JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Optimizing Conditions for the Extraction of Pigments in Cochineals (*Dactylopius coccus* Costa) Using Response Surface Methodology

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A simple method was developed for the extraction and determination of color pigments in cochineals (*Dactylopius coccus* Costa). The procedure was based on the solvent extraction of pigments in insect samples using methanol:water (65:35, v:v) as extractant. Two-level factorial design was used in order to optimize the solvent extraction parameters: temperature, time, methanol concentration in the extractant mixture, and the number of extractions. The results suggest that the number of extractions is statistically the most significant factor. The separation and determination of the pigments was carried out by high-performance liquid chromatography with UV−visible detection. Because the absorption spectra of different pigments are different in the visible region, it is convenient to use a diode array detector to obtain chromatographic profiles that allow for the characterization of the extracted pigments.

KEYWORDS: Natural colorants; *Dactylopius coccus* Costa; food analysis; solvent extraction; experimental design; high-performance liquid chromatography with UV−visible detection

INTRODUCTION

Because the appearance of food is as important as its taste, modern food manufacturers have become greatly concerned with conserving the aspect of foods that have lost their natural colors during processing. Adding colorants preserves the aspect of foods while also reducing batch-to-batch color variations and enhancing the natural color (*1*). Nevertheless, synthetic colorants have increasingly been perceived as undesirable or harmful (*2*, *3*) by consumers. At the same time, the European Union and the United States have restricted the use of synthetic colorants as food additives (*4*, *5*). These restrictions on the use of artificial colorants have increased the need to study the use of natural pigments in the food industry.

The cochineal (*Dactylopius coccus* Costa) is an insect that is widely used as a natural food pigment in the food industry (*6*). The most common pigment in the cochineal is carminic acid (a red hydroxyanthraquinone pigment), which is approved by legislation as a natural colorant (*4*, *5*). Several analytical methods have been reported for the determination of carminic acid using spectrometry (*7*, *8*), enzyme immunoassay (*9*), capillary electrophoresis (*10*), and liquid chromatography (*11*−*13*).

The determination of less common pigments in the cochineal has received less attention, although it is also very important, because precise knowledge of pigment composition helps to identify the authenticity of carmine lake (an aluminum/calcium salt complex containing between 50 and 65% of carminic acid). Pigment patterns can be used to detect adulterations in natural colorants in food products (*14*, *15*). Although many chromatographic methods have been developed for the determination of carminic acid, to our knowledge, only one method has been reported that determines the composition of the less common pigments in *Dactylopius coccus* Costa. However, this method was developed to distinguish between closely related species of insects (*16*).

A critical step in the analytical determination of cochineal pigments by high-performance liquid chromatography (HPLC) that has not been studied in detail is the extraction of these pigments from the insect's body. In this study, for the first time, the optimization of the extraction of color pigments from the cochineal using solvent extraction is proposed. The use of factorial experiment designs to optimize four variables (extraction temperature, extraction time, methanol concentration in the extractant mixture, and the number of extractions) determines an optimal set of operational conditions. Furthermore, it establishes experimental conditions for the efficient separation

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10.1021/jf025756r CCC: $22.00
Published on Web 10/25/2002

of the pigments using HPLC and highlights the importance of diode array detection in the characterization of colorants.

EXPERIMENTAL PROCEDURES

Cochineal Samples. Adult female cochineals (*Dactylopius coccus* Costa) were collected in Valle de Guerra, Tenerife, Canary Islands, from several wild cactus pear cultivars, *Opuntia ficus-indica* Mill. The cochineals were cleaned over a sieve to eliminate dust and heterogeneous materials such as molt residuals or plant material. The insects were then dried at 60 °C in a Selecta (Barcelona, Spain) heater until all water was completely eliminated (this was indicated when constant weight was reached).

Solvent Extraction Method. Dried insects were finely ground in a ceramic mortar, and an amount accurately weighed at ca. 0.125 g was mixed with 10 mL of methanol:water (65:35, v:v). The mixture was homogenized in an Omnimixer model ES-207 high-speed blender (Omni International Inc., Gainesville, VA) for 1 min, and pigments present in the sample were extracted for 30 min in a Selecta water bath at 80 °C in a sealed vessel. The sample was cooled and centrifuged at 7000 rpm for 15 min. This procedure was repeated twice, and the resulting two supernatants were mixed together. The collected supernatants were then diluted to 25 mL with water.

