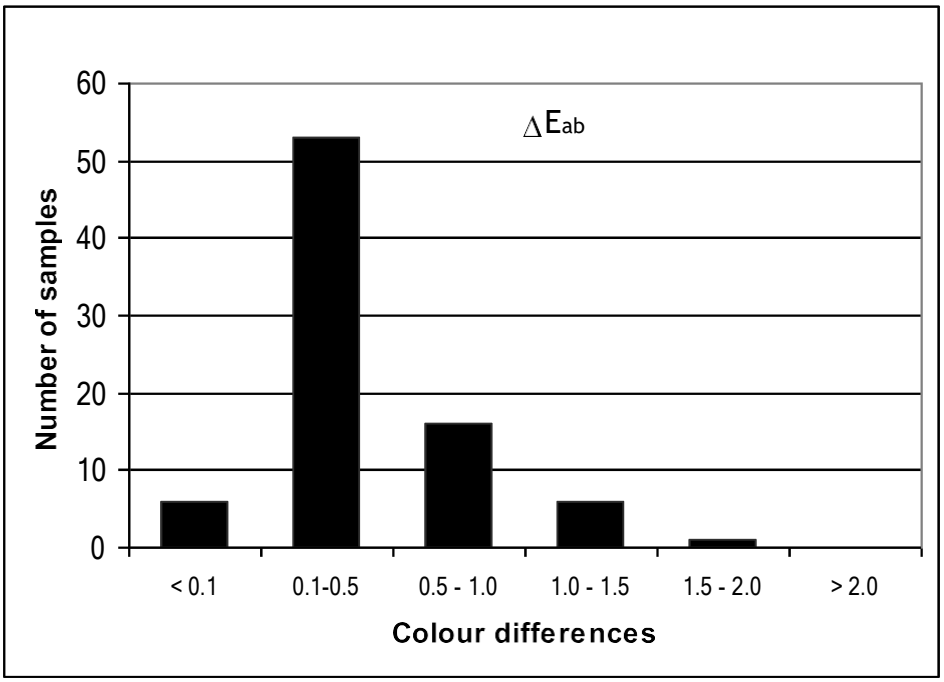
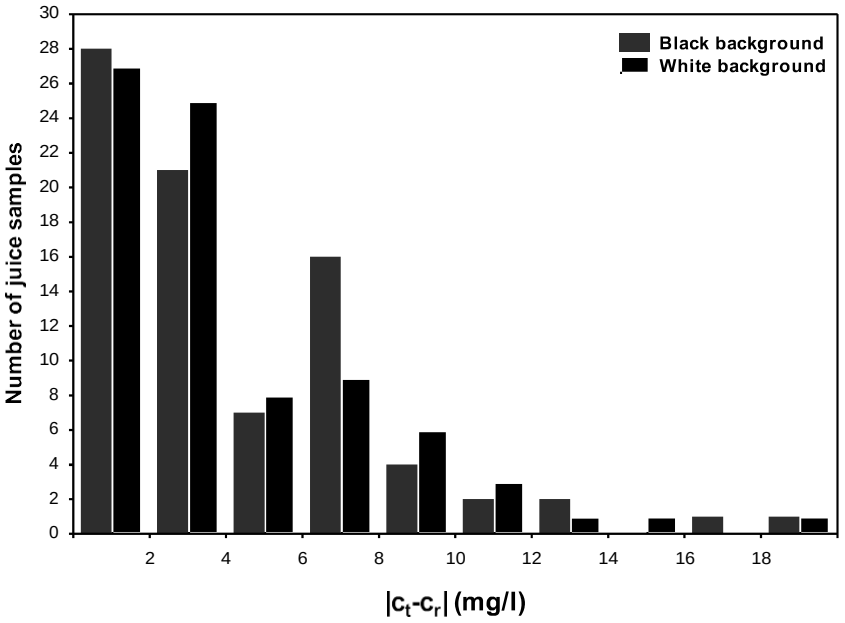


Figure 3. Frequency histogram of carotenoid content differences between original data and reconstructed spectra data obtained for white and black background. (c_t: Total carotenoid content; c_r: Carotenoid content calculated from reconstructed spectra): a) carotenoid content calculated with L*a*b* regression equation; b) carotenoid content calculated with reflectances regression equation.

a)



b)