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Unraveling a Historical Mystery: Identification of a Lichen Dye Source in a Fifteenth Century Medieval Tapestry

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Abstract: As part of a long-term campaign to document, study, and conserve the *Heroes* tapestries from The Cloisters collection at The Metropolitan Museum of Art, organic colorant analysis of *Julius Caesar* (accession number 47.101.3) was performed. Analysis with liquid chromatography–quadrupole time-of-flight mass spectrometry (LC-qToF-MS) revealed the presence of several multiply chlorinated xanthones produced only by certain species of lichen. Various lichen dye sources have been documented in the literature for centuries and are classified as either ammonia fermentation method (AFM) or boiling water method (BWM) dyes based on their method of production. However, none of these known sources produce the distinctive metabolites present in the tapestry. LC-qToF-MS was also used to compare the chemical composition of the dyes in the tapestry with that of several species of crustose lichen. Lichen metabolites, including thiophanic acid and arthothelin, were definitively identified in the tapestry based on comparison with lichen xanthone standards and a reference of *Lecanora sulphurata*, confirming the presence of a lichen source. This finding marks the first time that lichen xanthones have been identified in a historic object and the first evidence that BWM lichen dyes may have been used prior to the eighteenth century.

Keywords: lichen dye; xanthones; medieval art; textiles; LC-qToF-MS; dye analysis

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1. Introduction

Prior to 1856, the year Perkin's discovery of mauveine brought about the synthetic dye industry, dyers turned to the natural world to satisfy a deeply human obsession with color. Centuries of demand, trial and error, and global exchange led to a rainbow of hues attainable from a relatively limited range of sources, used both alone and in infinite combinations [1,2].

Natural dyes can be derived from various components of plants, such as the roots of madder (*Rubia tinctorum*), the bark of brazilwood (*Paubrasilia echinata*), or the leaves of weld (*Reseda luteola*). They are also present in insects, such as cochineal (*Dactylopius coccus*) or kermes (*Kermes vermilio*). A limited number of dyes come from animals, such as the famous "Tyrian purple" extracted from mollusks in the *Muricidae* family.

A fourth category of natural dyes is derived from lichens and includes orchil (*Roccella* spp.), cudbear (*Ochrolechia tartarea*), and others. Despite their historical importance in the textile [3–8], culinary [9], and medical [10] industries, lichens are poorly understood by the general population. A lichen is not a single organism but rather a symbiotic partnership between a mycobiont (a fungus, also known as "lichenized fungus") and a photobiont (usually a species of algae, though in some cases cyanobacteria are present instead or in

addition to algae). The photobiont provides nutrients for the fungal partner via photosynthesis. In exchange, the mycobiont provides physical protection for the photobiont and produces secondary metabolites, such as xanthones, depsides, and chromones, with antibiotic, anti-microbial, UV-protective, and herbivore-deterrent properties (Figure 1) [11,12]. Most of the known lichen metabolites, of which there are currently more than one thousand, are unique to the lichenized fungus and are not produced by other groups of fungi not involved in lichen symbioses [12–14]. Their collaborative relationship allows both partners to occur in extreme environments, such as extreme temperatures, ultraviolet (UV) radiation, or high concentrations of salt [15,16].

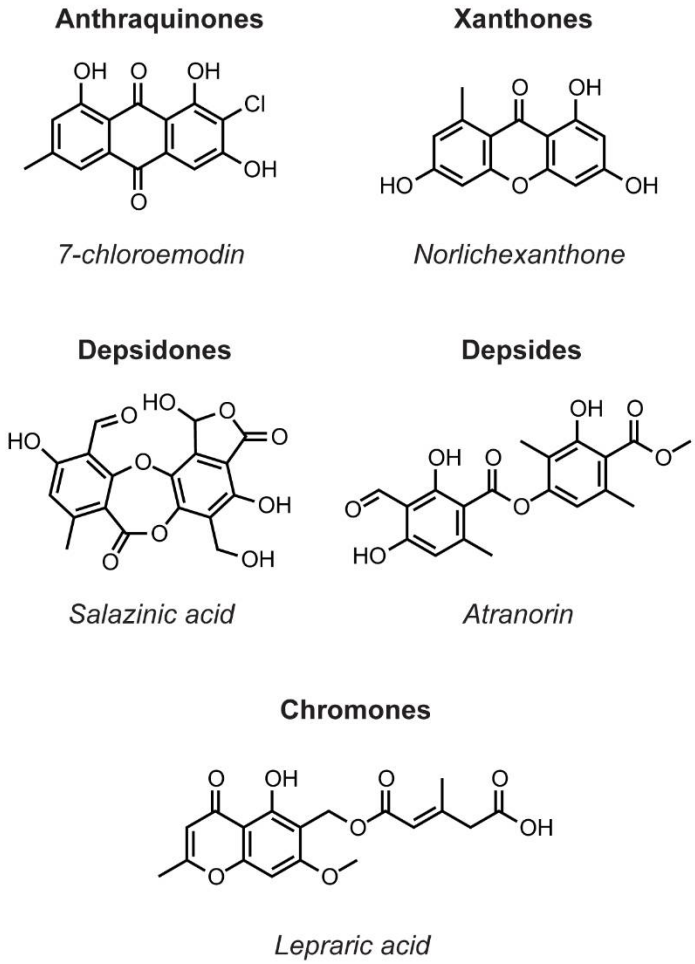

Figure 1. Several classes of secondary metabolites found in lichen.

Depending on their chemical composition and method of preparation, lichen dye preparation can be separated into two categories: the ammonia fermentation method (AFM) or the boiling water method (BWM). With AFM lichen, orsellinic acid depsides (the colorless precursors to orchil dyes) are converted into a bright purple or pink dye through a lengthy fermentation process using ammonia or, historically, urine. In contrast, BWM lichens produce their own colorants, such as atranorin, parietin, or usnic acid, which can be extracted in boiling water [8,17]. To the best of our knowledge, despite centuries of documentation indicating the global importance of lichen dyes, only AFM dyes have been identified in historic objects, using techniques such as Raman or SERS [18,19], fluorimetry [20], FORS [21], and LC-MS [22–24]. The absence of BWM dyes detected in historic objects may be explained by a combination of several factors, including the availability of reference materials, the relative stability of pigments, and a lack of research into the aging and degradation patterns of BWM-dyed materials.

