RESEARCH

Combined, sequential dye analysis and radiocarbon dating of single ancient textile yarns from a Nazca tunic

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Abstract

The accessioning of ancient textiles into museum collections often requires objective information regarding the object's appropriateness and authenticity before purchase or gift acceptance. In the case of colored fabrics, the identification of dyestuffs consistent with the attributed time period and culture builds confidence and reduces the chances of the object being a simple forgery or fake produced using modern materials. Moreover, this information adds to the technical, cultural, and conservation knowledge regarding the object. Increasingly, chronometric age estimates in the form of radiocarbon dating are also needed to establish the object's age or to further prove the materials match the purported date range of the textile. Each of these analyses consumes a small sample of the object, and typically they are conducted separately by different laboratories on individual sample yarns. This report demonstrates for the first time the sequential, combined analysis of dyes by liquid chromatography-diode array detection-mass spectrometry and radiocarbon dating of the same residual dye-extracted sample. The chemicals and solvents used in various dye extraction protocols are shown not to contaminate the extracted yarns for radiocarbon dating purposes. The approach was used in the authentication study of an ancient Nazca tunic made from natural fibers (wool) and dyes (indigoids, anthraquinones, and flavonoids) shown to have most likely been produced between 595 and 665 CE.

Keywords: Dye analysis, Radiocarbon dating, Technical art history, Peruvian textile, Nazca culture, Archaeometry, Authenticity

Introduction

The purchase or gift consideration of ancient textiles entering museum collections can be fraught with concerns regarding the authenticity of the artifacts or the accuracy of information associated with the object. Forgeries entering museum collections waste valuable museum resources, pollute the art historical record, and deceive the public. As a result, textiles under consideration are usually inspected closely by curators and conservators for any "red flags" that might raise concerns over the appropriateness of the object before they are accepted into the collection. In some instances, diagnostic imaging and materials characterization are undertaken to understand the construction, condition, and composition of the textile. Dye analysis is increasingly used to assess the colorants present in objects to identify the artistic choices and materials utilized in ancient cultures, but also with the goal of recognizing anachronistic materials that could indicate a forgery, fake, or misattribution. Dye analysis can be telling for dating purposes since a plethora of synthetic colorants have been introduced since the late-1800s, and the first introduction date of most of these dyes is accurately known [1]. These modern dyestuffs can help to refine the dating of authentic museum textile artworks [2] or can unmask clever forgeries of purportedly ancient textiles [3, 4]. Despite significant progress in molecular

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techniques like Raman [5, 6] and UV–Vis-NIR reflectance spectroscopies [6], liquid chromatography with diode array detection coupled to mass spectrometry (LC–DAD–MS) has been described as the technique par excellence [7] for this type of analysis because of its small sample requirement, high sensitivity, and comprehensive analysis of all extractable components [8–11].

When only natural dyes are found, however, no reliable date can be established because these colorants have been generally known since antiquity and are still available and used today. In these instances, a chronometric date can help to verify that the object likely comes from the anticipated time period. Despite a long running debate following the dating of the Shroud of Turin [12, 13], radiocarbon dating of textiles is widely used to provide missing information about an object's temporal origins including from medieval relics [14, 15], Coptic textiles [16], Tibetan fabrics, and pre-Columbian ponchos [17]. With the development of accelerator mass spectrometry (AMS), radiocarbon dating laboratories commonly ask for 10–20 mg of material for routine analysis of textiles. This sample requirement, which represents an undesirably large area of textile, precludes sampling of many priceless objects. Continuous technological developments in ^{14}C analysis, particularly regarding sample introduction, now allow measurements from minute quantities of carbon on the order of tens of micrograms prepared as carbon dioxide gas [18, 19] and thus resolves to an extent the limited access to sample material in cultural heritage objects. In the context of canvas paintings, canvas yarns of 0.5 to 1 cm proved to be sufficient in providing reliable ages [20, 21], revolutionizing the microsampling strategy for artworks. The achieved sample size reduction now offers feasible sampling amounts on a similar size scale to that required for dye analysis.

The combination of dye analysis and radiocarbon dating is extremely powerful as together they give knowledge of the technology and artistic choices for coloration of textiles with an objective measure of the object's age. This makes the approach particularly useful in the study of museum objects through the field of technical art history. This combined application can also on occasion provide the required evidence of authenticity for purportedly ancient objects when only natural dyes have been used. For example, radiocarbon dating is exquisitely sensitive to modern (post 1950) natural organic materials that show elevated ^{14}C levels resulting from above-ground thermonuclear weapon tests in the 1960s. This "bomb pulse" carbon presents a twentieth century marker allowing accurate dating of contemporary art [22–24] but also particularly fruitful in unmasking modern forgeries or

modern alterations to an otherwise historic artifact [21, 25–28].

Dye analyses and ^{14}C dating are commonly conducted in separate specialized labs on individual samples collected specifically for one or the other analytical techniques. For instance, the highly decorative clothing of the Egyptian embroideress mummy known as Euphemia was recently analyzed by both radiocarbon dating of 21 samples from the fabrics and LC–DAD dye analysis of 29 additional embroidery threads [29]. Similarly, De Moor and coworkers distinguished mollusk purple dyes from common madder/indigo purples through chromatography of 19 samples taken from 21 Roman tunics that were previously radiocarbon dated [30]. Splitstoser et al. used the same approach to identify the indigoid dyes present on eight pre-Hispanic Peruvian textiles that had been separately radiocarbon dated [31]. Kramell and coworkers used both LC–DAD and LC–MS/MS techniques in their study of 27 dyed yarns from a Late Bronze Age site in Turfan, China with 10 of those textiles additionally being sampled for radiocarbon dating [32].

