Rapid Spectrophotometric Determination of Red and Yellow Isochromic Carotenoid Fractions in Paprika and Red Pepper Oleoresins

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A rapid method has been developed for the determination of the red (R) and yellow (Y) isochromic carotenoid pigments fractions in paprika and oleoresins, based on UV−visible spectrophotometric measurement at two characteristic wavelengths and application of the Lambert−Beer law for multicomponent mixtures. The wavelengths 472 and 508 nm were selected as the most appropriate for simultaneous quantification of these fractions in the acetone extract of pigments. Experimental determination of the specific absorption coefficients (ϵ) for the two pigment fractions (R, Y) at 472 and 508 nm yielded equations to calculate the concentration of the two fractions, the total pigment content, and the ratio between the two fractions. The error in the determination of the isochromic fractions by the proposed spectrophotometric method was <5% when the results were compared with those obtained by HPLC analysis. The method can be applied to the direct extract of pigments, thereby avoiding saponification and minimizing errors from pigment degradation and sample manipulation as well as shortening the time of analysis (5 min in the case of oleoresins).

Keywords: *Spectrophotometry; Capsicum annuum; carotenoids; paprika; oleoresin; quality*

INTRODUCTION

Color is one of the main characteristics of a foodstuff noted by consumers. It predetermines certain expectations of quality and flavor, and a product with a color other than the "correct" one will be rejected. The food industry, with the knowledge of this natural color−quality relationship, attempts to adapt the industrial processes of transformation and preparation of foodstuffs to preserve the integrity of compounds responsible for acceptable color. It is normal practice to add colorants to enhance, homogenize, or even change the color of the foodstuff to make it more attractive to consumers (*1*). The carotenoid pigments, either in isolation or together with other natural pigments (chlorophylls and anthocyanins), are mainly responsible for food color. The carotenoids are, without doubt, the most widely distributed pigments in nature, being found throughout the plant kingdom and in bacteria, fungi, and animals, although the latter are unable to synthesize them but incorporate them from dietary plants. In animals, the carotenoids are compounds of great dietary importance, not only as precursors of vitamin A but also as antioxidants in cell protection and in the prevention of degenerative diseases. Today, some 650 carotenoid pigments are known (*2*).

The red pepper (*Capsicum annuum* L.) has been used since ancient times as a source of pigments to enhance or change food color. Pepper has traditionally been used as a food colorant in the form of paprika, although, today, oleoresins are widely used. The ripe fruits of the pepper owe their intense red color to carotenoid pigments synthesized massively during ripening. Noteworthy among these carotenoid pigments are capsanthin, capsorubin, and capsanthin 5,6-epoxide, which are almost exclusive to the genus *Capsicum* and are responsible for the final red color (*3*). The commercial value of the pepper for paprika depends solely on its "red coloring power", so that quantification of the carotenoid pigment content has always been of great interest. Both the transforming industry and potential buyers need analytical methods that are simple, quick, and readily available, enabling a rapid evaluation of product quality. The most important of the usual methods are ASTA-20.1 (American Spice Trade Association) (*4*) for extractable color and that of chromatic attributes (L^*, a^*, b^*) proposed by the Commission Internationale de l'Eclairage (CIE) (*5*). Both methods furnish measurements of color, although not necessarily that from carotenoids. Other, more reliable, methods include chromatographic separation prior to quantification. Some methods employ TLC (*6*, *7*) and others HPLC (*8*, *9*), but their complexity makes them unsuitable for routine industrial control. Such methods yield information about individualized pigment composition and total carotenoid content of the sample.

In an earlier work (*10*), a spectrophotometric method was established to estimate the concentration of the red (R) and yellow (Y) isochromic fractions and the total carotenoid content in oleoresins, which could be used as an index of their quality. The method defined independent equations for the red fraction and the total carotenoid content, and the yellow fraction was calculated as the difference. However, these equations can be applied only to samples in which the two isochromic fractions have a similar content and not to situations in which one or the other pigment fraction is dominant.

