

An Indian-Icelandic connection revealed by UHPLC-MS/MS

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Abstract

A precious dye produced in the Indian subcontinent, lac dye, extracted from insects of the *Kerria lacca* species, has been identified in the miniatures of a 14th -century Icelandic manuscript, AM 227 fol., kept at the Árni Magnússon Institute for Icelandic Studies in Reykjavik, Iceland. The precise identification of the dye, firstly hypothesised by means of Fiber Optic Reflectance Spectrophotometry (FORS) analysis on the pink and purple areas of the manuscript, was obtained after the collection of a micro sample of paint (weighing ca. 0.2 µg), an instance rarely allowed on manuscripts. On such a sample it was possible to carry out a thorough characterisation by means of Ultra High Performance Liquid Chromatography - tandem Mass Spectrometry (UHPLC-MS/MS) working in selected reaction monitoring (SRM) mode. The method relied on the extra sensitive identification of laccaic acids A, B and C which are known to be specific markers of the dye derived from *Kerria lacca*. Despite the extremely low amount of sample available, this method allowed to selectively identify lac dye and to open new perspectives in the study of dyes in illuminated manuscripts. Moreover, it revealed unexpected hypotheses regarding the trade network between Iceland and the rest of Europe (and through it of the world), attesting that Icelandic artists had access to such exotic colourants in a way similar to the artists of other countries whose commercial relationships with Asia were more consolidated.

1 Introduction

The study of colourants in painted artworks can reveal important details for other aspects of the artworks themselves, such as the origin of the raw materials used and the networks through which artists or workshops acquired them. In addition, the quality of the colourants used influences the assessment of the artwork and its commission. For example, the presence - or absence - of ultramarine blue in a painting always reveals the social status of the commissioner, the pigment being among the most expensive on the painters' materials market.

Of particular interest is the identification of colourants produced in a geographic area far from the area where the artwork itself was made. This is typically true of colourants originating in Asia and found in painted artworks created in Europe, such as gamboge [1], dragon's blood [2] and brazilwood [3], used by European artists since the Middle Ages.

A remarkable case is the dye known as lac dye or Indian lac. This dye is obtained from the exoskeleton of insects of the *Kerria lacca* species, a member of the superfamily Coccoidea, also known as scale insects. The species is native to the Indian subcontinent, but the dye extracted from it has been used in the Mediterranean basin at least since the 1st millennium BC [4], attesting to commercial relationships between Europe and the Indian subcontinent since long times ago. The dye could have travelled, together with many other goods, along the Silk Road to reach Europe. Considering the influence of the Republic of Venice, the European trade of painters' materials was most probably controlled by Venetian merchants. The high number of identification of lac dye in painted artworks produced in Europe attests

once more to the extensive trade network between Europe and Far Eastern Asia. This topic has been recently reviewed by Crippa et al. [5]

However, in the identification reported by the scientific literature the cases cited are mostly limited to continental Europe and Great Britain. The use of lac dye in Scandinavia is attested in very few cases in panel and church paintings on wood produced in Norway, dated to the 13th century [6, 7]. Proceeding further north and west towards the Atlantic Ocean, that is towards Iceland, no identification is reported of either lac dye or other insect-derived dyes in any kind of artworks.

In addition, it must be considered that, as far as illuminated manuscripts are concerned, very rarely it is possible to take samples that allow for the thorough characterisation provided by liquid chromatography coupled with mass spectrometry, which can reach the level of the animal or vegetal source of provenance of the dyes. Therefore, part of the lack of knowledge on specific colourants such as the insect dyes could be due to the lack of investigation carried out with powerful enough techniques, usually involving micro-invasive methods.

In this study, we report on the identification of lac dye in a 14th century Icelandic manuscript, AM 227 fol., which is kept at the Árni Magnússon Institute for Icelandic Studies in Reykjavik, Iceland. The manuscript contains partial translations of the Old Testament into the Old Norse language and is one of the finest manuscripts preserved from medieval Iceland. It is a large-format book (ca. 355 x 275 mm) of over 120 high-quality parchment leaves, skilfully decorated in a range of colours. Two anonymous scribes wrote the manuscript in the middle of the 14th century, while three artists contributed to the book painting [8, 9]. Scholars believe AM 227 fol. to originate from the North of Iceland, likely the Benedictine monastery at Þingeyrar [10] which was a prominent religious and cultural centre at the time.

A pink-purple dye was used extensively in the manuscript's elaborate major initials depicting biblical scenes, extending far into the margins. The diagnostic study focused mainly on these areas, considering that such hues are unusual in manuscripts produced in the Scandinavian countries. The identification of an insect dye, firstly suggested by Fiber Optic Reflectance Spectrophotometry (FORS) analysis on the pink and purple areas of the manuscript, was obtained after the collection of a micro sample of paint. On such a sample it was possible to carry out a thorough characterisation by means of Ultra High Performance Liquid Chromatography - tandem Mass Spectrometry (UHPLC-MS/MS), that eventually allowed the precise identification of the dye derived from *Kerria lacca*.

2 Materials and Methods

2.1 Reagents

Samples of *Kermes vermilio* and *Dactylopius coccus* were purchased from Kremer Pigmente (Aichstetten, Germany). Pure lac dye, extracted and purified from *Kerria lacca*, was kindly provided by Cheryl Porter. For the extraction of the dyes, ultrapurewater (UHPLC-MS grade) purchased from Carlo Erba (Milan, Italy) was used.