As normally practiced, the combined use of chromatographic dye analysis and radiocarbon dating requires at a minimum two samples, one for each analysis, although as mentioned previously, often many more are taken. While the informational value of these larger sampling campaigns is not to be questioned, repeated sampling may ultimately compromise the structure of a woven textile and is not always possible nor permitted in cases of little original material or a poor state of conservation. However, dye analysis normally leaves intact the dye extracted yarn, provided strong mineral acids are not used, and this residual material could be sufficient for subsequent ^{14}C dating using modern microanalytical methods. If the decolorized fiber bundle is retained and submitted for radiocarbon analysis, then further damage to the artifact could be avoided, or more samples overall could be completely analyzed for composition and age with less collateral impact on the artifact. Moreover, materials and dating information are then derived for the exact same sample location, ensuring the two types of information are properly correlated for the area sampled.

To this end, Armitage and Jakes [33] performed a novel experiment using direct analysis in real time mass spectrometry (DART-MS) on the pretreatment (cleaning) solution used to prepare yarns for plasma oxidation prior to radiocarbon dating. A 3 mg sample of red yarn was cleaned several times in a phosphate buffer prior to conversion of some of the organic carbon of the treated fibers by plasma oxidation into CO_2 for ^{14}C analysis by tandem AMS. A few microliters of the reserved phosphate wash liquid containing extracted dye was collected onto a glass capillary tip and placed into the ionization

gap of the time-of-flight mass spectrometer. This method was used to confirm the anthraquinones derived from bedstraw root (*Galium* spp.) were present on a Hopewell Period red dyed textile from the Sip Mound Group in Ohio.

Although this sequential analysis methodology yielded a combined radiocarbon date and major dyestuff identification for a single yarn, the MS technique lacks the separation step of a chromatographic method, struggles to ionize dye glycosides [11], and only identifies the pseudomolecular ion of the dye [7, 11]. As a result, molecules with identical masses like structural isomers (e.g., alizarin and xanthopurpurin, MW = 240.21 [7, 11]), conformational isomers (e.g., cis–trans Basic Red 46 [3], or anomers (e.g., dcIV and dcVII in cochineal [34]) would be indistinguishable by DART-MS but can be separated and identified by LC–DAD–MS. Extensive knowledge of all dye components present, including isobaric species like indigotin and indirubin (MW = 262.07), adds to the technical knowledge surrounding a dyed artifact and can yield a deeper understanding of this important artistic and cultural practice. This is exemplified, for instance, by Splitstoser et al.'s study of the earliest vat dying in ancient Peru, which identified unusually high levels of indirubin relative to indigotin in blue-dyed textiles, suggesting the use of natural dye plant species distinct from those available in Europe, or possibly vat conditions that promoted the uptake of indirubin in these dye baths [31].

Although the LC–DAD–MS technique is more involved, it yields additional colorant discrimination by providing a characteristic retention time, UV–vis spectrum, molecular weight, and fragmentation pattern for each resolved component [8]. Additionally, the separation of eluting species provides a visual indicator of the number of extracted components, even minor ones, that might be missed in a DART-MS spectrum of a dye mixture [7, 11]. As such, the combined, sequential use of LC–DAD–MS and radiocarbon dating on a single textile yarn could be a powerful combination that maximizes data from a single sample. However, if used prior to radiocarbon dating, the typical dye extraction protocols utilized in LC analysis introduce numerous sources of potential carbon contamination since additional glassware, numerous solvents, and organic acids are routinely used. The research described here explores the potential of conducting radiocarbon dating on yarns retained following a few typical dye extraction methods for LC–DAD–MS analysis of the colorants.

The proposed analytical approach was applied to the study of a Nazca dyed wool tunic, Fig. 1, purportedly dated to the period 100 BCE–600 CE, that was a purchase consideration at the Indianapolis Museum of Art at Newfields (IMA). The Nazca (or Nasca) culture prevailed along the coastal desert of modern-day Peru between ~ 1 and 700 CE [35, 36] and produced a rich textile history [35]. The tunic is rectangular in shape with fringe along both short edges and an opening in the center where two long rectangles are hand-sewn together. The stepped pattern, Fig. 1b, is created in rows of off-white, brown/black, red, and orange and aligns nicely across the two pieces of fabric. The weaving technique of the textile was identified as a complex form known as interlocking of discontinuous warps and wefts [37].

Fig. 1 **a** *Tunic*, Nazca culture, purportedly 100 BCE–600 CE, camelid wool, 84 × 50 in., Roger G. Walcott Fund, 2021.177. **b** Detail of pattern

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Although provenance information regarding its archaeological context is now lost, this Nazca artifact has a reputable legal standing. It was purchased in the second quarter of the twentieth century by a wholesale coffee trader and collector, Hans-Jurgen Westermann. In preparation for an auction of the collection, a tariff classification ruling was obtained on December 19, 2002 (control number 9706.00.0060), which declared that all of the items, including the Nazca tunic, were obtained prior to the 1973 UNESCO Treaty relating to pre-Columbian exportation regulations. It was purchased for a Hawaiian collection prior to being offered for purchase consideration to the IMA. Upon arrival at the museum, the condition of the textile, ostensibly from a burial context [35], was noted as remarkable due to the intense coloration, scarcity of stains, lack of extensive restorations, and the supple nature of the fibers for its purportedly 1500-year-old history. Despite the solid legal provenance, the exceptional condition of the textile for its purported age justified scientific analysis, which was agreed to by the dealer for a limited number of samples, thus spurring the research described here on combined dye analysis and radiocarbon dating of single, small yarns.

Experimental

Yarn samples

Five yarn tails were removed with tweezers and embroidery scissors from the sides and lower edges of the tunic in areas where their removal would not compromise the structural integrity of the textile, Fig. 2. These areas did not appear to have any restorations. The textile samples were all z-twisted, S-plied double yarns (2zS). The samples included colorless, orange, red, dark brown, and black wool, numbered 1–5, respectively. The yarns ranged in size from ~1–2 cm, more than the minimum needed for dye analysis, but sufficient to allow each sample to be subdivided for at least two extraction

Fig. 2 Yarns sampled from the Nazca tunic

experiments and to retain an unextracted portion as a control for radiocarbon analysis.