In 2018, The Metropolitan Museum of Art (the Met) initiated a long-term project to study and conserve the *Heroes* tapestries, a series of hangings that depict notable figures from Christian, Jewish, and Classical traditions. The tapestry hangings with the five *Heroes* that survive of the original nine are a highlight of the permanent collection at The Cloisters and have been on display since their acquisition by the Met in the mid-twentieth century [25]. Little is known about the origins of the tapestries; though they were likely made for a wealthy patron connected to the French royal court in the early fifteenth century, it remains unclear exactly where, when, or for whom they were woven. Treatment and dye analysis of *Julius Caesar* ("*Caesar*") commenced in September 2022.

Scientific analysis of tapestries has been used to study a work's historical context [24,26,27], trace the history of intervention and repair [28,29], study degradation of dyes and fibers [30,31], and provide insight into how a work may have originally appeared [32]. Thirty-seven samples of dyed wool of various colors were removed from across the *Caesar* tapestry and analyzed by LC-qToF-MS. The results of the full dye analysis and additional information regarding *Caesar's* conservation and acquisition history are beyond the scope of this article and will be published elsewhere [33]. In the three dark brown samples that were analyzed, chlorinated metabolites deriving from an unknown lichen source were detected. Through a collaboration between researchers at The Metropolitan Museum of Art, The Field Museum of Natural History, and The Rennes Institute of Chemical Sciences, the identities of these compounds were confirmed by comparison to standard lichen metabolites and by analysis of a reference specimen. This study, which highlights the power of an interdisciplinary approach to art history, marks the first time that genuine lichen xanthones have been definitively identified in a historic object.

2. Materials and Methods

2.1. Julius Caesar (from the Heroes Tapestries), Accession Number 47.101.3

Among the oldest surviving medieval tapestries in the world, *Julius Caesar* belongs to the set of *Heroes* tapestries depicting notable figures from Christian, Jewish, and Classical traditions, only five of which have been recovered. Well before their acquisition, these tapestries had been cut apart into fragments and shaped into window curtains [25]. The fragments had to be re-pieced together before they could be displayed at The Cloisters in their current configuration [34]. This work shows Caesar, seated in the center of an elaborate architectural setting with a saber, spear, and crown, identifiable by the double-headed eagle of the Roman empire on his shield (Figure 2). He is flanked by musicians, foot soldiers, and attendants. Likely woven circa 1400–1410 in France or the Southern Netherlands, the tapestry spans 420.4 cm × 238 cm and was woven with wool warps and wefts. The tapestry is composed primarily of bright blues, greens, reds, and yellows, with darker blues, beiges, and browns providing visual contrast and dimensionality.

2.2. Materials

All commercially available reagents and solvents were used as received. All solvents are of analytical or LC-MS grade. High-purity water was provided by a Milli-Q water purification system.

2.3. Reference Material

A specimen of *Lecanora sulphurata* (Ach.) Nyl.: *Lecanoroid Lichens exs. 14* (F) was used for comparison. Reference standards of 3-*O*-methyl-2,4,5-trichloronorlichexanthone (thuringione) and 3-*O*-methyl-2,5,7-trichloronorlichexanthone, as well as acetone extracts of *Lecanora alboflavida* Taylor and *Lecidella asema* var. elaeochromoides (Nyl.) Nimis and Tretiach were provided by Joël Boustie at the Université de Rennes.

2.4. On-Fiber Dye Extraction Methodology

Dye extraction was performed with a process adapted from Mouri and Laursen [35]. Yarn samples of original weaving (approximately 2–5 mm in length) were distinguished

from restoration yarns by a textile conservator. Samples were then extracted with 40 µL of a 0.01 M oxalic acid (aqueous)/pyridine/methanol solution (3:3:4 $v/v/v$) in a 6 × 50 mm glass test tube. The sample was left to extract at room temperature for 30 min and was then heated at 55–60 °C for 30 min. The extract was then transferred to a microcentrifuge tube, and the sample in the test tube was rinsed with 40 µL methanol, which was added to the same microcentrifuge tube. A total of 60 µL of extraction solution was added to the test tube containing the sample, which was heated at 90–100 °C for 10 min, cooled, and transferred to the microcentrifuge tube. Again, the tube was rinsed with 40 µL methanol and the rinsate was transferred to the microcentrifuge tube. The extract in the microcentrifuge tube was dried inside a vacuum desiccator with a water aspirator. Once fully dry, the residue was vortexed with 4 µL DMF, followed by 16 µL of 0.1% formic acid in methanol/acetonitrile (1:1, v/v) and 20 µL 0.1% formic acid in water (for a total of 1:4:5 $v/v/v$ reconstitution solution). The tube was centrifuged at 12,000× g for 10 min, and 20 µL of supernatant was transferred to a 0.2 mL micro-insert autosampler vial (Shimadzu 220-97331-63) for injection (8 µL) onto the LC-MS system. For LC-MS analysis of the other polarity, 10 µL of reconstitution solution was added to the insert and vortexed and then transferred to the original microcentrifuge tube (with the remaining 20 µL of the sample), and the tube was again centrifuged at 12,000× g for 10 min. A total of 20 µL of supernatant was transferred to a new 0.2 mL micro-insert for injection (8 µL) into the LC-MS system.

Figure 2. *Julius Caesar (from the Heroes Tapestries);* wool warp, wool wefts; South Netherlandish, ca. 1400–1410; 165 1/2 × 93 11/16 in. (420.4 × 238 cm); The Metropolitan Museum of Art, New York, Gift of John D. Rockefeller Jr., 1947 (47.101.3). Image © The Metropolitan Museum of Art. The following annotations to the original photograph were added by the author: the individual fragments from which the tapestry was recreated are numbered. White boxes correspond to areas from which the dark brown fibers were sampled.

