Color Quality of Pigments in Cochineals (Dactylopius coccus Costa). Geographical Origin Characterization Using Multivariate Statistical Analysis

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The commercial value of a cochineal (Dactylopius coccus Costa) sample is associated with its color quality. Because the cochineal is a legal food colorant, its quality is generally understood as its pigment content. Simply put, the higher this content, the more valuable the sample is to the market.

In an effort to devise a way to measure the color quality of a cochineal, the present study evaluates different parameters of color measurement such as chromatic attributes ($L^*$, and $a^*$), percentage of carminic acid, tint determination, and chromatographic profile of pigments. Tint determination did not achieve this objective because this parameter does not correlate with carminic acid content. On the other hand, carminic acid showed a highly significant correlation ($r = -0.922, p = 0.000$) with $L^*$ values determined from powdered cochineal samples. The combination of the information from the spectrophotometric determination of carminic acid with that of the pigment profile acquired by liquid chromatography (LC) and the composition of the red and yellow pigment groups, also acquired by LC, enables greater accuracy in judging the quality of the final sample. As a result of this study, it was possible to achieve the separation of cochineal samples according to geographical origin using two statistical techniques: cluster analysis and principal component analysis.

KEYWORDS: Carminic acid; pigment profile; natural colorants; cluster analysis; principal component analysis; food quality

INTRODUCTION

The cochineal (Dactylopius coccus Costa) is an economically profitable insect because the bright natural pigment obtained from the dried bodies of the adult females is one of the natural colorants approved by legislation (1, 2) for use in foods. The main parameter used for commercial evaluation of cochineal pigment quality is its pigment content. Because the quality of a cochineal increases when its pigment content is higher, its measurement ensures the quality of the product as the basis of commercial transactions (3). The most common pigment in the cochineal is carminic acid. The cochineal is commonly used as food colorant in the form of carminic acid extract or carmine lake (an aluminum/calcium salt complex containing between 50 and 65% carminic acid) (4). Quantification of the carminic acid in these secondary products of cochineals is usually done by pigment extraction with hydrochloric acid, followed by spectrophotometric quantification (5, 6).

The transforming industry needs simple, quick, and readily available analytical methods to enable a rapid evaluation of cochineal quality. In other food products, color quality is measured by using different simple parameters, such as the chromatic attributes ($L^*$, $a^*$, $b^*$), proposed by the Commission Internationale de l’Eclairage (CIE) (7), and tint determination, which is an attempt to interpret the sample’s value by considering the ratio between yellow and red pigments. Both methods are widely used in the measurement of carotenoids in carrots (8) and in grapefruit juices (9, 10) and anthocyanins in wines (11, 12).

On the other hand, the determination of less common pigments in the cochineal is also very important, because precise knowledge of pigment composition helps to identify the authenticity of the colorant. More complex methods are necessary to determine pigment composition, including determination by liquid chromatography (LC) (13). Such methods can provide information about individualized pigment composition and total pigment content of the sample, but their complexity makes them unsuitable for routine industrial control.

Determination of geographical origin is a crucial issue in food quality control and safety. Peru, the Canary Islands (Spain) (14), and Chile produce the greatest amount of cochineals for commercial use (15, 16). Other countries, such as Mexico, Bolivia, South Africa, and Argentina also produce cochineals,
but in smaller quantities. Color quality depends on the climate, the host plant (wild cactus pear, *Opuntia ficus-indica* Mill), cultivation techniques (such as irrigation, fertilization, or the use of plastic covering), and the cochineal harvesting techniques in which the cochineal has been developed (17). Thus, because color quality varies with geographical origin, it is very important to have methods to distinguish cochineals as a function of their geographical origin. Multivariate analysis has traditionally been employed for food quality evaluation. These methods make the interpretation of physicochemical data easier when a large number of variables are analyzed in a set of samples because they emphasize the variables that best characterize the samples. Cluster analysis and principal component analysis have been successfully applied to analytical results to authenticate and classify food products according to their geographical origin or variety (18–21).

