Chapter 13. Preliminary analysis of cochineal insects (*Dactylopius coccus* Costa) with high performance liquid chromatographic (HPLC)

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1. INTRODUCTION

The nine recognized species confined to the genus *Dactylopius* in the family Dactylopiidae (Hemiptera: Coccoidea) are well-know as cochineal insects. Commonly, the name cochineal is associated to species that produces pigments. The cochineal insects are a very interesting example of the biochemical relationships between plants and animals. The chemistry of the cochineal insects is no less unusual than their biology. They may be classified as useful to man yielding important commercial products as dyes and as potential biological control agents of certain species of *Opuntia*. Cochineal insects are considered probably the first organisms used deliberately and successfully in biological control, in India.

One of the species with great economic importance, since ancient times, is the 'American cochineal' *Dactylopius coccus* Costa (which some publications still refer to as *Coccus cacti* L.) because from the dried bodies of the adult females is obtained an expensive red dye, called carminic acid the most common pigment, widely used as red colouring agent (CAS Reg. No.: 1260-17-9; EINECS: 215-023-3; No. Shoultz: 1381; C.I.: 75470; Natural Red 4; E120). It is one of the most light and heat stable of all the colorants and is more stable than many synthetic food colors.

Carminic acid is a hydroxyanthraquinone with a lateral chain of C- glycosyl and only one position free on the aromatic nucleus. The composition of carminic acid was proposed to be $C_{22} H_{20} O_{13}$ but with reference to chemical structures have continued to appear in the literature with regularity, causing considerable confusion. The structure has been the subject of numerous researches in the 19th and at the beginning of the 20th century. Carminic acid was first isolated in crystalline form in 1858 (Allevi *et al.*, 1998) and subsequently by Schunk & Marchlewsky (1894). Through early studies the structure was established by Dimroth & Kämmarer (1920) and

successively has been modified with the addition, obviously of some new details, thanks to availability of improved techniques.

Carminic acid has been widely used to colour foods, beverages, textiles, pharmaceuticals and cosmetics products due a its relatively high chemical and biological stability and its apparent lack of mutagenic, carcinogenic or genotoxic effects. This stability and the concomitant absence of toxicity has been attributed to the presence of the C-Glycosyl moiety. Besides the colour attributes, recently, also has been reported to beneficial to health with potential antibiotic and antitumor properties. At the beginning of the 21st. century it is predicted that many colours will be used for both their additional beneficial functions in the body, as well as, colouring effect.

Various high performance liquid chromatographic methods (HPLC) have been frequently used for the separation and quantitative determination of a wide variety of natural pigments. This technique combines speed with high resolution and reproducibility and has the possibility for quantitative evaluation the opportunity for combination with techniques such as mass spectrometry for the peak identification. Due to the considerable commercial importance of pigment composition much effort has been devoted to the development of HPLC methods suitable for the separation of colour pigments of cochineal insects, but according to our knowledge the pigments of the cochineal insects have not been studied in detail. The more profound knowledge of the character and quantity of pigments present in the cochineal insects facilitates the understanding of the biochemical procedures exploited in various biotechnological processes, advances the development of molecular biology.

In this paper we describes, for the first time, the extraction of colour pigments from the cochineal insects using solvent extraction, and the pigments composition in cochineal insects (*DACTYLOPIUS COCCUS* C.) reared on nine different cactus pear cultivars (*OPUNTIA FICUS-INDICA* Mill.) using multivariate statistical methods.

2. EXPERIMENTAL

2.1. Optimizing conditions for the pigments extraction in cochineals insects using response surface methodology

Cochineal Samples. Adult female cochineals (*D. COCCUS* Costa) were collected in Valle de Guerra, Tenerife, Canary Islands from several wild cactus pear cultivars *O. FICUS–INDICA* Mill. The cochineals were cleaned over a sieve to eliminate dust and heterogeneous materials. Then the

insects were dried at 60°C in a Selecta (Barcelona, Spain) heater until all water was completely eliminated.

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 a high–speed blender Omnimixer Model ES–207 (Omni International Inc., Gainesville, VA) for 1 min and pigments present in the sample were extracted for 30 min in a sealed vessel using a Selecta water bath at 80 °C. 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 design, regression analysis and to obtain the response surface plots.