Dye extraction

Dyed yarns were extracted using three different approaches common in the cultural heritage field in order to study the impact of various preparation methods on the resulting radiocarbon dates. These extraction methods have evolved to deal with issues surrounding the need to hydrolyze mordant dyes to free them from the fiber while considering different dye component's solubilities and their sensitivity to strongly acidic treatments [9]; moreover, sometimes multiple different sequential treatments are needed to fully extract a mixture of dyes [2]. Table 1 lists the samples and their extraction steps. All glassware was heated to 500 °C in a furnace for 30 min and then stored in aluminum foil prior to use to reduce carbon contamination. All materials that could not withstand the furnace temperature were washed with methanol (MeOH, Fisher Optima), shaken dry, and stored in aluminum foil.

For the first extraction approach, a small yarn was pretreated with gaseous HF, which is considered a "mild" treatment in that it is only weakly acidic and preserves glycosides that can be important for species identification of botanical dyes [38, 39] while still reacting strongly with mordant metals, freeing the dye [40]. The yarn was placed on a PTFE sheet next to a single drop of 50% aqueous HF (Acros) and covered with a PTFE lined vial cap to create a small vapor chamber overnight. The fiber bundle was then placed in a 2 mL glass vial and extracted with 200 µL of 1:1 mixture of MeOH and water (MilliQ 18.2 MΩ) at 80 °C for 1 h with occasional shaking. The extract was pipetted into an autosampler vial and centrifuged to remove any interfering solids and was reserved for analysis. For the second extraction procedure, the MeOH/water solvent was used to prepare a 4 mM solution of oxalic acid (Acros, extra pure). A second, fresh yarn sample was then extracted with this solution in an identical way to the previous example and the extract solution was stored separately.

For yarns that were suspected to be colored with an indigoid, e.g. brown and black, a second sequential extraction was undertaken using a third method. The previously HF-treated yarn was re-extracted with 200 µL of dimethyl sulfoxide (DMSO, Thermo LCMS grade) heated to 80 °C for 2.5 h. Vat dyes like indigoids show increased solubility in DMSO compared to other common solvents like methanol, water, or even dimethylformamide [41]. The DMSO extraction liquid was then removed and stored separately, and the fiber was washed with MeOH:water to remove the residual DMSO, which itself is slow to evaporate, and then dried under vacuum.

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Table 1 Sample identifiers, observed colors, extraction protocols, and dyes identified by LC–DAD–MS

Sample	Color	Sample	Fiber weight/mg	1st Extraction		2nd Extraction		Possible dyestuff
				Method	Dyes identified	Method	Dyes identified	
1	Colorless	A	1170	Not attempted				
2	Orange	A	386	HF–MeOH/water	6-methoxyquercetin-3-O-glucoside, quercetin-3-O-glucosides, unk. anthraquinone 1, kaempfereol-3-O-glucoside, isorhamnetin-3-O-glucoside, unk. anthraquinone 2, lucidin primeveroside, quercetin-3-O-sulfate, quercetin, 6-methoxyquercitin, kaempferol, isorhamnetin, pseudopurpurin, munjistin, xanthopurpurin, purpurin, rubiadin	None attempted		*Relbunium, F. haumanii*
		B	488	Oxalic–MeOH/water	6-methoxyquercetin-3-O-glucoside, quercetin-3-O-glucosides, unk. anthraquinone 1, kaempfereol-3-O-glucoside, isorhamnetin-3-O-glucoside, unk. anthraquinone 2, lucidin primeveroside, quercetin-3-O-sulfate, quercetin, 6-methoxyquercitin, kaempferol, isorhamnetin, pseudopurpurin, munjistin, xanthopurpurin, purpurin, rubiadin	None attempted		
3	Red	A	1110	HF–MeOH/water	6-methoxyquercetin-3-O-glucosides, quercetin-3-O-glucosides, unk. anthraquinone 1, kaempfereol-3-O-glucoside, isorhamnetin-3-O-glucoside, unk. anthraquinone 2, lucidin primeveroside, quercetin-3-O-sulfate, quercetin, kaempferol, pseudopurpurin, munjistin, xanthopurpurin, purpurin, rubiadin	None attempted		*Relbunium, minor flavonoid yellow*
		B	1322	Oxalic–MeOH/water	6-methoxyquercetin-3-O-glucosides, quercetin-3-O-glucosides, unk. anthraquinone 1, kaempfereol-3-O-glucoside, isorhamnetin-3-O-glucoside, unk. anthraquinone 2, luciden primeveroside, quercetin, 6-methoxyquercetin, kaempferol, pseudopurpurin, munjistin, xanthopurpurin, purpurin, rubiadin	None attempted		

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Table 1 (continued)

Sample	Color	Sample	Fiber weight/mg	1st Extraction		2nd Extraction		Possible dyestuff
				Method	Dyes identified	Method	Dyes identified	
4	Brown	A	456	HF–MeOH/water	6-methoxyquercetin-3-O-glucoside, quercetin-3-O-glucoside, isatin, kaempfereol-3-O-glucoside, isorhamnetin-3-O-glucoside, quercetin-3-O-sulfate, quercetin, 6-methoxyquercitin, kaempferol, isorhamnetin, indigotin, indirubin	DMSO	indigotin, indirubin	natural indigoid, flavonoid yellow
		B	526	Oxalic–MeOH/water	6-methoxyquercetin-3-O-glucoside, quercetin-3-O-glucoside, isatin, kaempfereol-3-O-glucoside, isorhamnetin-3-O-glucoside, quercetin-3-O-sulfate, quercetin, 6-methoxyquercitin, kaempferol, isorhamnetin, indigotin, indirubin	None attempted		
5	Black	A	546	HF–MeOH/water	isatin, pseudoindirubin, pseudoindirubin-2, indigotin, indirubin	DMSO	isatin, pseudoindirubin, indigotin, indirubin	natural indigoid
		B	570	Oxalic–MeOH/water	isatin, pseudoindirubin, pseudoindirubin-2, indigotin, indirubin	None attempted		

Tabulated and graphic examples of retention times, absorption maxima, and MS data for the compounds identified are available in Additional file 1: Table S1 and Fig. S2

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None of the threads were completely decolorized by these extraction procedures. In each instance, the fiber bundle came unwound, making retrieval of the individual yarn impossible. Instead, the fiber mass was washed several times in the storage vial with deionized water using a Hamilton syringe to remove the wash water. The fiber mass was then dried using heat and a gentle stream of nitrogen before being recapped and shipped to the radiocarbon dating facility at ETH-Zürich.

