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Organic colorants based on lac dye and brazilwood as markers for a chronology and geography of medieval scriptoria: a chemometrics approach

Paula Nabais[1], Maria J. Melo[1*], João A. Lopes[2*], Márcia Vieira[1], Rita Castro[1] and Aldo Romani[3]

Abstract

This work presents the first proof of concept for the use of molecular fluorescence signatures in medieval colours based on lac dye and brazilwood lake pigments. These two important medieval dyes were tested as markers using their UV–Visible emission and excitation spectra. These medieval paints had been previously fully characterized through a multi-analytical approach. In this work, molecular fluorescence spectra were acquired in manuscripts dating from 12th to 15th c., which were produced in monastic scriptoria or workshops. First, the spectral distribution and relative intensity of the emission and excitation spectra were discussed in detail by comparison with reference compounds, including reproductions of paints based on medieval technical texts. It was possible to group the spectra according to recipe specificities. Then, statistical methods (principal component analysis and hierarchical cluster analysis) were applied to the same fluorescence spectra and the generated clusters were compared with the previous ones. Principal component analysis was initially employed to eliminate redundancy in fluorescence data, so minimizing bias on the hierarchical cluster analysis results. Except for some misplaced spectra, the placement of samples per group was confirmed. The outliers resulted from either a poor signal to noise ratio or occurred because certain paints were unique, such as the colour produced by mixing lac dye and brazilwood, which was found in manuscripts from the Alcobaça monastic scriptorium. Previously, by using infrared or Raman spectroscopies, only lac dye could be detected. Notably, these paints compare well with a recipe that was reproduced from the text by Jean Le Begue, in which both dyes were required.

Keywords: Lac dye, Brazilwood, Historical dyes, Brazilein, Laccaic acid, Fluorimetry, HCA, PCA, Conservation, Medieval manuscripts, Photoluminescence

Introduction

Colour, a fundamental attribute of our heritage, is fading in precious artworks. Thus, in the last decade, progress has been made in understanding the complex mechanisms of degradation of historical dyes anchored in the study of their photophysics and photochemistry [1–16]. Another vital ingredient for the understanding of colour stability relies on the reproduction of these ancient colours, which allows for a detailed characterization of these intrinsically heterogeneous systems [6, 7]. In this context, in recent years, molecular fluorescence spectroscopy has become a powerful analytical technique in the field of cultural heritage [5, 7–23]. In addition to its ability to identify organic dyes in complex matrices, by probing its environment it collects unique data linked to the paint formulation (other admixed pigments and dyes,

*Correspondence: mjm@fct.unl.pt; jlopes@ff.ulisboa.pt
[1] LAQV-REQUIMTE and DCR, Faculty of Sciences and Technology, NOVA University of Lisbon, 2829-516 Caparica, Portugal
[2] Faculty of Pharmacy, iMed.ULisboa-Research Institute for Medicines, University of Lisbon, Av. Prof. Gama Pinto, 1649-003 Lisbon, Portugal
Full list of author information is available at the end of the article

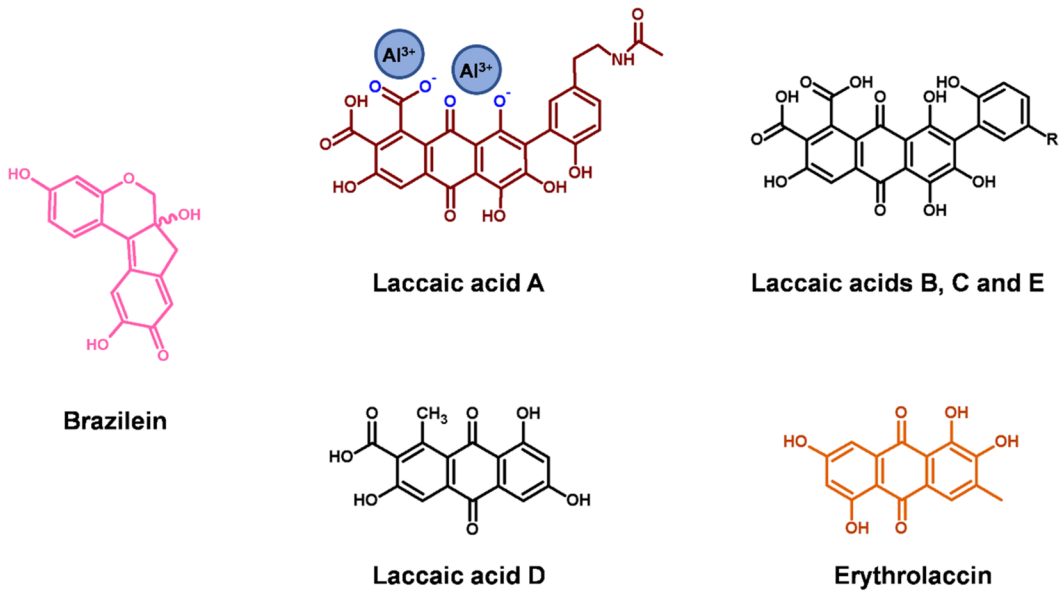

Fig. 1 Chemical structures of brazilein, laccaic acid A, laccaic acid B, C and E (B, R = CH_2CH_2OH; C, R = CH_2CHNH_2COOH; E, R = $CH_2CH_2NH_2$), laccaic acid D, and erythrolaccin. For laccaic acid A the main sites for complexation with a metal ion are shown

binding media and other additives, e.g., as fillers, and their relative proportions) [21, 22]. Thus, based on previous applications of microspectrofluorimetry in heritage science [5, 17–20, 24], in this work, we intend to develop a data-driven based method to assist the identification of organic dyes in medieval artworks, built from a database of fluorescence excitation and emission spectra, in the UV–VIS [5, 17–20]. Through the specificities of the paint formulation (the original recipe and its making), we also intend to extract useful details on their conservation conditions and their place of production. The study of these complex systems aged naturally for eight to six centuries, is supported by previous research, in which we built a database of molecular fluorescence spectra of reconstructions of medieval colours that included lake pigments and paints for the following colorants: kermes, lac dye, brazilwood, and cochineal [19]. The photoluminescence reference database that will be built for the present work, combines for the first-time molecular emission and excitation data acquired in medieval illuminations for lac dye[1] and brazilwood[2] chromophores, Figs. 1, 2, 3. What motivates us to test a reference database based on original medieval colours, and why lac dye and brazilwood paints [25, 26]? First, our interdisciplinary studies demonstrate that the colour we see today, in the selected manuscripts, has not been restored nor retouched [5, 7, 17–20, 24, 27, 32–36]. Second, we have studied them extensively because they represent some of the most important reds/pinks/purplish colours used in medieval illuminations [24, 26, 27, 32–37], which was one of the major arts of the European Middle Ages. By providing an in-depth knowledge of these systems, this research will contribute to overcoming the challenge of the identification and preservation of organic dyes in works of art.

In the past fifteen years, through a focus on colour, our team conducted studies of the selected manuscripts integrating contributions from molecular sciences, history, art history, codicology, religion and culture [7, 18–20, 24–27, 32–35, 38–51]. This research, especially on monastic collections, has demonstrated their cultural and

[1] Lac is part of a resinous cocoon secreted by parasitic insects, from the genus *Kerria*, on twigs of branches of host trees; for more details please see pp 665 in [25], pp 6–7 in [26] and pp 159–161 in [27]. These insects are native to the countries of the southern and south-eastern Asia [25, 28]. The female lac insect secretes a red resin, sticklac, from which are obtained both the lac dye and the shellac resin. The complex nature of lac reflects its composition: a terpenoid resin, which normally represents 68% of the entire matter, a dye that only represents 10%, and other less representative constituents, such as wax, gluten, foreign bodies and impurities [29]. The red colour extracted is named lac dye, which is made of laccaic acids A and B; laccaic acids C, D and E in minor quantities, Fig. 1. The refined resin, known as shellac contains the yellowish orange erythrolaccin, which is responsible for its colour, Fig. 1 [20, 27, 30, 31].

[2] Brazilwood is a soluble redwood that was used to prepare lake pigments (dye-metal complexes) for manuscript illumination. The lake pigments were based on brazilein, Fig. 1. In medieval Europe, sappanwood and other soluble redwoods were known commercially and technically under the name brazil. For more details see please [24] (p. 256) and [25] (pp. 274–75).

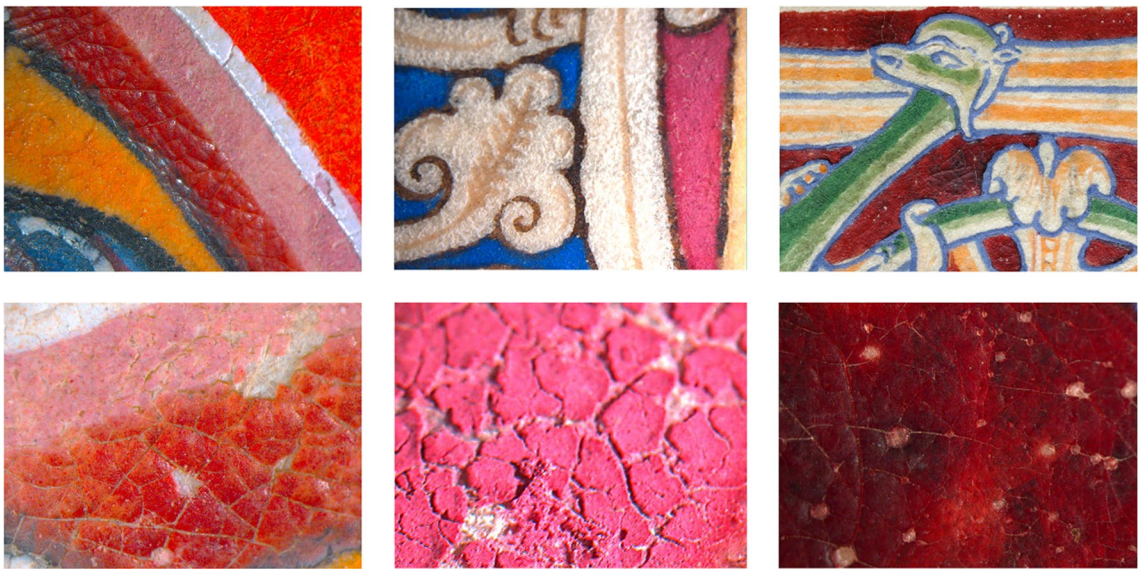

Fig. 2 Lac dye details from manuscripts showing the differences in hues of original paints: from left to right, lac dye in Lv 15 *f.* 26, SC 1 *f.* 21v, and SC 20 *f.* 191. The figures below are macro details of the top row figures (ANTT and BPMP collections)