Chromatographic Determination of Pigments. All measurements were made on a Shimadzu modular liquid chromatographic system (Kyoto, Japan), equipped with a LC–10 AD pump, a SPD–M6A UV–visible diode array detector, a SPD–10AV UV–visible detector and a Rheodyne (Cotati, CA) Model 7725i injector with a sample loop of 20 μL. Class LC–10 software (also from Shimadzu) was used for acquisition data. Separations were carried out on a Spherisorb ODS–2 RP–C_{18} (Alltech, Deerfield, IL), 5 μm, 25 cm x 4.6 mm I.D. column fitted with a Spherisorb ODS–2 RP–C_{18} (Alltech), 5 μm, 7.5 x 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. Then the mixture was 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 mobile phase was 1.2 mL/min.

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 efficiency of peak separation was checked by the peak purity test carried out at maximum absorbance.

Determination of 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 de–ionized 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 colour pigment pattern. The relative concentration (%) is defined as the relation 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).

2.2. Chromatographic determination of nine cochineal samples

Preparation of cochineal samples. Adult female cochineals were collected from different localities and host plant (Lanzarote, Portolano, Offer, Sicilia Bianca, Colorado Moscatel, Blanco Moscatel, Fagnia, ICIA and Tenerife). The cochineal insects were cleaned over a sieve; then the insects were dried at a 60°C in a Selecta (Barcelona, Spain) heater until the water was completely eliminated.

Extraction of pigments. Pigments were extracted from cochineal samples using the same method described in the section 2.1.

Instrumentation and column. All HPLC analysis were performed using a Shimadzu modular liquid chromatographic system, equipped with a LC-10 AD pump, a SPD-M6A UV-Visible diode array detector (DAD) and controlled with a Class LC-10 data acquisition software, also from Shimadzu. A reverse phase C_{18} Spherisorb ODS-2 column (5μm, 25 cm x 4.6 mm I.D) was used. A precolumn (5μm, 7.5 cm x 4.6 mm I.D)was fitted to protect the main column. The injection valve was a Rheodyne Moedel 7725i with an injection loop of 20 μL.

Chromatographic conditions The HPLC method used for the determination of pigments consisted of a gradient elution procedure with UV–visible detection. The mobile phase consisted of a mixture of water, methanol and orthophosphoric acid (5% in water). The elution program 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. Detection wavelengths for the DAD were set at 275, 420 and 500 nm. Because it is not possible to have access to standards for the cochineal pigments (except for carminic acid) the relative concentrations of pigments obtained after normalization was used to characterize to colour pigments patter. The relative concentration (5) is defined as the relation between peak area of each pigment and the total peak area.

Statistical evaluation of data Two multivariate techniques cluster analysis (CA) and principal component analysis (PCA) were used to characterize pigment pattern of cochineal insects from different cactus pear cultivars. Statistical analyses were carried out with the NTsys-pc Versione 2.11a (Exeter Software Setauket NY, USA).

3. RESULTS

3.1. Extraction of cochineal pigments

The cochineal pigments were well separated in many fractions under the reversed phase HPLC conditions applied as demonstrated in Figure 1. Initially, the chromatographic conditions described by Wouters & Verhecken (1989) were followed to separate the pigments. Then different solvent mixtures and gradient programs were 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.
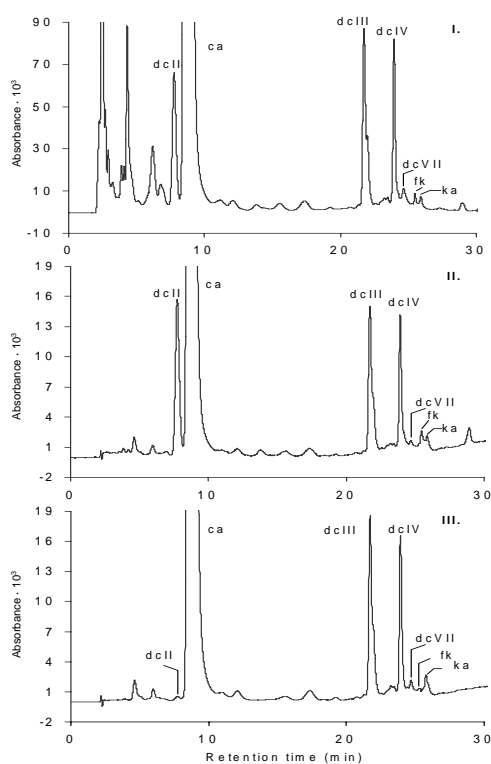

Figure 1. Chromatographic separation of cochineal (*D. coccus* Costa) pigments by RP–HPLC with UV–visible detection at 275 nm (I), 420 nm (II) and 500 nm (III).

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 in the different chromatograms. It also shows how the relations 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 co–eluted 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. Each concentration level was injected in triplicate. The repeatability of the chromatographic procedure, expressed as relative standard deviation (RSD), was checked on eleven consecutive injections of a standard solution containing 50 μg/mL of carminic acid.

