European Journal of Science and Theology, June 2020, Vol.16, No.3, 151-164

ON THE USE OF FIBRE OPTIC REFLECTANCE SPECTROSCOPY IN THE UV-VIS RANGE FOR THE ANALYSIS OF ORGANIC LAKE-PIGMENTS OF A MID-16TH CENTURY ILLUMINATED ANTIPHONARY A CHEMOMETRIC APPROACH

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(Received 19 November 2019, revised 18 February 2020)

Abstract

The dye source of the organic pink-lake pigments used to produce reddish-to-pink paints present in six representative illuminated capital letters of a mid-16th century illuminated Antiphonary housed in the Biblioteca Pública de Évora, Portugal - the Manizola 116c - was studied in-situ by non-invasive Fibre Optic Reflectance Spectroscopy (FORS) in the UV-VIS range. Historically accurate reproductions of pink lake pigments were used for calibrating the Principal Component Analysis (PCA) where historical FORS spectra were projected. The chemometric approach indicated the use of cochineal to produce the reddish (cochineal acidic form) and pink-purple (cochineal basic form) hues to obtain the painting layer. Two representative micro-samples were analysed by LC/DAD/MS, confirming the use by the illuminator of cochineal in its acid-basic equilibrium to produce different shades of reddish-to-pink colours across the manuscript.

Keywords: Antiphonary, illuminations, FORS, chemometrics, LC/DAD/M

1. Introduction

Medieval and Renaissance monasteries were remarkable centres of knowledge and culture. The *armarium* of the Portuguese Cistercian female Covent of São Bento de Cástris, located outside the Évora walls (southern Portugal) comprised, at the time of its closure in 1890, an interesting collection of

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Gospels Books and *Collationes*, collections of texts for the private use of some nuns and manuscripts of sacred music [1]. In particular, the Choir Books comprised of seven antiphonaries, one *Sanctorale* antiphonary, one *Hymanrium*, two Graduals and two Books of Invitatórios [1; ORFEUS, A Reforma tridentina e a música no silêncio claustral: o mosteiro de S. Bento Cástris, EXPL/EPH-PAT/2253/2013, http://www.orfeus.pt, accessed 4.11.2019]. Among the antiphonaries, the Manizola 116c stands out for the magnificence of its illuminations. Attributed to the mid-16th century, this large-size Antiphonary (measuring approximately 550 mm x 390 mm) is written in parchment and bound in embossed leather-covered wooden boards. There is no reference regarding the place of its production. However, the fact that one of its miniatures represents Saint Bernard of Clairvaux (*f.*4r), the founding abbot of the Cistercian Abbey of Clairvaux, suggests that it might have been produced for São Bento de Cástris Covent, the oldest female monastery in southern Portugal. It was founded in 1169 by D. Urraca Ximenes, but only obtained the regular legitimacy of the community in 1275. In 1278, the Pope confirmed the institution, imposing the Benedictine Rule, under the obedience of and with affiliation to the nearest male Cistercian abbey, Alcobaça [2].

Besides the antiphons and responsories with its related plainsong and other religious songs, the Manizola 116c contains at the end an addition related to the Office of the Dead [1]. The manuscript is comprised of 62 folia, although the analysis of the full text and the quires of the book suggests that it might have been composed of 65 folia. From the 123 miniatures present in this Antiphonary, 121 are illuminated capital letters.

The study of an illuminated manuscript provides not only crucial information concerning contemporary liturgical practices, but also outstanding information on the chemical knowledge of the time.

During the 15th-16th centuries, with new overseas trade markets and the widespread use of organic lake pigments, a new brighter colour palette became available for producing illuminated manuscripts. For producing these organic lake pigments, the dye was either extracted directly from the dyestuff or from dyed textiles, using an alkaline solution, such as lye. After the extraction, the dye was commonly precipitated by adding alum (a potassium aluminium sulphate, $AlK(SO_4)_2 \cdot 12H_2O$) to the solution [3]. The resulting pigment was then washed, dried and ground.