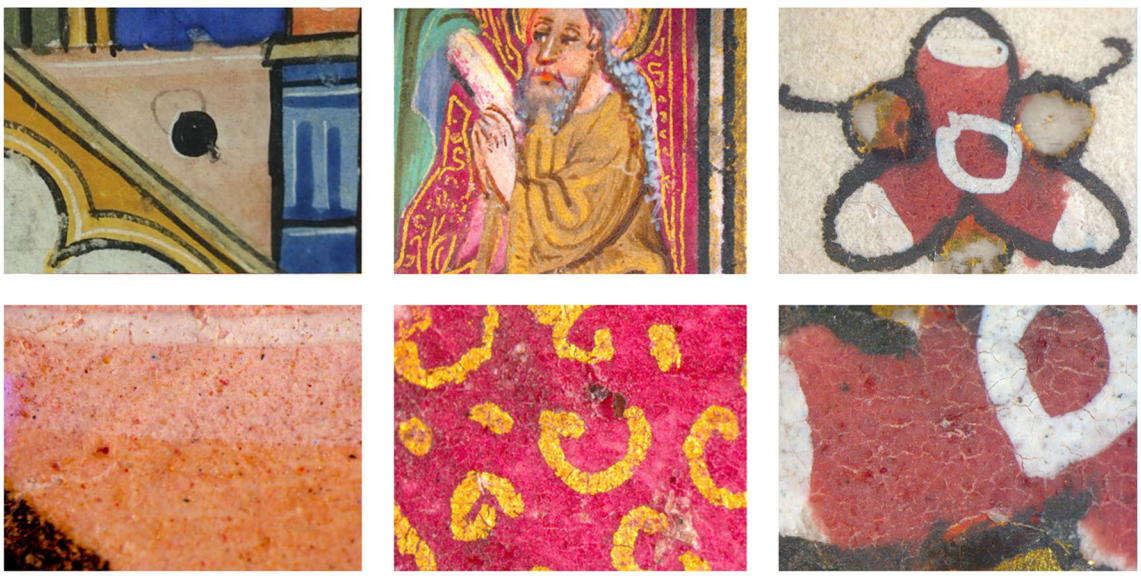

Fig. 3 Brazilwood details from manuscripts showing the differences in hues of original paints: from left to right, Ajuda Songbook *f.* 59, IL 15 *f.* 66, and ms 22 *f.* 76v. The figures below are macro details of the top row figures (PNA, BNP and PNM collections)

artistic importance, both within Iberian as well as European context [33, 38–42, 44–50]. Our database combines spectral information acquired in manuscripts preserved in Portuguese collections, having a Portuguese, French, or Flemish provenance, and dating from the 12th c. into the 15th c., Additional file 1: Figure S1. The Portuguese manuscripts were produced in the scriptoria of three important monasteries, whereas Flemish and French books of hours in lay workshops, possibly active in flourishing medieval cities [33, 36]. As an example of the monastic production, the monumental bible of the monastery of the Holy Cross (thereafter Santa Cruz) is one of the most important medieval manuscripts preserved in Portugal. Santa Cruz 1 (**SC1**) is a work of art created

by the same monastery that prepared Saint Anthony for the world, its illuminations ornament the Old testament. Also included in this work, and of uncertain provenance, is the Ajuda Songbook, the only surviving medieval songbook of Galician-Portuguese secular poetry, which dates to the end of the thirteenth or beginning of the fourteenth century [45]. Accurate dating of the manuscripts is possible when a colophon is present, but most of the manuscripts are dated by scholars based on the illuminations' style [47–50]; this information is provided in Additional file 1: Tables S1–S5. Molecular fluorescence spectra were acquired both in situ and in micro-samples (invisible to the naked eye, see in the Experimental Section for the average dimensions). We tested both sets and, although we had a greater number of spectra acquired in situ, we chose to present in this work the data acquired in micro-samples, as it is possible to guarantee a greater reproducibility in this set of samples.

Our *modus operandi* will be as follows. We will start by grouping the data, correlating the spectral distribution (shape) and relative intensity of the molecular fluorescence spectra of medieval paints with reference samples that have been prepared with different concentrations of H^+ and Al^{3+}, different fillers, etc. This experimentation, testing different concentrations of proton and aluminium ion as well as different fillers, is based on our research in medieval recipes: we show that these are the essential parameters that have been manipulated to obtain different hues (from deep red to purples and pinks), as well as different degrees of opacity; for more details please see [7, 24, 32, 35]. Then, we will apply a combination of chemometric methods (principal component analysis and hierarchical cluster analysis) and compare the results with our previous grouping. We will summarize our main results, stepwise, providing the relevant information for the non-expert in photophysics. The results obtained from the data processing with the chemometric methods will also be discussed taking into consideration the paint formulation previously characterized through a multi-analytical approach using Fourier Transform Infrared microspectroscopy (μFTIR), Raman microscopy (SERS), X-Ray Fluorescence microspectroscopy (μXRF), FORS (UV–VIS), colorimetry [24, 27, 32–35, 38–51].

The statistical methods employed in this work have the objective of disclosing consistent patterns that help us to differentiate and to cluster the profiles captured by the analytical methodologies, providing a systematic analysis of the collected data. As molecular fluorescence spectra, in the solid-state, may suffer the influence of external factors other than those relevant to this work (the signals of lac dye and brazilwood chromophores), the methods were selected to provide a reliable and robust analysis of the signals. To increase this robustness, a previous analysis of all spectra is carried out to detect and avoid potential interferences that vary from the existence of external impurities to a spectrum that has been acquired with a low signal-to-noise ratio. In this work, hierarchical cluster analysis and principal component analysis were used in sequence. Both methods are unsupervised techniques (so they do not require a "teaching" algorithm) and they are relatively simple to implement and use. Principal component analysis was initially employed to eliminate redundancy in fluorescence data, so minimizing bias on the hierarchical cluster analysis results. The simplicity of these methods when compared to other potential approaches allow for a better interpretation of results and increases the robustness of the obtained results. Other methods such as linear discriminant analysis or partial least squares discriminant analysis (both supervised) would require a more elaborated validation purpose to avoid overfitting and therefore the generation of spurious results. Three-way methods such as PARARAC or some versions of support vector machines would be better fitted if the data were 3D fluorescence (which is not the case). Hierarchical cluster analysis (HCA) also has some limitations as the results are confined to the samples used in the agglomerative or divisive algorithm. Different algorithms may also condition the method's output. The potential effects of data collinearity, noisy data, non-systematic data artifacts or even the existence of missing data can be minimized by applying a method like principal component analysis (PCA), as performed in this work, before employing an HCA algorithm. Additionally, HCA is not a model in the usual sense, so it cannot be preserved and used to assess new samples. Examples of the applications of these methods in related works can be found elsewhere [19, 52, 53].

Experimental

Artworks

The manuscripts studied have been kept in Lisbon, National Library (*Biblioteca Nacional de Portugal,* BNP), National Archives (Arquivo Nacional da Torre do Tombo, ANTT), Palace of Ajuda Library (Palácio Nacional da Ajuda, PNA); Municipal Library of Porto (Biblioteca Pública Municipal do Porto, BPMP); Mafra National Palace, (Palácio Nacional de Mafra, PNM), Additional file 1: Figure S1. Sample description and representative spectral information that was used in this work is provided in Additional file 1: Tables S1–S5 and S7–S12.

Seventeen manuscripts from the twelfth to the thirteenth century from scriptoria of the three main Portuguese monasteries,[3] São Mamede of Lorvão (ANTT),

[3] The nomenclature of the manuscripts was chosen in accordance with previous publications. Currently, the nomenclature used within the institutions is as follows:
Lv 5—Ordem de Cister, Mosteiro de Lorvão, códice 5 (ANTT); ALC 238—alc-238 (BNP); SC 20—Santa Cruz 20 (BPMP).

Santa Cruz of Coimbra (BPMP), and Santa Maria of Alcobaça (BNP), were selected. Lv 5 (1183–4), Lv 12 (13th c.), Lv 13 (13th c.), Lv 15 (1201–50) and Lv 50 (1183); SC 1 (1151–1200), SC 20 (early 13th c.) and SC 21 (early 13th c.); ALC 238 (late 12th c.), ALC 247 (12th c.), ALC 249 (13th c.), ALC 347 (12th–13th c.), ALC 412 (1257), ALC 419 (12th–13th c.), ALC 421 (12th–13th c.), ALC 427 (12th–13th c.) and ALC 446 (13th c.) [32, 38–44].

Other eight manuscripts dating from the thirteenth to the fifteenth century were included. The Ajuda Songbook (PNA, 13th–14th c.), the winter Breviary ALC 54 (BNP, 14th–15th c.) and six books of hours, ms 22 (PNM, 1400–20), ms 24 (PNM, 1420/1470), IL 15 (BNP, ca. 1450), IL 19 (BNP, 1420–30), IL 21 (BNP, 1460–70) and IL 42 (BNP, ca. 1470) [45–51].

Micro-sampling

Micro-sampling of the manuscripts was performed with a microchisel from Ted Pella microtools under a Leica KL 1500 LCD microscope, (7.1× to 115× objective) and a Leica Digilux digital camera, with external illumination via optical fibers. The dimension of the micro-samples ranges from 20 and 50 μm and, as such, invisible to the naked eye. As we have not yet obtained their weight, although micro-scales were used, we can use their detection limit to conclude that they weigh less than 0.1 μg. Micro-samples are stored in microscope slides with single cavity and covered with a microscope glass slide. They are closed with tape (magic tape 3 M) and used as sample holders. In situ spectra are collected directly from the sample by opening the cover. These sample holders are then stored in a microscope slide tray cabinet, in a dust-free enclosure (Ted Pella). The cabinet outer shell is white polypropylene, and the tray rails are polystyrene.