The relative concentration of pigments at UV–visible wavelengths of 275, 420 and 500 nm and retention times are summarized in Table 1. In the selected elution conditions, the chromatograms confirm the presence of 14 fractions in the cochineal insects. Although seven of the 14 pigments were not identified, their absorption spectra and chromatographic behavior seem to indicate important structural similarities between them. Several pigments analogous to CA can be present in cochineal insects that could be considered valuable commercially or this new information might be helpful to the better understanding of the identity and taxonomic relations of cochineal insects. The pigment components are anthraquinones and the most important one is carminic acid, other aglycones presents are kermesic acid (3,5,6,8-

tetrahydroxy-1-methyl-2-anthraquinone carboxylic acid) and flavokermesic acid o laccaic acid D (3,6,8-trihydroxy-1-methyl-2-anthraquinone carboxylic acid). 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–day 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.

Table 1. Retention times (t_R) and relative concentrations (n = 5) of pigment fractions of cochineal insect extracts.

| peak | T_R (min) | relative concentration (%) | | |
| | | UV detection | visible detection | |
		λ = 275 nm	λ = 420 nm	λ = 500 nm
1	4.4 ± 0.2	2.67 ± 0.18	0.29 ± 0.03	0.34 ±0.02
2	6.0 ± 0.2	–	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

3.2. Multivariate statistical analysis of nine cochineal samples

The pigment pattern (standardized area) at 275, 420 and 500 nm were used in multivariate statistical analysis.   Cluster analysis (CA) looks for groups of samples according to their similarities. Clustering methods are used to classify objects, characterized by the values of a set of variables, into groups. With this method the objects are aggregated stepwise according to the similarity of their features. A cluster describes a group of objects (samples) that are more similar to each other than to objects outside the group. The euclidean distance was chosen as the measure of similarity and the UPGMA method was the clustering algorithm. The results of the CA made over all studied cochineals are presented as dendrogram in Figure 2. In this tree

generated, two main clusters can be visualized, one of them consists of cochineal samples which geographical origin is Tenerife Island (bottom of dendrogram). This cochineal is a commercial sample bought in Santa Cruz's city (were collected from several cultivars). The second cluster contains cochineals samples reared in cactus pear cultivars which geographical origin is the Experimental Station of Instituto Canario de Investigaciones Agrarias. The cochineal samples Colorado and Fagniaare related to each other; they could not be separated. This second cluster seems to be split out into two principal subgroups. One of them includes cochineal samples obtained from Lanzarote Island.

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) that can also show which variables account for most of the variability in the data, was done. PCA is appropriate to extract the relevant information from the original variables into fewer new variables called principal components. The visualization of the scores and loadings from PCA, through the biplots, was necessary to obtain a clear and complete picture of the pigments pattern. When using PCA, it is essential to note that the score vectors provide the principal components composition related to the objects (samples), while the loading vectors provide this same composition related to the variables.The PCA also supports the aforementioned grouping as illustrated in Figure 3.The PCA showed three interpretable components, chosen on the basis of Kaiser's criterion (eigenvalues higher than 1.0 are chosen), extract together 86.72 % of the of the total variance.
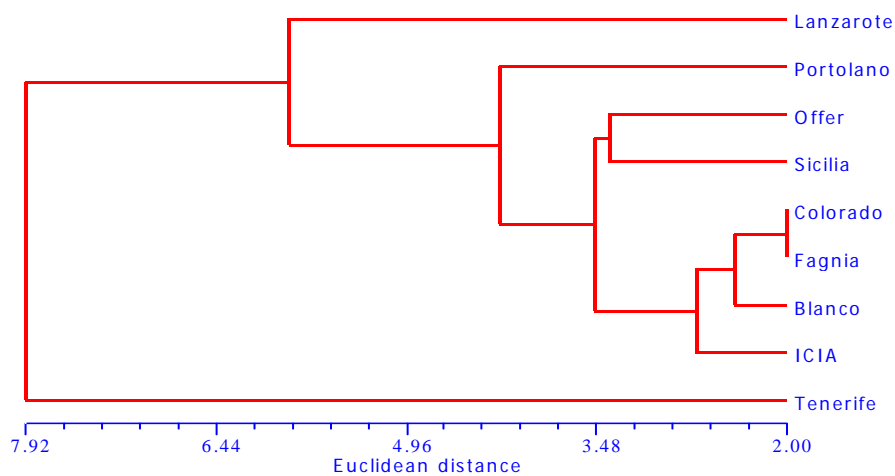

Figure 2. Dendrogram resulting of cluster analysis selecting the euclidean distance as similarity through UPGMA method applied to all cochineal samples studied.