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In the present work, the extension of the Lambert−Beer law for multicomponent mixtures has been applied to develop a quick method of spectrophotometric measurement for the simultaneous quantification of the R and Y isochromic fractions and of the total carotenoid content in paprika and oleoresins. This will enable the industry to establish quickly and simply quality criteria for the control of both raw material and finished product.

MATERIALS AND METHODS

Samples. Paprika powders from La Vera county (Cáceres, Spain) and red pepper oleoresins from different Spanish producers were used in the study.

Pigment Extraction for HPLC Analysis. One gram of paprika powder was extracted four times with 50 mL of acetone until the complete exhaustion of all color. In the case of oleoresin, 0.1 g was dissolved in diethyl ether. All extracts were pooled in a separator and shaken with diethyl ether. A sufficient quantity of 10% NaCl was added at the end to aid in the separation of the phases. The organic phase was saponified with 25 mL of 20% KOH/methanol during 1 h. The pigments were subsequently extracted with diethyl ether, evaporated in a rotary evaporator, and taken up in a maximum of 25 mL of acetone. A 1 mL aliquot was centrifuged at 12000 rpm and stored at −30 °C until analyzed. Losses occurring during the process were monitored using a β-apo-8′-carotenal internal standard, a known quantity of which was added to the sample at the start of the extraction process. All of the analyses were carried out in quadruplicate. Samples were cleaned before injection into the HPLC by using a benchtop centrifuge model Micro-Centaur (MSE Scientific Instruments, Sussex, U.K.). The whole extraction procedure was carried out in dimmed light.

HPLC Separation and Quantification of Carotenoids. For HPLC analysis, a Waters 600E quaternary pump equipped with a diode array detector (PDA 996, Waters) and controlled with a Millennium data acquisition station was used. The injection valve was a Rheodyne model 7125 with an injection loop of 10 μL. The HPLC system was equipped with a reversed phase C18 Spherisorb ODS-2 (5 μm, 0.46 cm × 25 cm) column. A precolumn (0.5 cm × 0.4 cm) of the same material was fitted to protect the main column. Monitoring and quantification of the carotenoid pigments was carried out using a method previously developed by the authors (*8*). Quantification was carried out using calibration curves for the main individual carotenoids and *all-trans*-β-apo-8′-carotenal as internal standard.

Pigment Extraction for UV−Visible Spectroscopy Analysis. UV−vis spectra were collected with a UV−vis diode array spectrophotometer 8452 A (Hewlett-Packard). The samples were prepared similarly to extracts for HPLC but without saponification and the addition of an internal standard. In the case of paprika, 0.5 g of sample was extracted with 75 mL of acetone for 1 h, and the extract was filtered and transferred to a volumetric flask and made up to 100 mL. For oleoresin samples, 0.025 g was dissolved in a volumetric flask with 100 mL of acetone. Depending on the total carotenoid content, a dilution might be necessary before spectrophotometric measurement.

Isolation and Preparation of the Red and Yellow Isochromic Fractions. R and Y isochromic fractions were isolated using semipreparative TLC on plates of silica gel 60 GF_{254} (20 × 20 cm glass plate, 0.7 mm thickness) (Merck, Darmstadt, Germany). A direct carotenoid extract (obtained without saponification during extraction) was applied to the plate and developed with the mixture hexane/ethyl acetate/ethanol/acetone (95:3:2:2) (*7*). The Y fraction comprised bands with R_f from 0.96 to 0.69 and consisted mainly of β-carotene, esterified β-cryptoxanthin, and esterified zeaxanthin. The R fraction comprised bands with R_f from 0.37 to 0.21 and consisted mainly of esterified capsanthin and capsorubin. Both fractions were scraped from the plate and eluted with acetone. The process was repeated several times (∼25), and the collected fractions were evaporated to dryness and then taken to volume with 50 mL of acetone. R and Y isochromic fraction solutions were stored at −30 °C and used as stock solution for the rest of the study. Both stock solutions were quantitatively analyzed by HPLC and at the same time checked for purity. Table 1 shows the quantitative composition of the two isochromic fractions.