Micro-samples collection under a microscope, ensures a selective sampling of the dye paint; that is, the micro-sample will not include parchment support, ground layers (mainly applied to metallic colours), or any other external layers. So, we can certify that fillers and other additives present in the medieval colours belong to the paint formulation.

Microspectrofluorimetry measurements

Fluorescence excitation and emission spectra were recorded with a Jobin–Yvon/Horiba SPEX Fluorog 3–2.2 spectrofluorometer coupled to an Olympus BX51M confocal microscope, with spatial resolution controlled by a multiple-pinhole turret, corresponding to a minimum 2 μm and maximum 60 μm spot, with 50× objective. Beam-splitting is obtained with standard dichroic filters mounted at 45°, in a two-place filter holder. For a dichroic filter of 570 nm, excitation may be carried out until about 560 nm and emission collected after about 580 nm ("excite bellow, collect above"). The optimization of the signal was performed daily for all pinhole apertures through mirror alignment, following the manufacturer's instructions, using a rhodamine standard (or other adequate references). Fluorescence spectra were corrected only for the wavelength dependence of the excitation-source intensity. For the study of red dyes, two filter holders with two sets of dichroic filters are employed, for lac dye the set of 500 and 600 nm and for brazilwood the set of 540 and 600 nm. This enables both the emission and excitation spectra to be collected with the same filter holder. A continuous 450 W xenon lamp, providing an intense broad spectrum from the UV to near-IR, is directed into a double-grating monochromator, and spectra are collected after focusing on the sample (eye view) followed by signal intensity optimization (detector reading). The pinhole aperture that controls the area of analysis is selected based on the signal-to-noise ratio. For weak to medium emitters, it is set to 8 μm, in this work for very weak signals 30 μm spot was also used (pinholes 5 and 8, respectively) with the following slits set: emission slits = 3/3/3 mm (6 nm bandpass) and excitation slits = 5/3/0.8 mm (final bandpass of 2 nm). Emission and excitation spectra were acquired on the same spot. With our experimental set, usually, excitation spectra are acquired with a higher S/N than emission spectra. For more details on the experimental set-up please see [5, 18, 19].

Fourier transform infrared microspectroscopy (microFTIR)

Infrared analyses were performed using a Nicolet Nexus spectrophotometer coupled to a Continuμm microscope (15× objective) with a MCT-A detector cooled by liquid nitrogen. The spectra were collected in transmission mode, in 50 μm areas resolution setting 4 or 8 cm^{-1} and 128 scans in the 4000–650 cm^{-1} spectral range, using a Thermo diamond anvil compression cell. For some infrared spectra, the system was purged with nitrogen prior to the data acquisition; for all infrared spectra the CO_2 absorption at circa 2400–2300 cm^{-1} was removed from the acquired spectra. To improve result robustness, more than one spectrum was acquired from different sample spots.

Data analysis

Spectral pre-treatments

Excitation and emission spectra were tested, separately and in combination. Each spectrum was pre-processed by normalization to unit area. Spectra of different samples were then arranged vertically forming two data blocks (excitation and emission) forming two-way matrices (samples versus wavelength). These data blocks were analysed in separate and in combination. In the latter

Table 1 A total of 70 emission and 70 excitation spectra on 37 lac dye paints were collected from 17 illuminated manuscripts produced in Portuguese monasteries (12th–13th c.). Based on their spectral features and intensity these spectra were assembled in 4 groups, Lac 1 to 4

	Lac 1	Lac 2	Lac 3	Lac 4
Excitation maxima	*473 nm* Band at 430 nm	*535–555 nm*	*525 nm* *sh* 500 nm (resin chromophores) *sh* 550–555 nm	*554 nm*
Intensity				
Max	4.2×10^5	4.8×10^5	6.5×10^5	1.0×10^6
Min	1.3×10^5	2.6×10^5	2.2×10^5	4.6×10^5
Emission maxima	*588–593 nm* *sh* 562 nm *sh* 615 nm	*585–590 nm* *sh* 563 nm *sh* 610–616 nm	*585–590 nm* *sh* 610–616 nm	*600–610 nm* *592–594 nm* *sh* 610 nm
Intensity				
Max	8.1×10^4	9.2×10^4	1.3×10^5	9.3×10^4
Min	2.1×10^4	3.4×10^4	2.0×10^4	3.6×10^4

The values for the emission and excitation maxima are in italics

sh shoulder

situation, the two blocks were merged by horizontally concatenating the matrices. The best results were accomplished with the combination of the excitation and the emission spectra. Further details on the selection of preprocessing methods can be found in [54].

Chemometric methods

Hierarchical cluster analysis (HCA) was applied to the spectral data towards the discrimination and classification of artworks using different dye families. Dendrograms were developed considering data from the entire artworks' dataset including the whole wavelength range. The HCA method was applied not directly to the fluorescence excitation and emission spectra but, to the result of a principal component analysis of these data. The first three components encompassing slightly more than 95% of the total variance were used by the HCA method. The Ward's algorithm was used to perform the clustering approach and the Euclidean distance selected. Prior to the application of all chemometric methods, the datasets were mean centred. All chemometric analyses and data manipulations were performed with Matlab Version 8.6 (R2015b) (The Mathworks, Natick, MA) and the PLS Toolbox Version 8.2.1 (Eigenvector Research, Manson, WA).

Results and discussion

Lac dye identification in medieval manuscripts by molecular fluorescence: probing the influence of the manufacturing processes and additives

The main spectral features used to establish the four groups of lac dye-based paints are shown in Table 1. Next, they will be discussed in detail and compared with the reference paints in our database [19, 20, 27]. It should be noted that most of the molecular fluorescence spectra obtained in medieval manuscripts belong to one of these two main groups, ***Lac 1*** and ***Lac 3***, or have some characteristics of one or both as will be discussed below, Fig. 4.

In a previous publication, and based on medieval reproductions of lac dye paints, we proposed that lac dye reds can be produced as "free lac dyes" and Al^{3+}-lac complexes [20]. For this reason, fourteen recipes for lac dye were selected from eight technical sources dating from the tenth century to the end of the sixteenth century, including four that do not use an aluminium salt[4] [32]. These reproductions will support our analysis of the original medieval paints.

Analysis of the emission and excitation spectra of lac dye paints

"Free lac" paints are not complexed with Al^{3+} and display the spectral features of ***Lac 1 group***; (i) a broad excitation spectrum with a maximum at 473 nm (and possibly also a band at 430 nm, in the same region as we get an instrumental artefact), Fig. 4; (ii) an emission spectrum with a maximum at 588 nm and shoulders at 563 nm and 600 nm, Fig. 4. From eight original manuscripts (from a total of seventeen), twelve paints are included in this

[4] Ms. of Ibn Bādīs (c.1025), Chapter 6; Ms. O Livro de como se fazem as cores (fifteenth century), Chapter 13; Ms. Paduan (late 16th–seventeenth century), Recipe 90 and 113 [7, 55–57].

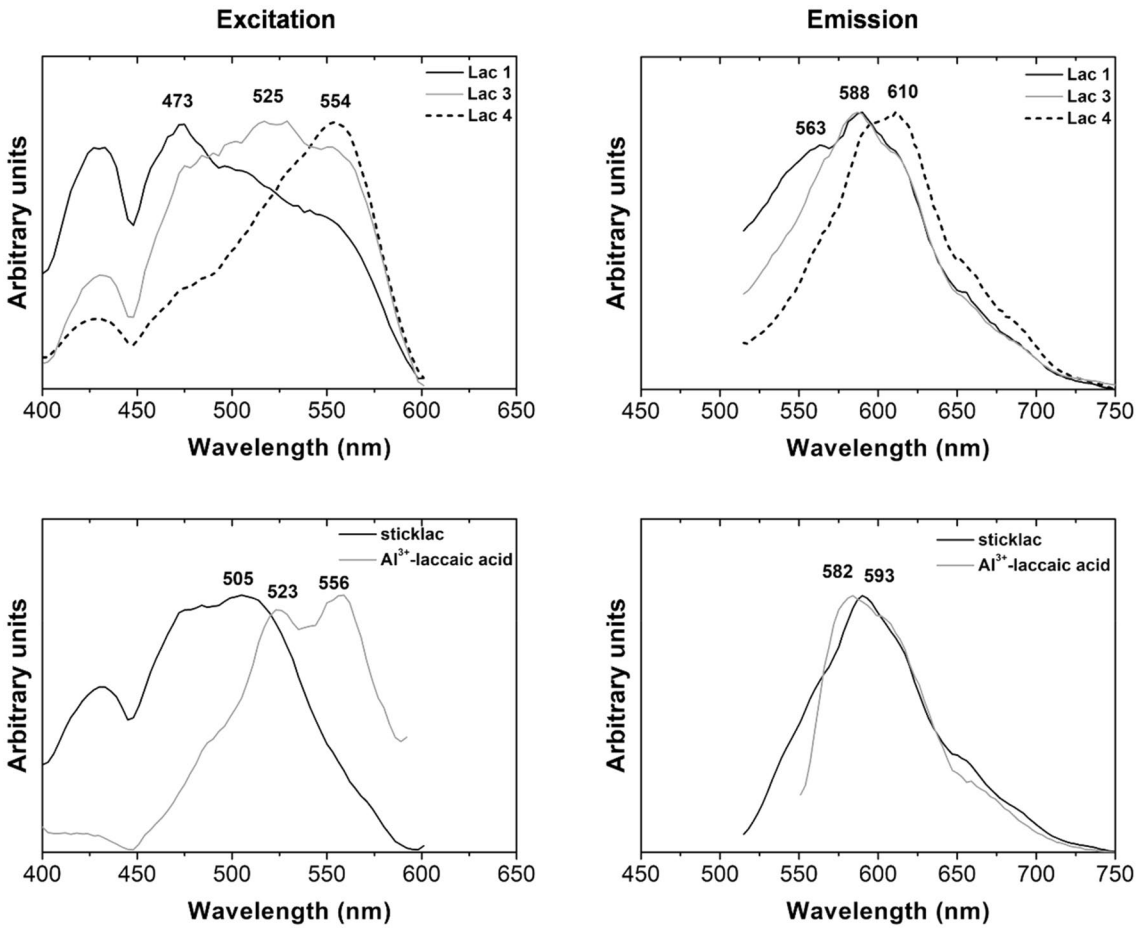

Fig. 4 Lac dye paints, excitation and emission spectra from: 12th c.–13th c. Portuguese manuscripts that are represented as groups Lac 1 (▬), Lac 3 (▬), and Lac 4 (▪▪▪▪); sticklac (raw material, resin + chromophores); Al^{3+}-laccaid acid A complex, applied on filter paper, pH = 3.5. For more details, please, see the text

group, ***Lac 1***,[5] Additional file 1: Table S1. Their spectra can be compared to those obtained from the colours described in Ibn Bādīs (c.1025), chapter 6; the Ms O Livro de como se fazem as cores (15th c.), chapter 13; and manuscript Paduan (late sixteenth to seventeenth century), recipes 90 and 113 [7, 27, 32, 55–60], Fig. 5.