Liquid chromatography–diode array detection–mass spectrometry (LC–DAD–MS)

LC–DAD–MS analysis was performed separately on the dye solution from each sample extraction method using a Thermo Accela LC system coupled in sequence to a DAD and an LTQ linear ion trap MS detector with an electrospray ionization source. The entire system was under the control of Thermo Xcalibur v4.0 software. Because historic sample sizes were necessarily limited and the nature of the colorants and their mixtures were unknown, the gradient method was intentionally generic, capable of identifying a wide array of natural and synthetic colorants. Dye separation was carried out using a Restek Ultra C18 reverse phase column (150 mm × 4.6 mm, 5 μm particle) eluted at 0.2 mL/min using a water (MilliQ 18 MΩ)-acetonitrile (Fisher Scientific Optima) gradient system containing 0.1% formic acid (Fisher Scientific Optima). The column was equilibrated at 10% acetonitrile, and after 6 min following sample injection, analyte separation was achieved by applying a linear gradient of acetonitrile increasing from 10 to 90% over 70 min. The DAD detector was set to record spectra in the range 200–800 nm at 20 Hz. The MS collected a full-scan mass spectrum (FSMS) in the m/z range 50–1000 followed by two MS/MS spectra of fragment ions resulting from helium collision-induced dissociation (CID), respectively of the highest and second highest abundant ions detected in the FSMS. The sequence alternated rapidly between positive and negative ionization modes. Dye extract solutions were centrifuged, and 25 μL was injected for analysis. Dye components were identified based on comparisons to literature data and/or the analysis of authentic reference samples purchased from natural dye suppliers (e.g. Maiwa) or chemical warehouses (e.g. Sigma Aldrich).

Radiocarbon dating

The typical sample preparation for textiles submitted for ^{14}C analysis involves the traditional acid–base-acid (ABA) preparation, which here comprised an initial acidic wash (0.1 M HCl, p.a. Supelco) for 15–30 min at 60 °C, followed by an alkali wash (0.5 M NaOH, p.a. Supelco) at room temperature for 10 min, and a final acid wash [42], where the reaction time and temperature can be adjusted depending on the sample size, textile type, and state of conservation. Due to the observed pronounced fiber degradation (vide infra), only the three red samples (>1 mg) were subjected to the whole ABA procedure while none of the other samples went beyond the initial acidic wash.

All ^{14}C measurements were carried out on the Mini Carbon Dating System MICADAS [43]. The cleaned samples were directly measured as carbon dioxide after combustion in an elemental analyzer (EA) coupled to the gas ion source of the MICADAS [19]. The entire microsample is consumed in this process preventing any further reanalysis of the exact same material. Data reduction was performed using in house BATS software [44] prior to constant contamination correction ($F^{14}C_C = 0.90 \pm 0.14$ and $m_c = 1.0 \pm 0.4$ μg C) [45]. The corrected radiocarbon ages were further calibrated to real calendar ages with the calibration software OxCal v.4.4 (https://c14.arch.ox.ac.uk/oxcal/OxCal.html) [46] with the SHCal20 southern hemisphere calibration curve [47].

Results and discussion
Dye analysis

The chemical analysis of ancient Peruvian textile dyes has a long history that parallels the development of ever more sophisticated instrumental techniques in cultural heritage. Some of the earliest comprehensive work on pre-Columbian dyes was conducted by Max Saltzmann using thin-layer chromatography and UV–visible spectroscopy [48], and the same approaches were used by Wallert and Boytner in their study of ancient, dyed textiles on the southern coast of Peru [49], while Tiedemann and Yang used spectroscopy alone to assess red dyes in textiles from a number of pre-Columbian civilizations [50]. Wouters improved upon those studies by using HPLC–DAD analysis of HCl/MeOH/water extracts of dyed yarns from textiles originating from a number of ancient South American cultures, including the Nazca [51]. Michel and coworkers used mass spectrometry to investigate blue and purple dyes in Peruvian textiles [52], and an array of Raman spectroscopies have also found their place in the analysis of dyestuffs from pre-Columbian Peru [5, 6] as has reflectance spectroscopy [6]. The combination of LC separation and MS detection of Peruvian textile dyes has been recently practiced by several researchers [11, 38, 53, 54]. The latest developments include fast ambient ionization MS approaches without prior dye component separation by techniques like DART-MS, paperspray MS, or flowprobe-MS to quickly assess blue and red Peruvian textile threads for their colorants [7, 11, 33, 55], although yellow dyestuffs have been more difficult to identify [7].

Through these studies, a significant amount of information has been compiled regarding the dyes used throughout pre-Columbian Peru where a large number of dye plants and insects have been utilized for textile coloration [56]. In general, blue colors tend to be sourced from sea snails or indigoid plants [49, 51, 52], the latter of which is often noteworthy for the unusually high level of extracted indirubin in comparison to European examples [54, 57]. Similarly, very dark colors such as blacks and dark browns can also come from the use of indigoids for very deep dying [54]. White, tan, cream, and brown colors can be attributable to the natural coloration of the fibers, although the use of tannins for darker colors is also possible [7, 54, 56]. Reds and oranges arise from both insects (cochineal) [11, 49–51, 53] as well as anthraquinone bearing plants, especially *Relbunium* and *Galium* species. The *Relbunium* species is distinct from the traditional European madder plant (*Rubia* sp.) and the native Peruvian *Galium* by the absence of alizarin and predominance of purpurin [7, 11, 49, 51, 54, 58]. Peruvian yellow dye sources are plentiful [56] and many rely on flavonoids for their coloration. As a result, specific identifications of yellow dye plant species used to dye textiles have been harder to come by [7, 38, 54].