Table 1. Carotenoid Composition of the Red (R) and Yellow (Y) Isochromic Stock Solutions

fraction/pigment	concn[a] (μg/mL)
R	3.7324 ± 0.0432[b]
capsanthin	2.9416 ± 0.0340
capsorubin	0.7908 ± 0.0092
Y	3.4332 ± 0.0940[b]
zeaxanthin	1.0546 ± 0.0289
β-cryptoxanthin	0.7103 ± 0.0194
β-carotene	1.6683 ± 0.0457

[a] Mean ± standard deviation of four determinations. [b] Sum of the carotenoids listed.

Preparation of Standard Solutions and Mixtures of Red and Yellow Isochromic Fractions. From the stock solution of each isochromic fraction, 10 standard solutions were prepared by successive dilution in acetone, with concentrations ranging from 0.25 to 3.75 μg/mL. Similarly, solutions were prepared containing mixtures of the two fractions with R/Y ratio between 9:1 and 1:9. All of the standard solutions were prepared and analyzed in triplicate.

RESULTS AND DISCUSSION

All of the carotenoid pigments present in the pepper have chromophore properties that allow their grouping in two isochromic families: red (R) and yellow (Y). The R fraction contains pigments exclusive to the genus *Capsicum*—capsanthin, capsanthin-5,6-epoxide, and capsorubin—whereas the Y fraction comprises the remaining pigments (zeaxanthin, violaxanthin, antheraxanthin, β-cryptoxanthin, β-carotene, and cucurbitaxanthin A), which act as precursors of the former (*11*). Figure 1 shows the structures and electron absorption spectra in acetone of the most representative carotenoids of the two fractions. Earlier works on the red pepper (*7*, *10*, *12*) established that the total carotenoid content and R/Y ratio can be used to define the quality of the raw material (fruits) and derived products (paprika and oleoresins). Fluctuations in the total carotenoid content and the R/Y ratio with respect to those found in the source fruit can indicate poor processing, the existence of degradative reactions (acting preferentially on one of the isochromic fractions), or even possible color adulteration.

The extensive system of conjugated double bonds in the carotenoid pigments makes them excellent chromophores, absorbing considerable amounts of visible light. The color of the carotenoids varies from yellow to red, as the region of light absorption is displaced depending on the length of the conjugated double-bonds system and the presence of different functional groups. The carotenoids present characteristic electron absorption spectra that distinguish them from other compounds and enable their quantitative determination by UV−vis spectrophotometry, applying the Beer−Lambert law.