The excitation spectrum for Al^{3+}-lac complexes is different from "free lac", being characterized by two bands at *ca.* 554–56 nm and 523–25 nm as well as by a shoulder at *ca*. 500 nm which is characteristic of the yellow chromophores present in sticklac, Fig. 4. ***Lac 3***, combines both features in its excitation spectrum, the Al^{3+}-lac complex, and the resin signature [20]. The emission spectra for this group is characterized by a maximum at 588 nm, like ***Lac 1*** group, but is easily distinguished from the latter because the shoulder at 563 nm is absent, Fig. 4. From six original manuscripts (from a total of 17), seven paints are included in this group ***Lac 3***,[6] Additional file 1: Table S3.

Lac 3 spectra compare well with those obtained from the colours described in Ms Mappae Clavicula (twelfth century), recipe 253; Ms Bolognese (fifteenth century), recipes 129, 131, 137 and 140; Strasbourg manuscript (fifteenth century), recipe for *Bright Paris Red*; Montpellier Ms (fifteenth century), recipe 1.9; and Jean le Begue (1431), recipe 36, Figs. 5 and 6 [20, 27, 32, 55–60]. The spectra from the reconstructions, generally, have a better signal-to-noise ratio, Fig. 6.

In group ***Lac 2*** (Additional file 1: Table S2), the emission spectra are similar to group ***Lac 1***, but the excitation

[5] Lac 1 group—SC 20, *ff.* 191 and 197v; Lv 13, *ff.* 21, 30 and 44v; ALC 412, *ff.* 10v and 12; ALC 421, *f.* 202v; ALC 446, *f.* 96v, Lv 12, *ff.* 17 and 94; and SC 21, *f.*19.

[6] Lac 3 group—Lv 5, *ff.* 6 and 73v; Lv 13, *ff.* 44v; Lv 15, *f.* 26; SC 21, f. 2; ALC 247, *f.* 21v, and ALC 421, *f.* 193v.

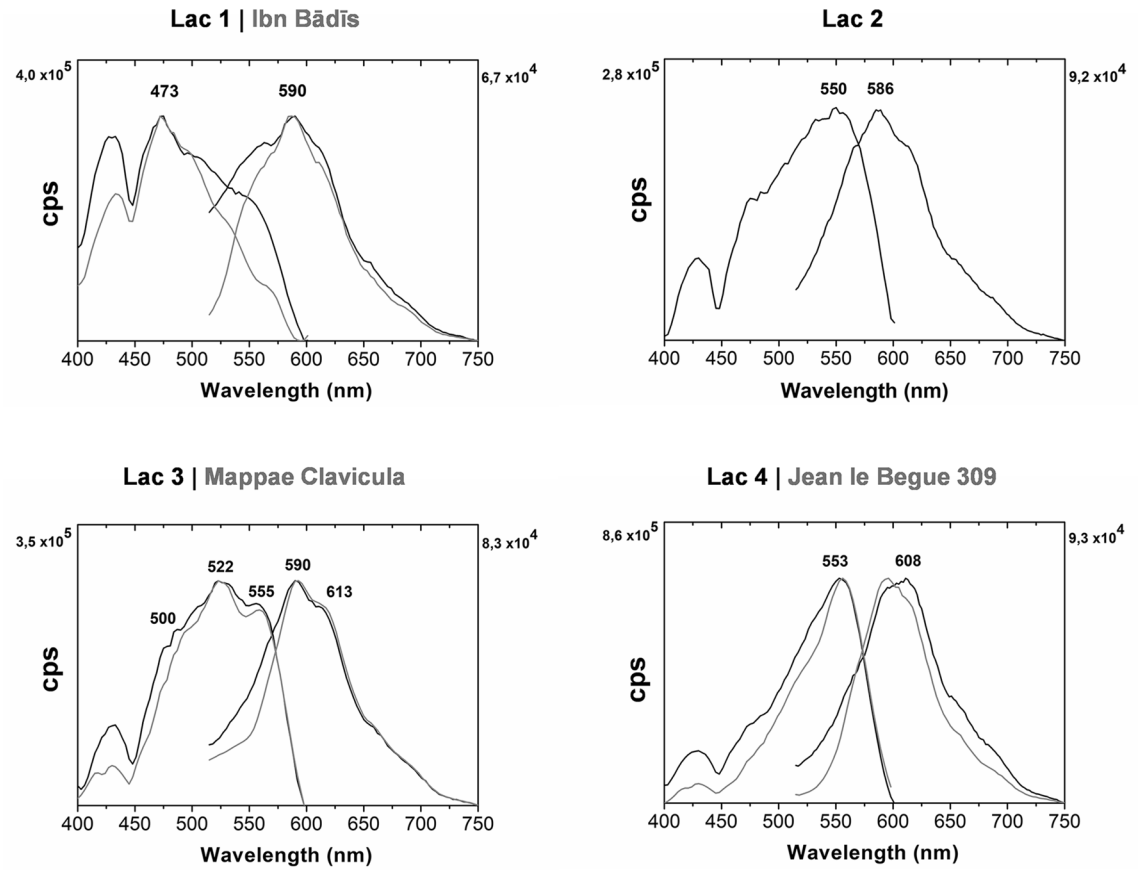

Fig. 5 Lac dye paints, representative excitation and emission spectra for each group compared with reference paints applied, using glair as binding medium, on filter paper; for Lac 2 group it was not possible to find a match with a medieval reconstruction. *Lac 1*, ALC 412 *f.* 10v with an ibn Bādīs reconstruction. *Lac 3*, ALC 247 *f.* 21v with a Mappae Clavicula reconstruction. *Lac 4*, ALC 238 *f.* 206v Jean le Begue, recipe 309, reconstruction

spectra are different, Fig. 5. These excitation spectra are still characterized by a very broad band, but the maxima fall at higher wavelengths, between 535 and 555 nm (the region in which the first band of the complex Al^{3+} -laccaic acid A emits). It resembles, to a certain degree, the excitation spectra obtained for brazilwood lake pigments (see Sect. 2). From the original manuscripts, nine paints are included in group ***Lac 2***.[7] In this case, we could not find a medieval recipe for a lac dye colour with these specific spectral features.

The paints from the monumental bible Santa Cruz 1 (**SC1)** are possibly the most complex in the collection. In *ff*. 2v, 37 and 77, the excitation spectra can be compared with the ***Lac 2*** group, but the emission spectra show a well-resolved envelope, with two maxima at *ca* 560 and between 580 and 586 nm (closest to the ***Lac 1*** spectra, where the shoulder has turned into a band), Additional file 1: Table S5. We could not find a match with any of our historical reconstructions. Different spectra, from those described above, were acquired in two other folios: that of folio 14v can be compared to that of ***Lac 1*** group, with an excitation maximum at 474 nm and an emission maximum at 593 nm, while that of folio 24 has the same excitation maximum as the latter, but an emission maximum shifted to 560 nm with a shoulder at 580 nm, similar to the emission spectra obtained in folio 37.

Finally, the spectra of the ***Lac 4*** group (Additional file 1: Table S4), were found only in four paints of three folios from two manuscripts (ALC 238 and ALC 347), displaying a different spectral distribution, with maxima shifted to higher wavelengths when compared to the other groups. In Fig. 5, two representative spectra are depicted, being characterized by an excitation maximum at 554 nm and emission at ca 592–610 nm. In this case, it was possible to find a very good match with a lac dye paint reproduced following the instructions of the recipe 309 from *De diversis coloribus*, which is included in the manuscript compiled by Jean Le Begue (1431), employing both lac

[7] Lac 2 group—ALC 249, *f.* 109v and ALC 419, *f.* 98; ALC 427, *f.* 115v; Lv 15, *ff.* 16 and 50; Lv 50, *ff.* 1v and 64v, and SC 20, *ff.* 78 and 86.