The present study evaluates the capacity of different parameters (chromatic attributes, percentage of carminic acid, tint determination, and pigment content determined by LC) to determine color quality in cochineals. Cochineal samples from different geographical origins were analyzed by multivariate statistical analysis (cluster analysis and principal component analysis) to determine if it is possible to characterize cochineals samples according to their geographical origin, and consequently, by their color quality.

**MATERIALS AND METHODS**

**Cochineal Samples.** Adult female cochineals were obtained from different geographical origins: Mexico (from three regions: Jalisco, Hidalgo, and Tepeitic), Peru, Chile, and Spain (from Canary Islands: Tenerife and Lanzarote). The cochineals (50 g) were collected from wild cactus pear cultivars (*Opuntia ficus-indica* Mill) and cleaned over a sieve to eliminate dust and heterogeneous materials such as molt residuals or plant material. Then, the insects were dried at 60 °C in a Selecta (Barcelona, Spain) heater until all water was completely eliminated.

**Cochineal Extraction.** Dried insects were finely ground in a ceramic mortar, and an amount accurately weighed at ca. 0.125 g was mixed with 30 mL of 2 N HCl. The mixture was homogenized in an Omnimixer model ES-207 (Omni International Inc., Gainesville, VA) high-speed blender for 1 min, and pigments present in the sample were extracted for 35 min in a Selecta water bath at 65 °C in a sealed vessel. Then, the sample was cooled and centrifuged at 7000 rpm and 4 °C for 15 min. This procedure was repeated twice, and the resulting two supernatants were mixed together. The collected supernatants were then diluted to 250-mL with water. This extraction procedure permits the spectrophotometric determination of percentage of carminic acid, although other less common pigments, such as deVII pigment, flavokermesic acid, and kermesic acid, are destroyed when HCl is used in the extractant (13). For this reason, another extraction procedure was developed to determine the chromatic attributes and tint value of the extract, as well as the liquid chromatographic pigment profile. The samples were extracted similarly to those to measure carminic acid, but with 10 mL of methanol/water (65:35, v/v) as extractant, 80 °C as extraction temperature, and 30 min as extraction time. For the determination of chromatographic profile, 25-mL was used as final volume of the extract (13). All extractions were carried out in quintuplicate.

**Evaluation of Chromatic Attributes.** Chromatic attributes were measured with a Minolta Chroma Meter model CR-300 (Wheeling, IL) color difference meter, using ca. 5.0 g of the sample placed in a glass flask. *L* *, a* color parameters were measured in the whole dried insect and in dried insects finely ground. Both chromatic attributes (*L* *, a* *) were also measured in the cochineal methanol/water (65:35, v/v) extract.

**Carminic Acid Percentage and Tint Determination.** Absorbance measurements, for the determination of percentage of carminic acid and tint, were made on a Shimadzu UV–vis 160A recording spectro-photometer (Kyoto, Japan) with 1-cm path length glass cells. The percentage of carminic acid in cochineal samples was calculated by absorbance measurement at 494 nm. The absorbance was also recorded at 420 and 500 nm, and tint was calculated as the ratio of absorbance at 420 to absorbance at 500 nm ($A_{420}/A_{500}$).

