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High-resolution mass spectrometry identification of dye compounds and their degradation products in American cochineal from a historic shipping cargo

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ABSTRACT

Cochineal dyes constitute paradigmatic organic compounds that guide in the elucidation of historic cultural and economical exchanges. This study combines liquid chromatography and high-resolution tandem mass spectrometry to assess the degradation products of the organic dyes of American cochineal collected from the cargo of a 16th century sunken wreck. The identification of biological materials of historical and archeological origin is often challenging due to molecular degradation. In this scenario, the mapping of the chemical routes underlying the fate of the organic and biochemical compounds employed as taxonomic biomarkers becomes of crucial importance. This work shows that, under harsh environmental conditions, the original carminic and (flavo) kermesic acid structures of cochineal dyes are prone to chemical transformation, typically losing the carboxylic acid and glucoside groups. The anthraquinone core is preserved in the majority of degradation products identified in this study, with eventual side chains reminiscent of a partial degradation of the glucoside moiety. The comparison of these observations with the analysis of modern cochineal samples allows us to lay out an updated chart of dye compounds and degradation products which should constitute a seminal benchmark for future biomolecular analysis of historic and archeological dye materials.

1. Introduction

The identification of natural dyes and pigments in ancient materials is of paramount importance to track cultural and technological evolution throughout History. The incursion of tandem and high resolution mass spectrometry in the biomolecular analysis of archeological and heritage samples is currently driving outstanding advances in the level of chemical knowledge accessible to research in this field. Such progress is fueling paradigmatic strategies of multidisciplinary investigation involving experimental sciences, humanities and social sciences [1–4].

Modern mass spectrometry provides particularly powerful tools for the identification of (bio)organic compounds of moderate and low molecular weight (typically < 1000 Da) [5,6], which is of enormous interest to archeological research [7,8]. However, chemical degradation constitutes a major challenge for the characterization of aged biological samples based on low-molecular weight organic signatures [8]. In order to face systematic bioanalytical procedures and anticipate realistic historical reaches, the field needs to stablish kinetic routes of chemical degradation for common organic and biomolecular families, and assess the influence of the physicochemical environment in which the materials have been preserved.

This study aims at contributing to this ambitious task by providing insights into the degradation routes of dye compounds in American cochineal (*Dactylopius coccus*), which constitute one of the most highly appreciated group of natural dyes in historic times [9–11]. The crimson tonality of cochineal colorants is dominantly associated to carminic acid

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(above 90% of dye composition) and to minor components, mainly (flavo)kermesic acids and related derivatives. The work of different groups over the past decade has jointly laid out systematic methodologies for the analysis of natural dyes based on liquid chromatography and tandem mass spectrometry [2,4,12–18]. The methods have been applied to the identification and structural elucidation of a remarkably large ensemble of dyes of historic relevance extracted from american and eurasian insect species (*Dactylopius*, *Porphyrophora*, *Kermes*, *Kerria*). Based on this background, extensive efforts have been devoted to the profiling of historic materials, aimed at the elucidation of the taxonomic and geographical origin of the dyestuff employed in their manufacture [12,13,16,19–21]. While the focus of the previous studies has been mostly on the sensitive detection of the typically low concentrations of dye molecules in historic materials, a limited attention has been drawn towards the determination of their degraded chemical forms [19,20]. We show here that the abundances of molecular aging products may become overwhelmingly larger than those of the original organic compounds. It is nevertheless scarcely straightforward to identify a given detected analyte as a degraded species and trace it back to the original organic compound. A good example of the common difficulties is found in the controversial assessment of hydroxybenzoic acids as markers of dye aging, as they may be formed from the photooxidation of flavonoid dyes, but also from degraded peptidic components of undyed biological fibers and tissues [19].

In the preset work, we compare the dye profiles extracted directly from modern and aged cochineal specimens. We specifically analyze the dye profile of cochineal insects collected from the cargo of a 16th century sunken shipwreck, much affected by aging from the underwater environment. While dyestuff from historic artwork and textiles preserved in more favourable conditions can be expected to undergo a less extensive degradation, the present results should constitute a benchmark for a case study with a composition dominated by degradation products which plausibly constitute valid markers for historical research.