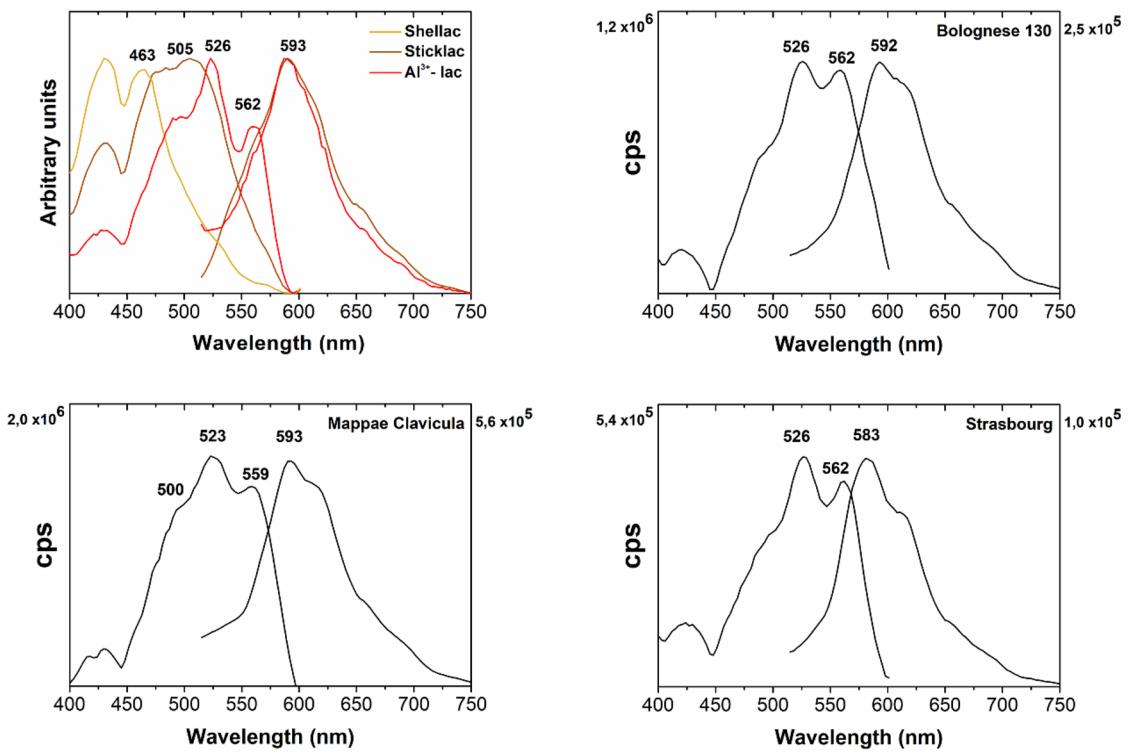

Fig. 6 Excitation and emission spectra of: shellac (processed resin without lac dye chromophores), sticklac (raw material, resin + chromophores), and Al^{3+}-lac complex, pH = 3.5; lac dye reconstructions from recipes in Bolognese 130, *Mappae Clavicula* and Strasbourg, applied, using glair as a binding medium, on filter paper

dye and brazilwood [32, 56]. The excitation spectrum is what best distinguishes this recipe from all other medieval lac dye paints, and the spectral envelope acquired in the original manuscripts compares very well with the Jean le Begue 309 recipe, both in spectral distribution and intensity. This paint combines spectral features from two different chromophores, which are medium to weak emitters, so, depending on the raw materials, their quantities, and processing as well as on the acquisition conditions, one can dominate over the other. It should be noted that, of the fourteen recipes selected to be reproduced, two included a mixture of lac with brazilwood; in one of the recipes, the quantity of both materials is indicated,[8] while in the second, the recipe Jean le Begue 309, only a vague description is given: "*Take an ounce of lake, and rasp finely a little Brazil wood*" [32, 56]. The spectra of the historical reconstructions are included in Additional file 1: Table S6.

In our previous publications, of these medieval lac dye paints, Surface-enhanced Raman spectroscopy (SERS) identified only lac dye chromophores [20, 27], including in ALC 238 and ALC 347. Although brasilein was not identified by SERS in these manuscripts, it is likely that in ALC 238 and ALC 347 the red colour was obtained admixing lac dye and brazilwood colorants, as indicated by molecular fluorescence. By SERS it would have been very difficult to detect brasilein in a mixture with lac dye.

The intensity of the emission is also characteristic of a molecular fluorescence spectrum, and for historical reconstructions, it has been observed that it increases with the amount of alum used in the recipe. Thus, under certain conditions, the signal intensity can be correlated with the presence of an Al^{3+}-complex and the use of alum. However, emission intensity can be strongly affected by the morphology of the analysed surface. For this reason, meaningful conclusions are only possible when relevant changes are observed. Based on our experience of applying microspectrofluorimetry to the study of works of art, for an experienced user, it is possible to acquire spectra with a consistent S/N for the same colour/micro-sample, so it is possible to include the signal intensity in the spectral characteristics. The effect of surface morphology is minimized by selecting the same type

[8] Ms. Bolognese (fifteenth century), Recipe 130, To make lake as before in another manner: Take of gum lac 5 lbs., (…) take 6 oz of verzino in very fine powder [56].

of surface (usually smooth/homogeneous surfaces) and optimizing the S/N before acquiring the signal.

In the medieval paints the differences in intensity observed are small and, for the time being, do not allow us to conclude if alum was not used in these medieval colours. The greatest variations are detected in the excitation spectra, with the lowest intensities found in Lac 1 and Lac 2 groups, 4.2×10^5 and 4.8×10^5, respectively; while for Lac 3 and Lac 4 groups, they are 6.5×10^5 and 1×10^6, respectively. Interestingly, in the SC1 group, the excitation spectra close to Lac 1 group, acquired in *ff.* 14v and 24, also present the lowest intensities of this group.

Correlating the groups of lac dye paints based on molecular fluorescence spectra with the paint formulation given by infrared spectroscopy

As will be detailed below, for lac dye paints, the diversity observed in the paint formulations by infrared spectroscopy is not directly reflected in the photophysical properties of the main chromophores that allowed us to distinguish four groups for medieval lac dye paints. On the other hand, the two analytical techniques offer relevant complementary information. In these paints, infrared spectroscopy detects the presence of shellac resin, proteinaceous binder, and fillers as gypsum and calcium carbonate. The latter may be used as fillers or as opacifiers and/or to create lighter colours, such as when adding lead white (rarely applied to create lighter tones, but extensively used to "heighten the colours" [40]). It is also clear, from the infrared spectra, that their relative concentration varies. However, neither specific charges nor relative proportions could be associated with the four groups, created based on the analysis of the molecular fluorescence spectra.

Representative infrared and molecular fluorescence spectra are available as Additional file 1: Tables S1 to S5. Most of the infrared spectra of the paints in group ***Lac 1*** show the presence of the shellac resin through its distinctive C-H stretching bands, but in some, its presence is barely visible. All spectra are dominated by the proteinaceous binder. Fillers such as calcium carbonate and gypsum are also clearly visible, Additional file 1: Table S7. When compared with the binder, they are usually present in low concentrations, with one exception, the paints in folios 30 and 44v in Lv 13, as it is visible in Additional file 1: Table S8, where the $CaCO_3$ percentage is more than 6 times as higher as the rest of the manuscripts. Most excitation spectra displayed consistently a broad band with a maximum at ca 472–474 nm and shoulders at 508–514 and at ca. 550 nm. On the other hand, the emission spectra display a higher diversity, being characterized by a maximum at *ca* 590 nm or in the interval 605–615 nm, and a more or less pronounced shoulder at 563 nm. However, no meaningful correlation between the fillers and the fluorescence emission spectra could be detected.

The infrared spectra in group ***Lac 2*** are characterized by a lower amount of shellac resin and a higher proportion of proteinaceous binder and fillers when compared with group Lac 1; ALC 249 and 419 (*ff.* 109v and 98, respectively) display a high proportion of calcium carbonate and gypsum; Lv 15 and Lv 50 of calcium carbonate and lead white. For the latter, it is possible that the difference in the excitation maxima may be due to the presence of lead white in a relatively high amount [27].

In the infrared spectra of the paints in group ***Lac 3***, it is possible to assess the presence of the shellac resin through its distinctive C–H stretching bands, Additional file 1: Table S7. Lv 5, *f.* 6 displays a spectrum dominated by the shellac resin whereas in the other spectra the proteinaceous binder is clearly visible. Gypsum was detected in three paints (Lv 5, *f.* 6; SC 21 and ALC 247); whereas, in Lv 13, ALC 421 and Lv 15, calcium carbonate was found, the latter with lead white. Both gypsum and calcium carbonate were employed as fillers (improving the paint mechanical performance) and, depending on the concentration, as opacifiers. So, although the paint formulations differ, the recipe used to produce the lac dye chromophore was probably similar. In this case, microspectrofluorimetry could group the recipes according to the chromophore and infrared spectroscopy was able to discriminate the paints' formulation (SC 21 and ALC 247 are very similar).

In **SC1** the infrared spectra are dominated by the protein fingerprint and the shellac resin is not visible, except for folio 14v; a small amount of calcium carbonate was also detected. There is no information in these spectra that explains the differences observed in the molecular fluorescence spectra.

The two paints in group ***Lac 4*** were found in manuscripts from the collection of the Monastery of Alcobaça, ALC 238 (*Book of birds*) and ALC 347 (*Sermones de verbis Domini*); in ALC 238 paint, the shellac resin and the proteinaceous binding medium are clearly visible (C–H and amide stretching, respectively), and a high amount of gypsum as a filler (when compared with the binder) is also observed. On the other hand, the infrared spectrum of the ALC 347 paint is dominated by the protein fingerprint and a high amount of calcium carbonate. So, again, the diversity found in the paint formulations, detected by the infrared spectra, did not leave a visible mark in the chromophore emission. In this case, as already discussed, the excitation spectrum points to the presence of a brazilwood lake pigment (please, see next section and Fig. 6). Considering that the presence of shellac is very clear in the infrared spectra, we propose this paint to be included

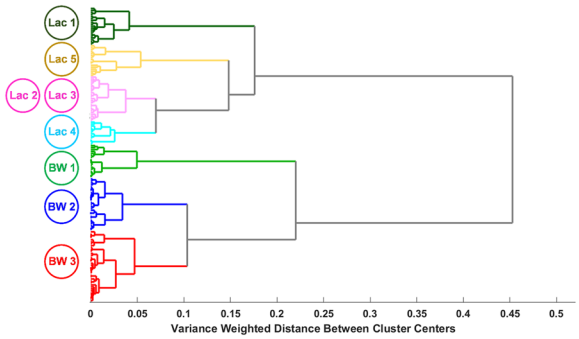

Fig. 7 Dendrogram generated by HCA applied to excitation and emission spectra of all the historical samples for lac dye (Lac) and brazilwood (BW) based paints

in the recipes that ask for a mixture of lac and brazilwood, in the proportion used to reproduce the Jean le Begue 309 recipe, Fig. 6.