**Chromatographic Determination of Cochineal Pigments.** The liquid chromatographic method used for the determination of pigments consisted of a gradient elution procedure with UV–vis detection. Measurements of pigments were made on a Shimadzu modular chromatographic system, equipped with a LC-10 AD pump, a SPD-M6A UV–vis diode array detector (DAD), and controlled with a Class LC-10 data acquisition software (also from Shimadzu). The injection valve was a Rheodyne (Cotati, CA) Model 7725i with an injection loop of 20 μL. The chromatographic system was equipped with a reversed phase C8 Spherisorb ODS-2 (Alltech, Deerfield, IL) column (5-μm, 25-cm × 4.6-mm i.d. D.). A precolumn (5-μm, 7.5–× 4.6-mm i.d.) of the same material was fitted to protect the main column. Separation and quantification of the cochineal pigments was carried out using a method previously developed by the authors (13). The mobile phase consisted of a mixture of water, methanol, and orthophosphoric acid (5% in water). The elution program, which lasted 30 min, consisted of an initial mixture of 50% water/40% methanol/10% orthophosphoric acid maintained for 11 min. The mixture was then changed with a linear gradient over the next 13 min to 0% water/90% methanol/10% orthophosphoric acid and finally maintained for an additional 6 min. The flow rate of the mobile phase was 1.2 mL/min. Detection wavelengths for the DAD were set at 275, 420, and 500 nm. The concentrations of yellow (*Y*) and red (*R*) pigment groups were calculated as the concentration of all the pigments that absorb at a wavelength of 420 nm or at 500 nm, respectively (22). Because it is not possible to have access to standards for the cochineal pigments (except for carminic acid, which was supplied by Sigma (Madrid, Spain)), the absolute area of pigments was used to characterize the color pigment pattern and to compare its profile between samples.

**Statistical Analysis.** Data analysis was carried out with the Statgraphics Plus software version 5.1 (Statistical Graphics, Rockville, MD). Simple linear correlation analysis was used to measure the correlation between chromatic attributes (*L* *, a* *) or tint determination with the percentage of carminic acid. Data were processed by one-way analysis of variance (ANOVA) for color quality parameters in five replicates (*p > 0.05*), with geographical origin or pigment as fixed effects. Fisher’s Least-Significant-Difference test (LSD) was used for assæ differences between individual samples (*p > 0.05*).

Two multivariate techniques [cluster analysis (CA) and principal component analysis (PCA)] were used to characterize quality cochineals from different geographical origins on the basis of its chemical composition. The CA has been used for searching natural groupings among the studied variables. Clustering techniques are an unsupervised classification procedure that involves a measurement of either the distance or the similarity between objects to be clustered. The initial assumption is that nearness reflects similarity. The simplest distance between two points is well defined, being the simplest distance the Euclidean distance. Ward’s method was applied in this test as clustering algorithm that indicates the distance between two groups (23, 24). Due to its unsupervised character, cluster analysis can only be used to perform preliminary, essentially descriptive, scans of the data to be analyzed. An explanatory examination of the auto-scaled data was performed using the PCA (25), a technique to extract, rationalize, and visualize all useful information from the data set. It involves an orthogonal rotation that transforms the original variables in an m-dimensional space, into uncorrelated variables called principal components (PC), in a space of two or three dimensions, ordered according to their explained variance. These new PCs are linear combinations of the original variables calculated to maximize the dispersion of individuals. The coefficients of the original variables defining each PC are called loadings, and the projections of the samples on the new axes are called scores.

**RESULTS AND DISCUSSION**

**Percentage Carminic Acid Determination in Cochineal Extract.** Carminic acid determination is used for commercial
purposes. This analysis is usually carried out in carminic acid extract or carmine with UV-vis spectrophotometric methods described by the Instituto de Investigación Tecnológica Industrial y de Normas Técnicas de Perú (ITINTEC) (5) and Joint FAO/WHO Expert Committee on Food Additives (JECFA) (6). Both methods consist of the extraction of the sample with 2 N HCl at 100 °C until complete dissolution followed by UV-vis spectrophotometric determination at 494 nm. In accordance with previous studies in the treatment of cochineal samples done by our research group (13) and the results obtained by other authors (25), carminic acid is destroyed when exposed to temperatures above 80 °C for a long time. For this reason, the optimization of carminic acid extraction conditions, previous to the UV-vis spectrophotometric determination, was carried out. Several variables that can potentially affect the extraction efficiency were studied: temperature, time, extractant volume, and number of extractions. Other factors implicated in the extraction were kept constant: amount of dried cochineal (0.125 g), extractant (2 N HCl), and final volume of the extract (250 mL). The effect of extraction temperature on the carminic acid extraction was studied over the range of 30–80 °C. The maximum extraction was obtained at temperatures between 60 and 70 °C; meanwhile higher temperatures decreased the extraction efficiency. An extraction temperature of 65 °C was chosen as the optimum. The extraction time was varied between 15 and 60 min, and 35 min was found to be optimal for the maximum extraction of carminic acid. A sequence of experiments was performed to select the extractant volume (2 N HCl) varying it between 20 and 40 mL and keeping the other factors constant. The best extractant volume was 30 mL of 2 N HCl. The optimum number of extractions was 2, because the extraction efficiency remained practically constant above this value.