Experimental Design. The extraction method described in the above section was optimized using an experimental design. The entire experiment was executed in two phases using two central composite designs. The objective of the first phase was to study the simultaneous effect of four variables (temperature, time, methanol concentration in the extractant mixture, and number of extractions) on extraction efficiency using a factorial Draper−Lin central composite design. The experimental results obtained in this step demonstrated how the variables influenced pigment extraction. Using this information, two of the variables were fixed (extraction time and number of extractions) in the next phase. In the second phase, a two-level full factorial design was superimposed on a face-centered star design with two center points. The two remaining variables (extraction temperature and methanol concentration in the extractant mixture) were set at three separate coded levels.

The Statgraphics Plus for Windows version 4.2 package (Statistical Graphics, Rockville, MD) software program was employed to generate a design, perform regression analysis, and obtain the response surface plots.

Chromatographic Determination of Pigments. The HPLC method used for the determination of pigments consisted of a gradient elution procedure with UV−visible detection. All measurements were made on a Shimadzu modular liquid chromatographic system (Kyoto, Japan), equipped with an LC-10 AD pump, an SPD-M6A UV−visible diode array detector, an SPD-10AV UV−visible detector, and a Rheodyne model 7725i injector (Cotati, CA) with a sample loop of 20 μL. Class LC-10 software (also from Shimadzu) was used for acquisition of data. Separations were carried out on a Spherisorb ODS-2 RP-C_{18} (Alltech, Deerfield, IL), 5 μm, 25 cm × 4.6 mm i.d. column fitted with a Spherisorb ODS-2 RP-C_{18} (Alltech), 5 μm, 7.5 × 4.6 mm i.d. guard column. Each analysis was performed at room temperature. 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 maintained for 6 additional min. The flow rate of the mobile phase was 1.2 mL/min. Solutions and samples were not filtered before HPLC injection because it was observed that filter membranes adsorb carminic acid and other pigments of the cochineal.

Detection wavelengths for the UV−visible detector were set at 275 nm and for the diode array detector at 420 (yellow pigments) and 500 nm (red pigments) simultaneously. The spectra (detection wavelengths from 200 to 700 nm) were recorded for all peaks. The identities of the different chromatographic peaks were confirmed by comparing their visible spectral characteristics with retention times and the standard of carminic acid. The spectra of the other pigments of the cochineal (flavokermesic acid, kermesic acid, dcII, dcIII, dcIV, and dcVII) were compared with the results published by Wouters and Verhecken (*16*). The efficiency of peak separation was checked by the peak purity test carried out at maximum absorbance.

Determination of the pigment profile was done in quintuplicate, and the relative standard deviations (RSD) of the retention time, peak height, and peak area were then calculated (intra-day reproducibility). The inter-day reproducibility was calculated from the five parallel determinations carried out for three consecutive working days.

Chemical and Reagents. Carminic acid was supplied by Sigma (Madrid, Spain). All other reagents (methanol, hydrochloric acid, and orthophosphoric acid) were purchased from Merck (Darmstadt, Germany). For chromatographic analysis, HPLC-grade methanol, supplied by Merck, and deionized water of 18 MΩ/cm resistivity, purified with a Milli-Q system (Millipore, Bedford, MA), were used throughout.

In the analysis of natural products, it is relatively normal not to have access to standards for some of the compounds separated chromatographically. In this situation, the relative concentrations of pigments obtained after normalization can be used to characterize the color pigment pattern (*14, 15*). The relative concentration (%) is defined as the relationship between peak area (or height) of each pigment and the total peak area (or height). This is true if the linearity between concentration and peak area (or height) is ensured and if the proportionality constant between the detector response and the concentration is similar for the different analytes. The linearity for carminic acid was determined using the linearity test. For this purpose, stock standard solution containing 1 mg/mL of carminic acid was prepared in water and stored in a glass-stoppered bottle at 4 °C in the dark, and solutions of variable concentrations were prepared by diluting the stock standard solution in methanol:water (65:35, v:v).