For the preparation of the mobile phase, acetonitrile (UHPLC-MS grade), methanol (UHPLC-MS grade) and formic acid and methanol were purchased from Carlo Erba (Milan, Italy).

2.2 Sample preparation

The withdrawal of the micro sample from AM 227 fol. was carried out in Reykjavik at the Árna Magnússon Institute for Icelandic Studies, where the manuscript is kept, inside the conservation laboratory. When seeking locations from which to remove a micro sample, paint applications that under magnification appeared uniform in colour and were slightly marred and not obviously treated, were chosen as sampling points. These criteria were used to avoid collecting a sample that contained additional colourants or added materials while limiting the sampling to an area that had been previously damaged. It was chosen to collect the micro sample from the dark pink line at f. 23v (Fig. 1).

To collect the sample, first a fine white tipped brush was lightly tapped over the sampling point to gather any loose particles and to find a possible weak point in the paint layer from which to take the sample. Then a modified beading needle was used to remove the micro sample by minutely tapping the needle's point into the paint layer and using it to lift particles from the parchment leaf. Once a micro sample was collected, it was immediately placed onto a small piece of blotter, which was put into a custom paper envelope. The collection process was carried out under magnification and in a stable environment.

The opening of the paper envelope was carried out under magnification inside the chemistry laboratory in Italy. The sample resulted to be composed of two main fragments of a paint layer (Fig. 2); no residues of parchment were present. According to the graduated scale (below in the figure), the diameter of the fragments can be estimated as 0.06 mm (left in the figure) and 0.09 mm (right in the figure).

Due to the risk of losing the micro sample, it was not possible to weigh it; however, an estimate can be done based on the size of the fragments and the density of the paint layer. There are no specific studies dealing with the binding media used in Icelandic miniature painting; however, one can guess that egg white was the most probable candidate, with fish glue and gum Arabic being other possibilities. Considering that liquid egg white has a density close to that of water (approximately 1.03–1.05 g/cm³) and that the paint contained an organic lake, which keeps the overall density low, but also lead white (see later) which increases it, an estimated value of 2.0 g/cm³ could be used for the density. Approximating the two fragments to small cylindrical platelets with a height of 0.01 mm, an estimated total weight of 0.18 µg would result.

The two fragments were carefully collected from the paper envelope and transferred into a LLG-Labware (Meckenheim, Germany) short thread vial ND9 model, capacity 1.5 mL, dimensions 11.6 x 32 mm, equipped with a 0.25 mL conical insert.

2.3 Fibre Optic Reflectance Spectrophotometry (FORS)

FORS analysis was performed with an Avantes (Apeldoorn, The Netherlands) AvaSpec-ULS2048XL-USB2 model spectrophotometer and an AvaLight-HAL-S-IND tungsten halogen light source; detector and light

source were connected with fibre optic cables to an FCR-7UV200-2-1,5x100 probe. In this configuration, both the incident and detecting angles were 45° from the surface normal, in order not to include specular reflectance. The spectral range of the detector was 200–1160 nm; the overall operational range of the device (combination of lamp + detector) was 375–1100 nm. Depending on the features of the monochromator (slit width 50 µm, grating of UA type with 300 lines/mm) and of the detector (2048 pixels), the best spectra resolution was 2.4 nm calculated as FWHM. Diffuse reflectance spectra of the samples were referenced against the WS-2 reference tile provided by Avantes and guaranteed to be reflective at 98% or more in the spectral range investigated. The investigated area on the sample had a 1 mm diameter. In all measurements the distance between probe and sample was kept constant to 0 mm. The instrumental parameters were as follows: 10 ms integration time, 100 scans for a total acquisition time of 1.0 s for each spectrum. The whole system was managed by means of AvaSoft v. 8 dedicated software, running under Windows 11™.

2.4 X-ray Fluorescence Spectrometry (XRF)

XRF measurements were performed with an EDXRF Thermo (Waltham, MA, USA) NITON spectrometer XL3T-900 GOLDD model, equipped with an Ag tube (max. 50 kV, 100 µA, 2 W), a large area Silicon Drift Detector (SDD), with an energy resolution of about 136 eV at 5.9 keV. The analysed spot had an average diameter of 3 mm and was focused by a CCD camera with a working distance of 2 mm. The total time of analysis was 120 s. The instrument was held in position with a moving stage allowing millimetric shifts in order to reach the desired probe-to-sample distance; the stage was attached to a tripod. The obtained spectra were processed with the commercial software BAxil (Brightspec NV/SA, Belgium) derived from the academic software QXAS from IAEA.

2.5 Optical Microscopy

A USB Dino-Lite (New Taipei City, Taiwan) AM413TL-FVW model microscope was used to acquire digital images in the 20x-90x magnification ratio range. The instrument is equipped with 375 nm and visible LED lights and a digital camera with 1.3 Megapixel resolution.