To calculate the carminic acid percentage, calibration graphs for carminic acid were constructed by plotting the absorbance against the carminic acid concentration at seven concentration levels (5–75 μg/mL). Standards of each concentration level were analyzed in triplicate. The equation obtained from the calibration graph was: absorbance = −0.0016 + 0.0201 · carminic acid concentration (μg/mL). The detection limit, defined as three times the standard deviation of the background noise, determined using 2 N HCl, divided by the calibration graph slope, was 0.5 μg/mL. The repeatability of the spectrophotometric method, expressed as relative standard deviation, was checked on 11 individual samples containing 50 μg/mL of carminic acid and was found to be ca. 3.1%.

**Color Quality Determination in Cochineals.** A set of seven samples of different geographical origin was analyzed for color quality. One group was composed of Canary Islands cochineals: Tenerife (n = 1) and Lanzarote (n = 1). The other group was composed of America cochineals: Mexico (n = 3), Peru (n = 1) and Chile (n = 1).

The mean and standard deviations (obtained from five sub-samples of each of the seven samples analyzed) for the quality parameters studied, chromatic attributes (measured in dried whole cochineal, powered insects, and in a pigment extract), percentage of carminic acid, and tint value, are shown in Table 1. The L* parameter was significantly different with dried cochineal samples (whole insects or in powder). Dried whole cochineal samples presented higher values of L* than those from the powder or extract, meanwhile a* values were higher for powdered cochineals showing significant differences within powder and extract samples. Positive values of a* parameter indicate red coloration; therefore, the increase in this parameter indicates that the pigments were freed when the insect structure was modified. There were significant differences in the carminic acid content on the different samples. The carminic acid percentage was between 12.5 and 21.0%. The Jalisco (Mexico) cochineal presented the lowest percentage of carminic acid (12.8 ± 0.3%), and the Tenerife (Canary Islands) and Chile cochineals showed the highest content, 19.7 ± 1.0% and 19.4 ± 1.6%, respectively. The amount of carminic acid found in the analyzed cochineals is similar to that described by other authors (17, 25, 26). Wouters and Verhecken (26) established a percentage of carminic acid of 18–20% and 13% for Tenerife and Mexico cochineals, respectively. There were no significant differences in the tint value on the different samples analyzed. Therefore, tint value, which is based in the ratio between yellow and red pigments, did not indicate quality and may even lead to confusion, because it is not possible to distinguish among cochineals with very different carminic acid contents.

<p>| Table 1. Average Quantities and Standard Deviations (n = 5) of Quality Parameters for Different Cochineals from Different Geographical Origins |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>quality parameter</th>
<th>Chile</th>
<th>Peru</th>
<th>Tenerife</th>
<th>Lanzarote</th>
<th>Hidalgo</th>
<th>Tepetic</th>
<th>Jalisco</th>
</tr>
</thead>
<tbody>
<tr>
<td>L* value</td>
<td>44.92 ± 1.40</td>
<td>40.55 ± 3.13</td>
<td>43.42 ± 2.37</td>
<td>40.72 ± 1.95</td>
<td>42.41 ± 4.71</td>
<td>37.14 ± 2.63</td>
<td>40.71 ± 0.92</td>
</tr>
<tr>
<td>a* value</td>
<td>2.36 ± 0.17</td>
<td>3.03 ± 0.30</td>
<td>2.32 ± 0.44</td>
<td>2.60 ± 0.52</td>
<td>2.43 ± 1.10</td>
<td>2.11 ± 0.13</td>
<td>2.84 ± 0.40</td>
</tr>
<tr>
<td>carminic acid (%)</td>
<td>19.4 ± 1.6</td>
<td>16.0 ± 0.3</td>
<td>19.7 ± 1.0</td>
<td>15.8 ± 0.8</td>
<td>17.9 ± 0.3</td>
<td>15.9 ± 0.2</td>
<td>12.8 ± 0.3</td>
</tr>
<tr>
<td>tint value (A00/A000)</td>
<td>0.44 ± 0.44</td>
<td>0.62 ± 0.74</td>
<td>0.44 ± 0.75</td>
<td>0.62 ± 0.75</td>
<td>0.44 ± 0.75</td>
<td>0.44 ± 0.75</td>
<td>0.44 ± 0.75</td>
</tr>
</tbody>
</table>