RESULTS AND DISCUSSION

Chromatographic Conditions. The cochineal pigments were separated into many fractions under the optimal HPLC conditions as demonstrated in **Figure 1**. Initially, the chromatographic conditions described by Wouters and Verhecken (*16*) were followed to separate the pigments. Different solvent mixtures and gradient programs were then optimized to reduce the analysis time while keeping a good resolution between all pigment peaks. Good results were obtained using a mixture of water, methanol, and orthophosphoric acid (5% in water) as the mobile phase. The concentration of orthophosphoric acid was studied over the range 0.1−8%, and a concentration of 5% orthophosphoric acid was found to be optimal for resolution of the pigments. A sequence of experiments was performed for flow rates between 0.25 and 2.0 mL/min, keeping the other factors constant. The flow rate significantly influenced the retention times of the pigments; the best flow value was 1.2 mL/min (column pressure, 200 N/m^2) due to the better retention times and resolution for all the colorants. Higher flow rates created overpressure in the system.

The similarity between their UV−visible absorption spectra of carminic, flavokermesic, and kermesic acids and those of the unidentified pigments (referred to as dcII, dcIII, dcIV, and dcVII) could indicate that the structures of the different pigments are similar. In the UV region, all of the pigments display spectra constituted by various maximums, with one that stands out for its intensity at 275 nm. In the visible region, the different compounds show an absorption band that has a maximum intensity at wavelengths between 420 and 500 nm. This difference can be associated with the presence of yellows and reds in the colorant. To detect components separated by chromatography, a wavelength of 275 nm seems adequate. However, detection at different wavelengths could be useful to achieve chromatographic profiles that allow for the detection of adulterations in food colorants. With this in mind, additional chromatograms were made using detection at 420 and 500 nm. **Figure 1** shows the significant difference in the size of the peaks

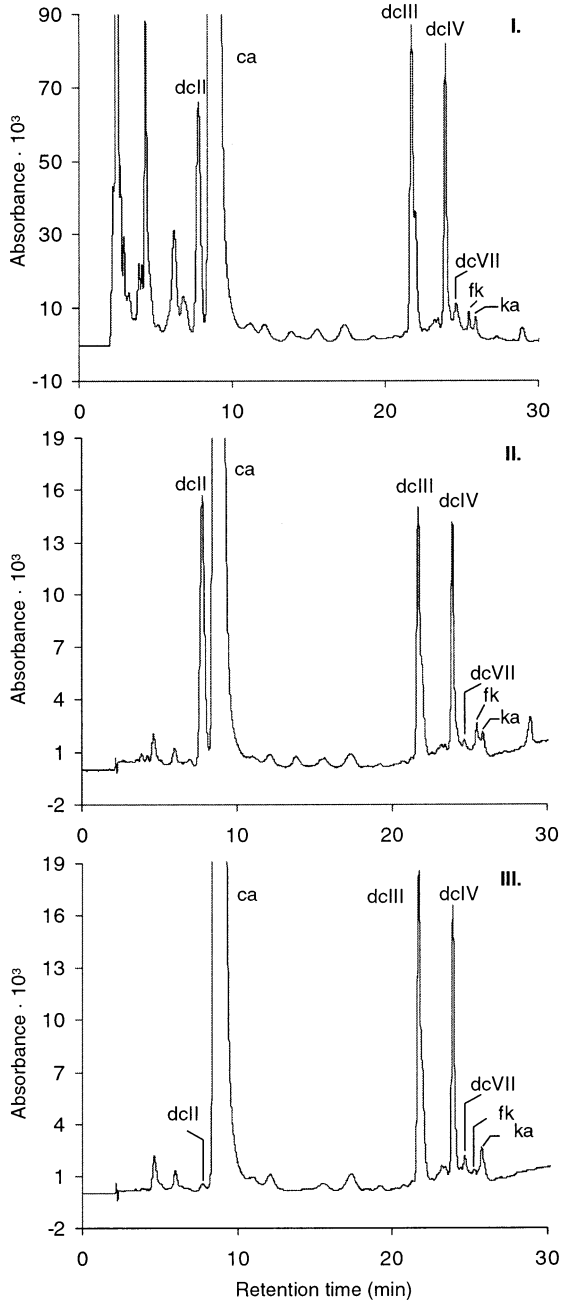

Figure 1. Chromatographic separation of cochineal (*Dactylopius coccus* Costa) pigments by HPLC with UV–visible detection at 275 (I), 420 (II), and 500 nm (III). Cochineal pigments: dcII, dcIII, dcIV, and dcVII, unknown pigments of *Dactylopius coccus* Costa; ca, carminic acid; fk, flavokermesic acid; and ka, kermesic acid.