2.6 Chromatographic and mass spectrometric conditions

The UHPLC analyses were carried out by Nexera 40 (Shimadzu, Kyoto, Japan) system equipped by a DGU-405 Degasser, two LC-40D X3 Pumps, a SIL-40C X3 Autosampler, a CTO-40C column compartment and a SCL-40 controller. The system was coupled with the Qtrap 6500 + mass spectrometer (Sciex, Framingham, USA) through an electrospray ionization (ESI) source. Instrument control and data acquisition were performed by Analyst 1.7.3 software (Sciex, Framingham, USA), whereas data reprocessing and quantification were carried out by Sciex OS 3.3.1 (Sciex, Framingham, USA). The stationary phase used was an Acquity UPLC HSS T3 (2.1 x 100 mm, 1.8 mm) column (Waters, Milan, Italy). The mobile phase was a mixture of ultrapure water with the addition of 1% of formic acid (A) and 90/10 (v/v) acetonitrile/methanol with the addition of 0.1% of formic acid (B), eluting at flow rate 0.500 mL min⁻¹. The final gradient conditions of UHPLC-MS/MS were the following: 0.0-0.5 min 20% B, 0.5–1.9

min 50% B, 1.9–2.8 min 50% B, 2.8-4.0 min 99% B, 4.0-4.5 min 99% B, 4.6 min 20% until to 8.0 min. The injection volume was 10.0 µL and the oven temperature was set at 40°C.

The ESI source worked in positive ion mode. The instrumental parameters were set as follows: curtain gas (N2) at 40 psig, nebuliser gas GS1 (N2) and GS2 (N2) at 70 and 75 psig, respectively, desolvation temperature (TEM) at 500°C, collision activated dissociation gas (CAD) at 6 units of the arbitrary scale of the instrument and ion spray voltage (IS) at + 5000 V. The mass spectrometer was used in scheduled multiple reaction monitoring (sMRM) considering the transitions of each species at a prefixed retention time (Table 1). Unit mass resolution was established and maintained in each mass-resolving quadrupole by keeping a full width at half maximum (FWHM) of about 0.7 u.

Table 1
Scheduled Selected Reaction Monitoring (sSRM) positive ion transitions (Q1 and Q3 masses), time (min), and mass spectrometry parameters: DP (Declustering Potential), EP (Entrance Potential), CE (Collision Energy), CXP (Collision cell eXit Potential). Quantifier and qualifier transition are reported for each analyte.

Analyte	Q1	Q3 quant / Q3 qual	time (min)	DP (V)	EP (V)	CE (V)	CXP (V)
Carminic acid	493.3	355.2 / 373.2	1.1	28	5.5	39.9 / 20.6	3.5 / 3.3
Kermesic acid	331.2	257.1 / 229.1	2.9	34	2.7	37.2 / 47.9	3.2 / 3.1
Laccaic Acid A	538.2	443.1 / 461.2	1.9	43	4.5	49.9 / 32.3	3.4 / 3.5
Laccaic Acid B	497.2	479.2 / 461.1	1.9	42	4.0	24.0 / 29.9	3.4 / 3.8
Laccaic Acid C	540.0	476.1 / 458.1	0.7	50	4.0	30.3 / 43.4	3.4 / 3.4

INSERT HERE Table 1

2.7 Preparation of standard and sample solutions

Carminic and kermesic acids were extracted from *Kermes vermilio* and *Dactylopius coccus* by using 100 mg and 10 mg of dried insects, respectively. The difference is due to the content of anthraquinone acids which is at least 10 times higher in *Dactylopius coccus* than in *Kermes vermilio* [11]. They were weighed, ground in a mortar, and placed in a 50/50 (v/v) water/methanol solution, then sonicated in boiling water for 30 min and subsequently vortexed and filtered on PTFE filters (0.20 µm, VWR, Radnor, USA). The final concentration of kermesic acid and carminic acid can be estimated in 17 and 13 mg/L, respectively, considering a yield of 1% and 10% of dye extracted from the insects. A solution of laccaic acids A + B+C was prepared by dissolving the pure dye, obtained after extraction and purification of dried *Kerria lacca* insects, in a 50/50 (v/v) water/methanol solution and sonicated for 30 min to obtain a final concentration of 20 mg/L expressed as the total of A + B+C acids.

A mixture of the three solutions was prepared at 1 mg/L and kept at -20 °C in an amber glass vial until its use.

For the extraction of the dye from the sample taken from ms. AM 227 fol., we adapted a method developed by Zhang and Laursen [12] modified to make it more compatible with the mobile phase. 100 μ L of a mixture of water/ACN/MeOH/formic acid 79/18/2/1 were introduced into the insert in the ND9 vial, which was vortexed for 2 min and incubated at 40°C for 40 min. It was subsequently vortexed for 1 min and subjected to UHPLC-MS/MS analysis.

3 Results

The diagnostic study in the manuscript AM 227 fol. began with non-invasive measurements carried out with FORS and XRF analyses. The study aimed at characterising the palette used to decorate this remarkable example of Icelandic medieval miniature painting art.

3.1 Non-invasive measurements

The identification of an insect dye on the pink and purple areas of ms. AM 227 fol. was previously obtained by means of FORS analysis, which yielded spectra typical of anthraquinone dyes of animal origin according to the characteristic spectral features [13], with two absorption bands at 530 and 565 nm accompanied by inflection points at 500, 540 and 595 nm. An example of a FORS spectrum from a pink miniature of ms. AM 227 fol. is shown in Fig. 3.