2.5. Preparation of Lecanora sulphurata for HPLC-ESI-qToF-MS Analysis

A total of 19.4 mg of lichen was removed from the reference sample with a clean razor blade and placed in a clean, 4 mL glass vial. A total of 1 mL acetone (HPLC grade) was added, and the sample was crushed and extracted at room temperature for 1 h. The extract was filtered with an Ultrafree-MC centrifugal filter (0.2 µm pore size, hydrophilic PTFE membrane, Millipore, Burlington, MA, USA), and the solvent was removed with an SP Genevac EZ-2 4.0 centrifugal evaporator (ATS Scientific Products; Warminster, PA, USA. Once fully dry, the residue was vortexed with 4 µL DMF, followed by 16 µL of 0.1% formic acid in MeOH/ACN (1:1, *v*/*v*) and 20 µL 0.1% formic acid in water (for a total of 1:4:5 *v*/*v*/*v* reconstitution solution).

2.6. High-Performance Liquid Chromatography Electrospray Ionization, Quadrupole Time-of-Flight Mass Spectrometry (HPLC-ESI-qToF-MS)

The HPLC-ESI-qToF-MS system consists of a Bruker Impact II quadrupole Time-of-Flight mass spectrometer with an electrospray ionization source (Billerica, MA, USA) and a NexeraXR high-performance liquid chromatograph with two LC-20ADxr HPLC pumps, an HPLC gradient mixer, an SPDM30A diode array detector, a CTO-20AC column oven, a DGU-20A5R degassing unit, an SIL-20ACxr autosampler, and a CBS-20A communications bus module (Shimadzu, Columbia, MD, USA).

A Zorbax SB-C18 reversed-phase column (3.5 µm particle size, 2.1 mm I.D. × 150.0 mm, Agilent Technologies, Santa Clara, CA, USA) was used with a Zorbax SB-C18 guard column (3.5 µm particle size, 2.0 mm I.D. × 15.0 mm, Agilent Technologies, Santa Clara, CA, USA). A pre-filter (Upchurch ultra-low Volume pre-column filter with 0.5 µm stainless steel frit, Sigma-Aldrich, St. Louis MO, USA) was attached to the guard column. Chromatography was performed at a flow rate of 0.2 mL/min and a column temperature of 40 °C, with a gradient of 0.1% formic acid in Millli-Q water (mobile phase A) and 0.1% formic acid in methanol/acetonitrile (1:1, *v*/*v*) (mobile phase B). The gradient system was as follows: initial conditions of 90% A for 1 min, a linear slope from 90% to 60% A over 6 min, a second linear slope from 60% to 1% A over 23 min, holding at 1% A over 3 min, and then a linear slope to return to 90% A over 1 min and holding at 90% A for 18 min.

Internal calibration of the mass spectrometer was performed daily using 10 mM sodium formate solution. Sodium formate clusters were formed using a mixture of Milli-Q water, 2-propanol, formic acid, and 1 M sodium hydroxide (250:250:5:1, *v*/*v*/*v*/*v*).

ESI operating parameters were as follows: capillary voltage 4500 V, dry gas 8.0 L/min, dry heater 220 °C, nebulizer 1.8 Bar.

Chromatograms were smoothed using a Gaussian algorithm, with a width of 2s.

Operation of the HPLC and MS systems was performed using Compass otofControl Version 6.3.106 and Compass Hystar Version 6.2.1.13. Data analysis was performed using Compass DataAnalysis software Version 6.1.

3. Results

Dyes were extracted from the fiber using mild acid hydrolysis according to the method described in Section 2.4 and analyzed by LC-qToF-MS as described in Section 2.6. Dye analysis was performed to identify the dyes in all colors across all fragments of the tapestry, with a primary focus on the original weaving [33]. In total, thirty-seven samples were analyzed. Whereas most of the colorants used were typical medieval dyes (madder for red and weld or dyer's broom for yellow), three dark brown areas of the tapestry were identified as containing chlorinated norlichexanthone derivatives and subjected to further investigation (Figure 3). Though the dark brown yarns are particularly fragile and much of the original dark brown weft (including nearly all the double-headed eagle on Caesar's shield) has been lost, these samples were determined to be original and likely had a similar dark brown color originally. The chemical components related to the dark brown areas of the tapestry have been identified in Table 1.

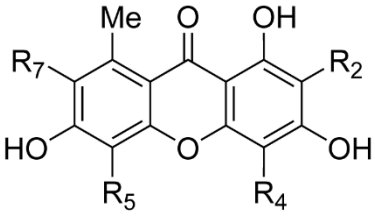

Figure 3. Basic structure of lichen metabolites derived from the norlichexanthone scaffold.

Table 1. Chemical composition of the brown dyes in fragments 3, 4, and 6 and the reference of *Lecanora sulphurata* extracted with acetone. Components are indicated as present (+), present in trace amounts (t), or not detected (-). [a] Is where references were not available and the concentration in the tapestry was too low for absorption measurements and UV data are left blank. [b] Due to the low solubility of indigo and its poor ionization in negative mode, indigotin was identified by the presence of isatin and by matching the retention time and the UV-visible spectrum.