Of the samples from the Nazca tunic described here, the colorless yarn appeared to be an undyed camelid wool and was therefore not extracted for dye analysis. It served as a control in studying the potential impacts of

dye extraction on radiocarbon dating. Figure 3a shows the chromatogram generated in the LC–DAD–MS analysis of the extract from the orange yarn sample, 2A, which was extracted with MeOH:water following vapor phase HF pretreatment. The chromatogram shows major peaks whose DAD spectra and MS data (Additional file 1: Table S1 and Fig. S2) are consistent with anthraquinones commonly found in *Relbunium* plant dyestuffs including pseudopurpurin, munjistin, xanthopurpurin, purpurin, and rubiadin as reported in the literature [7, 11, 51, 54]. However, minor anthraquinones reported by Armitage et al. were not observed in the extract even with selected ion monitoring; namely, 1-methoxy-2-methylanthraquinone (m/z 251), 2-methoxy-2-methylanthraquinone (m/z 237), and the primeverosides of xanthopurpurin (m/z 533), rubiadin (m/z 547), and pseudopurpurin (m/z 593) [11]. Lucidin was also not detected, but its glycoside, lucidin primeveroside was observed due to the mild extraction procedure [59].

Moreover, two previously unreported anthraquinone-like compounds, labelled unk. anthraquinones 1 and 2, were found in the orange yarn extract (Fig. 3a) and also in the red yarn extract (Additional file 1: Fig. S3). These peaks give even numbered [M–H]$^-$ ions in the FSMS spectrum, suggesting compounds having an odd number of nitrogens, and yield MS/MS fragment ions that show a mass loss of 17 (Additional file 1: Table S1 and Fig. S2). Based on the mass similarities to other anthraquinones
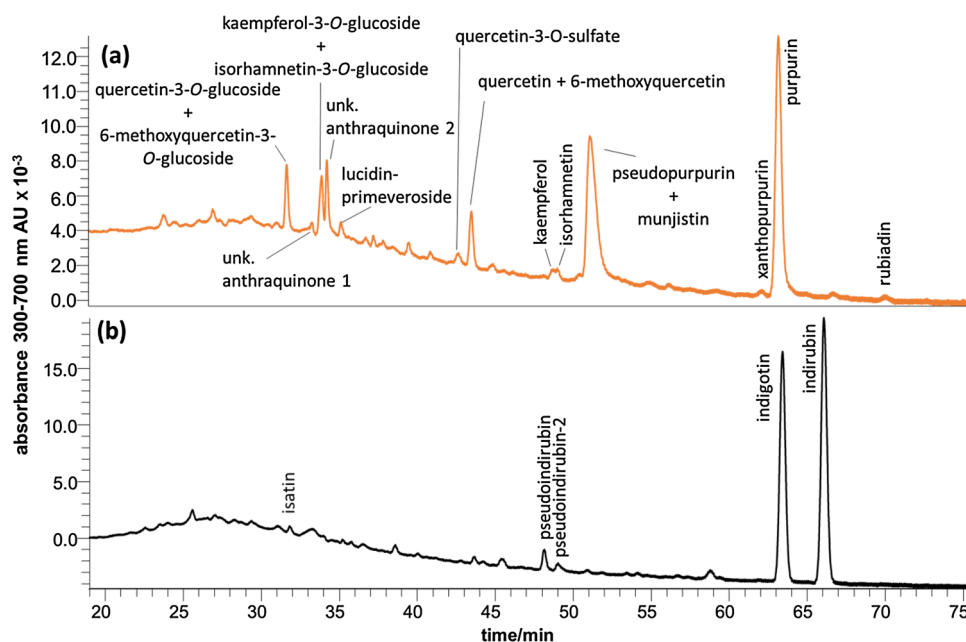

Fig. 3 Chromatograms from the analysis of **a** the orange yarn 2A after HF-MeOH/water extraction and **b** the black yarn 5B after oxalic acid-MeOH/water extraction

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observed in the extracts, it is possible that unknown anthraquinone 1 could be an isomer of amino rubiadin (m/z $253 + 15 = 268$) while unknown anthraquinone 2 could be an isomer of amino lucidin (m/z $269 + 15 = 284$), although these assignments are purely speculative. The source of these previously undetected compounds, whether present in the dyestuff originally or produced through the dying process, requires further investigation.

The orange extract also contained yellow flavonoid compounds, The same collection of nine separated and identified aglycones, glycosides, and sulfate compounds were observed by Zhang et al. in *Flaveria haumanii* studied as part of their investigation of yellow pre-Columbian dyed textiles [38]. The orange yarn is therefore a mixture of a red anthraquinone plant dye, most likely a *Relbunium* species based on the significant presence of purpurin and lack of evidence of alizarin, combined with a yellow flavonoid plant dye, possibly F. *haumanii*. The portion of the yarn extracted with oxalic acid provided nearly identical results, showing that either of the "mild" acid hydrolysis procedures used here is viable for comprehensive dye analysis. The use of *Relbunium* and a yellow dye plant to produce orange colors in ancient Peruvian textiles has been documented by Campos Ayala et al. [7], although in some instances orange appears to have been generated with an anthraquinone dye alone. It is important to note that the use of different mordants can also impact the color of anthraquinone dyes. The red yarn, Sample 3 (Additional file 1: Fig. S3), regardless of its preparation method, provided the same grouping of primary anthraquinones, along with much lower relative levels of some of the same flavonoids, indicating the likely use of *Relbunium* to produce deep red shades as commonly observed in other published studies of historic Peruvian textiles, but with either some minor intentional addition of perhaps the same F. *haumanii* yellow flavonoid dyestuff or possibly chance contamination or bleeding from adjacent areas containing yellow flavonoid dyes.