The position of the two absorption bands was unusual for an insect dye, being the bands more commonly located at 520–528 and 550–560 nm respectively, according to the literature [13, 14]. The insect dye could be one among five possibilities: lac dye from *Kerria lacca*, a species native to the Indian subcontinent; kermes from *Kermes vermilio*, common in nearly all the Mediterranean basin; Armenian cochineal from *Porphyrophora hamelii*, native to the Caucasian area; Polish cochineal from *Porphyrophora polonica*, native to northeastern Europe. For historical reasons, Mexican cochineal from *Dactylopius coccus*, native to South America, could be safely excluded as it was firstly taken to Europe by the Spanish Conquistadores after the conquest of Mexico in 1521, that is two centuries later than the production of ms. AM 227 fol.

The dye was found to be used either pure or in mixture with red lead for darker hues (example in Fig. 3, dashed line). The mixture was suggested by an additional inflection point at 560 nm in the FORS spectrum and confirmed by the presence of lead in XRF analysis. The simple addition of lead white would not have caused the appearance of an additional inflection point in the FORS spectrum.

A 90x image taken with a USB microscope at a pink area (Fig. 4) showed that the paint had a discrete hiding power and suggested that, rather than being a pure dye juice, it could be a lake, that is a dye fixed on an inorganic substrate.

The substrate could be lead white - $\text{PbCO}_3 \cdot \text{Pb}(\text{OH})_2$ - rather than alum - $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ - the classical agent used since the Middle Ages to transform dye juices into lakes according to the ancient technical treatises [15]. After XRF analysis, this was evidenced by the presence of lead in the spectrum (Fig. 5, left

spectrum) and by the fact that the signals of aluminium and potassium were not higher in the paint than in the parchment (Fig. 5, right spectrum). It was not possible to assess the presence of sulphur, due to interference of the M line of lead on the K and K lines of sulphur.

Besides the insect dye, other colourants were identified in ms. AM 227 fol. by means of FORS and XRF analysis, the list of whom is reported in Table 2.

Table 2
List of colourants identified in ms. AM 227 fol.

Colour	Colourants	Identification method
black	tannin-like pigment carbon based pigment	FORS
blue	azurite	FORS, XRF
brown	red ochre	FORS
green	verdigris azurite/orpiment	FORS, XRF
orange	red lead	FORS
pink	insect dye	FORS, UHPLC-MS/MS
purple	insect dye	FORS, UHPLC-MS/MS
red	cinnabar/vermillion red lead red ochre	FORS
violet	insect dye	FORS, UHPLC-MS/MS
white	lead white bone white	XRF
yellow	gold orpiment	XRF

INSERT HERE Table 2

The rich palette, including high priced pigments such as cinnabar/vermillion, orpiment and – in a later addition - gold, which is rare in medieval Icelandic manuscripts, suggests that the manuscript AM 227 fol. was a luxury book. The presence of an insect dye from far away lends further support to that assumption.

3.2 UHPLC-MS/MS analysis

After the non-invasive measurements, it was possible to withdraw a micro sample of paint from a dark pink area of the miniature at f. 23v. The precise characterisation of the insect dye relied on the identification of the anthraquinone acid considered as the chemical marker of the animal species from which the dye is obtained [11]. Laccaic acids A, B and C are the typical markers of the *Kerria lacca* species, and therefore of lac dye. Kermesic acid is the marker of *Kermes vermilio*, from which the kermes dye is obtained. Carminic acid is the common marker of *Porphyrophora hamelii*, *Porphyrophora polonica* and *Dactylopius coccus*, the insect species from which Armenian cochineal, Polish cochineal and Mexican cochineal, respectively, are obtained. Although the identification of carminic acid does not allow the three cochineals to be distinguished (other chemical markers must be sought [16, 17]) and the results of chromatographic analysis can be influenced by the extraction conditions [18], in this study we were interested only in determining whether the dye present in the paints of AM 227 fol. was lac dye. Therefore, since laccaic acids are specific markers of lac dye only [19], the chromatographic conditions were optimised in order to have a good separation between laccaic acids, carminic acid and kermesic acid, without trying to identify further molecules.

Due to the extremely small amount of sample ($\sim 0.2 \mu\text{g}$), it was necessary to use a method with extra sensitivity. Therefore, UHPLC coupled with tandem mass spectrometry was chosen, looking for specific chemical markers: laccaic acids A, B and C, kermesic acid and carminic acid, as explained before. A rapid UHPLC-MS/MS method was developed, allowing the identification of the cited anthraquinone acids in less than 3 min. A typical chromatogram obtained from a mixed standard solution is shown in Fig. 6b. As it is apparent, laccaic acids A and B could not be separated, nevertheless they are clearly separated from carminic and kermesic acids. Laccaic acid C also is well separated from the other molecules.

UHPLC-MS/MS analysis on the sample taken from ms. AM 227 fol. identified unambiguously the presence of laccaic acids A + B and C (chromatogram in Fig. 6c), while carminic and kermesic acids are not present. The confirmation was obtained by the retention time of the standard, the presence of quantifier and qualifier MRM transition and the positive match with qualifier/quantifier ion ratio with respect to the standard.

4 Discussion

The identification of lac dye in the paints of ms. AM 227 fol. open up interesting views on trade and the circulation of goods in the Middle Ages. This is, to the authors' knowledge, the first identification of lac dye in an Icelandic manuscript, but also the first overall identification of an insect dye in artworks created in pre-modern Iceland. It is further the only known occurrence of an insect dye in medieval Scandinavian manuscripts. However, due to the scarcity of data available so far (the only diagnostic studies on Scandinavian manuscripts were those of Best et al. [20], Winther et al. [21] and Glindvad [22]), it is possible that future analyses will bring further evidence to light.