in the different chromatograms. It also shows how the relationships between the peaks are different in each chromatogram. For example, dcII pigment and flavokermesic acid showed a very small molar absorptivity at 500 nm, while kermesic acid and dcVII pigment showed differences in the absorption of radiation at 420 and 500 nm. In this sense, the use of a diode array detector is especially useful. The matching values of the peak purity test varied between 85.2 and 97.3%, indicating that the color pigments of the cochineal were satisfactorily separated and that the quantity of coeluted pigment impurities was fairly low.

Calibration graphs for carminic acid (the only standard colorant available) were constructed by plotting the peak area against the carminic acid concentration at seven concentration levels. Samples of each concentration level were injected in triplicate. Quality parameters for the chromatographic determination of carminic acid are reported in **Table 1**. The detection limit, defined as the minimum concentration capable of giving a chromatographic signal 3 times higher than background noise, is also listed in **Table 1**. The repeatability of the chromatographic procedure, expressed as relative standard deviation (RSD), was checked on 11 consecutive injections of a standard solution containing 50 μg/mL of carminic acid.

Table 1. Quality Parameters for the Chromatographic Determination of Carminic Acid

	UV detection	visible detection	
	$\lambda = 275$ nm	$\lambda = 420$ nm	$\lambda = 500$ nm
slope[a]	19.217 ± 0.938	$5.94 \times 10^{-2} \pm 0.23 \times 10^{-2}$	$7.20 \times 10^{-2} \pm 0.33 \times 10^{-2}$
intercept[a]	0.791 ± 0.038	$2.25 \times 10^{-3} \pm 0.11 \times 10^{-3}$	$2.56 \times 10^{-3} \pm 0.12 \times 10^{-3}$
r^2	0.997	0.999	0.995
$S_{y/x}$	3.324	0.160	0.412
linear range[b] (μg/mL)	5–250	–	–
detection limit[b] (μg/mL)	2	–	–
RSD (%)	2.4	–	–

[a] Mean ± standard deviation. [b] The linear range and detection limit correspond to the most sensitive wavelength.

Optimization of the Solvent Extraction: Factorial Design. The use of a factorial design to explore the variables that affect the solvent extraction of cochineal pigments gives a clear idea of the overall number of experiments and possible interaction effects between the variables. In accordance with our previous experience in the treatment of cochineal samples, several variables that can potentially affect the extraction efficiency were chosen: temperature, time, methanol concentration in the extractant mixture, and number of extractions. A Draper−Lin fractional factorial design superimposed on a face-centered star design with two central points involving 18 runs was the first approach used to predict the response surface of the solvent extraction process. The lowest and highest values given to each factor were chosen: 50 and 70 °C (for the extraction temperature); 15 and 30 min (for the extraction time); 5% and 50% (for the methanol concentration in the extractant mixture); and 1 and 3 (for the number of extractions). Other factors implicated in the extraction were kept constant: amount of dried cochineal (0.125 g), volume of extractant (10 mL), and final volume of the extract (50-mL).

The experimental design parameters and the elemental response are shown in **Table 2**. The design's response was studied for all the pigments: carminic acid (ca), flavokermesic acid (fk), kermesic acid (ka), dcII, dcIII, dcIV, and dcVII. The relationship between peak area for each run and the peak area for the run with maximal response was used as the normalized response value for each pigment as a way to measure the extraction efficiency. The chronological listing of the experimental design parameters represents the statistically randomized order in which the experimental treatments were undertaken.