#	Compound Name	RT (min)	$[M-H]^-$, m/z	Product ions, m/z	λ_{max}, nm [a]	Frag. 3	Frag. 4	Frag. 6	*Lecanora sulphurata* Reference
1	isatin	8.4	146.024	118	-	-	-	+	-
2	luteolin 7-*O*-glucoside	9.4	447.093	285	197, 253, 346	-	+	-	-
3	ellagic acid	9.6	300.999	284, 257, 245, 229, 173, 145	252, 366	-	-	+	-
4	*O*-methyl ellagic acid	11.3	315.015	300	-	+	+	+	-
5	luteolin	12.7	285.04	241, 199, 175, 151, 133, 107, 83	204, 253, 346	-	+	-	-
6	$C_{14}H_4Cl_4O_7$	14.8	422.863	381, 346, 318, 307, 289, 279, 251, 224	198, 249, 320, 366	-	-	-	+
7	alizarin	17.5	239.035	211, 195, 183, 167	247, 428	-	+	-	-
8	munjistin	17.6	283.025	239, 211, 195, 167	209, 246, 288, 430	-	+	-	-
9	indigotin [b]	19.5	261.067	260, 233, 217, 156	200, 239, 284, 609	-	-	t	-
10	$C_{14}H_6Cl_4O_6$	20.0	408.885	393, 381, 357, 329	211, 246, 317, 355	+	+	+	+
11	4,5-dichloronolichexanthone	20.6	324.967	290, 261, 233, 183	211, 246, 317, ~366	-	-	-	+
12	2,5-dichloronorlichexanthone	21.1	324.967	290, 261, 233, 183	197, 247, 316	-	-	-	+
13	2,4,5-trichloronorlichexanthone (arthothelin)	22.7	358.929	323, 295, 267, 259, 231	212, 249, 315	+	+	+	+
14	4,5,7-trichloronorlichexanthone (asemone)	23.8	358.929	324, 295, 267.0	248, 314, 352 (sh)	t	t	+	+
15	2,5,7-trichloronorlichexanthone (isoarthothelin)	24.5	358.929	324, 295, 267, 181, 163.0, 137.1	201, 250, 315, 350 (sh)	t	t	t	+
16	2,4,7-trichloronorlichexanthone	25.0	358.929	323, 295, 287, 267, 259, 231, 177, 149	201, 247, 314, 350 (sh)	-	-	-	+
17	2,4,5,7-tetrachloronorlichexanthone (thiophanic acid)	25.8	392.89	360, 329, 321, 301, 293, 264, 303, 265, 149	200, 250, 316, 355 (sh)	+	+	+	+

Table 1. *Cont.*

#	Compound Name	RT (min)	[M − H]⁻, m/z	Product ions, m/z	λ_{max}, nm [a]	Frag. 3	Frag. 4	Frag. 6	*Lecanora sulphurata* Reference
18	$C_{15}H_9Cl_3O_5$ A	26.4	372.944	358, 330, 294, 266, 97	-	+	+	+	-
19	$C_{15}H_9Cl_3O_5$ B	27.5	372.944	358, 340, 294, 266	-	+	+	+	-
20	atranorin	27.5	373.093	177, 163, 133, 119, 105	208, 250, 319	-	-	-	+
21	chloroatranorin	28.5	407.054	211, 167, 163, 139, 131, 119	211, 248, ~350	-	-	-	+

All three dark brown samples that were sampled from the tapestry included components with isotopic patterns, indicating the presence of multiple chlorine atoms (Figures 4 and 5).

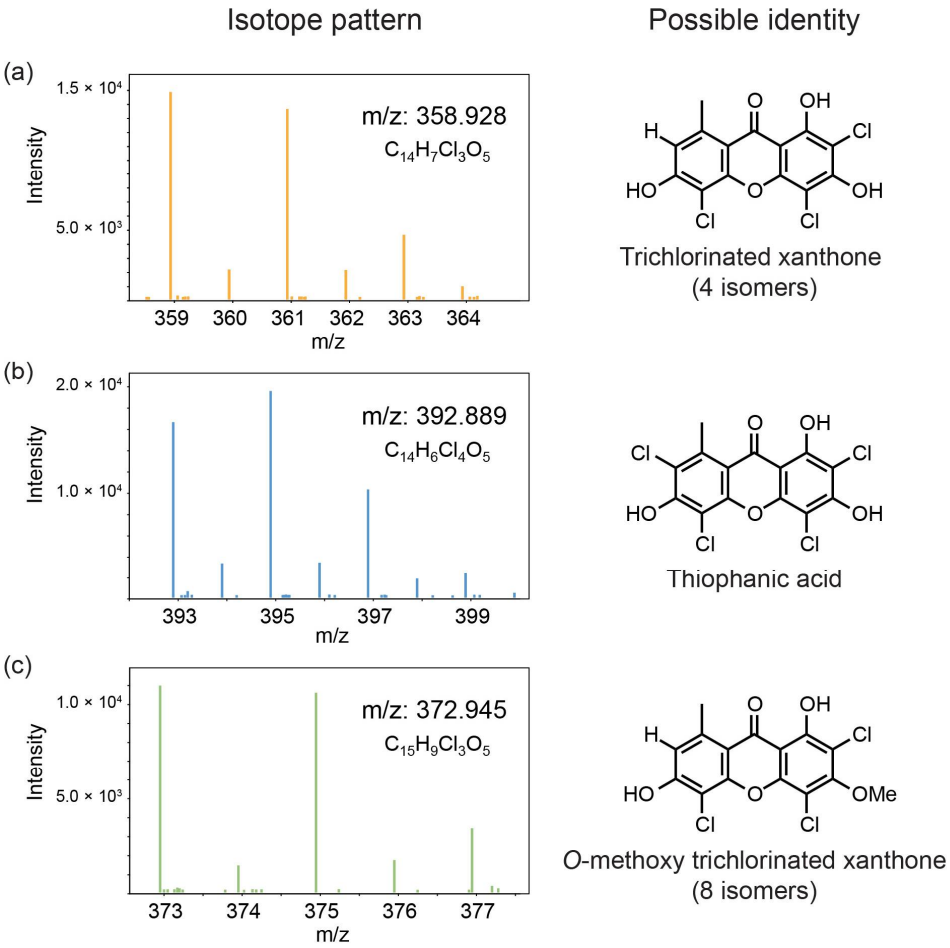

Figure 4. Isotopic pattern, exact mass and [M − H]⁻ formula, possible chemical structure, and number of possible isomers for the components at 22.7, 23.8, 24.5, and 25.0 min: (**a**), 25.8 min (**b**), and 26.4 and 27.5 min (**c**).

Halogenated metabolites are not uncommon in nature [36,37], but few are present in common sources of natural dyes. Perhaps the best-known exception to this rule is 6,6′-dibromoindigo, a colorant found in shellfish purple and derived from mollusks from the *Muricidae* family [38].