Outside the manuscript tradition, lac dye appears to have been used rarely in mainland Scandinavia. White and Kirby [6] and Plahter [7] identified it in 13th -century Norwegian altar frontals. Additionally, it is known that the Vikings used kermes and cochineal [23, 24] to dye their textiles - testifying to the vast scope of their trade - but the presence of lac dye has not yet been reported in textiles produced in Scandinavia.

The lac dye used in the manuscript AM 227 fol. was certainly imported to Iceland from abroad, given the impossibility of any insect species of the Coccidae family surviving in the sub-Arctic climate of the island. Iceland had close ties with Norway at the time, and Bergen was the most important market harbour in medieval Norway and one of the main centres of North Atlantic trade [25]. An Icelandic manuscript from the second half of the fifteenth century preserves a list of some foreign painting materials, detailing their selling prices in Bergen [26]. It is therefore highly probable that the dye arrived in Iceland from Asia via Bergen.

Compared to other Icelandic manuscripts, even of comparable contents, AM 227 fol. stands out as being exceptionally well-made with no expense having been spared [9]. Its parchment is virtually flawless and significantly lighter than typical Icelandic parchment, which has led Guðbjörg Kristjánsdóttir to speculate that it could have been imported [8]. Its decoration is also among the finest preserved in vernacular Icelandic manuscripts [27]. Generally, large-format religious manuscripts, together with law codices, tend to be the most lavishly decorated of the Icelandic manuscripts [28]. The making of a high-status luxury book like AM 227 fol., containing translations of biblical texts, thus constituted an undertaking prestigious enough to merit the use of a rare and exotic colourant such as lac dye.

5 Conclusions

The method proposed allows for a more in-depth study of the dyes present in illuminated manuscripts, for whom the knowledge is sometimes limited by the difficulty in doing analytical investigation. Despite the extremely small sample size, the HPLC-MS/MS procedure enabled the selective identification of lac dye in a 14th-century Icelandic manuscript. As the first known evidence of this rare colourant being used in Iceland, the finding provides new insight into medieval painting materials and the extensive trade networks that connected even remote regions of Europe. It demonstrates that Icelandic artists had - at least in some cases - access to exotic colourants in comparable ways to those available to artists elsewhere in Europe, highlighting the far-reaching circulation of artistic materials and the integration of Iceland into wider medieval networks of exchange.

Declarations

Author Contribution

M.A., V.R. and G.Z. conceived and planned the experiments. E.C., F.G., and A.A. carried out the experiments. E.C. and F.G. planned and carried out the simulations. M.A., F.G., and V.R. contributed to

sample preparation. M.A., G.Z., and B.S. contributed to the interpretation of the results. G.Z. and B.S. supervised the project. M.A. took the lead in writing the manuscript. All authors provided critical feedback, helped shape the research, analysis and manuscript, and reviewed the final version before submission.

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Figures



Figure 1

Left: the miniature at f. 23v from which a micro sample was taken. Right: the area sampled; the blue pen indicates the point of withdrawal.