A–D Within a row, different letters denote significant differences (p < 0.05) between cochineals.

The correlation coefficients indicated a moderately strong relationship between the carminic acid percentage and a* parameter from the powdered cochineal.
for carminic acid ranging between 93 and 97%. The standardized compound in the cochineal extract. At 275 nm (where the pigment Chile Peru Tenerife Lanzarote Hidalgo Tepeitic Jalisco

Table 2. Absolute Area (n = 5) of Pigments for Cochineals from Different Geographical Origins

<table>
<thead>
<tr>
<th>pigment</th>
<th>Chile</th>
<th>Peru</th>
<th>Tenerife</th>
<th>Lanzarote</th>
<th>Hidalgo</th>
<th>Tepeitic</th>
<th>Jalisco</th>
</tr>
</thead>
<tbody>
<tr>
<td>dcII</td>
<td>167 ± 17 A</td>
<td>196 ± 8 A</td>
<td>88 ± 8 A</td>
<td>148 ± 2 A</td>
<td>203 ± 7 A</td>
<td>94 ± 5.8 A</td>
<td>102 ± 4 A</td>
</tr>
<tr>
<td>ca</td>
<td>6609 ± 446 B</td>
<td>5814 ± 72 B</td>
<td>5097 ± 40 B</td>
<td>4808 ± 80 B</td>
<td>5914 ± 216 B</td>
<td>4949 ± 93 B</td>
<td>4464 ± 65 B</td>
</tr>
<tr>
<td>dcII</td>
<td>212 ± 22 B</td>
<td>167 ± 14 C</td>
<td>202 ± 1 A</td>
<td>226 ± 2 C</td>
<td>211 ± 14 B</td>
<td>169 ± 9 C</td>
<td>148 ± 15 C</td>
</tr>
<tr>
<td>dcIV</td>
<td>143 ± 13 C</td>
<td>126 ± 10 D</td>
<td>133 ± 10 B</td>
<td>159 ± 4 D</td>
<td>115 ± 1 C</td>
<td>90 ± 3 A</td>
<td>98 ± 3 A</td>
</tr>
<tr>
<td>dcVII</td>
<td>30.5 ± 1.0 D</td>
<td>26.8 ± 2.5 E</td>
<td>37.1 ± 3.9 E</td>
<td>37.6 ± 2.3 E</td>
<td>23.7 ± 0.9 D</td>
<td>6.2 ± 0.1 D</td>
<td>22.3 ± 2.0 D</td>
</tr>
<tr>
<td>fk</td>
<td>17.5 ± 0.9 E</td>
<td>21.0 ± 0.7 F</td>
<td>19.2 ± 0.6 F</td>
<td>29.3 ± 2.0 F</td>
<td>14.5 ± 0.6 E</td>
<td>5.7 ± 0.3 E</td>
<td>13.9 ± 0.9 E</td>
</tr>
<tr>
<td>ka</td>
<td>9.3 ± 1.0 B</td>
<td>14.3 ± 0.3 G</td>
<td>20.6 ± 1.0 G</td>
<td>30.3 ± 1.0 E</td>
<td>8.7 ± 0.3 F</td>
<td>9.7 ± 0.1 F</td>
<td>8.5 ± 0.8 F</td>
</tr>
<tr>
<td>Visible Detection: λ = 420 nm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dcII</td>
<td>30.6 ± 3.1 A</td>
<td>35.9 ± 0.4 B</td>
<td>16.7 ± 0.1 A</td>
<td>26.0 ± 0.6 A</td>
<td>36.8 ± 1.0 B</td>