The presence of a brazilwood lake pigment is not visible in the infrared spectrum, although the high gypsum concentration is indicative of brazilwood recipes [24]. The use of this mixture of lac dye and brazilwood is an interesting discovery concerning the production and experimentation in the Alcobaça scriptorium. It provides a link from an earlier period in which lac dye paints were used to produce dark reds, pinks and carmine hues to its substitution by brazilwood lake pigments as observed in the winter Breviary ALC 54 dated from the fourteenth century [46]. A missing link, but which does not explain why a glossy lac dye paint, was substituted by a matte colour: the same hues are obtained with the two chromophores, but not the same brightness. This can open new perspectives for the accurate dating of medieval manuscripts.

The chemometrics approach for lac dye paints

The molecular fluorescence spectra (excitation and emission) were paired side-by-side before analysis. Before alignment, each spectrum was processed using the normalization by unit area. Paired pre-processed spectra encompassing all samples for analysis were mean-centered and subjected to PCA. PCA results show that the first three components encompassed over 95% of the total original data variance, thus a sound basis for the variability observed in the spectra. These three components were stored and used for further analysis with the HCA method (Ward's algorithm and Euclidean distance), see Additional file 1: Figure S2. The results of the cluster analysis using both the excitation and emission spectra are plotted in Fig. 7. This figure offers a general view of the separation of the groups for lac dye (Lac) and brazilwood (BW). For a close-up of the groups of each colorant please see Figs. 8 and 11. Except for the SC1 set, the grouping followed closely our proposal based on the discussion of the fluorescence emission and excitation spectra. Although most of the folia (and colours) are within the expected groups, the clustering method was unable to differentiate group Lac 2 and Lac 3, since they share common spectral features already discussed, and hence are represented together in Fig. 7.

Lac 1 group is represented at the extremity of the cluster, being considered the most different among the groups, which is in accordance with our previous analysis, Table 1. Lac 4 group is the nearest to the brazilwood cluster, which agrees with the hypothesis of a possible mixture of the two chromophores. Because of the use of both excitation and emission spectra, the obtained dendrogram correctly positions this group within the lac dye cluster. Such a result would not have been possible using only the excitation spectra, due to similarity with brazilwood signals. Moreover, groups Lac 2 and Lac 3, although being in a separate cluster, are close to group Lac 4, while SC1 is a separate cluster close to groups Lac 1 and Lac 2 & 3; SC1 is named Lac 5 group in Fig. 7. This may be due to the excitation spectra of folios 2v, 37, and 77 resembling group Lac 2, with an emission signal like what was found for group Lac 1, as discussed previously.

Some misplacements can be observed in Fig. 8 when compared with our previous grouping based on molecular fluorescence spectra, Additional file 1: Tables S1–S5. In group Lac 1, the following spectra are misplaced: SC 21, *f.* 2v, and ALC 421, *f.* 193. The first might be due to a higher intensity of the band at ca. 483 nm, when compared to the rest of group Lac 3, where the sample supposedly belong. The placement of ALC 421 in group Lac 1 instead of group Lac 3, may be explained by poor resolved spectral features, with a higher predominance of the band at 475 nm.

In group SC1, named group Lac 5 in Fig. 7, the following spectra are misplaced: Lv 50, *f.* 64v; Lv 15, *f.* 26; Lv 13, *f.* 21; Lv 12, *f.* 94; and ALC 412, *ff.* 10v and 12. Both the folios misplaced of Lv 15, Lv 50, Lv 12, and ALC 412 show a shoulder ca 560 nm in the emission spectra, similar to what was found in SC1, which caused the incorrect positioning within the cluster. This was expected as this is the manuscript displaying the more complex and diverse features.

In groups Lac 2 & 3, the only misplaced paint is that of folio 2v of SC 1. It is placed on the cluster Lac 2, indicating some similarity between excitation spectra.

Finally, in group Lac 4 the misplaced spectra are: ALC 427, *f.* 115v, and Lv 15, *f.* 50. The predominance of the band at ca. 550 nm, with a well-resolved emission spectra with bands at 586 and 614 nm have placed them next to folio 3, lac of ALC 347.

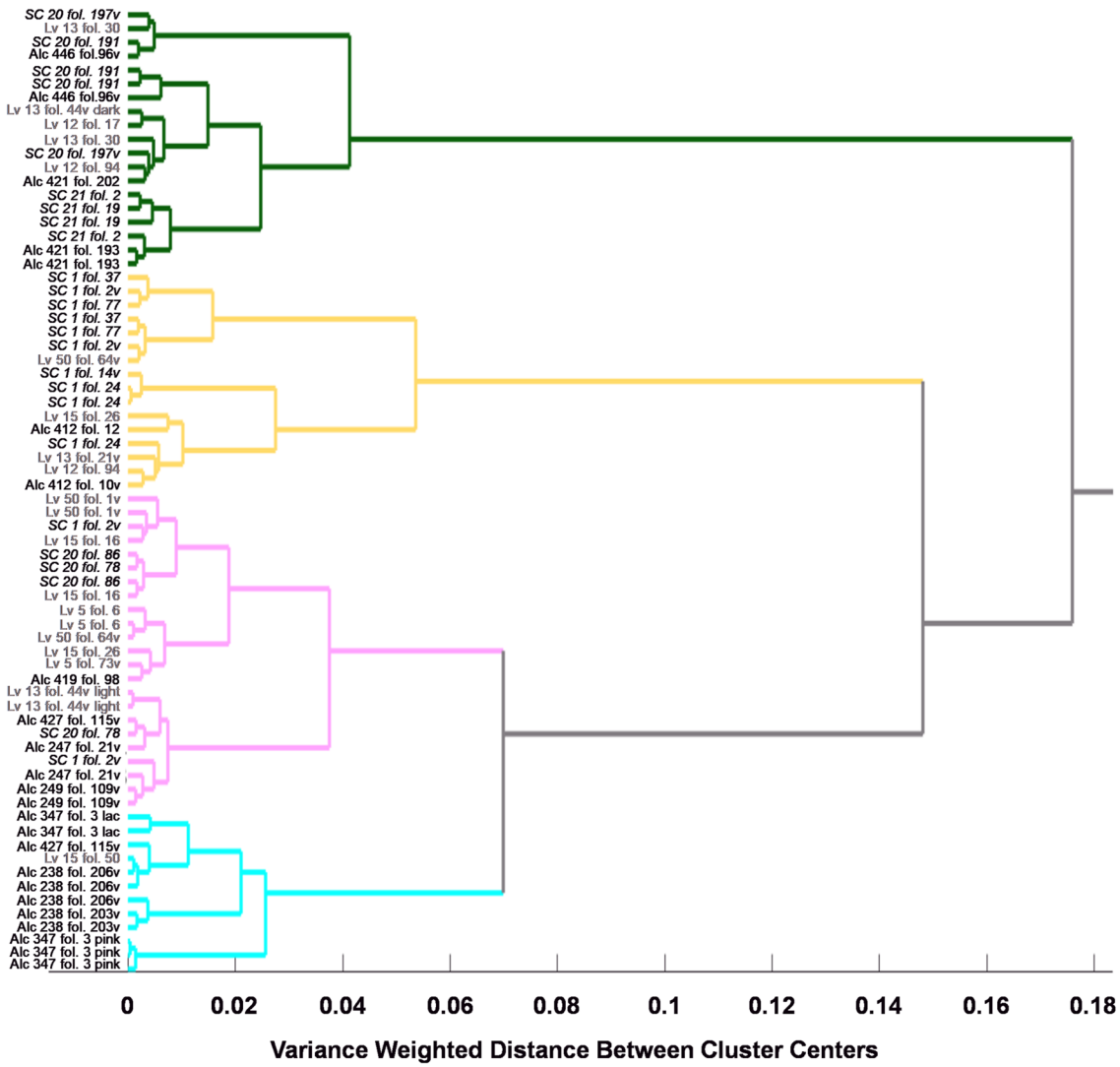

Fig. 8 Close-up for the lac dye cluster of the dendrogram generated by HCA applied to excitation and emission spectra of all the historical samples of lac dye-based paints from all three Portuguese Monasteries: Alcobaça (*black*), Lorvão (*grey*), and Santa Cruz (*italic*): Lac 1 (*green*), Lac 2 & 3 (*pink*), Lac 4 (*light blue*) and Lac 5 (*yellow*)

Brazilwood in medieval manuscripts

The differences observed in the molecular fluorescence spectra of brazilwood medieval paints are much smaller than those found for lac dye paints, so compared to them, brazilwood colours can be considered a single group, Fig. 9. Likewise, for the four recipes of brazilwood in the *O Livro de como se fazem as cores*, Fig. 10. However, based on our previous experience applying chemometrics to historical reconstructions [19], we think that it is possible to extract more information from the brazilwood cluster. Besides the position and intensity of the maxima in a spectrum, band broadening and other less visible features will be processed in the chemometrics analysis. In fact, a spectrum of molecular fluorescence is very rich in information, and with the support of chemometrics, the way is open for an in-depth analysis of the complexity of medieval painting.