From the range of organic lake-pigments used during the 15th-16th centuries, pink-lake pigments were among the most widely used for producing illuminations. Vegetable or animal matter could be used as dye sources. Regarding vegetable dye sources, the most common were brazilwood (extracted from the trunk bark of *Caesalpinia* trees) and madder (extracted from the roots of *Rubia* genus) [4]. For the former, the main chromophore is the red brazilein, oxidized from the colourless homo-isoflavonoid brazilin, whereas for madder lake, the main chromophores are alizarin and purpurin [4, p. 274-288]. As for animal dye sources, insect dyestuffs extracted from the female eggs of scale insects such as lac insects (*Kerria lacca*), kermes (*Kermes vermilio*), Polish

cochineal (*Porphyrophora polonica*), Armenian cochineal (*Porphyrophora hameli*), and Mexican cochineal (*Dactylopius coccus*) were the most commonly used [4, p. 619-654]. Laccaic acids are the main chromophores present in lac insects, whereas kermesic and carminic acids are the main chromophores found in kermes and cochineal, respectively [4, p. 619-654]. As a pH sensitive compounds, lake pigments' molecular structures change according to the pH [5-7]. As a consequence, different colours are achieved, ranging from purple to red according to the pH used during the lake pigment preparation: in acidic conditions (lower pH), the colour shows reddish shades, whereas in more basic conditions (higher pH), the colour acquire a purplish hue.

In this work, a set of reddish-to-pink-purple lake paints was found in six illuminations of the Manizola 116c (*ff.* 13v, 19r, 35r, 39r, 41r and 42r) by non-invasive Fibre Optic Reflectance Spectroscopy (FORS) in the UV-VIS range. As an easy-to-use technique requiring a short time of analysis, FORS is a powerful in-situ and non-invasive technique for analysis. However, concerning the analysis of illuminated paints, the use of FORS becomes a less effective tool for the identification of organic lake-pigments in the UV-VIS range, as for this region these class of materials present low fingerprint features [8]. The use of chemometric methodologies is a powerful tool to assist in the FORS spectral analysis of organic lake-pigments. In this sense, to better discriminate the dye source used to produce the selected reddish-to-pink-purple lake paints from Manizola 116c, an unsupervised approach using Principal Component Analysis (PCA) following a calibrated approach was used. For this, historically accurate reconstructions of the most common reddish-to-pink lake pigments used at the time were produced. Different parameters that might affect the lakes final colour, such as the effect of pH and dye concentration, were evaluated. Illuminated paints were produced and its FORS spectra were used to calibrate the PCA model, on which the FORS spectra of historical paints were projected. To confirm the PCA results, two micro-samples representative of the reddish-to-pink-lake historical paints were analysed by HPLC/DAD/MS.

2. Materials and methods

The study started with the FORS analysis of the pink-to-reddish paints present in the illuminated capital letters found in *ff.* 13v, 19r, 35r, 39r, 41r and 42r. Following a similar approach, namely the number of spots of analysis and the experimental conditions, FORS spectra of historically accurate reconstructions of madder, brazilwood and cochineal lake paints were analysed. To interpret the influence of the binder, two classes of binders were used for the paints' reconstruction: proteinaceous binders (parchment glue, egg white and egg yolk) and polysaccharides binders (gum Arabic). To evaluate the effect of concentration and pH sensitivity on the discrimination of the PCA model, four cochineal lake paints were prepared: one following as accurately as possible the historical recipe for cochineal lake from the Bolognese manuscript (cochineal lake #1), one testing the increasing concentration of the solution on the lake final colour (cochineal

lake #2), and two others prepared with different amounts of vinegar added: one prepared with a lower amount of vinegar (to evaluate the influence of a more basic environment) and another with a higher amount of vinegar added (to evaluate the influence of a more acidic environment) - cochineal lake #3a and cochineal lake #3b, respectively. For the PCA analysis, the model was calibrated with the FORS spectra of historically accurate reconstructions of madder, brazilwood and cochineal lakes' paints produced with proteinaceous (parchment glue, egg white and egg yolk) and polysaccharides binders (gum Arabic), onto which was projected the FORS spectra of pink-to-reddish paints from the illuminations present in the Codex Manizola 116c. To test the PCA model discrimination, two micro-samples representative of a reddish (*f.*19r) and of a pink (*f.*41r) paint from Manizola 116c were run in the HPLC/DAD/MS.

2.1. Pink-lake paints from the Manizola 116c

From the 123 illuminations present in the Manizola 116c, only two are isolated depictions: the representation of Infant Jesus in His Majesty, related to Christmas day (*f.*1r), and the already-mentioned representation of Saint Bernard of Clairvaux, *f.*4r. The remaining 121 miniatures are all illuminated capital letters. Besides vegetable and zoomorphic representations, those of anthropomorphic motifs stand out for their elegance and movement. From these, a set of six illuminated capital letters (*ff.* 13v, 19r, 35r, 39r, 41r and 42r) having reddish-to-pink-purple lake paints were chosen to be characterized in this work (Figure 1).