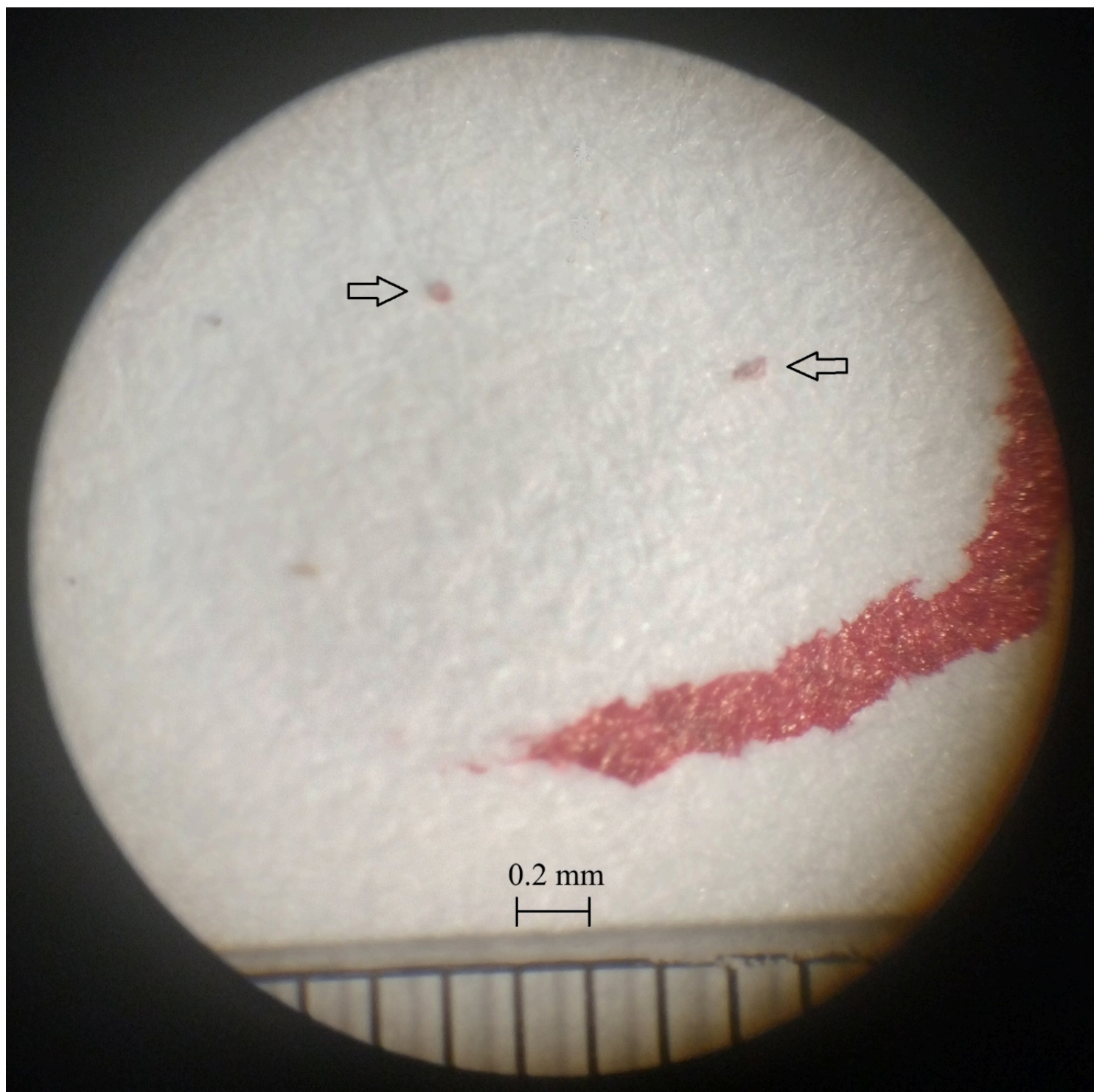


Figure 2

5x image of the micro sample of paint taken from ms. AM 227 fol. (the red curve is the line made with a marker on the paper to better recognise the particles). On the graduated scale below, the distance between two lines is 0.2 mm

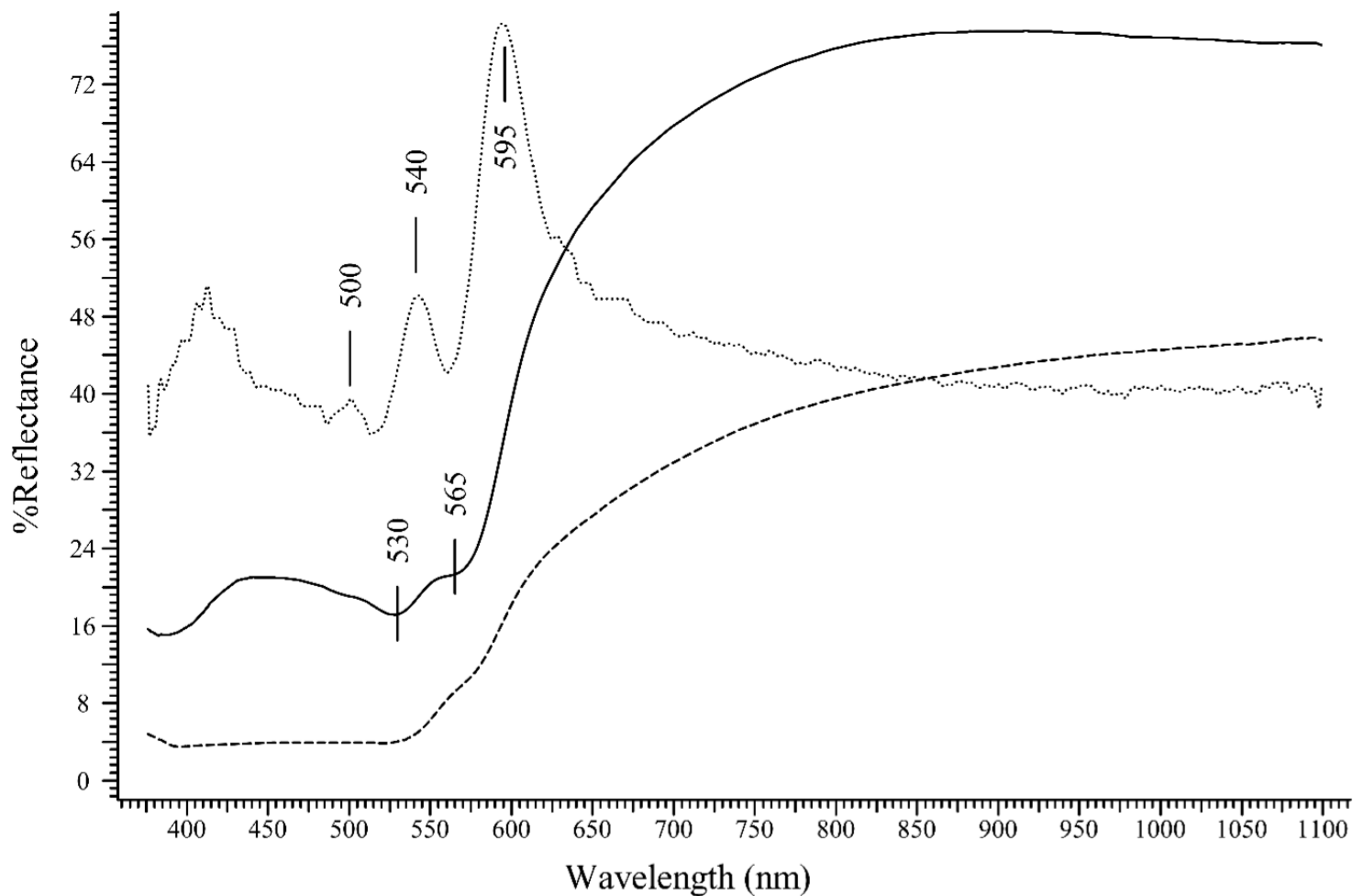


Figure 3

Example of FORS spectrum from a pink paint containing an insect dye (solid line) and a dark pink paint containing a mixture of insect dye and red lead (dashed line). The dotted line is the first derivative of the spectrum of the insect dye.