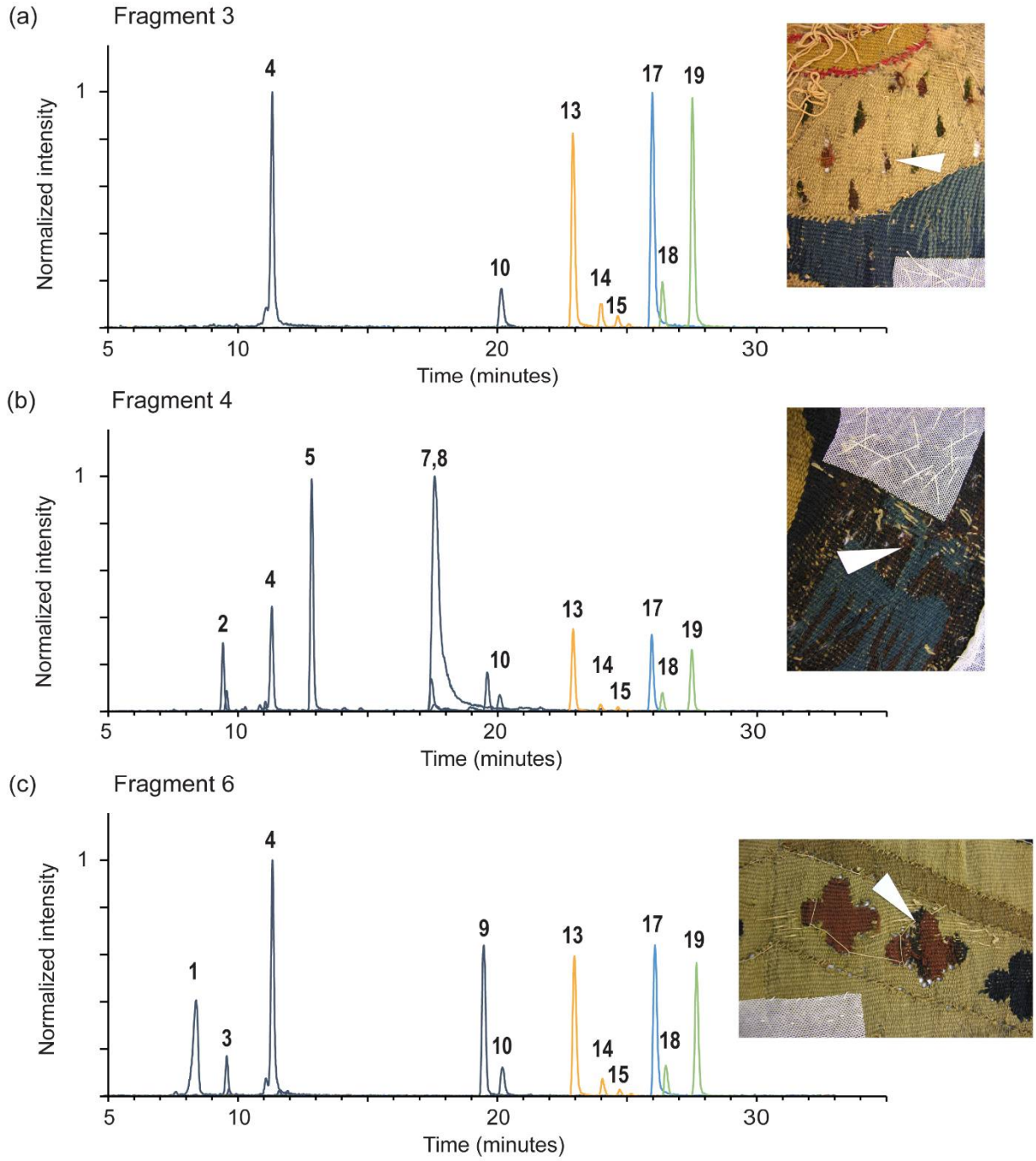

Figure 5. Extracted ion chromatogram (EIC) for colorants and lichen xanthones identified in the dark brown samples from fragments 3 (**a**), 4 (**b**), and 5 (**c**). The components are numbered corresponding to Table 1. The colors for compounds **13**–**15** and **17**–**19** correspond to the chlorinated xanthones in Figure 4. The white arrows in the image indicate the location from where the samples were taken (see Figure 2).

Based on accurate mass measurements and the isotopic pattern (Figure 4b), the component at 25.8 min was determined to have a chemical formula of $C_{14}H_6Cl_4O_5$ (error = 0.8 ppm), which corresponds to thiophanic acid (TA) **17**, a tetrachlorinated xanthone that is specific to certain species of crustose lichens, primarily those of the genera *Lecanora* and *Lecidella* [39,40]. In addition to potential fungicidal properties, TA absorbs light in the UVA range (315–400 nm) and is believed to function as a UV-protectant compound (Figure 6) [41]. In lichen, UV protectants often localize in the thallus, or upper portion of the lichen,

and act as chemical filters of UV light without blocking the visible light necessary for photosynthesis [16].