Figure 1. Normalized size images of capital letters from Manizola 116c: *ff.*13v (60 mm x 68 mm), 19r (40 mm x 52 mm), 35r (87 mm x 107 mm), 39r (89 mm x 107 mm), 41r (93 mm x 106 mm) and 42r (120 mm x 116 mm). White circles show the FORS spots of analysis.

2.2. Pink-lake historically accurate reconstructions

For producing pink-lake historical reconstructions, lake pigments and binders were produced based on recipes from historical treatises. Paints were than produced accurately following the same approach.

2.2.1. Lake pigments

Lake pigments were produced following the interpretation developed for the workshop on the preparation of historical lake pigments organized at the Doerner Institut in 2011 in the framework of the EU FP7 CHARISMA (Cultural Heritage Advanced Research Infrastructures: Synergy for a Multidisciplinary Approach to Conservation/Restoration) project [Back to the roots - workshop on the preparation of historical lake pigments, 23-25 March 2011, Doerner Institut, Munich; https://cordis.europa.eu/project/rcn/92569, accessed 4.11.2019], and are based on two manuscripts from the 15th century: the Nuremberg *Kunstbuch* and the Bolognese Manuscript [https://cordis.europa.eu/project/rcn/92569; 9]. Table 1 resumes the key aspects for the production of the six reddish-pink lakes used as standards for the calibration of the PCA model.

Table 1. Key aspects for the production of the six reddish-to-pink lakes used as standards for the calibration of the PCA model.

<table>
<tr><th></th><th>Madder</th><th>Brazilwood</th><th>Cochineal lake #1</th><th>Cochineal lake #2</th><th>Cochineal lake #3a</th><th>Cochineal lake #3b</th></tr>
<tr><td>Recipe book</td><td>Nuremberg Kunstbuch, 15th century</td><td colspan="5">Bolognese Manuscript, 15th century</td></tr>
<tr><td rowspan="2">Key aspects</td><td>Mordanted wool</td><td rowspan="2">Alum added to the dye solution; K_2CO_3 was used for lake pigment's precipitation</td><td colspan="3">Mordanted wool</td></tr>
<tr><td>Wheat bran was used as mordant solution</td><td>Alum and sodium tartrate were used as mordant solution</td><td>Similar to cochineal lake #1, but precipitated from a more concentrated solution</td><td>Amount of vinegar added: #3b > #3a</td></tr>
</table>

Madder lake was reproduced based on the Nuremberg *Kunstbuch* manuscript - a manual used by the sisters of St. Catherine's Church in Nuremberg in their "daily work of making the rich liturgical vestments worn by the Dominican priests, cleaning a variety of fine textiles, and painting and gilding both manuscripts and cloth" [10]. The recipe was reproduced and explained by W. Jacobs [11]: a mordant called 'semelwater' was produced boiling a mixture of 20 g of wheat bran and 450 mL of water. After letting it cool, 300 mL of water was added, and the container covered. The liquid was stirred every hour, 7 repetitions in total. It was left to sit and after 6 days, 400 mL was removed and replaced with 400 mL of hot water. It was stirred once more and left to sit for 24 hours. 0.75 g alum was added to 250 mL of the above 'semelwater' and dissolved. 5 g of undyed wool yarn was added to this solution and boiled for 2 hours, after which the yarn was drained, but not wrung out. 3 g of madder was added to 100 mL

'semelwater' and 150 mL water. While stirring, the solution was brought to 40°C. The mordanted wool was added and kept at 90 °C for 3 hours. The yarn was then drained, rinsed, and wrung out before being placed for 30 minutes into a solution of 0.5 g K_2CO_3 and 250 mL water. The yarn was rinsed again and left to dry.

Brazilwood lake was produced based on the recipe described in the Bolognese Manuscript as recipe B 136 [9, p. 452-454]. Again, the recipe was reproduced by W. Jacobs [11]: 4 g of brazilwood, ground in a coffee grinder, was added to 130 mL water and heated until the solution had reduced by approximately half of the volume. The solution was filtered to remove the plant matter. 2.5 g alum was dissolved in 20 mL water and added to the dye solution. A solution of 2 g potassium carbonate and 10 mL water was slowly added to the previous solution, stirring, until effervescence stopped. It was covered and left to sit overnight. The mixture was filtered and lyophilized for four days until all the liquid was removed.