Figure 3b shows the chromatogram of the oxalic acid-MeOH/water extract of the black yearn, sample 5B. Indigoid related species like isatin, indigotin, and indirubin were identified. The presence of both isomers is indicative of a natural indigo dye since indirubin is not found in synthetic indigo. The ability to resolve and identify these isobaric indigoid species demonstrates the power of the LC–DAD–MS technique. Moreover, the relative intensity of the indirubin peak with HF or oxalic acid extraction was always significant compared to similar analyses of European indigo dyed fibers, an observation routinely commented on by researchers studying historic South American indigoid plant dyes [7, 51, 54, 57]. However, some researchers report variability in this indigoid ratio

based on sample extraction and treatment [7, 41], an observation also made here (vide infra).

In addition, the relatively newly described indigoid compound, pseudoindirubin, was detected in the strongly dyed black yarn. This compound was first identified by Laursen and Mouri as a possible marker for woad-based indigo in European textiles [60] and may also be the "indirubin-like" compounds observed later by Splitstoser et al. [31]. Laursen and Mouri also reported pseudoindirubin in two brown/black Nazca funerary textiles from Peru as well as from a native indigo producing tree *Cybistax antisyphilitica*, which has been used historically as a source for blue dye as well as medicine in Peru [56]. Interestingly, pseudoindirubin has not been identified in the common *Indigofera* species analyzed so far, nor in synthetic indigo. A second, related peak, coined pseudoindirubin-2 and thought based on its UV–vis spectrum to be an isomer of pseudoindirubin [60], also appears in the chromatogram of the black yarn extract. Importantly, this research further supports its identity as an isomer since the FSMS spectrum showed the same $[M–H]^-$ molecular ion (m/z 500, Additional file 1: Table S1 and Fig. S2) as pseudoindirubin.

While both initial "mild" extraction protocols allowed for identification of the plant-based indigoid dyestuff in the black yarns, the second extraction of Sample 5A in hot DMSO removed significantly more indigotin, which is less soluble than indirubin in MeOH/water. When a fresh sample of the black yarn was extracted with oxalic acid/water/DMSO, the resulting chromatogram revealed that indigotin, seen to overload the column and present a large tailing peak, is still the dominant chromophoric dye species (Additional file 1: Fig. S4), although the indirubin component remained significant.

The same major indigoid dye components were also detected in the brown dyed tunic yarn, Sample 4, using either the HF or the oxalic acid extraction methods (Additional file 1: Fig. S5). However, no pseudoindirubin or its possible isomer were observed, perhaps due to the lower total levels of indigo dye present. In addition, significant relative amounts of flavonoid compounds were also extracted, suggesting that the brown color could arise from the intentional combined use of indigo and a yellow flavonoid-based dye plant. This is somewhat unusual in that brown colors in Nazca dyeing so far analyzed come from the natural wool color [7], from combinations of anthraquinone reds and indigoid blues [51], or from the use of tannins [7]. It is also possible that the major brown colorant is either melanin from the use of dark wool or tannins from dyeing, both of which can be difficult to extract and analyze by normal LC–MS procedures [7], although special methods have been developed

to ascertain their presence. If these unextracted colorants are present in the brown fibers, then the indigoids and flavonoids detected might actually be minor contributors to the yarn's color.
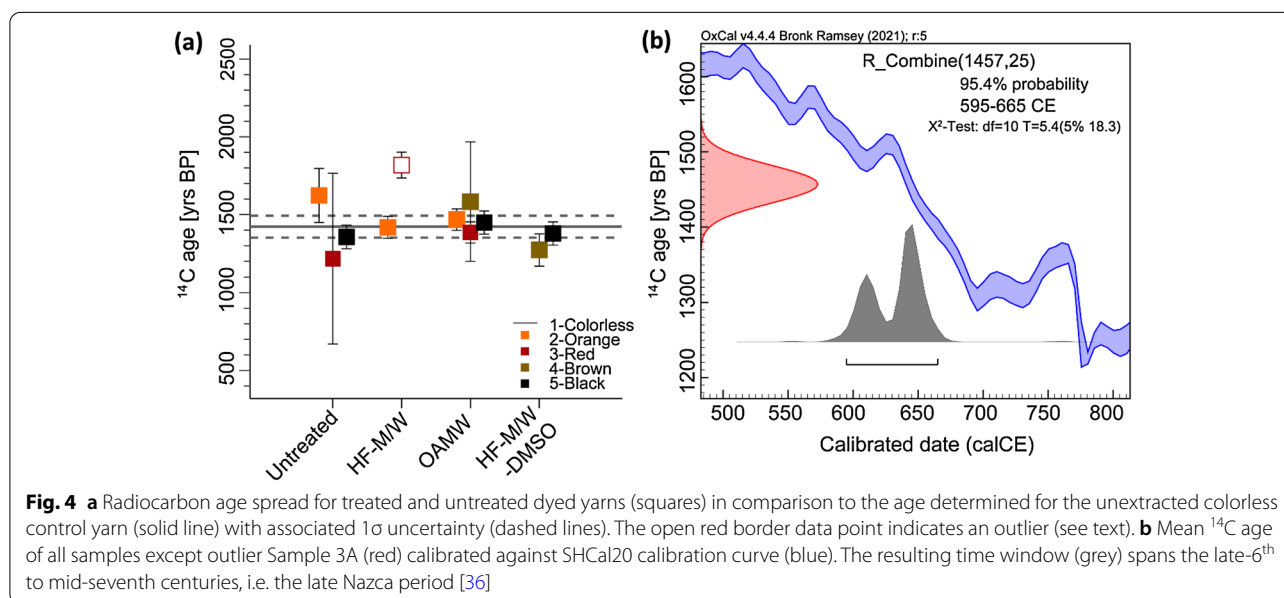
Radiocarbon dating

A total of 13 samples from 5 threads taken from the tunic were prepared for radiocarbon dating. The residual fiber samples from the dye analysis experiments along with unextracted portions of each colored thread and

the untreated, colorless yarn (Sample 1) were analyzed. The ^{14}C ages and associated data are reported in Table 2 and visually displayed in Fig. 4. The impact of the three extraction procedures is discussed, including hydrofluoric acid hydrolysis with MeOH/water extraction (HF-M/W), oxalic acid hydrolysis in MeOH/water extraction (OAMW), and DMSO extraction of yarns previously extracted with MeOH/water (HF-M/W-DMSO).