Variance analysis (ANOVA) was used to estimate the statistical significance of the main effects and interactions (**Table 3**). Analysis of the experimental results showed that, in the majority of cases, the most notable effect was caused by the number of extractions, its contribution ranging between 21% for kermesic acid and 88% for carminic acid and dcIV. The extraction temperature was statistically significant for flavokermesic acid, and the interaction between the number of extractions and methanol concentration in the extractant mixture was significant for carminic acid and dcIV pigment. The other

Table 2. Design Matrix in the Draper–Lin Small Central Composite Design and Response Values of the Pigments from Cochineal

run	temp (°C)	time (min)	no. of extractions	methanol (%)	normalized response (%)[a] dcII	ca	dcIII	dcIV	dcVII	fk	ka
1	50	30.0	3	50	93	97	100	96	85	76	23
2	60	22.5	1	27.5	73	77	48	62	52	29	14
3	50	30.0	1	50	71	74	65	71	64	56	20
4	70	15.0	3	50	100	100	75	100	100	100	100
5	60	15.0	2	27.5	82	90	69	85	76	46	21
6	60	22.5	3	27.5	82	95	63	87	78	45	49
7	60	22.5	2	27.5	84	90	61	81	70	42	38
8	70	22.5	2	27.5	91	94	84	89	90	92	42
9	50	15.0	3	5	94	95	74	92	81	92	47
10	60	22.5	2	5	98	95	79	91	87	89	47
11	70	15.0	1	50	74	73	74	70	63	56	31
12	50	22.5	2	27.5	88	91	74	85	78	55	51
13	60	22.5	2	27.5	84	90	59	85	81	56	57
14	70	30.0	1	5	84	84	69	79	75	60	63
15	60	30.0	2	27.5	88	92	73	85	75	44	50
16	50	15.0	1	5	83	81	53	74	67	54	46
17	70	30.0	3	5	91	88	63	90	93	65	49
18	60	22.5	2	50	98	98	86	98	97	66	59

[a] Normalized response (relationship between area for each run and the area for the run with maximal response) of each cochineal pigment: dcII, dcIII, dcIV, and dcVII, unknown pigments of *Dactylopius coccus* Costa; ca, carminic acid; fk, flavokermesic acid; ka, kermesic acid.

Table 3. Estimation Effects and Interactions in the Draper–Lin Small Central Composite Design

effects	probability values[a] dcII	ca	dcIII	dcIV	dcVII	fk	ka
temperature (T, °C)	0.537	0.312	0.449	0.349	0.245	**0.043**	0.627
time (t, min)	0.293	0.427	0.742	0.981	0.904	0.885	0.174
no. of extractions (n)	**0.008**	**0.001**	0.083	**0.001**	**0.007**	**0.015**	0.085
methanol (m, %)	0.999	0.292	0.589	0.159	0.305	0.127	0.549
$T \times t$	0.584	0.241	0.642	0.213	0.309	0.111	0.379
$T \times n$	0.890	0.434	0.078	0.752	0.316	0.711	0.221
$T \times m$	0.204	0.270	0.967	0.996	0.809	0.484	0.087
$t \times n$	0.453	0.098	0.804	0.222	0.532	0.079	0.094
$t \times m$	0.787	0.306	0.393	0.625	0.780	0.058	0.152
$n \times m$	0.070	**0.011**	0.432	**0.035**	0.192	0.430	0.083

[a] Probability values for each cochineal pigment: dcII, dcIII, dcIV, and dcVII, unknown pigments of *Dactylopius coccus* Costa; ca, carminic acid; fk, flavokermesic acid; ka, kermesic acid. Boldface denotes significant effects or interactions on the pigment extraction method at the 95% confidence level ($p < 0.05$).

factors and the interactions between them were not statistically significant ($p < 0.05$). Extraction time was the least important factor in the extraction efficiency. Two response surfaces are shown in **Figure 2**, selected from among those obtained using the experimental model. In these graphics, the variable with the greatest effect on the extraction, number of extractions, is shown along with the other two factors that have some influence: the composition of the extractant solution and the temperature. Both graphics show that the optimum number of extractions was 2.5, and the estimated values for extraction efficiency were similar for 2 and 3 extractions This conclusion is similar for all of the pigments detected in the cochineal. In the case of the extractant composition, when the number of extractions was equal to or greater than 2, the extraction efficiency was improved by increasing the percentage of methanol. Moreover, the response surface obtained by plotting the temperature versus the number of extractions shows that the extraction efficiency remained practically constant between 50 and 60 °C, while the efficiency increased upon increasing the temperature. The results obtained show that the ranges previously established to vary the percentage of methanol and temperature did not include the optimum. Even when the optimum was not included, the shape of the surfaces indicated the direction in which the new experiments should be performed.