<td>170 ± 0.8 C</td>
<td>20.1 ± 3.2 A</td>
</tr>
<tr>
<td>ca</td>
<td>1026 ± 74 B</td>
<td>834 ± 8 B</td>
<td>710 ± 3 B</td>
<td>682 ± 9 B</td>
<td>852 ± 38 B</td>
<td>699 ± 12 C</td>
<td>654 ± 42 B</td>
</tr>
<tr>
<td>dcII</td>
<td>29.6 ± 1.6 A</td>
<td>20.3 ± 0.4 C</td>
<td>26.2 ± 0.3 C</td>
<td>30.6 ± 0.4 A</td>
<td>24.3 ± 3.3 D</td>
<td>21.9 ± 0.1 C</td>
<td>19.1 ± 0.6 B</td>
</tr>
<tr>
<td>dcIV</td>
<td>21.1 ± 1.5 A</td>
<td>16.6 ± 1.1 D</td>
<td>17.4 ± 0.2 D</td>
<td>21.7 ± 0.7 D</td>
<td>16.4 ± 1.1 D</td>
<td>12.1 ± 0.3 C</td>
<td>13.8 ± 1.6 C</td>
</tr>
<tr>
<td>dcVII</td>
<td>2.3 ± 0.3 D</td>
<td>2.4 ± 0.2 E</td>
<td>3.3 ± 0.1 E</td>
<td>2.3 ± 0.1 E</td>
<td>2.5 ± 0.2 E</td>
<td>0.99 ± 0.03 E</td>
<td>1.8 ± 0.1 D</td>
</tr>
<tr>
<td>fk</td>
<td>3.3 ± 0.1 E</td>
<td>2.6 ± 0.2 E</td>
<td>2.4 ± 0.1 F</td>
<td>3.8 ± 0.2 F</td>
<td>2.7 ± 0.2 E</td>
<td>0.57 ± 0.02 E</td>
<td>1.8 ± 0.1 D</td>
</tr>
<tr>
<td>ka</td>
<td>1.4 ± 0.1 F</td>
<td>1.4 ± 0.1 F</td>
<td>1.5 ± 0.1 G</td>
<td>2.9 ± 0.2 G</td>
<td>1.3 ± 0.2 E</td>
<td>0.65 ± 0.02 E</td>
<td>1.0 ± 0.1 E</td>
</tr>
</tbody>
</table>

(λ = 275 nm) and from the pigment extract (r = −0.831, p = 0.000) and from the pigment extract (r = −0.583, p = 0.006). The highly significant correlation (r = −0.922, p = 0.000) between carminic acid percentage and L* parameter from the powdered cochineal can be emphasized. This correlation defines the following regression line that permits the calculation of the percentage of carminic acid in a cochineal sample with a previous determination of L* value: L* (powdered cochineal) = 33.56–0.38 · carminic acid (%). Thus L* color value from the powdered insect can be used as a rapid parameter from the powdered cochineal can be emphasized. This correlation allows the complete pigments characterization in the cochineals. But many other compounds can influence the final quality of the color value from the powdered insect can be used as a rapid parameter from the powdered cochineal can be emphasized. This correlation allows the complete pigments characterization in the cochineals.