Contrarily to what was observed for lac dye paints, for brazilwood pigment lakes, it is the spectral distribution of the emission spectra that most differentiates them. It is important to note that the differences are in the range of a few nanometres, being the intensities observed very similar and in the range to what observed for lac dye paints, Table 2. Based on the emission and excitation

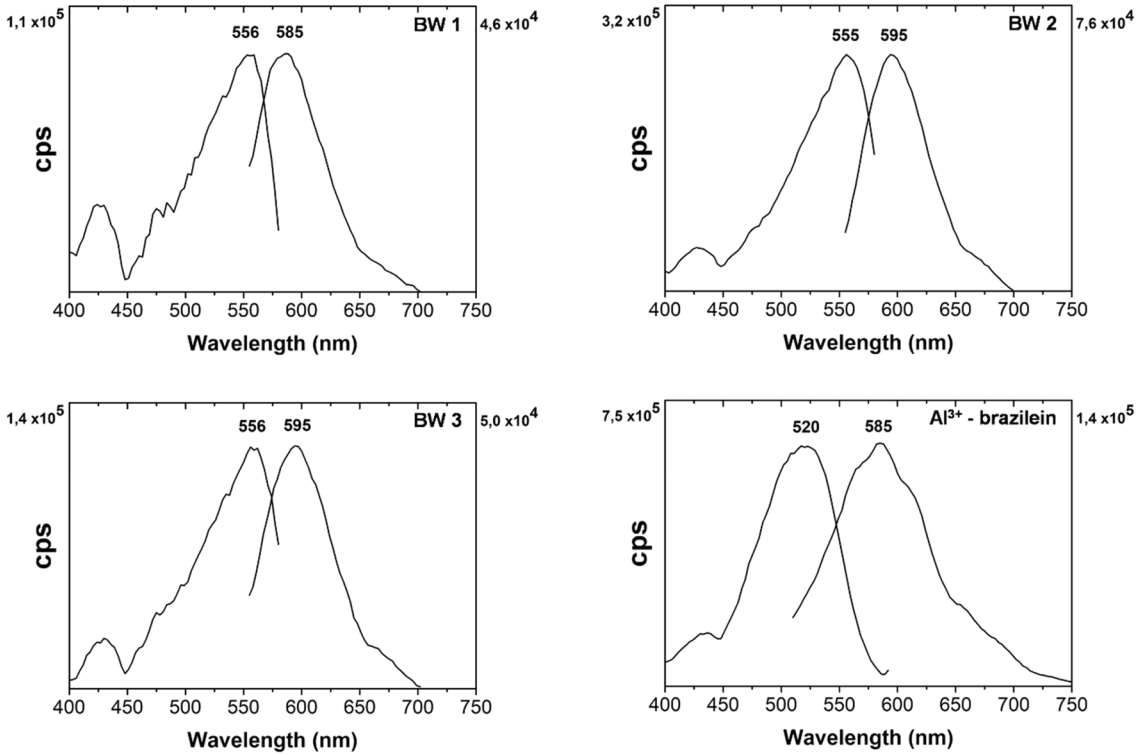

Fig. 9 Brazilwood paints, excitation and emission spectra representative for BW 1 group, Ajuda Songbook *f.* 59); BW 2 group, IL 42 *f.* 113; BW 3 group, ms 24 *f.* 60; and brazilein 5×10^{-5} M in MeOH:H_2O (70:30, v/v) with Al^{3+} (× 1000) at pH 3.2, applied on filter paper

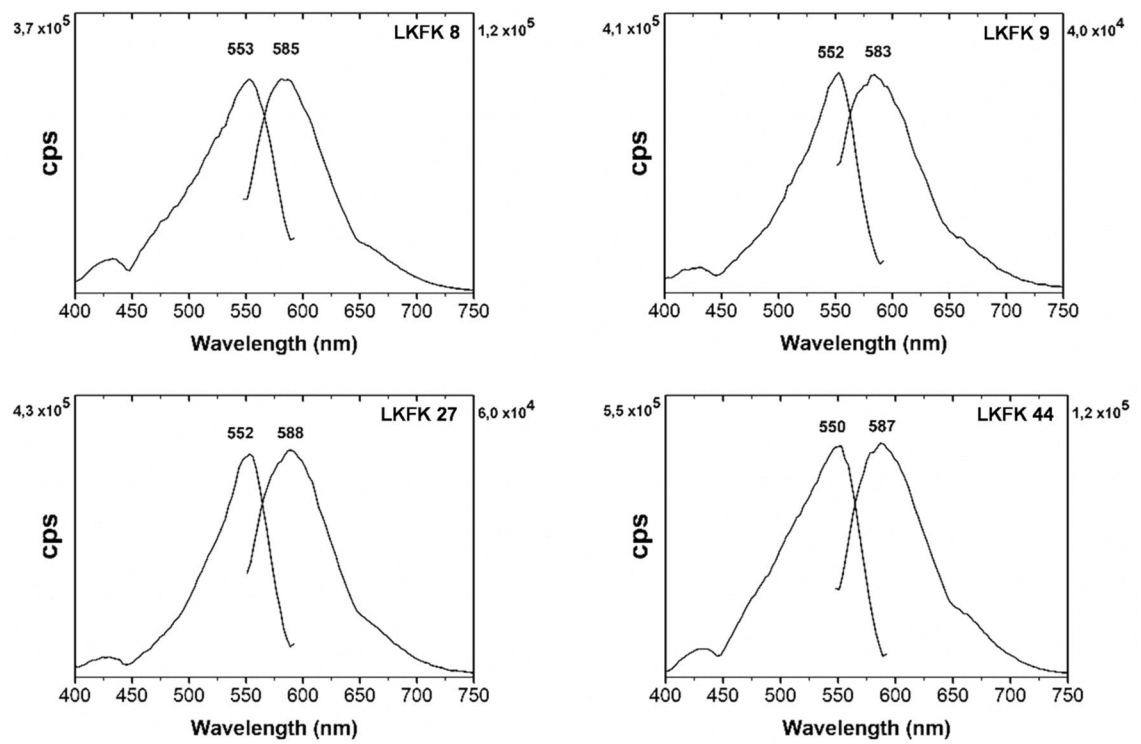

Fig. 10 Excitation and emission spectra of brazilwood lake pigment reconstructions from the *Livro de como se fazem as cores*: recipe 8, 9, 27, and 44

Table 2 A total of 80 emission and 80 excitation spectra on 18 brazilwood paints were collected from 8 illuminated manuscripts produced during the 13th—15th c. Based on their spectral features these spectra were assembled in 3 groups

	Group BW 1	Group BW 2	Group BW 3
Excitation maxima	*552–561 nm*	*555–562 nm*	*553–564 nm*
Intensity			
Max	2.2×10^5	3.2×10^5	1.7×10^5
Min	9.1×10^4	1.0×10^5	9.9×10^4
Emission maxima	*582–586 nm*	*594–602 nm*	*590–600 nm*
Intensity			
Max	9.5×10^4	8.6×10^4	6.2×10^4
Min	3.5×10^4	3.0×10^4	2.4×10^4
Infrared	$CaCO_3$ Lead White	$CaCO_3$ $CaSO_4 \cdot 2H_2O$	$CaCO_3$ » $CaSO_4 \cdot 2H_2O$
Reconstruction	LKFK 8		LKFK 9

The values for the emission and excitation maxima are in italics

spectral distribution, we propose three groups that will be next discussed.

Analysis of the emission and excitation spectra of brazilwood paints

In group ***BW 1***[9] are included all the spectra collected from Ajuda Songbook, which are characterized by excitation maxima within 552–556 nm and emission maxima at ca. 585 nm, Fig. 9, Table 2 and Additional file 1: Table S9. These spectral features compare well with those obtained from the four recipes described in the *O Livro de como se fazem as cores,* in particular with recipe 8, as well as the Al^{3+}–brazilein reference sample, Figs. 9 and 10 and Additional file 1: Table S13.

The spectra frombrazilwood paints applied in the Books of hours of French or Flemish production may be assembled in two groups. In group ***BW 2***,[10] the excitation maxima are shifted to slightly higher wavelengths, 555–562 nm, and the band is not so broad when compared with group BW 1, Additional file 1: Table S10. Emission maxima are also shifted to higher wavelengths, being found in the interval 594–602 nm. The excitation spectra that represent group ***BW 3***[11] are found between 553 and 560 nm, falling in the interval of group BW 2, being differentiated by their emission maxima that is shifted to slightly lower wavelengths, 589–600 nm, Additional file 1: Table S11. Usually, also a lower S/N was obtained for BW 3 signals. As already pointed out, the differences between these two groups are smaller than those found for lac dye. However, they can reveal unique information that can be linked to a different original recipe or the aging of the paint. We will further discuss this issue when analysing the infrared data.

Finally, the paints from the winter breviary (ALC 54, *f.* 92) may be compared to the French book of hours ms 22, *f.* 76v that is included in group BW 3, Additional file 1: Tables S11 and S12.

Correlating the groups of brazilwood paints based on molecular fluorescence spectra with the paint formulation given by infrared spectroscopy

For medieval brazilwood paints, it is possible to correlate the differences observed in the molecular fluorescence spectra with the paint formulation. We will start by analysing the pigment reconstructions to support our discussion of the medieval paints. For both, reconstructions and original paints, infrared analysis showed extenders and binders and, in certain cases, the pigments' aluminate substrate, Table 2 and Additional file 1: Table S13. The infrared spectrum of recipe 8 is mainly characterized by the presence of gypsum, while both recipes 9 and 27 present a mixture of gypsum and calcium carbonate. The infrared spectrum of recipe 44 however, is characteristic of the pigments' substrate, showing the aluminate compound [24], Additional file 1: Table S13. All these data are relevant as we show that added extenders can change the colour of the paint [24]. Pigments may appear redder or more violet, darker, or lighter depending on the complexing metal ion, Al^{3+}, Ca^{2+}, or Pb^{2+}, with the latter two displaying a bluer shade.

Group BW 1 is characterized by the presence of both calcium carbonate and lead white. This is similar to what was found in recipe 8 of the *Livro de como se fazem as cores*, where the extraction is done in the presence of alum and white lead. Calcium carbonate may be found in the pigment's substrate if the filtration is done over a chalk bow [24]. However, as indicated in the recipe, a gypsum bowl can also be used, in which case, no calcium carbonate would be present, Additional file 1: Table S13. This is the only group to which lead white is added and, considering that Pb ions can quench the fluorescence emission [18], this would explain the low signal-to-noise ratio found in fluorimetric data.

[9] BW 1 group – Ajuda Songbook, *ff.*4, 17, 21 and 59; IL 15, *f.* 60.

[10] BW 2 group –IL 15, *f.* 15; IL 19, *f.* 91, IL 21, *f.* 88, IL 42, *ff.* 9, 85 and 113.

[11] BW 3 group – ms 22, *f.* 76v; ms 24, *f.* 60; IL 15, *f.* 66; IL 19, *f.* 21; IL 42, *ff.* 23 and 133.