Using these results, a new central composite design was established. The number of extractions and extraction time were kept constant. The number of extractions was fixed at 2 (instead of 3) to obtain the maximum extraction efficiency in the least amount of time. Due to its small influence on extraction efficiency, the extraction time was eliminated in the new experimental design and set at 30 min for the following experiments. Other factors, such as the amount of dried cochineal, volume of extractant, and final volume of the extract, were kept at the values mentioned earlier. A two-level full factorial design superimposed on a face-centered star design, 2^2 + star, with two center points was used, resulting in 10 randomized runs. **Table 4** shows the levels used, and **Table 5** shows the experimental results obtained and the design matrix. It should be noted that modification of the experimental conditions used for the extraction did not affect all of the pigments equally. In fact, different chromatographic profiles were obtained for different extraction conditions.

Extraction temperature was the most important factor for all pigments, being statistically significant for dcII, dcVII, flavokermesic acid, and kermesic acid. The Pareto chart for kermesic acid is shown in **Figure 3**. The response surface estimated for carminic acid, which is similar to those obtained for other pigments, is also represented in **Figure 3**, where the optimum extraction efficiency was reached at high extraction temperatures and high percentages of methanol in the extractant solution, especially for the compounds that are less soluble in water, such as dcVII pigment, flavokermesic acid, and kermesic acid. Higher

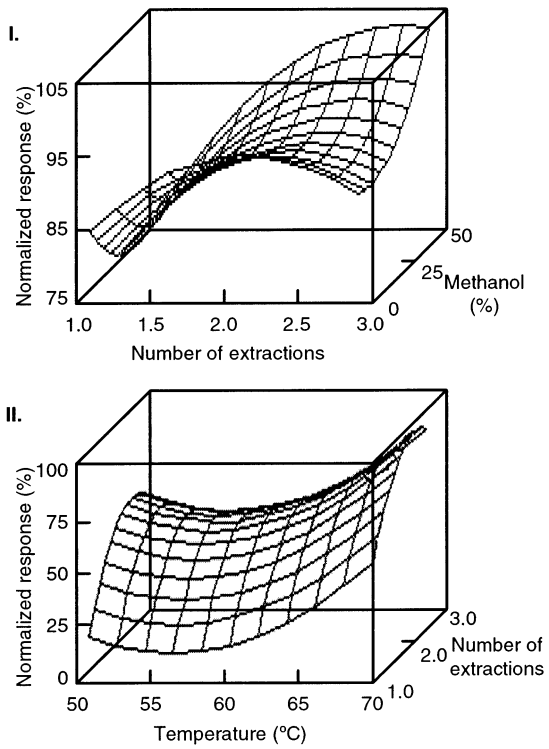

Figure 2. Estimated response surfaces obtained by plotting (I) the number of extractions and the methanol percentage in the extractant mixture for carminic acid, fixing temperature at 60 °C and time at 22.5 min, and (II) the number of extractions and extraction temperature for flavokermesic acid, fixing methanol percentage at 27.5% and time at 22.5 min.

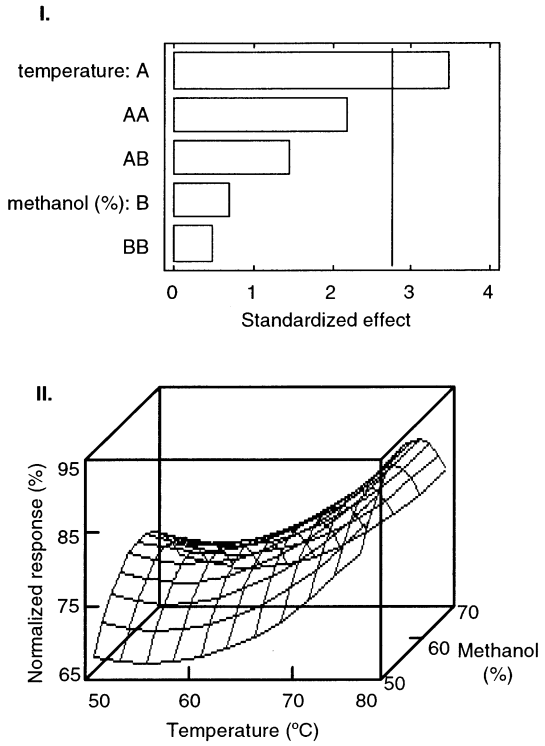

Figure 3. (I) Pareto chart for the standardized main effects in the second factorial design experiment for kermesic acid, where the vertical line indicates the statistical significance bound for the effects, and (II) response surface estimated for the carminic acid.