Cochineal lake #1 was produced based on the recipe described in the Bolognese Manuscript as recipe 110 [9]. Also, as is the case for madder and brazilwood lake, W. Jacobs [11] is the most recent literature reference for the preparation of the Cochineal lake. The procedure entails: 0.262 g alum ($AlK(SO_4)_2{\cdot}12H_2O$) and 0.131 g sodium tartrate ($Na_2C_4H_4O_6$) was added to 130 mL of water. This solution was brought to 40°C. 1.31 g of undyed wool yarn was added to the solution, heated to 95°C and kept at this temperature for 45 minutes. The solution was left to cool, and the wool was rinsed with water. 0.131 g of ground cochineal was added to 300 mL water and heated to 40°C. The mordanted wool was added, heated to 80°C for 60 minutes while stirring. The wool was then rinsed until the water ran clear and was left to air dry. The dyed yarn was cut into fine clippings approximately 3 mm in length and added to 50 mL of 0.1 M K_2CO_3 solution. This was heated to 60°C. The temperature was lowered to 50°C and kept at this temperature, while stirring, for one hour. The solution was filtered using Whatman 114 paper to remove the fibres and reheated to 50°C. A solution of 1.574 g alum and 7.9 mL water, heated slightly to dissolve, was slowly added to the dye solution, still stirring, until the effervescence stopped. It was then covered and left to sit overnight. The next day, the precipitate was filtered using a Buchner funnel and Whatman 114 filter and left to dry in a desiccator [11]. To infer the effect of the alum concentration on the final colour of the lake, a second cochineal lake (cochineal lake #2) was produced by increasing the amount of alum to 0.4 g and reducing considerably the amount of water used during the dyeing process. Recipes #3a and #3b were prepared using the same procedure followed for cochineal #2, but cochineal #3a received 40 drops from a glass Pasteur pipette of a commercially available white vinegar while cochineal #3b received 100 drops of the same vinegar.

2.2.2. Binders

For preparing parchment glue, 3.5 g of calf parchment (cut into small squares of 0.5 cm per side) was boiled in 50 mL of water (80-90ºC) for 4 hours.

After that, water was allowed to evaporate, until sticky consistency glue was obtained. Calf parchment was acquired from Museé du Parchemin (Rouillon, France) [12].

The egg white was beaten with a fork and allowed to stand for 6 hours. The deposited serum was used as binder [13]. The egg yolk was diluted in water (1:1, v/v) after withdrawal from its sheath [13, p. 70-81]. Eggs were acquired in a local market.

Gum Arabic was dissolved in water in a 10% solution (wt%). Gum Arabic was acquired from Kremer Pigmente (Aichstetten, Germany).

2.2.3. Paints production

To produce the paints, each organic-lake pigment was first ground in an agate mortar with a drop (approximately 25μL) of water, and then ground with the binder (approximately 80% of binding media in a dry-paint composition, %wt.). Paints were then applied with a paintbrush over parchment.

2.3. Analytical approach

2.3.1. Colorimetry

Colorimetric analysis was performed using a DataColor Check II Plus spectrophotometer with a diffused illumination 8º viewing, a pulsed xenon light source with a spectral range of 360-700 nm and effective bandwidth and wavelength bandwidth of 10 nm and 2 nm, respectively. Black and white calibration standards were used for calibration. Analyses were made with measuring times of <2.5 seconds with an aperture spot size of 3 mm. Each surface was analysed 3 times to create an average and identify variations within the painted surfaces.

2.3.2. FORS

FORS analysis was made using an ASEQ Instruments LR1-T v.2 compact spectrometer, with a spectral range of 300-1000 nm and a spectral resolution <1 nm (with 50 μm slit). Measurements were taken using the ASEQ CheckTR software. Calibration was made using Whatman filter paper. Samples were analysed at an exposure of 100-200 ms, five scans, a BoxCar smoothing of fifteen, and a spot size of 12 mm^2 (3 mm x 4mm). Each spot was measured three times for five seconds each.

2.3.3. Micro-sampling

For the chromatographic analysis, two micro-samples representative of a reddish paint (*f.*19r) and of a pink paint (*f.*41v) were sampled from lacunas with a micro chisel from Ted Pella micro tools (size ranging between 20 μm and 50 μm)

under a LEICA M205C stereomicroscope with a zoom range of 7.8x to 160x equipped with a Leica DFC295 camera and an external illumination by optical fibres. The same apparatus was used to acquire magnified images of historical reddish-to-pink paints of Manizola 116c.