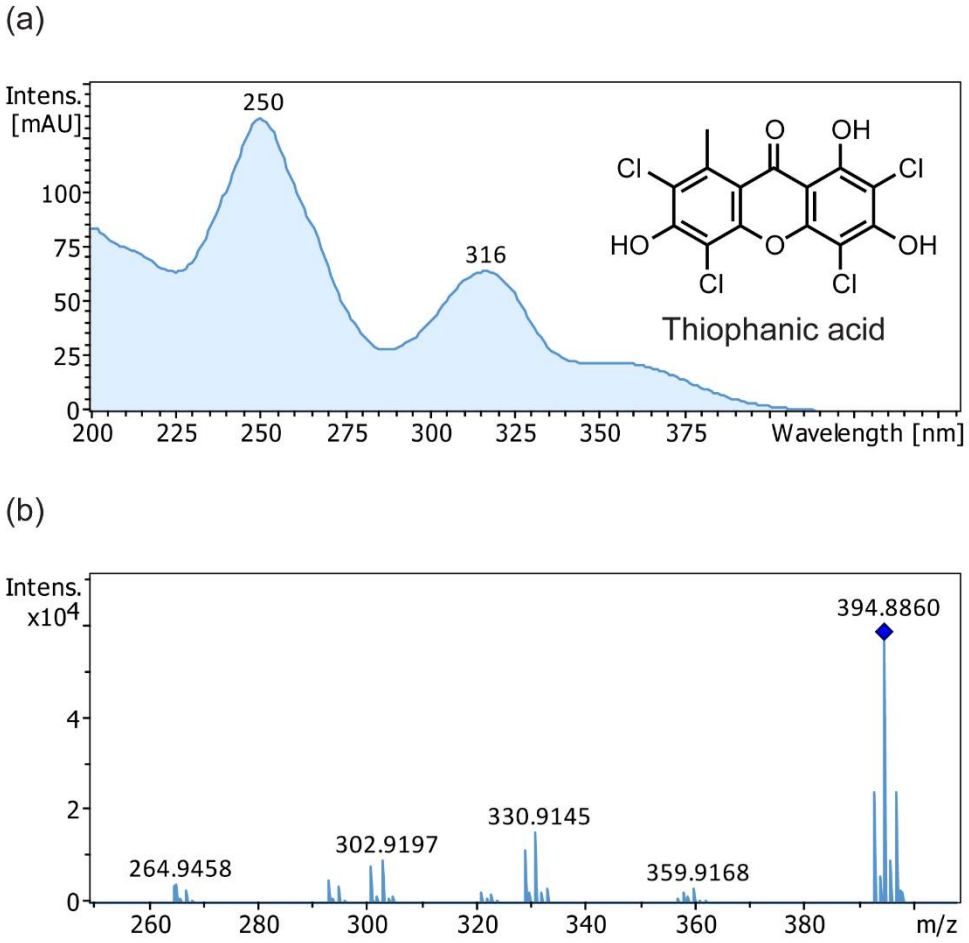

Figure 6. UV-visible spectrum (a) and MS/MS fragmentation spectrum (b) for thiophanic acid, **17**.

The other related components were also identified as trichlorinated xanthones, likely with similar functions, including three isomers of $C_{14}H_7Cl_3O_5$, (Figure 4a), and two isomers of $C_{15}H_9Cl_3O_5$ (Figure 4c).

Xanthones are a class of oxygenated heterotricycles present as secondary metabolites in plants, fungi, and lichens. Their pharmacological properties, including antimicrobial, anti-inflammatory, and antioxidant activity, have been studied in depth [42]. Although xanthones are not lichen-specific compounds, a rare biosynthetic pathway in lichen leads to substitution patterns not found in other species; the lichexanthone type pattern is a 1,3,6-trihydroxy-8-methylxanthone, whereas plants produce mostly 1,3,5- or 1,3,7-trihydroxyxanthones [43,44]. Furthermore, of the naturally occurring xanthones in nature, only a small percentage contain one or more chlorine atoms [45]. The majority of these chlorinated xanthones are synthesized exclusively by lichen [44,46].

For lichenologists, the identification of secondary metabolites has long been critical to the taxonomic classification of lichen species, with species identifiable by a "chemosyndrome", or set of characteristic metabolites. A chemosyndrome often consists of a major metabolite and minor "satellite" compounds with similar biosynthetic origins [39,47]. Researchers use spot tests, chromatography, or even mass spectrometry to identify characteristic components, allowing morphologically similar species to be distinguished, which in some cases is necessary for species identification. There is, therefore, a record of chemical information for known lichen species, including those that have been documented as commonly used for dyeing, such as *Ochrolechia tartarea* ("cudbear"), *Parmelia saxatilis* ("light crottle" or "salted shield lichen"), and *Letharia vulpina* ("wolf moss") [1,8].

Lichens cannot be easily cultivated, and are, therefore, geographically constant, though their populations can be threatened by overharvesting and environmental changes. Furthermore, because secondary metabolites allow lichens to adapt to their environments, chemical composition is often closely related to geography [48,49].

Considering the specificity of lichen metabolites and the little information available about the *Caesar* tapestry, we hypothesized that the identification of the metabolites could offer a rare look into where or how the materials of the *Caesar* tapestry were sourced. However, despite an abundance of literature detailing lichen dye sources across the world, to the best of our knowledge, there are no accounts of TA-containing lichen species used for dyeing, either industrially or domestically [1,6–8,50,51]. This conclusion can be made after cross-referencing the available literature on lichen dye species with the extensive literature detailing the chemical composition of lichen species [13,14,39,40,44,52–54]. Apart from orchil lichens, most of the lichen species used for dyeing were employed in small, local industries, and the visual similarity of many lichens makes differentiation between species difficult in the absence of modern analytical techniques. A species of lichen could certainly have been used without knowing or recording its precise identity.

Within Europe, one of the only TA-containing species with common and large enough populations to be identified and collected for dyeing is *Lecanora sulphurata*, a species that is common in the Mediterranean area and does not occur in Central Europe [53,55]. Critically, chemotaxonomic reports have identified TA and several trichlorinated xanthones as major and minor components of this species.

To investigate the chemical composition of the specimen of *Lecanora sulphurata*, a small area was scraped off and extracted for an hour in acetone. LC-qToF-MS was used to identify the metabolites. The major components present in the reference sample were chlorinated xanthones and two depsides: atranorin **20** (a pale-yellow pigment), and chloroatranorin **21**.

Due to the condition and size of the samples, the concentration of the metabolites present in the tapestry was generally not high enough to be identified by their UV-visible spectra but could be validated by retention time and MS/MS fragmentation (Figures 5 and 6b).