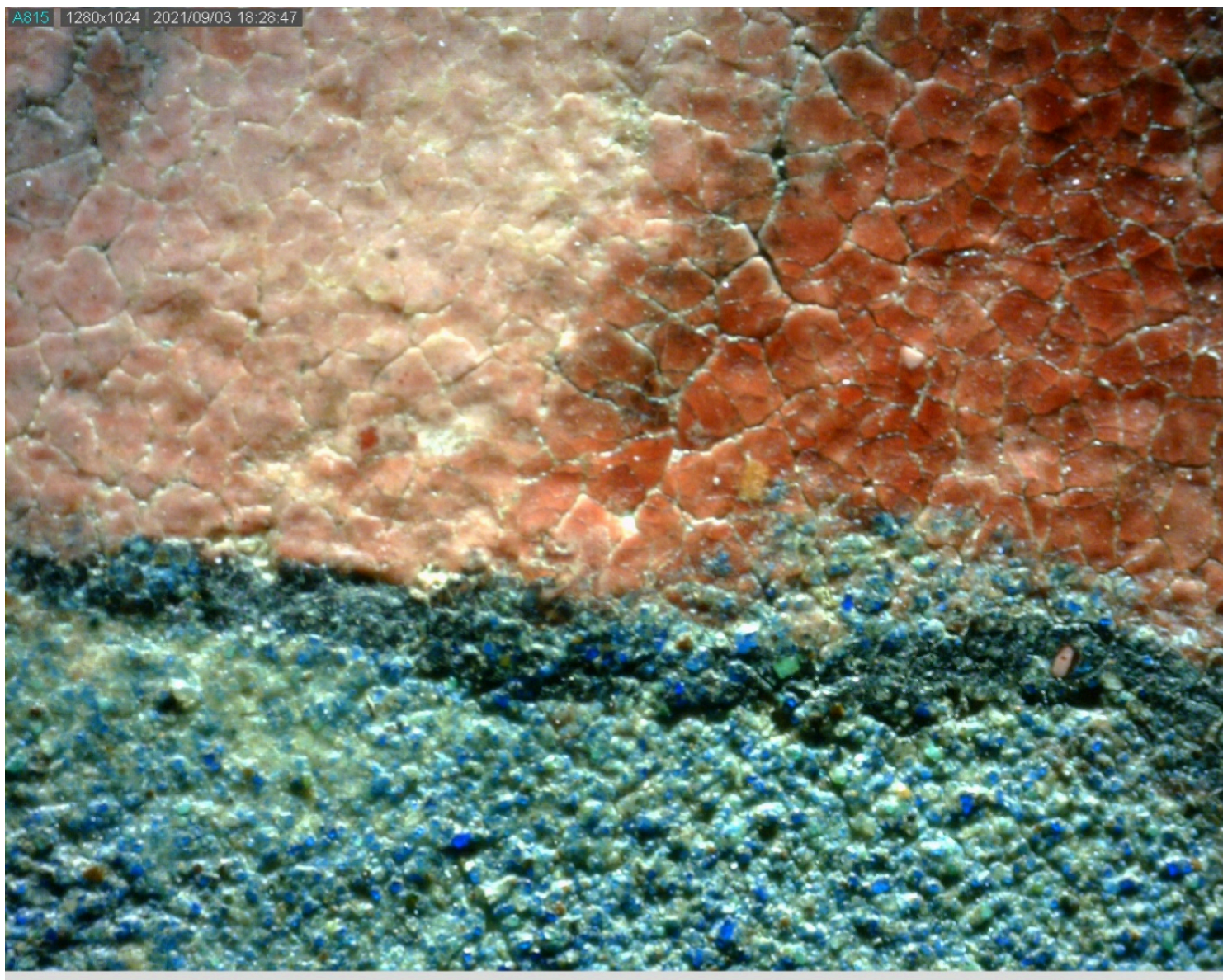


Figure 4

90x image of a pink area.