The Figure 3 show the plot of loadings by selecting the first two principal components (PC) as axes. The first PC that explains the higher percentage of variance (47.50%) is meanly related to standardized area for P1, P2 and P8 with a positive correlation. Thus cochineal from Tenerife were most correlated with this pigments. The standardized area of pigments P5, P7, dcVII, flavokermesic acid and kermesic acid with a negative correlation. The second PC (23.1% of the variance) is related of standardized area of pigment dcII and dcIV. Cochineal samples from Lanzarote were most correlated with the standardized area for dcIV and flavokermesic acid. The selection of cactus pear cultivar with a high level of colour pigments is a significant aspect of research on the improvement of carminic acid quality.

CONCLUSIONS

The above explanations allow us to conclude that the pigments found in the cochineal can be efficiently extracted using an adequate selection of the experimental conditions optimized in this study. As the cochineal insects reared on different cactus pear cultivars may produce a characteristic pigment profile, the separation and quantitative determination of these pigments may also help their identification, thus cochineal samples could be differentiated by CA and PCA techniques and may also indicates the best type of cultivar used for the production.
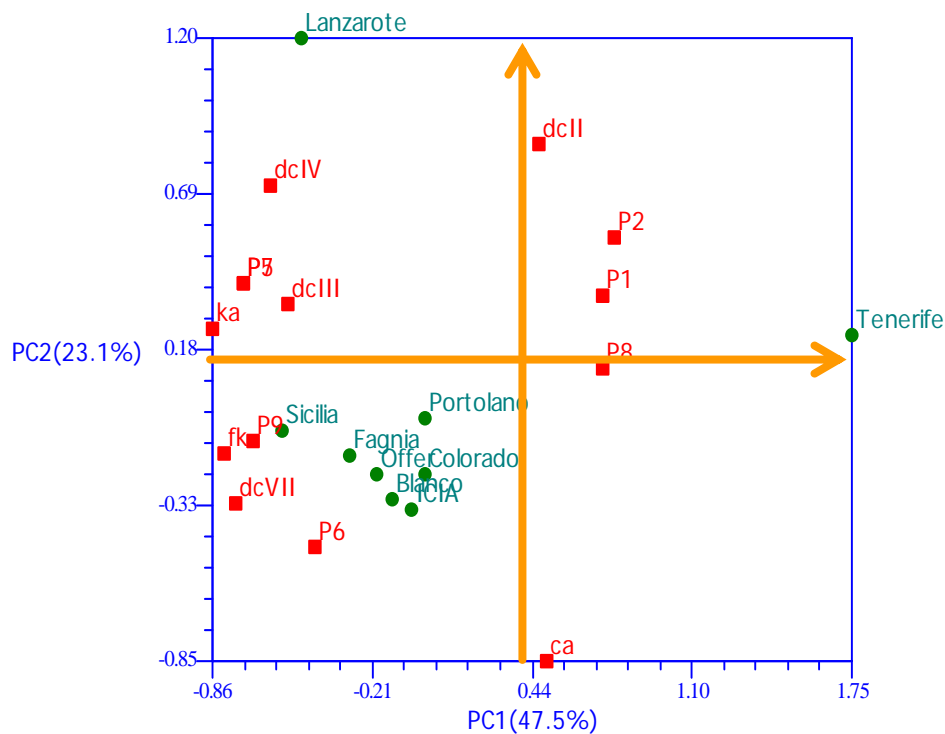

Figure 3. Results obtained by PCA PC1×PC2 biplots: for nine cochineal samples

REFERENCES

ALLEVI, P.; ANASTACIA, M.; BINGHAM, S.; CIUFFERDA, P.; FIECCHI, A.; CIGHETTI, G.; MUIR, M., & J. TYMAN. 1998. Synthesis of carminic acid, the colourant principle of cochineal. J. Chem. Soc. Perkin Trans. (1):575-582.

DIMROTH, O. & W. KÄMMERER. 1920. Über die Carminsäure. Ber. Dtsch. Chem. Ges. 53:471-480.

SHUNCK, E. & L. MARCHLEWSKI. 1894. Zur kenntniss der carminäure. Berichte Der Deutchen Chem. Gesellschaft.27:2979-2985.

WOUTERS, J. & A. VERHECKEN. 1989. The scale insect dyes (Homoptera: Coccoidea) species recognition by HPLC and diode-array Analysis of the dyestuffs. Annales de la Societe Entomologique Francaise. 25(4):393-410.