$$A = \epsilon CL \quad (1)$$

The additivity of the absorbances at a given wavelength

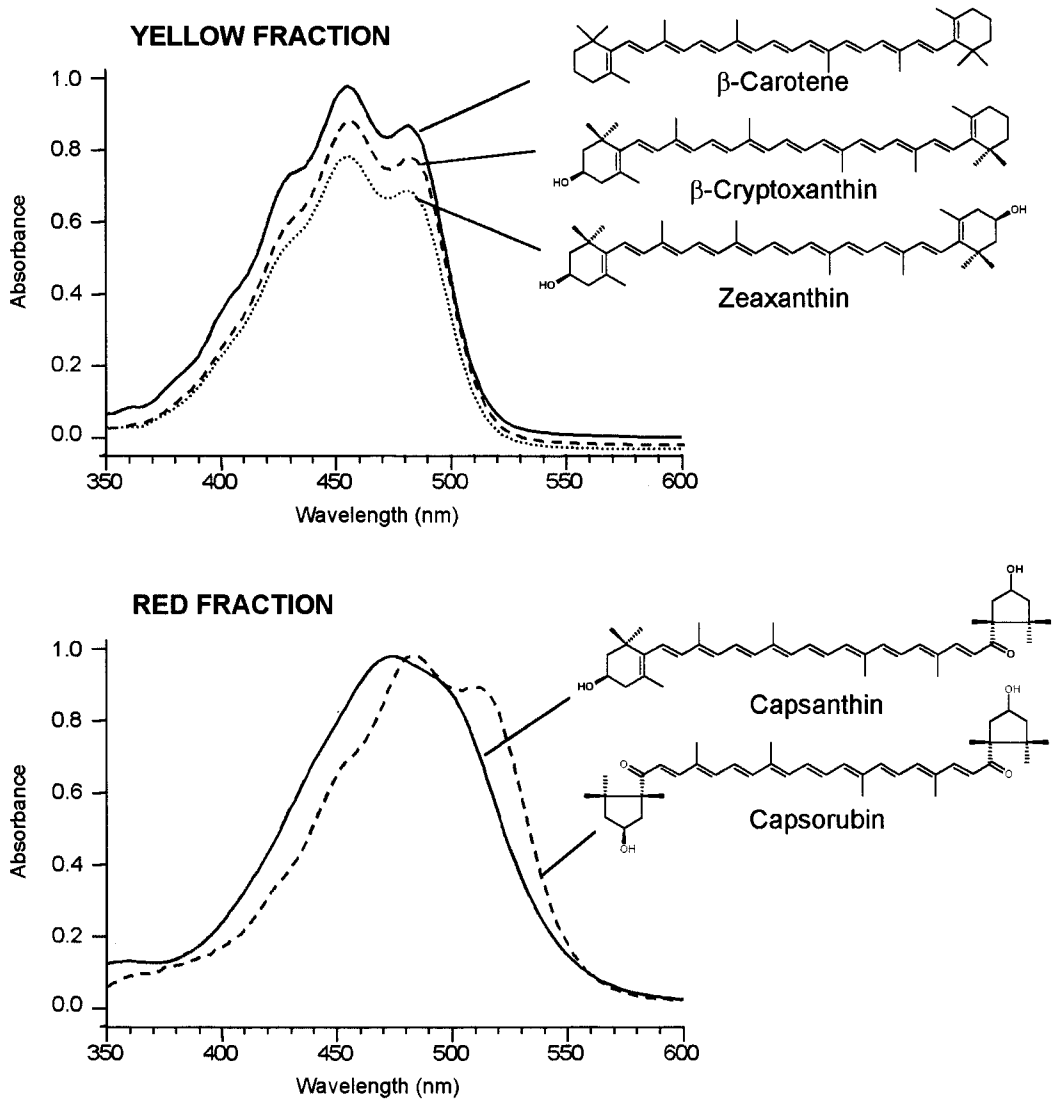

Figure 1. UV−vis absorption spectra and structures of the main carotenoid pigments present in red pepper fruit (*C. annuum*) and derived products (paprika and oleoresins).

enables individual analytes in multicomponent mixtures to be determined via an extension of the Beer−Lambert law (eq 2) whenever their absorption spectra differ significantly and measurements of absorbance are available at as many different wavelengths as analytes to be analyzed.

$$A_{\lambda_j} = \sum_{i=1}^{m} \epsilon_{\lambda_j}^{i} C^{i} L \qquad (2)$$

In eq 2 A_{λ_j} is the absorbance of a mixture of m components at the jth wavelength, C^i is the concentration of the ith component (g 100 mL^{-1}), $\epsilon_{\lambda_j}^{i}$ is the specific absorption coefficient of the ith component at the jth wavelength (cm^{-1} g^{-1} 100 mL), and L is the cell path length (cm).