2.3.4. Chromatographic analysis

Chromophore extraction was performed according to the methodology proposed by Wouters et al. [14]. Samples were extracted for 4h with 200 µL of a solution containing hydrofluoric acid, vacuum dried and re-dissolved in 50 µL of methanol/water (1:1, v/v). After centrifugation, the supernatant was injected in the LC/DAD/MS system.

A liquid chromatography system equipped with a diode array detector and a mass spectrometer (LC/DAD/MS) was used for analysis and identification of red lake pigments. Table 2 presents LC/DAD/MS conditions used during this work.

Table 2. LC/DAD/MS experimental conditions.

Mass spectrometer	**LCQ Fleet Thermo Finnigan**
Ion source	ESI
Mass analyser	Ion trap
Capillary temperature	300ºC
Source voltage	5.0 kV
Source current	100.0 µA
Capillary voltage	-17.0 V (negative mode)
MS mode	Single Ion Monitoring (SIM) (*m/z* 239, 243, 283, 329, 491, 536)
LC/DAD system	Finnigan Surveyor Plus, equipped with autosampler and quaternary pump
Column	Zorbax Eclipse XDB C18 (Rapid Resolution, 3.5 µm, 150 mm × 4.6 mm)
Temperature control	30ºC (column oven), 24ºC (sample tray)
Mobile phase	(A) 0.1% (v/v) formic acid solution, (B) acetonitrile
Elution programme	0-63% B (0-14 min), 63-90 % B (14-25 min), 90% B (25-30 min)
Flow rate	0.2 mL min^{-1}
Sample volume	20 µL
Wavelength range	200-800 nm

2.3.5. Data assessment

An unsupervised approach using Principal Component Analysis (PCA) of FORS spectra restricted to the 380-895 nm region was performed using MATLAB (version R2016a) and PLS toolbox (version 8.2.1) from Eigenvector Research Inc. FORS spectra of historically accurate reconstructions were pre-processed with Standard Normal Variate (SNV) for scaling the spectra (weighted normalization), followed by Mean Centre for removing mean offset from each

variable, and a Savitzky-Golay smoothing filter with 15-point window size, second-order polynomial and first derivative. The PCA model was calibrated with 69 FORS spectra of historically accurate reconstructions of madder, brazilwood and cochineal lakes' paints produced with proteinaceous (parchment glue, egg white and egg yolk) and polysaccharides binders (gum Arabic) on which 21 FORS spectra of pink-to-reddish paints from the illuminations present in the Codex Manizola 116c were projected. Finally, classes were connected with 90% confidence limit.

3. Results

3.1. Colorimetry

The historically accurate reconstructions allowed for the production of purple to pink and reddish lake pigments. Table 3 presents the CIELAB colour coordinates for these purple-pink-reddish lake paints' reproductions in egg white binder. Madder lake together with cochineal lake #2 presents higher values for a*, meaning that the red component is more pronounced in these lake pigments. However, the fact that cochineal lake #2 presents a more negative value for b* (blue component), explains the more purplish hue of this paint, whereas the more positive value of b* in madder lake is in accordance with its reddest hue. Interesting to note was the vinegar effect on the CIELAB colour coordinates, as the higher amount of vinegar (cochineal lake #3b) led to a more pronounced b* parameter, and not to a more pronounced a* - the red component. Yet, the fact that b* is more positive for cochineal lake #3b than for cochineal lake #3a is reflected by a more purple-reddish lake pigment. In fact, the addition of vinegar to both solutions turned the solutions red. It was just after adding K_2CO_3 that the resulting lakes turned into a dark purple precipitate. Considering the pH sensitivity of cochineal's molecular structure, this shift from purple-reddish to dark purple might be related to an increment of the pH caused by the reaction of carbonate ions with hydrogen ions present in the medium. In this sense, to achieve a final reddish hue, the pH should have been decreased after adding K_2CO_3, by adding an additional amount of vinegar to the lake pigment. Finally, brazilwood and cochineal lake #1 presented a purplish hue.

Regarding the CIELAB colour coordinates for pink-reddish paints of Manizola 116c, *f.*42r presents the purplish hue, with high contributions from the red and blue components, meaning a high positive value of the a* component and a low positive value of the b* component (Table 4). On the contrary, *f.*19r presents the higher value for the a* component and a high contribution of the yellow component (meaning a high positive value of the b* component), which is reflected in its reddish hue.