TA was indeed a major component of the reference, allowing for the confirmation of its presence in the tapestry sample (Figures 7 and S1). Three of the trichlorinated xanthones from the tapestry were also present in the reference species, which were compared with reference material and identified as trichloronorlichexanthone isomers arthothelin **13**, asemone **14**, and isoarthothelin **15** [39,53]. In contrast with the available literature, the reference specimen also contains the fourth isomer of $C_{14}H_7Cl_3O_5$, **16**, but the chemical composition of this species has been reported to differ slightly between populations. The reference was not found to contain any isomers of the methylated xanthones ($C_{15}H_9Cl_3O_5$) that are present in the tapestry. We ruled out the possibility that methylation in the tapestry samples could have resulted from the extraction process by treating the lichen reference with the same mildly acidic extraction process used to extract the dye from the fibers (Figure S2). Oxalic acid extraction led to the appearance of peaks that may correspond to monoaromatic depside hydrolysis products [56]. However, no methylation was observed.

Finally, a molecule with the chemical formula of $C_{14}H_6Cl_4O_6$, **10**, possibly an oxidized form of TA based on MS/MS fragmentation, was found to match a compound present in trace amounts in the tapestry (Figure S3). This compound has not yet been reported as a lichen metabolite, and further experimentation will be necessary to identify it.

With the goal of identifying the two isomers of $C_{15}H_9Cl_3O_5$ present in the tapestry, two of the eight isomers (3-*O*-methyl-2,4,5-trichloronorlichexanthone and 3-*O*-methyl-2,5,7-trichloronorlichexanthone) were obtained in pure form as reference standards. References of *Lecanora alboflavida* and *Lecidella asema*, which contain 3-*O*-methyl-2,4,5-trichloronorlichexanthone (thuringione) and 3-*O*-methyl-2,5,7-trichloronorlichexanthone, respectively, were prepared as extracts in acetone and analyzed for comparison (Figure S4). The chemical composition of these references can be found in Table S1. Based on retention time, compound **19** at 27.5 min could be 3-*O*-methyl-2,5,7-trichloronorlichexanthone. Unfortunately, the isomers have nearly identical

MS/MS fragmentation, and without knowing if the remaining six isomers co-elute, we are currently unable to definitively identify these components in the tapestry.

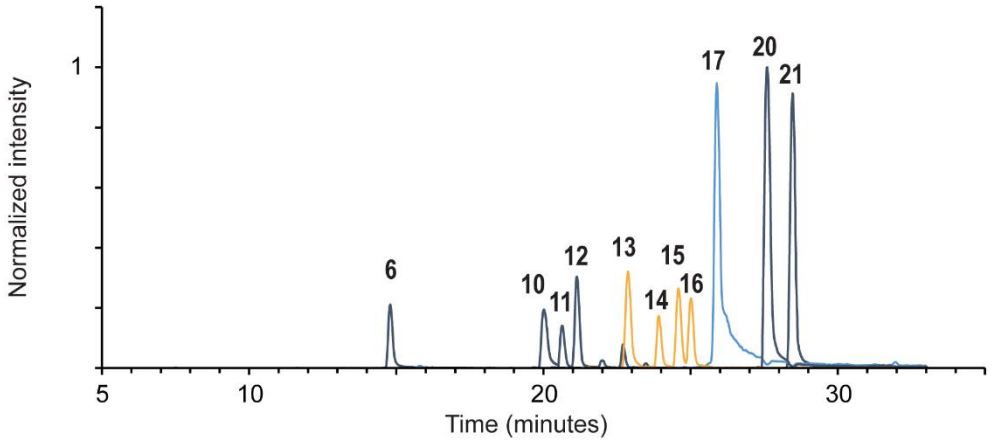

Figure 7. Extracted ion chromatogram (EIC) of acetone extract of *Lecanora sulphurata*.

Finally, since the process of dyeing can also lead to changes in composition, a small amount of lichen (*Lecanora sulphurata*) was heated with a sample of wool in Milli-Q water for several hours to observe the effects of the BWM process that may have been used. After boiling, the dye was then extracted from the fiber with a mildly acidic oxalic acid extraction and analyzed by LC-qToF-MS. The major component that was present in the wool sample was TA, with atranorin and chloroatranorin having almost completely degraded (Figure S5). This supports our hypothesis that the stability of chlorinated xanthones relative to lichen pigments may allow them to serve as markers for lichen dyes.

4. Discussion

Due to the age of the tapestry and the condition of the dyes, it may not be possible to definitively identify the lichen source (or sources) present in the dark brown dye. The reference specimen of *Lecanora sulphurata*, chosen as a candidate for its composition and population size, contained several of the chlorinated xanthones present in the tapestry, but not all. Most lichens containing these chlorinated xanthones are crustose lichens that grow on saxicolous or vegetal supports and would have to be scraped off the substrate to be used as a dye. Crustose lichens often form patches, and many species can have similar morphology. Therefore, more than one species could have been mixed, possibly along with parts of the support (see the composition of *Lecanora alboflavida* extract, Table S1).

Regardless of the species, the presence of distinct, lichen-specific xanthones raises the question of why a lichen source would be used to make the dark brown dye of the tapestry. No lichen dye source in the TA chemosyndrome has been documented, but it is possible that a lichen species could have been used as a dye without recording its identity. This is especially likely in the medieval era before the development of detailed taxonomy and chemical tests to distinguish between morphologically similar species of lichen.

Although chlorinated xanthones form yellow crystals, they have not been reported to function as dyes. However, the use of lichens that contain depside pigments, such as atranorin, is well-documented [6,8,57]. In fact, atranorin is one of the main colorants found in crottle, a famous Scottish lichen dye made from *Parmelia saxatilis* that can be used for dyeing golden or reddish brown on wool. Many lichen species contain atranorin, including those producing chlorinated xanthones. No such depsides were detected in the tapestry, but this could be due to the age and condition of the tapestry. Ester-linked (β)-orsellinic derivatives, such as atranorin, have been shown to be photolabile under high exposure to UVA and UVB [58]. It is, therefore, possible that the lichen species was selected as a dye for the presence of a pigment that has since faded.

Lichens are also “substantive” dyes, meaning they can be used on their own or combined with other dyes without the need for mordanting [8]. This quality may be

explained by their high iron content: lichens contain an average of 5.16 milligrams of iron per gram of dry material, in contrast with 0.30 milligrams for land plants [59]. Crustose lichens access iron from the substrate by disintegrating it through chemical and mechanical means [60,61]. Some lichen compounds possess chelating properties with regard to metals. The high iron content of the lichen may also explain the poor condition of the dark brown areas, some of which (in the case of the double-headed eagle on Caesar's shield) have almost completely disintegrated and could not be analyzed.