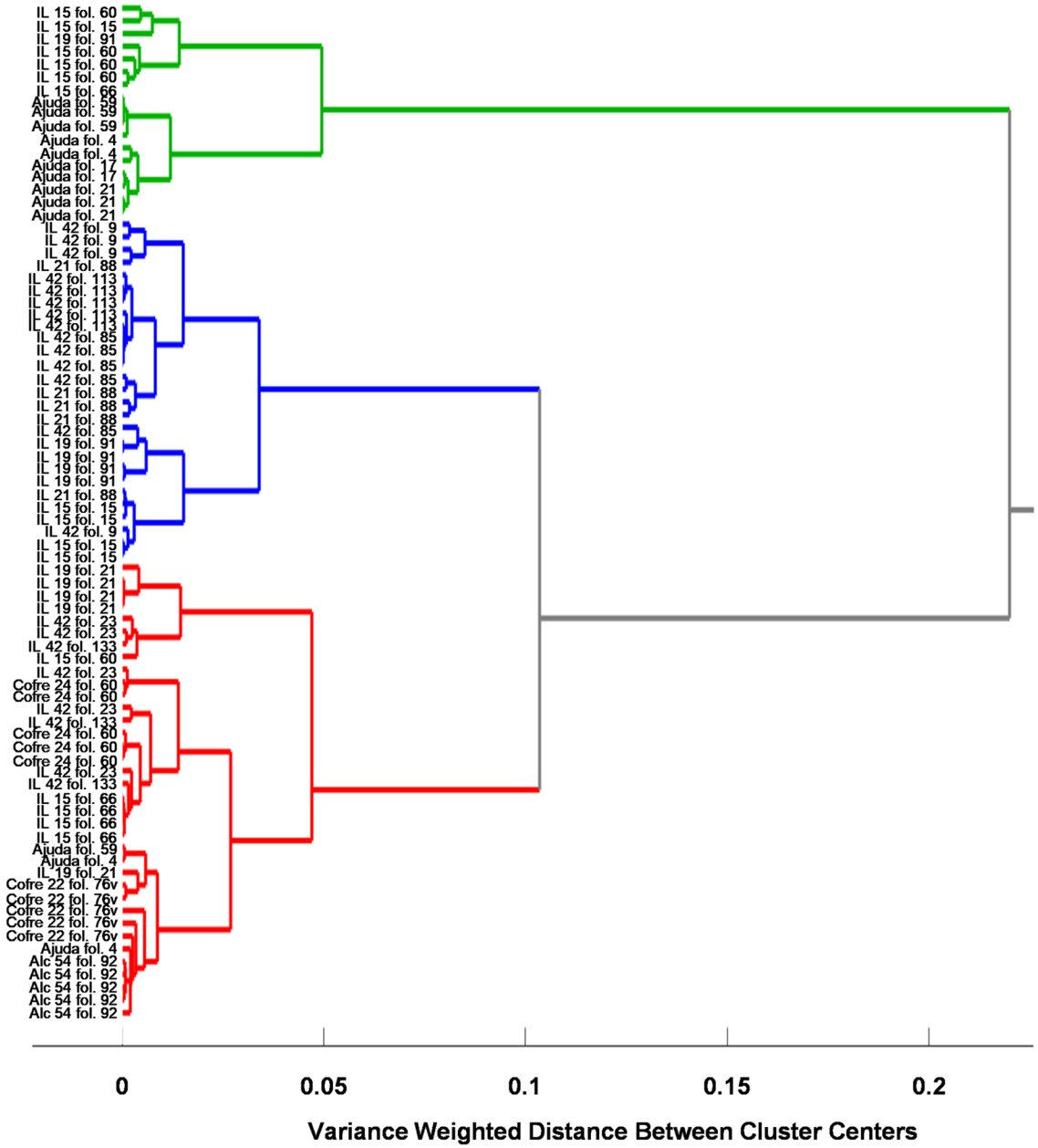

Fig. 11 Close-up for the brazilwood cluster of the dendrogram generated by HCA applied to excitation and emission spectra of all the historical samples of brazilwood based paints: group BW1 (*green*), BW 2 (*blue*) and BW 3 (*red*)

Groups BW 2 and ***BW 3*** are characterized by the presence of calcium carbonate and gypsum. The main difference is the higher amount of gypsum found in the latter, which may be responsible for the lower intensities observed with our acquisition set-up (less light will be absorbed by the chromophore, which will lead to a lower fluorescence emission), Fig. 9. The infrared spectra of group BW 3 are comparable with recipe 9 of the *Livro de como se fazem as cores*, in which is noticeable the presence of both gypsum and calcium carbonate.

Again, the winter Breviary, ALC 54, does not fit in any of the proposed groups. The infrared spectrum does not show the presence of lead white, calcium carbonate, or gypsum.

The chemometrics approach for brazilwood paints

The results of the hierarchical cluster analysis, using both the excitation and the emission spectra, are plotted in Fig. 11. The grouping followed closely our proposal based on the discussion for the fluorescence emission and excitation spectra, combined with infrared spectroscopy. The presence of specific extenders, proved by infrared spectra, influences the molecular fluorescence spectra and is detected by the statistical approach applied, Table 2. The exception is the paint in IL 19 *f.* 21; due to the presence of lead white it could be included in BW1, however, its emission maximum is shifted towards longer wavelengths, closer to BW2 & BW3.

ALC 54 was placed within the BW 3 cluster, essentially due to the emission spectrum shifted to longer wavelengths. However, ALC 54 presents a lower signal-to-noise ratio when compared with the other samples in group BW 2. Nevertheless, the clustering method identifies some differences, placing it on the limit of the dendrogram. Another challenging case is the book of hours IL 15, the only one of Flemish production. The three folios analysed are spread through all three groups. This can indicate that three different illuminators were at work, an aspect that we would like to explore in the future.

Conclusions

Molecular fluorescence data embodies the rich complexity that characterizes paints prepared with organic chromophores. Based on our knowledge of the reconstruction of medieval paints, we anticipated that lac dye formulations would be more complex than brazilwood lake pigments. On the one hand, from the source material several chromophores are extracted, although we have always found laccaic acid A as a main compound in our paints, Fig. 1 [32]. On the other, in lac dye paints we find variable amounts of shellac resin, a complex material that also emits in the visible, partially overlapping the molecular fluorescence spectra of laccaic acid A, the main chromophore. Possibly, due to this greater complexity, it has not yet been possible to fully correlate the influence of fillers and other additives, in the formulation of the paint, with its spectral signature.

The complete evaluation of the paints' composition, in particular, the data obtained in the semi-quantitative analyses based on the infrared spectra, allowed an in-depth discussion of the molecular fluorescence spectra. In general, the grouping based on the molecular fluorescence signature of the chromophores was correctly obtained from the chemometric methods, with few misplaced samples, Fig. 7. These deviations are explained by a low signal-to-noise ratio or because the sample was different from all the others.

Notably, in the spectral signature of brazilwood paints, it was possible to probe the presence of fillers and to differentiate between paints' formulation. Future work will systematically study these effects to provide a general rationale. It is important to note that molecular fluorescence pinpointed, for the first time, a colour in which both lac and brazilwood chromophores are present (Lac 4 group), in manuscripts from the Alcobaça scriptorium (*De avibus* and *Sermones de verbis Domini*, ALC 238 and 347, respectively). Being this one of the "different paints". The presence of the two chromophores could not be detected either in the infrared or Raman spectra. Remarkably, the use of these two chromophores to produce a medieval paint had been described in the text by Jean Le Begue and, indeed, these paints compare very well with Le Begue's recipe 309. This allows us to hypothesize that, with further developments, molecular fluorescence could be used as a tool to provide geographic information on the place of the manufacture of an illuminated manuscript as well as on its dating.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40494-021-00490-8.

Additional file 1: Figure S1. Typologies of the manuscripts found in Portuguese collections. **Table S1–S5.** Microspectrofluorimetry and infrared data of samples from lac dye in medieval manuscript illuminations (12th–13th c.). **Table S6.** Microspectrofluorimetry and infrared data of lac dye reconstructions. **Table S7.** Infrared spectra. **Table S8.** Percentage of calcium carbonate. **Table S9–S12.** Microspectrofluorimetry and infrared data of samples from brazilwood in medieval manuscript illuminations (end of 13th–15th c.). **Table S13.** Microspectrofluorimetry and infrared data of brazilwood reconstructions. **Figure S2.** Scatter plot representing the first three scores from the PCA model used to produce the dendrogram for lac dye (Lac) and brazilwood (BW) based paints. Link to video in: https://www.dropbox.com/s/4vfyc63cjsvn7j1/Heritage%20Sci%20Nabais%20P_%20Scatter%20plot.mp4?dl=0.

Abbreviations

SC: Santa Cruz Monastery/Mosteiro de Santa Cruz; Lv: Lorvão Monastery/Mosteiro do Lorvão; ALC: Alcobaça Monastery/Mosteiro de Alcobaça; SERS: Surface-enhanced Raman spectroscopy.

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Authors' contributions

PN contributed with the acquisition of emission and excitation spectra; conception of the models and treatments applied to the spectral data, and with the writing and revision of the version to be published. MJM contributed with the conception and design of the research work; acquisition, analysis and interpretation of data; writing and revision of the version to be published. JAL contributed with the conception of the data treatment and calculations, as

well as the revision of the version to be published. MV contributed with the acquisition and spectral interpretation of infrared spectra of brazilwood reconstructions and the revision of the version to be published. RC contributed with the reconstructions of lac dye and as well as with the acquisition of the data related to lac dye medieval paints. AR contributed with the conception of the work, data interpretation and revision of the version to be published. All authors read and approved the final manuscript.

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Availability of data and materials
Most of the data on which the conclusions of the manuscript rely is published in this paper, and the full data is available for consultation on request.

Competing interests
The authors declare that they have no competing interests.

Author details
[1] LAQV-REQUIMTE and DCR, Faculty of Sciences and Technology, NOVA University of Lisbon, 2829-516 Caparica, Portugal. [2] Faculty of Pharmacy, iMed. ULisboa-Research Institute for Medicines, University of Lisbon, Av. Prof. Gama Pinto, 1649-003 Lisbon, Portugal. [3] SMAArt Centre and Department of Chemistry Biology and Biotechnology, University of Perugia, via Elce di Sotto 8, 06123 Perugia, Italy.

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