Following dye extraction, the residual fibers showed different appearances, having lost between 20 to 80% of

Table 2 Sample name and treatment, respective ETH lab code, starting sample size, recovered mass following sample treatment, respective recovery, resulting carbon mass available for measurement, radiocarbon age and uncertainty, and final calibrated age range with OxCal and SHCal20 calibration curve (95.4% probability)

Sample	ETH label	Mass (mg)		Yield	C (µg)	^{14}C age $\pm$ σ (yrs BP)	Calibrated date (2 σ)
		Start	Cleaned	(%)			
1 Colorless untreated	113,951.1.1	0.830	0.217	26	92	1470 ± 67	438–773 CE
2 Orange untreated	113,952.1.1	0.770	0.048	6	11	1661 ± 165	26 BC–771 CE
2A Orange HF-M/W	113,953.1.1	0.386	0.275	71	130	1466 ± 67	442–773 CE
2B Orange OAMW	113,954.1.1	0.488	0.404	83	186	1513 ± 66	425–670 CE
3 Red untreated	113,955.1.1	2.400	0.010	0.5	4	1275 ± 522	515 BC–1669 CE
3A Red HF-M/W	113,956.1.1	1.110	0.096	9	38	1847 ± 79	30–412 CE
3B Red OAMW	113,957.1.1	1.322	0.440	33	100	1434 ± 65	530–827 CE
4 Brown untreated	113,958.1.1	0.488	0.000	0	0	–	–
4A Brown HF-M/W-DMSO	113,959.1.1	0.456	0.051	11	9	1328 ± 100	595 BC–987 CE
4B Brown OAMW	113,960.1.1	0.526	0.013	2	5	1623 ± 366	406 BC–1207 CE
5 Black untreated	113,961.1.1	0.450	0.131	29	57	1407 ± 72	543–871 CE
5A Black HF-M/W-DMSO	113,962.1.1	0.546	0.248	45	103	1428 ± 71	526–851 CE
5B Black OAMW	113,963.1.1	0.376	0.338	90	78	1495 ± 71	426–768 CE

Fig. 4 **a** Radiocarbon age spread for treated and untreated dyed yarns (squares) in comparison to the age determined for the unextracted colorless control yarn (solid line) with associated 1σ uncertainty (dashed lines). The open red border data point indicates an outlier (see text). **b** Mean ^{14}C age of all samples except outlier Sample 3A (red) calibrated against SHCal20 calibration curve (blue). The resulting time window (grey) spans the late-6th to mid-seventh centuries, i.e. the late Nazca period [36]

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their color and being mostly unraveled. Owing to the small sample sizes and poor sample integrity, the traditional ABA treatment had to be adjusted. The first strong acidic hydrolysis was shown to largely decolorize the red and orange yarns, but unfortunately it also destroyed the fiber structure with 10 to 100% mass loss. In fact, the untreated brown thread (113,958.1.1) was completely lost and therefore no date was obtained for this sample. Based on observations from applying the complete ABA protocol on the red dyed yarns, it was obvious that the alkali step decomposed the camelid fibers. This was deemed an unnecessarily harsh treatment and was not pursued on the other extracted fiber masses, which were subsequently only cleaned using the initial acid wash.

These findings have important implications for further development of the approach as not only the sample size, but also its integrity and fiber type are relevant. When the sample no longer exists as a yarn, but rather as an unwound mass of fibers, the handling becomes difficult and can result in substantial sample loss. Furthermore, unlike cellulosic fibers, proteinaceous fibers are much more prone to hydrolysis under both strongly acidic and alkaline conditions. Strong alkaline hydrolysis processes are known to result in degradation of wool fibers as the disulfide bonds are broken [61]. These results highlight the necessity to adjust reaction time and temperature to prevent irreversible sample destruction while still ensuring adequate removal of exogenous carbon contamination prior to ^{14}C analysis.

Radiocarbon dating of the uncolored control thread, Sample 1, with no extraction treatment yielded a ^{14}C age of 1470 ± 67 yrs BP, which upon calibration to real calendar dates provides a time window of 438–773 CE, consistent with the Nazca period in pre-Columbian Peru. Generally, the dates obtained from all colored threads that underwent different dye extraction procedures as well as the unextracted subsamples agree within 1 σ. Although LC analysis introduces numerous sources of potential carbon contamination through additional handling steps and numerous organic solvents and acids, none of the tested protocols were shown to interfere with ^{14}C dating. The washing of the extracted fibers with deionized water to remove the extraction solution, then drying under nitrogen is successful in preventing exogenous carbon cross contamination prior to ^{14}C analysis, validating the protocol developed here. Regardless of the treatment method, all returned dates agree, i.e. yielding the same conclusion, namely that the tunic dates to the Nazca culture. Furthermore, if only one of the yarns had been sampled and dated, overlap with the Nazca period would have still been confirmed in all cases (see Additional file 1: Table S6 and Fig. S7).