2. Materials and methods

Sample collection: Cochineal insects were collected from the cargo of the Genovese ship *San Giorgio e Sant'Elmo Buonaventura*, sunken at the harbour of Cádiz (Spain) during the assault of Francis Drake of April 29th, 1587 (wreck Delta II) [22]. The shipwreck was buried under 7–9 m of compact marine sediment. Upon excavation in archeological campaigns in 2012–13, a cochineal load was found in canvas packages stored inside partially fragmented wooden barrels. The collected material was kept in our laboratories at 5 °C without further treatment. The cochineal from the shipwreck is known to originate from South Mexican plantations, exported as dried insects to Spain by Oaxaca traders from the Veracruz Harbour [10]. Modern cochineal from a plantation in Lanzarote (Canary Islands, Spain) was analyzed as reference. Dried insects (*Dactylopius coccus*) were provided in 2019 by the "Milana Association", an institution devoted to the promotion of the value of cochineal as cultural heritage. Fig. 1 shows photographs of the two types of samples prior to the dye extraction treatment in our laboratory. Both samples could be visually recognized as a granulate material made of dried insects partially disintegrated into a coarse powder.

Dye extraction: Dye compounds were extracted from cochineal (20 mg) with methanol (10 mL, UHPLC grade). The sample solution was kept in an ultrasonic bath for 15 min at room temperature, then in a warm water bath (60 °C) for 30 min. The solution was finally filtered through a 0.45 μm PTFE (polytetrafluoroethylene) syringe filter. Fig. 1 shows photographs of the resulting dye extract solutions. The different colorimetric properties of the two extracts are discussed below.

UV–vis spectroscopy: UV–vis absorbances of the methanol extracts were measured in a Cary-100 Spectrophotometer (Agilent Technologies). The samples were appropriately diluted to keep absorbances below the saturation threshold.

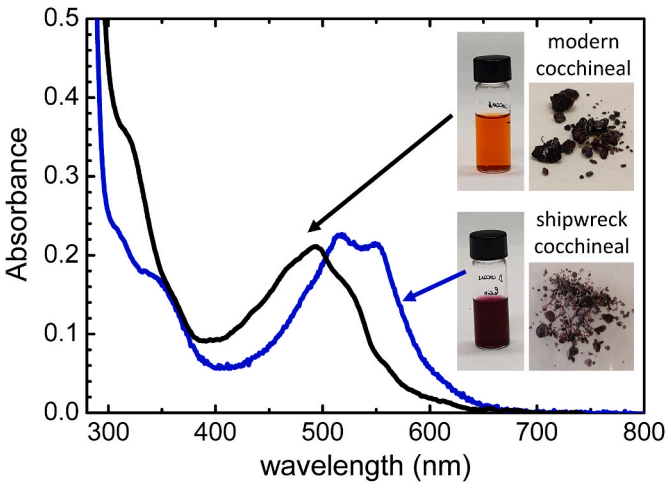

Fig. 1. Visual inspection of the modern and shipwreck cochineal samples analyzed in this work and UV–vis absorption spectra of the corresponding dye extracts. The different colorimetric features in the two samples are attributed to extensive alteration of the molecular structures due to aging.

Liquid Chromatography: Separation of the molecular constituents of the extracts was carried out using ultra high performance liquid chromatography (UHPLC) in an Ultimate 3000 equipment (Thermo Scientific). Reverse-phase separation was applied with a Waters Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm) and a similar precolumn (2.1 × 5 mm, 1.7 μm) at 25 °C. The mobile phase comprised (A) water with 0.15% formic acid and (B) acetonitrile (all solvents of UHPLC quality). An injection volume of 5 μL and a flow rate of 0.3 mL/min were applied with the following sequence of A/B mobile phase ratios: 0–1 min 5% B, 1–41 min linear gradient up to 60% B, 41–50 min 60% B.