Table 2 shows the results of the chromatographic determination of the pigment profile (compared as absolute area) in the samples corresponding to cochineals from different geographical origins. The results of statistical analysis are also included in this table for comparison. Seven pigments were used to characterize the pigment pattern of cochineal: carminic acid, flavokermesic acid, kermesic acid, and four structurally unknown pigments of Dactylopius coccus Costa: dcII, dcIII, dcIV, and dcVII (13, 26). As can be seen in the table, carminic acid is the principal compound in the cochineal extract. At 275 nm (where the absorbance is maximum for all the pigments) the standardized area, defined as the ratio between peak area of each pigment and the total peak area, for carminic acid was between 84 and 94%. Wouters and Verhecken (26) found a standardized area for carminic acid ranging between 93 and 97%. The standardized area for dcII, dcIII, and dcIV pigments (measured at 275 nm) ranged from 1.4 to 3.2%, from 2.4 to 4.1%, and from 1.6 to 2.9%, respectively. However, the standardized area for dcIII (0.1–2.4%) and dcIV (0.7–2.0%) pigments measured by Wouters and Verhecken (26) was lower. The less common pigments were dcVII pigment, flavokermesic acid, and kermesic acid, which confirms the results obtained by Wouters and Verhecken (26). At 275 nm, the area for dcVII pigment from Tepeitic (Mexico) cochineal was lower than those of Tenerife and Lanzarote (Canary Islands), which showed the highest area of this pigment. The Tepeitic cochineal also presented a lower area for flavokermesic acid than the area observed in the Lanzarote cochineal. The Chile, Jalisco, and Hidalgo (Mexico) cochineals presented the lowest area for kermesic acid. On the other hand, the area for kermesic acid of Lanzarote cochineal was significantly higher than the area obtained in the rest of cochineals. In most cases, it was also observed that dcII pigment and flavokermesic acid showed a smaller area at 500 nm than at 420 nm. Figure 1 shows the significant difference in the pigments peaks in the chromatograms from Lanzarote (Figure 1A) and Tepeitic (Figure 1B) cochineal samples.

Table 3 shows the pigment concentrations of the yellow and red pigment groups, as well as the yellow/red ratio for the seven cochineal samples studied. The pigment content in the different samples varied greatly. The results indicate that the differences were statistically significant for both yellow and red pigments within the samples. The yellow pigment concentrations were between 847 ± 34 and 1327 ± 53 mg/L, whereas red pigment concentrations were between 830 ± 39 and 1308 ± 45 mg/L. As can be seen in Table 3, the ratio of yellow/red pigment groups tends to be 1, remaining constant despite the differences found in the pigment content of the different cochineal samples (22). Statistical analysis did not show significant differences in the value of the ratio of yellow/red pigment groups.

Multivariate Statistical Analysis. To ensure that the different color quality variables analyzed in the cochineal samples can achieve a separation among samples, multivariate analysis was
carried out. The following chemical parameters analyzed were used in multivariate statistical analysis: percentage of carminic acid; carminic, flavokermesic and kermesic acids, and dcII pigment absolute area at 275, 420, and 500 nm; and yellow and red pigment groups (number of variables: 15). Samples from different geographical origin (Canary Islands and America; seven samples of different geographical origin and five sub-samples) were discriminated by color quality. The difference in the samples may be a response to a number of diverse factors: climate, host plant cultivation techniques, and cochineal harvesting techniques.

Cluster analysis (CA) was carried out with Euclidean distance as similarity measurement and Ward’s method as amalgamation rule. The results of the CA made over all studied cochineals are presented as dendogram in Figure 2. Taking as an arbitrary cutoff point a similarity level < 50, three main clusters can be

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**Table 3. Pigment Fractions (Yellow and Red), and Yellow/Red Ratio Determined by the Chromatographic Method (n = 5)**