According to this expression, to determine the concentration of n analytes in a mixture, we need n equations and n readings of absorbance at n different wavelengths. We also need to know the absorption coefficient of each analyte at the n wavelengths. As the number of analytes increases, the system of resulting equations will become more and more complicated, finally involving the resolution of a matrix system (assuming $L = 1$ cm):

$$\begin{bmatrix} A_{\lambda_1} \\ \vdots \\ A_{\lambda_n} \end{bmatrix} = \begin{bmatrix} \varepsilon_{\lambda_1}^{1} & \cdots & \varepsilon_{\lambda_1}^{n} \\ \vdots & \ddots & \vdots \\ \varepsilon_{\lambda_n}^{n} & \cdots & \varepsilon_{\lambda_n}^{n} \end{bmatrix} \cdot \begin{bmatrix} C^1 \\ \vdots \\ C^n \end{bmatrix} \qquad (3)$$

Because the pepper carotenoid pigments can be grouped in two isochromic fractions (R and Y) according to their chromophore or structure, the expression of multicomponents (eq 3) can be reduced to the minimum—a two-component mixture, in this case, the R pigment fraction and the Y pigment fraction. The expression then becomes

$$\begin{bmatrix} A_{\lambda_1} \\ A_{\lambda_2} \end{bmatrix} = \begin{bmatrix} \epsilon_{\lambda_1}^{R} & \epsilon_{\lambda_1}^{Y} \\ \epsilon_{\lambda_2}^{R} & \epsilon_{\lambda_2}^{Y} \end{bmatrix} \times \begin{bmatrix} C^R \\ C^Y \end{bmatrix} \qquad (4)$$

The resolution of this two-equation system gives the expressions (eq 5) for the calculation of the concentrations of the red (C^R) and yellow (C^Y) fractions. It also enables estimation of the total carotenoid content

Table 2. Specific Absorption Coefficient $\epsilon^i_{\lambda_j}$ (100 mL g^{-1} cm^{-1}) of the Yellow (Y) and Red (R) Isochromic Fractions at the Four Preselected Wavelengths

fraction	$\epsilon^i_{\lambda_j}$ (100 mL g^{-1} cm^{-1})			
	λ_{426nm}	λ_{448nm}	λ_{472nm}	λ_{508nm}
R	1365.7	2009.6	2450.1	1724.3
Y	1903.1	2377.7	2144.0	403.3

Table 3. Absolute Error (Percent) for the Determination of the Concentration of Yellow (Y) and Red (R) Isochromic Fractions Depending on the Wavelength Pair Used and the Fraction Concentration Ratio (R/Y)

ratio	wavelength pair (nm)					
R/Y	426, 448	426, 472	426, 508	448, 472	448, 508	472, 508
			Red Isochromic Fraction			
1/9	2.00	3.09	1.44	3.59	1.32	1.19
2/8	8.40	7.07	6.14	6.64	6.02	4.72
3/7	3.75	3.74	3.37	3.76	3.33	3.32
4/6	1.94	0.70	0.45	0.27	0.38	0.42
5/5	1.38	0.18	1.35	0.76	1.44	1.52
6/4	0.02	0.40	0.82	0.55	0.84	0.88
7/3	0.50	0.39	1.27	0.71	1.32	1.40
8/2	1.40	0.41	0.37	0.05	0.43	0.48
9/1	1.69	0.17	1.08	0.38	1.17	1.26
			Yellow Isochromic Fraction			
1/9	0.30	0.22	0.35	0.15	0.36	0.48
2/8	0.37	0.13	0.03	0.01	0.14	0.25
3/7	0.69	0.70	0.81	0.68	0.84	0.92
4/6	0.17	0.42	0.54	0.78	0.72	0.66
5/5	3.39	2.29	1.46	1.55	0.98	0.59
6/4	1.93	1.53	1.09	1.25	0.88	0.62
7/3	4.40	2.93	1.47	1.96	0.74	0.05
8/2	9.52	6.72	4.52	4.90	3.27	2.21
9/1	27.74	18.05	10.10	11.79	5.69	1.80

($C^T = C^R + C^Y$) and the ratio between isochromic fractions R/Y (C^R/C^Y).