The dark brown color of the wool and the co-occurrence of hydrolyzable tannins in all three of the samples raise the additional possibility that the lichen was considered a quality of the tannin dye rather than its own organism. Tannin dyes such as oak bark yield lighter, more beige hues unless combined with an iron mordant, which is necessary to achieve darker browns, grays, and blacks [1]. If the lichen was growing on the bark of a tree used for dyeing, the lichen and the tannin source could be added simultaneously to the boiling dye bath as an ingenious way to avoid spending time and material on a separate mordanting step. Not only would the iron contained in the lichen modify the color and improve its longevity, but any pigments produced by the lichen (such as atranorin) may have provided additional nuance to its appearance. Professional dyers were—and still are—experts at using the materials provided by nature to achieve a complex range of hues. Even if they did not understand the precise chemical processes at play, they would have recognized this combination as a source of a fast, dark brown dye.

Whether sought out for its own dyeing capabilities or combined with its substrate, the lichen source in the tapestry appears to be the first example of a non-orchil lichen dye that has been identified in a historic object. Though the earliest literature evidence of BWM dyes is from the eighteenth century, some experts believe they have been in use for much longer [8]. One possible explanation for this gap in the literature could be that medieval dyers had a different understanding of their materials than modern researchers. They may have considered the lichen and its substrate not as separate organisms but as a beneficial union, much like the symbiotic relationship between mycobiont and photobiont that allows the lichen to survive. Although it is not possible to establish a dyer's intent through scientific means, these findings present the first indication that BWM lichen dyes could have been employed as early as the medieval period, centuries before the first literature evidence appears.

Though an initial goal of this study was to investigate the tapestry's origins, the novelty of evidence supporting the use of BWM lichen dyes during the medieval period makes interpreting these findings challenging. It is not currently possible to precisely identify the species from which the lichen metabolites were derived. However, we hope these findings will initiate further research leading to new discoveries and additional information. We are currently investigating brown dyes from other medieval tapestries in the Met's collection to understand the prevalence and use of lichen dyes.

5. Conclusions

High-resolution LC-qToF-MS was performed on yarn samples from a rare fifteenth-century tapestry from The Cloisters collection at The Metropolitan Museum of Art, suggesting the presence of a lichen dye source. Comparison with reference material confirmed the identity of several lichen metabolites. Though we could not identify any known lichen pigments in the tapestry, chlorinated xanthones, such as thiophanic acid, may prove to be valuable markers for certain lichen dyes due to their high stability and the uniqueness of their isotopic patterning.

This study presents the first time that lichen xanthones, and possibly a BWM lichen dye, have been identified in a historic object. This is a significant finding that raises additional questions about the use of lichen and the production of artistic materials during the medieval period. This work would not be possible without high-resolution mass spectrometry (HRMS), which can be used to identify unknown colorants at low concentrations and enables molecular-level insights into the use of organic dyes and pigments in art objects.

The insights provided by HRMS highlight the strength of a micro-invasive approach which, while requiring sampling from a precious cultural object, can uncover chemical fingerprints, even at low concentrations, of a complex mixture of known and unknown dyes. These deep insights have profound implications for understanding how art objects were made centuries ago and are especially critical for objects with lost or non-existent records, such as *Caesar* and the remainder of the *Heroes* tapestries.

The question of which lichen species (or mixture of lichen species) could have ended up in a French or Southern Netherlandish tapestry presents a compelling mystery and the need for additional experimentation. LC-qToF analysis of tapestries from the South Netherlands or France, particularly those with well-documented provenance, may allow us to understand how common these dye sources were and if their use was geographically specific or widespread. Work is ongoing at the Met to analyze the brown dyes in medieval tapestries, including but not limited to the rest of the *Heroes* tapestries from the series. Trace amounts of arthothelin, a trichlorinated xanthone identified in *Caesar*, have already been detected in another tapestry from the Met's collection, this time from the sixteenth century (data not shown). The results of this research may provide connections between objects that were previously hidden and deepen our understanding of the ancient art of lichen dyeing.

Furthermore, though there has been extensive analysis of orchil/AFM dyes, BWM lichen dyes in general have received minimal attention. This is unfortunate, considering the local nature of BWM dyes and the chemical specificity of lichen species. A thorough investigation of these lichen species, using both non-invasive and micro-invasive techniques, may reveal markers for local lichen dyes, which can be useful in cases where an object's origins are uncertain. We hope that these findings will spark renewed interest in these fascinating colorants within the fields of conservation science and art history.

Finally, this work highlights the power of a collaborative approach to studying works of art. The detection of unusual molecules in a medieval tapestry led to the unexpected connection of researchers from across the globe with specializations in lichenology, natural product chemistry, conservation science, and art history. Like a tapestry born from individual warps and wefts, the breadth and depth of this collective expertise unites as we weave a new history of lichen dyes from a single serendipitous discovery.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/heritage7050112/s1, Figure S1: *Lecanora sulphurata* extracted with acetone; Figure S2: *Lecanora sulphurata* after extraction with 0.01 M oxalic acid (aqueous)/pyridine/methanol solution (3:3:4 v/v/v); Figure S3: UV-visible spectrum (a) and MS/MS fragmentation spectrum (b) for the unidentified compound **10** with chemical formula $C_{14}H_6Cl_4O_6$ at 20.2 min in both the tapestry, *Lecanora sulphurata,* and *Lecidella asema*; Figure S4: 350 nm UV chromatograms for *Lecanora sulphurata* (top), *Lecanora alboflavida* (middle), and *Lecidella asema* (bottom) extracted with acetone; Table S1: Chemical composition of three lichen references: *Lecanora sulphurata, Lecanora alboflavida,* and *Lecidella asema*; Figure S5: 350 nm UV chromatograph of *Lecanora sulphurata* after boiling with wool for 3 h followed by mild extraction with oxalic acid.

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