Table 4. Extraction Parameters and Factor Levels Used in the Central Composite Design (2^2 + Star) and the Optimum Values for the Solvent Extraction of the Cochineal Pigments

factor	fixed	low	high	center	optimum
no. of extractions	2				2
time (min)	30				30
temperature (°C)		50	80	65	80
methanol (%)		50	60	70	65

Table 5. Design Matrix in the Central Composite Design (2^2 + Star) and Response Values of the Pigments from Cochineal

run	temp (°C)	methanol (%)	normalized response (%)[a] dcII	ca	dcIII	dcIV	dcVII	fk	ka
1	80	70	84	77	38	56	93	82	100
2	80	60	82	100	100	100	100	100	64
3	50	70	91	74	25	31	33	32	34
4	65	60	78	74	28	43	34	30	29
5	80	50	47	76	36	48	43	34	54
6	65	50	74	75	28	50	35	30	41
7	65	60	80	76	29	38	28	26	32
8	50	60	100	71	23	28	27	22	27
9	50	50	79	71	42	32	27	22	31
10	65	70	75	74	49	54	21	16	17

[a] Normalized response (relationship between area for each run and the area for the run with maximal response) of each cochineal pigment: dcII, dcIII, dcIV, and dcVII, unknown pigments of *Dactylopius coccus* Costa; ca, carminic acid; fk, flavokermesic acid; ka, kermesic acid.

temperatures were not chosen because carminic acid is destroyed when exposed to high temperatures (above 80 °C) for more than 1 h, as was observed in previous experiments and as confirmed by the results obtained by other authors (*18*). To extract the different pigments contained in the cochineal, the variables of the method were set using the optimum values described in **Table 4**.

Table 6. Retention Times (t_R) and Relative Concentrations ($n = 5$) of Pigment Fractions of Cochineal Insect Extracts

peak	t_R (min)	relative concentration (%) UV detection $\lambda = 275$ nm	visible detection $\lambda = 420$ nm	$\lambda = 500$ nm
1	4.4 ± 0.2	2.67 ± 0.18	0.29 ± 0.03	0.34 ±0.02
2			0.29 ± 0.02	0.16 ±0.01
dcII	7.8 ± 0.2	2.53 ± 0.04	3.32 ± 0.06	0.09 ±0.01
ca	8.7 ± 0.2	87.57 ± 0.46	90.14 ± 0.32	93.90 ±0.23
5	12.4 ± 0.4	0.36 ± 0.03		0.29 ±0.01
6	14.2 ± 0.4	0.23 ± 0.02	0.26 ± 0.02	
7	16.0 ± 0.6	0.25 ± 0.02	0.27 ± 0.03	0.14 ±0.01
8	17.8 ± 0.5	0.39 ± 0.01	0.34 ± 0.03	0.33 ±0.02
9	21.4 ± 0.1	0.11 ± 0.01	0.04 ± 0.00	0.03 ±0.00
dcIII	21.8 ± 0.1	3.21 ± 0.24	2.95 ± 0.14	2.82 ±0.12
dcIV	24.3 ± 0.2	2.15 ± 0.07	1.64 ± 0.11	2.06 ±0.13
dcVII	25.5 ± 0.1	0.20 ± 0.02	0.03 ± 0.00	0.21 ±0.01
fk	26.0 ± 0.1	0.27 ± 0.02	0.17 ± 0.02	0.05 ±0.00
ka	26.6 ± 0.3	0.06 ± 0.00	0.14 ± 0.01	0.16 ±0.01

To determine the accuracy of the extraction method, five independent extractions were carried out using the newly established experimental conditions. The relative concentration of pigments at UV−visible wavelengths of 275, 420, and 500 nm and retention times are summarized in **Table 6**. No significant differences were found between the intra- and inter-day reproducibilities of retention times, peak heights, and peak areas, indicating that the method has good reproducibility and that the extraction−HPLC system is stable and reliable. The RSD values for both intra- and inter-day reproducibilities were 0.22−4.80% for retention times, 0.36−9.08% for peak heights, and 0.24−9.89% for peak areas.