$$C^R = \frac{A_{\lambda_2}\epsilon^Y_{\lambda_1} - A_{\lambda_1}\epsilon^Y_{\lambda_2}}{\epsilon^R_{\lambda_2}\epsilon^Y_{\lambda_1} - \epsilon^R_{\lambda_1}\epsilon^Y_{\lambda_2}}; \quad C^Y = \frac{A_{\lambda_1}\epsilon^R_{\lambda_2} - A_{\lambda_2}\epsilon^R_{\lambda_1}}{\epsilon^R_{\lambda_2}\epsilon^Y_{\lambda_1} - \epsilon^R_{\lambda_1}\epsilon^Y_{\lambda_2}} \qquad (5)$$

However, to obtain the expression of both equations, we must select the two optimal wavelengths (λ_1 and λ_2) of the whole spectral range and determine the specific absorption coefficients at both wavelengths for the yellow ($\epsilon^Y_{\lambda_1}$, $\epsilon^Y_{\lambda_2}$) and red ($\epsilon^R_{\lambda_1}$, $\epsilon^R_{\lambda_2}$) fractions.

Selection of the Measurement Wavelengths (λ_1 and λ_2). The choice of wavelength depends fundamentally on the values at which the spectra of the two fractions (R and Y) show the greatest difference, thereby minimizing errors. Figure 2 shows normalized spectra for the R and Y fractions, together with the curve of point-by-point difference between the two spectra. The wavelengths at which the two spectra are most different can be easily selected from the maxima on this curve. This procedure was used to preselect four wavelengths: 426, 448, 472, and 508 nm. The best wavelength pair was then chosen from these four values. For this, it is necessary to compare the theoretical functions of concentration (eq 5) for the isochromic fractions with the actual known values of the working solutions. However, to obtain these functions of concentration, we must first calculate the absorption coefficients (ϵ) for both fractions (R and Y) at each of the four preselected wavelengths. This calculation was performed by constructing calibration curves for the two fractions (R and Y) at 10 consecutive concentration levels (0.3732−3.7324 μg/mL for the R fraction and 0.3433−3.4332 μg/mL for the Y fraction). For each concentration level and each fraction, the complete UV−vis absorption spectrum in the range of 360−650 nm (with 2 nm resolution) was recorded. The absorption coefficients were calculated from the slope of the linear regression of concentration of each fraction against the absorbance measured at each of the four preselected wavelengths. Table 2 shows the values obtained for the absorption coefficients.

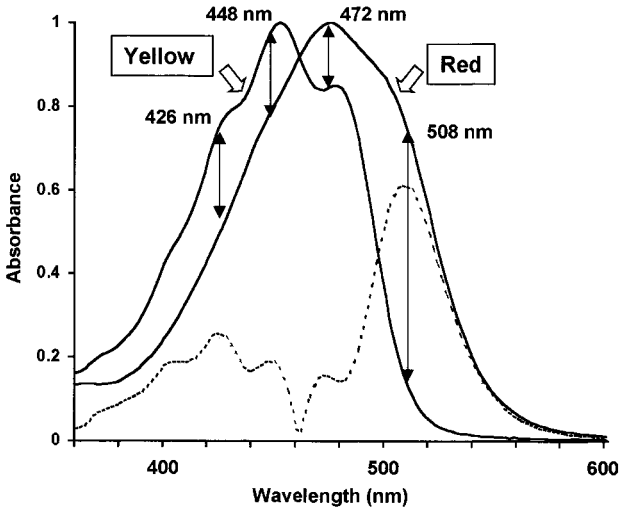

Figure 2. UV−vis absorption spectra of the red and yellow isochromic fractions in acetone. Dotted line represents the resulting plot after subtraction of both spectra.