The high standard normal deviations found for the b* component of *ff.*39r and 41r might be related the influence of the support (parchment) caused by some heterogeneity of the paint under analysis or to the spot size of analysis of the colorimeter.

Table 3. CIELAB colour coordinates for pink-reddish lake paints' reproductions in egg white binder.

Lake paint's reproduction	L*	a*	b*
Madder	43.30 ± 0.70	35.79 ± 0.72	3.45 ± 0.98
Brazilwood	30.82 ± 1.42	25.56 ± 0.36	1.30 ± 0.57
Cochineal lake #1	43.26 ± 1.93	27.63 ± 0.93	-9.21 ± 0.51
Cochineal lake #2	36.77 ± 1.57	37.17 ± 1.04	-4.77 ± 1.54
Cochineal lake #3a	27.74 ± 1.04	26.08 ± 1.41	1.69 ± 0.18
Cochineal lake #3b	30.16 ± 3.38	26.63 ± 2.73	3.22 ± 0.47

Table 4. CIELAB colour coordinates for pink-reddish paints of Manizola 116c (*ff*.13v, 19r, 35r, 39r, 41r and 42r).

Folium-colour	L*	a*	b*
13v - pink	51.03 ± 2.53	16.32 ± 2.37	1.81 ± 0.22
19r - reddish	44.15 ± 0.36	31.05 ± 2.62	9.11 ± 0.87
35r - pink	51.96 ± 1.29	25.45 ± 0.79	2.26 ± 0.16
39r - pink-reddish	50.90 ± 1.01	15.53 ± 0.70	3.55 ± 3.30
41r - pink	43.03 ± 1.02	10.45 ± 1.36	2.18 ± 1.86
42r - pink-purplish	50.30 ± 0.79	26.52 ± 2.04	3.84 ± 0.87

3.2. FORS and PCA analysis

FORS spectra of pink-lake historical reconstructions displayed the characteristic spectral profiles of red dyes with an anthraquinone structure, namely the absorption band structured into two sub-bands at 520-525 nm and 550-565 nm for those of animal origin such as cochineal lake, or at 510-515 nm and 540-545 nm for those of vegetable origin such as madder lake. Regarding brazilwood, a single absorption band centred at 550 nm is the fingerprint for this red dye [8].

PCA analysis of the FORS spectra of pink-lake historical reconstructions allowed for the discrimination between red lake pigments of vegetable origin (madder and brazilwood lakes) and of animal origin (cochineal lakes): red lake pigments' scores of red lakes of vegetable origin present negative PC2 values (which concerns 23.27% of the spectral variability), whereas cochineal lakes scores present positive PC2 values (Figure 2). Regarding the influence of the procedure used for the extraction of the dye (cochineal lake #1 versus cochineal lake #2 scores' projections) and the influence of the pH used for the dye production (cochineal lake #3a versus cochineal lake #3b scores' projections), PC1 (which concerns 35.23% of the spectral variability) discriminates well cochineal lake #3a and cochineal lake #3b (PC1 < 0) from cochineal lake #1 and cochineal lake #2 (PC1 > 0). The analysis of the loadings on PC1 demonstrated that the discrimination on this principal component is mainly due to the resolution of the two sub-bands at 520-525 nm and at 550-565 nm, which for PC1 > 0 are better resolved, than for PC1 < 0, that are less resolved.