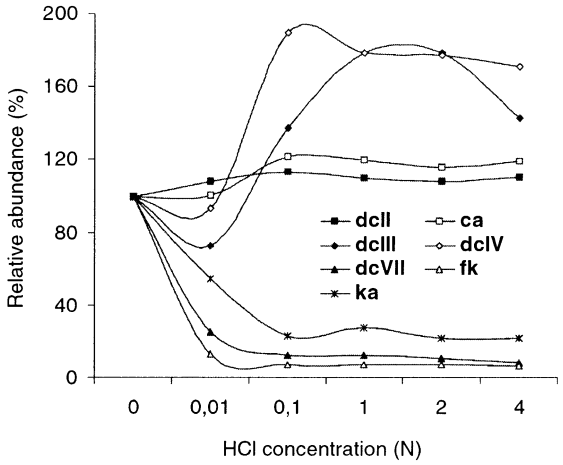

Figure 4. Influence of the acid concentration on the extraction efficiency of cochineal pigments. When the acid concentration in the extractant mixture was 0 mol/L HCl, a relative abundance of 100% was assumed for each pigment.

Influence of the pH of the Extractant Mixture on the Extraction Efficiency. It is possible that some pigments were fixed to the proteins of the insect's body (*16*). This link is broken by hydrolysis with HCl. On the other hand, pigments have functional groups in either ionic or molecular states according to the pH of the environment. As a consequence, it may be necessary to vary the acid concentration in the extractant mixture to reach optimum extraction conditions. To study this factor, a set of experiments was performed, working with acid concentrations in the extractant mixture (65% methanol:35% water) ranging between 0 and 4 mol/L HCl, and with the optimum conditions established in **Table 4**. **Figure 4** shows the influence of acid concentration on the extraction efficiency. Greater extraction efficiency was achieved for the dcIII and dcIV pigments using HCl concentrations higher than 0.05 mol/L, although their behavior was irregular. However, the extraction efficiencies for the rest of the pigments were either greatly diminished when the HCl concentration was increased above 0.01−0.1 mol/L, as for the dcVII pigment, flavokermesic acid, and kermesic acid, or hardly affected at all, as with carminic acid and the dcII pigment. In quantitative terms, for example, the presence of HCl diminishes the extraction efficiency of flavokermesic acid by 87% in relation to its extraction efficiency without HCl. Because of this, it was decided not to include an acid in the extractant mixture. With regard to extraction efficiency with HCl, the behavior of the different pigments described must be closely related to the structural characteristics that regulate their chromatographic retention factors. In fact, the pigments that constitute each of the three groups differentiated in **Figure 4** also appear grouped in the chromatograms in **Figure 1**.

Conclusions. The pigments found in the cochineal can be efficiently extracted using an adequate selection of the experimental conditions optimized in this study. The most important condition is the number of extractions, although high temperatures and high percentages of methanol in the extractant solution also notably contribute to the extraction efficiency, especially for the less common pigments such as flavokermesic and kermesic acids. Not all of the pigments are affected in the same way by the experimental variables used in the extraction process. As a consequence, the chromatographic profile of the cochineal can vary if the extraction conditions are modified. Under the selected elution conditions, the chromatograms confirm the presence of seven pigments in the cochineal. Although four of the seven pigments were not identified, their absorption spectra and chromatographic behavior seem to indicate important structural similarities between them. It was shown that carminic acid is the principal compound in cochineal extract. The less common pigments are kermesic acid, flavokermesic acid, and dcVII. It is advisable to use chromatographic profiles like those shown in **Figure 1** to detect adulterations in food colorants. Because detection at different wavelengths is important, the use of a diode array spectrophotometer is especially recommended.

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Received for review June 23, 2002. Revised manuscript received September 22, 2002. Accepted September 23, 2002.

JF025756R