From Fig. 4a, the colored thread ages scatter around the value of the colorless control yarn with only a small variance. The observed variability matches that observed in an earlier study [20] that showed that the measurements on individual yarns (10–50 μg C) presented a marginally larger variability, whereas larger samples (>200 μg C) averaged out individual characteristics. While all samples agree within a 1 σ interval, smaller samples bear larger uncertainties that are due to counting statistics. AMS measurements rely on direct counting of ^{14}C atoms present in the sample, which for samples>200 μg C allows precision down to 2‰ error [62], whereas samples below 100 μg C typically yield uncertainties ranging between 0.5 to 2% [63, 64]. These errors increase further from constant contamination because of error propagation (Additional file 1: Table S6). This effect is particularly visible in the dating of the brown yarn extract Sample 4B where the ^{14}C age was acquired on as little as 5 μg C. Following constant contamination correction, the associated error increases to several hundred years. Upon calibration to real calendar ages the achieved time window includes the period of interest (Additional file 1: Fig. S7), but it also extends beyond to both older and younger ages as a result of this uncertainty.

Regardless of the varying uncertainties and respective broader calibrated calendar ranges for the individual data points, all ^{14}C ages tend to the same mean value except one, Sample 3A, shown in Fig. 4a as an open red square. This subsample of the red yarn was extracted using HF-MeOH/water (ETH-113956.1.1) and gave a radiocarbon age of 1847 ± 79 yrs BP, which in comparison to the other 11 samples is older, i.e. an outlier. From the different hypotheses to explain this odd behavior, one can exclude the sample's size as a potential issue since it was one of the largest used in the study with 38 μg of C available for dating; this sample should not be affected by constant contamination. The applied constant contamination correction is shown to adjust the other samples' ages adequately, and so this potential source of error cannot be the source of the problem. The dye analysis extraction procedure is also not at fault since the other extractions involving HF-M/W or HF-M/W followed by DMSO yielded consistent results with the other extraction methods and the unextracted controls. While a robust explanation could not be found, Sample 3A is the only one showing a bias, and so the problem is most likely linked to that specific thread section (was it a portion contaminated with an exogenous material?) or to the handling of the specific sample (was the sample vial or pipette tip improperly cleaned?). Regardless of the source of the contamination, the exogenous material is most likely ^{14}C depleted, as upon constant contamination correction, the resulting age is overcorrected. Unfortunately,

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the combustion process used in the ^{14}C dating is destructive and so a repeat analysis of this yarn subsample is impossible.

A mean value may be calculated from the pooled ^{14}C ages using the "R-Combine()" function in OxCal. Figure 4b shows the resulting averaged ^{14}C age calibrated to real calendar ages. Within this approach, the validity of the final combined result is checked using a χ^2 test providing a measure for internal consistency, i.e., how well do the ^{14}C dates agree among each other. The χ^2 test proves Sample 3A to be an outlier, as when combining all 12 dates the test fails being that the value is larger than the expected 5% deviation given by the 11 degrees of freedom. In contrast, when considering only the other 11 sample dates and omitting Sample 3A, the test is passed, thus confirming the hypothesis. Leaving aside this suspect sample's date, a mean value radiocarbon age of 1457 ± 25 yrs BP is calculated at the 95% confidence level, which upon calibration to real calendar dates provides a time window of approximately 595–665 CE. Not only does the radiocarbon dating confirm the original Nazca attribution (100 BCE–600 CE), but it also provides a more definite origin for the object pointing to the waning years of the Nazca civilization [36].

Conclusions

Dye extraction by "mild" hydrolysis conditions allowed natural plant-based dyes to be identified in the four colored threads removed from the tunic. The presence of purpurin and no detectable alizarin is common in historic red Peruvian dyed textiles, likely indicating the use of a *Relbunium* species of plant. When mixed with a flavonoid rich dyestuff, orange color was produced. Dark browns and blacks were generated through deep dyeing with a plant-based indigoid dyestuff, notable for its significant indirubin content and the presence detected here of pseudoindirubin. These results are consistent with literature related to the analysis of dyes used by the Nazca during the period in which the tunic was presumably woven.

The subsequent ^{14}C analysis of the extracted threads showed that the dye analysis extraction procedures were compatible for subsequent dating, rendering the combined approach amenable to common practice in the field today. In this case, radiocarbon ages were acquired from sample threads weighing less than half a milligram, and their mean value provided a date for the artifact between 595 and 665 CE, or the late Nazca period [36]. Overall, the approach described here reduces artifact damage due to sampling compared to methods commonly employed while generating correlated compositional and age information from each single yarn. The multi-sample strategy

here builds confidence that the object is consistent in its dating, that heavy restorations are not present, and revealed outliers through statistical analysis.

Based on the demonstrated presence of period, natural, and widely reported dyes for the Nazca culture, combined with radiocarbon dates consistent with the purported age of the textile, the IMA purchased and accessioned the Nazca tunic into its permanent collection in 2021. As a result of these experiments, the "tombstone data" associated with the artifact and incorporated into wall didactics and online resources was changed to include the averaged date determination from the radiocarbon measurements, 595 to 665 CE. Current efforts are underway to reduce the size of the yarn sample used for sequential dye analysis and ^{14}C dating to further improve the technique described here.

Supplementary Information

Additional file 1: Table S1. Table of LC–DAD–MS data of dyes. **Figure S2.** Library of UV–vis, FSMS, and MS/MS spectra for dye compounds identified in this study sorted by retention time. **Figure S3.** Chromatogram of red fiber extract, Sample 3A. **Figure S4.** Chromatogram of black fiber, Sample 5, extracted with oxalic acid/water/DMSO. **Figure S5.** Chromatogram of brown fiber extract, Sample 4A. **Table S6.** Table of ^{14}C dating data. **Figure S7.** Calibration plot of ^{14}C dating data.

Author contributions
GDS Conceptualization; Original Draft; Reviewing and editing; Resources; Funding acquisition; Project administration VJC: Methodology; Investigation; Review and editing AH: Conceptualization; Review and editing; Funding acquisition LH: Conceptualization; Investigation; Formal analysis; Original draft; Review and editing NH: Investigation; Review and editing. All authors read and approved the final manuscript.

Availability of data and materials
The data is available upon reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

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