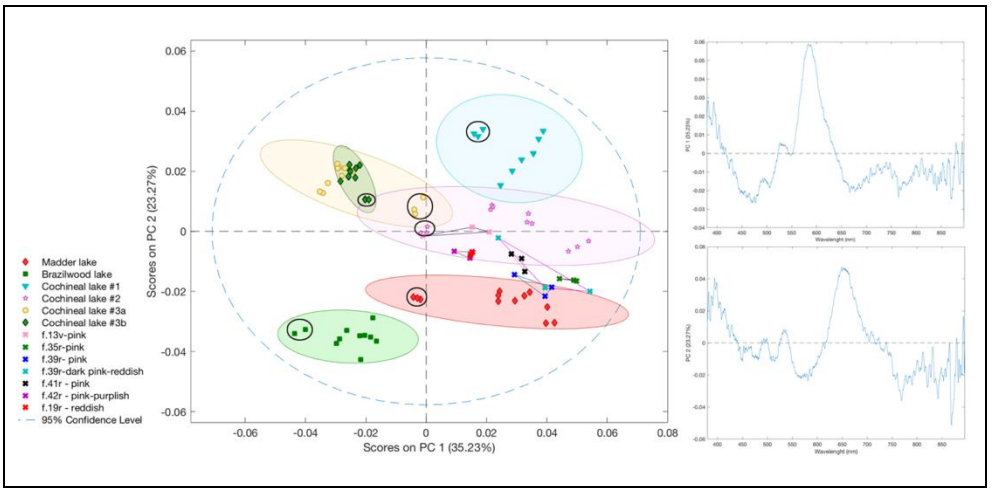

Figure 2. *Left*, scores plot of PCA analysis calibrated with 69 FORS spectra of historical accurate reconstructions of madder, brazilwood and cochineal lakes (#1, #2 and #3a and #3b) on which were projected the 21 FORS spectra of reddish-to-pink lake paints in Manizola 116c. *Right*, loadings on PC1 and PC2. Highlighted in black, scores of egg yolk reproductions.

Regarding the four binders used for the paints' reconstructions, egg yolk-based paints presented a higher score dispersion compared to the reproductions produced with the other three binders (Figure 2, black circles). This is likely due to the yellowish contribution of egg yolk for the paint's final colour, reflected mainly in the visible region, a fact that is not observed for the remaining three binders. In the projection of PCA scores of historical FORS spectra into the calibrated model, it is interesting to note that, from the six reddish-to-pink paints analysed, only those from *f.*39r (a pink paint and a dark pink-reddish paint) cluster closer to madder lake paints reproductions (Figure 2, light and dark blue crosses). However, to validate these results, more specific molecular spectroscopic techniques or a chromatographic analysis should be used. Equidistant from the confidence ellipse of madder lake and of cochineal lake #2 are the scores related to the pink paint FORS spectra of *f.*35r. The remaining FORS spectra cluster into the confidence ellipse of the scores' projections of cochineal lake #2. In fact, the FORS spectra of the pink-reddish paints of Manizola 116c from *ff.*19r and 41r reflected a similar spectral profile close to the one found for cochineal lake #2, with an absorption band structured into two sub-bands at *circa* 523 nm and 562 nm [8]. The small shift found between Manizola's paints and the cochineal lake #2 absorption bands might be due to the lake's production, since, according to Aceto et al. [8], these two absorption bands are dependent on the procedures used for dye extraction and its precipitation into a lake pigment.

3.3. LC-DAD/MS

Figure 3 displays DAD and MS chromatographic profiles and spectral data for sample *f.*19r.

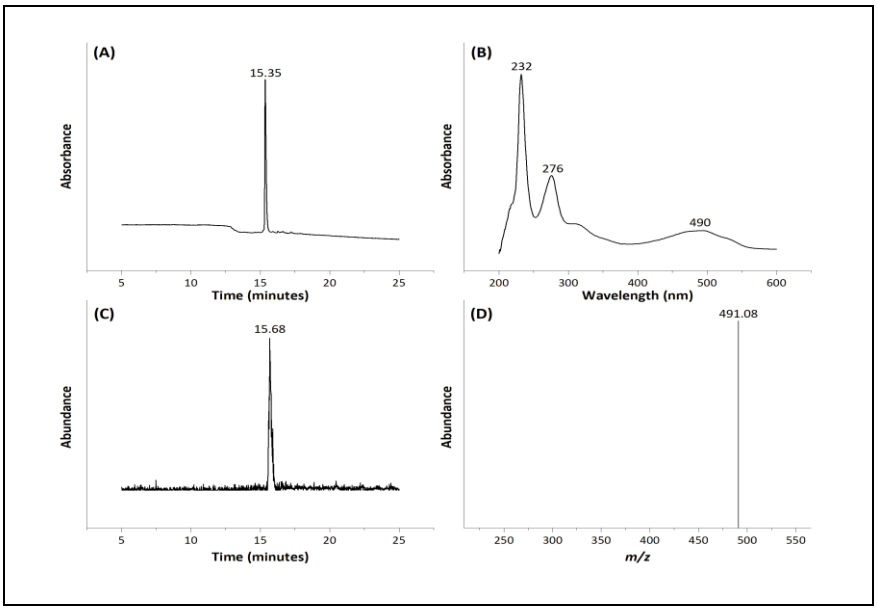

Figure 3. LC/DAD/MS chromatographic profiles and spectral data of sample *f*.19r: (A) LC/DAD chromatographic profile, recorded at 490 nm; (B) Absorbance spectra of chromatographic peak eluting at 15.35 min.; (C) LC/MS chromatographic profile; (D) MS spectra of chromatographic peak eluting at 15.68 min.