<table>
<thead>
<tr>
<th>Pigment Group</th>
<th>Chile</th>
<th>Peru</th>
<th>Tenerife</th>
<th>Lanzarote</th>
<th>Hidalgo</th>
<th>Tepetic</th>
<th>Jalisco</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yellow $^a$</td>
<td>1327 ± 53 $^a$</td>
<td>1088 ± 44 $^b$</td>
<td>926 ± 31 $^c$</td>
<td>916 ± 30 $^c$</td>
<td>1115 ± 45 $^b$</td>
<td>919 ± 46 $^c$</td>
<td>847 ± 34 $^c$</td>
</tr>
<tr>
<td>Red $^a$</td>
<td>1398 ± 45 $^a$</td>
<td>1098 ± 46 $^b$</td>
<td>910 ± 40 $^c$</td>
<td>991 ± 37 $^c$</td>
<td>1082 ± 62 $^b$</td>
<td>901 ± 34 $^b$</td>
<td>830 ± 39 $^c$</td>
</tr>
<tr>
<td>Yellow/Red</td>
<td>1.02 ± 0.05 $^a$</td>
<td>1.00 ± 0.04 $^a$</td>
<td>1.02 ± 0.03 $^b$</td>
<td>1.00 ± 0.03 $^b$</td>
<td>1.03 ± 0.02 $^a$</td>
<td>1.02 ± 0.04 $^a$</td>
<td>1.02 ± 0.06 $^a$</td>
</tr>
</tbody>
</table>

$^a-c$ Within a row, different letters denote significant differences (p < 0.05) between pigment fractions and yellow/red ratio. $^d$ Concentration (mg/L).

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**Figure 1.** Separation of cochineal (*Dactylopius coccus* Costa) pigments by liquid chromatography with UV−vis detection at 275, 420, and 500 nm for the Lanzarote (A) and Tepetic (B) samples. Cochineal pigments: dcII, dcIII, dcIV, and dcVII, unknown pigments of *Dactylopius coccus* Costa; ca, carminic acid; fk, flavokermesic acid; and ka, kermesic acid.
visualized, one of them consists of cochineals whose geographical origin is the Canary Island in the European continent (top of dendogram), and the second and third clusters contain cochineals whose geographical origin is the American continent. The second cluster includes samples obtained from Mexico (Jalisco and Tepeitic), whose percentage of carminic aid was lower. The third cluster consists of insects from Chile, Hidalgo, and Peru.

CA is not conclusive in and of itself, because it merely gives information on the similarity of the different samples. For this reason, principal component analysis (PCA), a technique that illustrates which variables account for most of the variability in the data, was employed. All variables were mean centered and scaled to unit variance prior to analysis. PCA showed three interpretable components, chosen on the basis of Kaiser’s criterion (eigenvalues higher than 1.0 are chosen), explaining together 92.96% of the total variance in color quality (Figure 3). Figure 4A shows the plot of loadings by selecting the first two principal components (PC) as axes. The first PC that explains the higher percentage of variance (44.74%) is mainly related to the yellow and red pigment fractions and to the areas for carminic acid detected at 275, 420, and 500 nm and for dcII pigment detected at 275 and 420 nm (with a positive correlation). The second PC (34.09% of the variance) is related to the areas of dcII pigment detected at 500 nm (with a positive correlation) and of flavokermesic and kermesic acids at the three wavelengths studied (with a negative correlation). On the other hand, Figure 4B also shows the corresponding scores onto the two first PC. As can be seen, samples are grouped according to geographical origin in three distinct groups: cochineals from the Canary Islands, from Jalisco and Tepeitic, and from Chile, Hidalgo, and Peru. Thus, cochineals from the Canary Islands scored high for area of flavokermesic and kermesic acids detected at 500 and 275 nm, respectively. Cochineals from
Jalisco and Tepeitic scored high for area for dcII pigment detected at 500 nm. Cochineals from Chile, Hidalgo and Peru were correlated with area for dcII pigment (detected at 275 and 420 nm), area for carminic acid (at the three wavelengths studied), and the yellow and red pigment fractions.

The variables studied are chemical descriptors useful in classifying cochineal samples according to geographical origin, because cochineal samples from Canary Islands and from America could be differentiated by CA and PCA techniques. Nevertheless, no definitive conclusions can be confirmed. The study of a greater number of samples will be necessary to check and validate these classification results.

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