With the absorption coefficients known for the preselected wavelengths, the concentration equations (eq 5) of the isochromic fractions can be resolved for each wavelength pair. The choice of best pair was made by preparing standard solutions of known concentration for both fractions in the R/Y ratio range from 9:1 to 1:9. Table 3 shows the absolute errors in determining the concentration of each isochromic fraction from the theoretical expressions of C^R and C^Y obtained for each wavelength pair. As can be observed, for each wavelength pair used, the errors are smaller when the concentrations of the two isochromic fractions are similar—that is, in the R/Y ratio range from 4:6 to 6:4. The error is then, in most cases, below 1%. For the R isochromic fraction, and over the whole range of relative concentration (R/Y), the errors are significantly smaller when the wavelength pairs 448,508 and 472,508 are

Table 4. Isochromic Fractions (R and Y), Total Carotenoid Content (Micrograms per Gram), and R/Y Ratio Determined by the Proposed Method, HPLC, and the Mínguez-Mosquera and Pérez-Gálvez Spectrophotometric Method (*10*)

sample	proposed spectrophotometric method				HPLC (*8*)				spectrophotometric method (*10*)			
	C^R	C^Y	C^T	R/Y	C^R	C^Y	C^T	R/Y	C^R	C^Y	C^T	R/Y
oleoresin 1	60266.95	40023.54	100290.49	1.506	61218.50	40943.73	102161.73	1.495	54488.99	34276.44	88765.44	1.600
oleoresin 2	26448.04	16081.22	42529.26	1.640	27502.15	17188.84	44691.00	1.600	24916.41	13340.89	38257.30	1.870
oleoresin 3	23742.77	14797.77	38540.54	1.600	24450.10	15092.65	39542.75	1.620	22061.20	12459.91	34521.12	1.770
oleoresin 4	60115.98	29531.72	89647.70	2.040	62287.05	29519.93	91806.98	2.110	55112.48	23880.18	78992.66	2.310
paprika 1	1029.12	432.65	1461.77	2.380	1065.02	451.53	1516.55	2.360	992.41	387.37	1379.79	2.560
paprika 2	904.10	412.08	1316.19	2.190	956.32	428.84	1385.16	2.230	840.83	356.75	1197.58	2.357

used. However, at a lower R/Y range (from 1:9 to 3:7)—that is, when the concentration of the Y fraction is significantly higher than that of the R fraction—the errors are smaller when the wavelengths 472 and 508 nm are used. Similarly, in the case of the Y isochromic fraction, the errors are greater with increasing R/Y ratio and are also significantly smaller when 472 and 508 nm are used. These are therefore the best wavelengths for quantification of the two isochromic fractions over the whole range of relative concentration.

Expressions for the Calculation of the Isochromic Fraction Concentration. When the values of the absorption coefficients for the two fractions at 472 and 508 nm are introduced into the general equations of C^R and C^Y (eq 5) and multiplied by 10^4 to express the data as micrograms per milliliter, the following equations are obtained:

$$C^R = \frac{A_{508} \times 2144.0 - A_{472} \times 403.3}{270.9} (\mu g/mL)$$

$$C^Y = \frac{A_{472} \times 1724.3 - A_{508} \times 2450.1}{270.9} (\mu g/mL)$$

To express the result in milligrams per kilogram, it is necessary to multiply by the final volume (milliliters) to which the sample was taken and divide by the weight (grams) of sample. If the sample was diluted prior to performance of the spectrophotometric measurement, this factor must be taken into account.

Analysis of Oleoresins and Paprikas. Different samples of paprika and oleoresins were analyzed using the proposed method. Table 4 shows the results obtained for the content of each isochromic fraction (C^R and C^Y), the R/Y ratio, and the total carotenoid content and compares them with the results obtained by HPLC and with the previously proposed method (*10*). As can be seen, the new method shows good correlation with results obtained by HPLC, with errors below 5%. In the case of the method used until now, the errors are ~10% for the R fraction and total carotenoid content and exceed 15% for the Y fraction. The reason for this greater error is because the concentration of the Y fraction is calculated as the difference between total content and the R fraction. The new method is substantially improved by obtaining expressions for the concentration of the two fractions from the simultaneous measurement at two wavelengths, enabling each pigment fraction to be quantified in the presence of the other.

The proposed method is quick and readily available and can be used on the direct extract of pigments, because esterification of the xanthophylls does not modify their chromophore characteristics, but avoids manipulation and possible losses during saponification.

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