Sample *f*.41v exhibited similar chromatographic profiles and spectral data.

One single major peak was identified in both reddish-pink samples, eluting at approximately 15 minutes, and showing an absorption maximum in the visible light spectrum at 490 nm (Figure 3B), characteristic of red chromophore compounds. For the MS analysis, SIM mode was used and six different ions, characteristic of chromophores from red natural dye sources, were selected. The analysis focused on major ions found in brazilwood (*m/z* 243, 283), madder (*m/z* 239), kermes (*m/z* 329), cochineal (*m/z* 491) and lac dye (*m/z* 536). From the six different ions that were selected for the MS analysis, only ion *m/z* 491 was detected in the LC/MS chromatographic profile, in a peak eluting at approximately 15 minutes. This compound was identified as carminic acid, the major chromophore in cochineal insect dyes. Mexican, Polish and Armenian cochineal insect dyes present carminic acid as main chromophore. The distinction between these three insect dyes is only possible when minor chromophores, such as kermesic and flavokermesic acids and dcII, are detected [13]. Besides carminic acid, no other chromophore was detected on the analysed samples and, therefore, it was not possible to identify the cochineal species that was used to produce these reddish-pink lakes.

4. Conclusions

The approach followed in this work combining FORS spectroscopy with PCA analysis allowed to reach interesting results concerning the use of organic

lake pigments both in their acidic and in their basic form. In fact, to the best of our knowledge, this was the first time that it was possible to characterize the use of cochineal lakes both in its reddish and pink-purple hues to produce illuminations in the same manuscript. As pH sensitivity compounds, it is expectable that these range of colours have been achieved by changing the pH of the environment of these lakes at the time of the paints' production. The option of range the colour during the lake's production is, thus, less probable, as our experiments provided evidence that, following accurately the recipe described in the Bolognese Manuscript, a change in the pH during cochineal lake's production does not result in a reddish lake, due to the increment of pH achieved after adding K_2CO_3 at the end of the recipes (Figure 4).

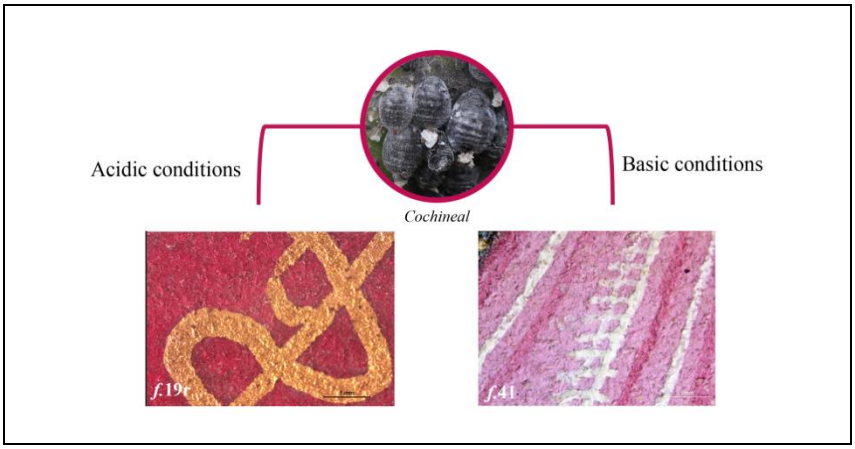

Figure 4. The use of cochineal lake in its acid-basic equilibrium in Manizola 116c.

In this sense, the range of hues achieved across the manuscripts using this acid-basic manipulation suggest, somehow, that the illuminator should have had an (al)chemical knowledge of the behaviour of these organic molecules. It is thus expected that the man who illuminated this manuscript was not only an impressive artist, but also a profound connoisseur of the chemical properties of the painting materials - a truly *Renaissance man*.

Acknowledgement

This work was financially supported by FCT - Fundação para a Ciência e a Tecnologia, I.P., through the projects UID/Multi/04449/2013 (POCI-01-0145-FEDER-007649) and Norma Transitória - (DL 57/2016/CP1372/CT0012). The authors would like to thank to the Portuguese Research Infrastructure for Heritage and Science (ERIHS.pt) for the access to the Mobile facilities (MoLab), financially supported by FCT through the project ALT20-03-0145-FEDER-022115, and to the Biblioteca Pública de Évora for the access to Codex Manizola 116c.

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