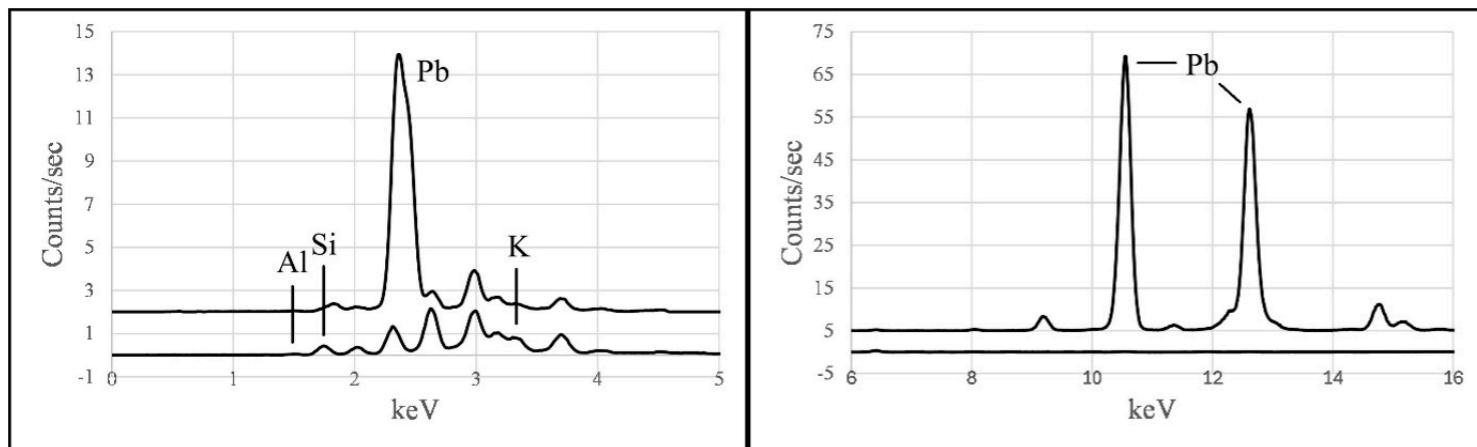


Figure 5

XRF spectra of a pink paint (top) and of parchment (bottom). Spectra are offset on the Y axis for clarity of comparison. Left: low energy range; right: high energy range.

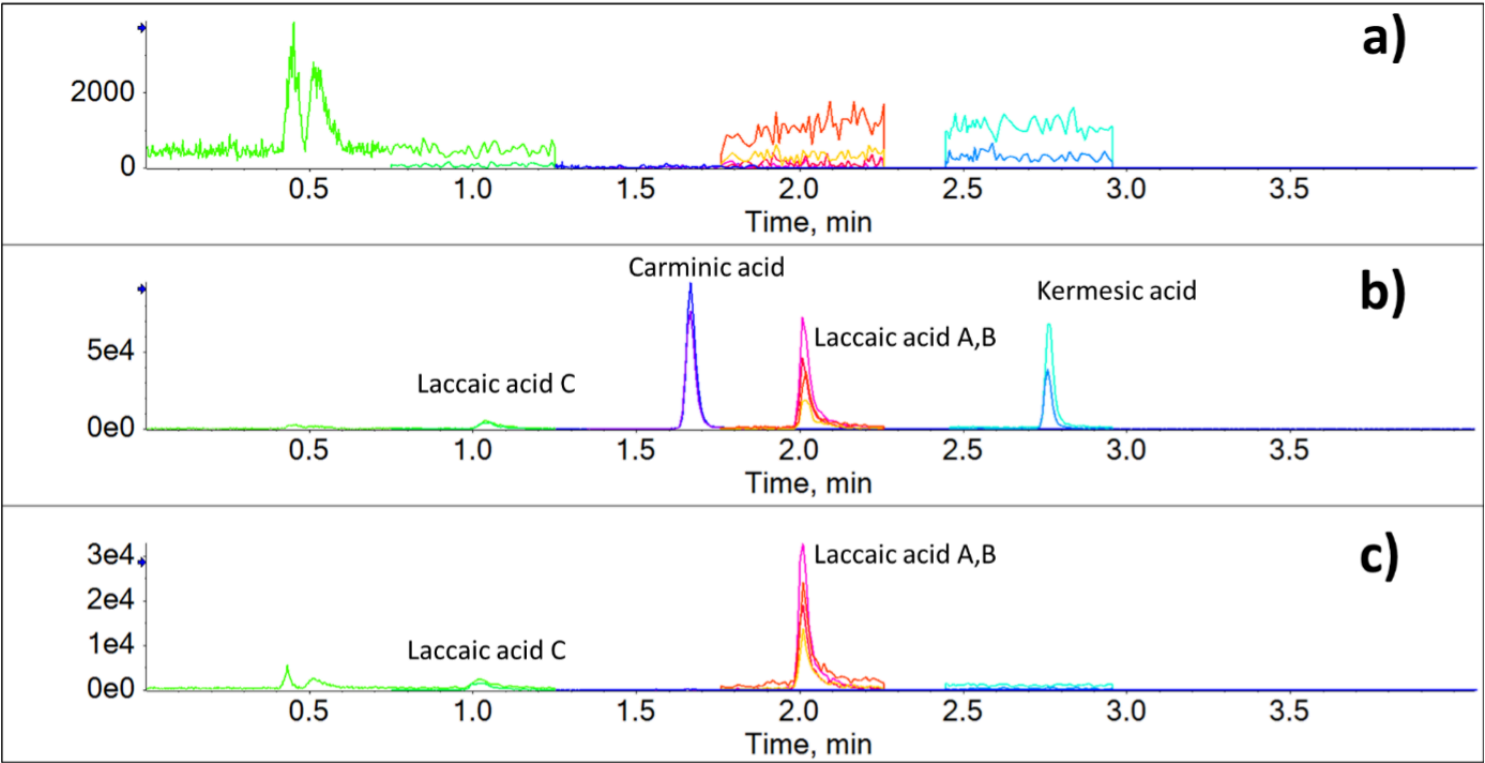


Figure 6

Extracted ion chromatograms of carminic acid, kermesic acid and laccaic acid in a) blank sample; b) standard mixture; c) sample from ms. AM227 fol.