Mass spectrometry: A high-resolution Exactive Focus orbitrap mass spectrometer (Thermo Scientific) was employed in the analysis. The dye compounds eluting from the UHPLC stage were electrosprayed in negative polarity, leading to $[M-H]^-$ deprotonated anions. The electrospray source was operated with the following settings: seath gas temperature 320 °C; sheath, auxiliary and sweep gas flows 45, 8 and 3.5 $mL{\cdot}min^{-1}$, respectively; spray voltage 2.7 kV. Higher-energy collisional dissociation (HCD) was performed for MS/MS fragmentation pattern identification of the analytes [23], with a N_2 background gas at effective collision energies of 30 eV. The spectrometer was calibrated with a solution of standard compounds in the range m/z 100–800 Da. The relative analyte intensities reported in Tables 1 and 2 below, result from the time integration of the recorded ion signals over the corresponding mass-selected chromatography peak.

Diode array UV–vis absorbances: UHPLC-DAD measurements were performed under the same chromatographic conditions employed for the MS experiments, though on dye extracts with five-fold higher concentrations. A detector Acquity UPLC PDA (Waters) was employed for the inline monitoring of UV–vis absorbance spectra of the eluting products. The absorption spectrum of the most abundant degradation product, dcka, could be registered with this technique and it is shown as Supplementary Information to the manuscript.

Elemental analysis: Traces of metals in the cochineal samples were analyzed by means of energy dispersive spectroscopy (SEM-EDX), in a Zeiss EVO 25 electron microscopy equipment.

3. Results and discussion

3.1. Colorimetric characterization of the cochineal samples

The visual inspection of the extracts from the Lanzarote and shipwreck cochineal already revealed distinct color tonalities, namely orange-red and purple, respectively. Near UV–vis spectrophotometric

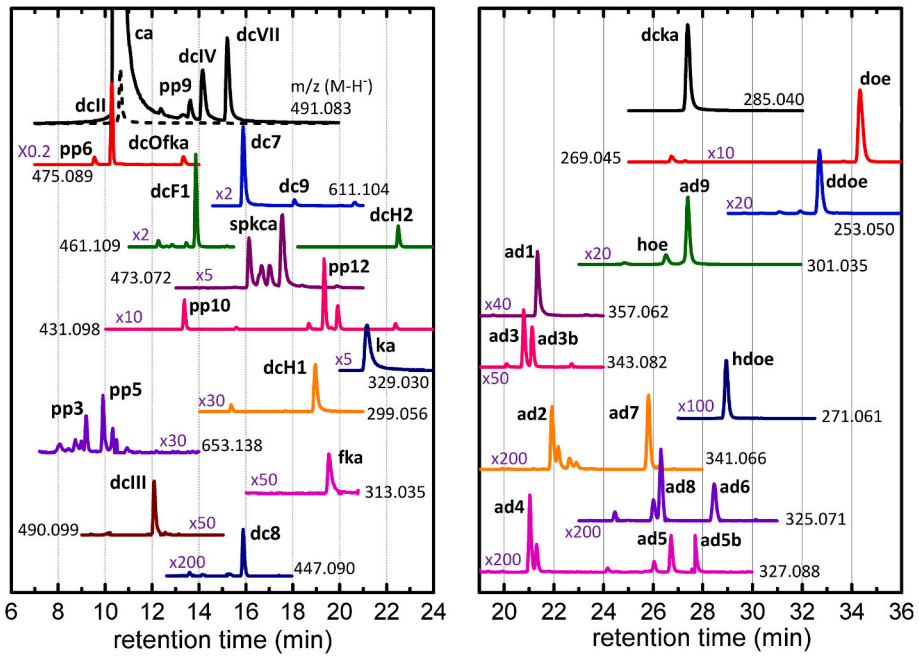

Fig. 2. Mass-selected chromatograms of the dye extracts from the modern (left) the shipwreck (right) cochineal samples recorded in negative ion mode. The indicated masses (m/z) correspond to the anionic deprotonated $[M - H]^-$ forms of the analytes. The weak signal detected for carminic acid (**ca**) in the shipwreck sample is included in the left panel for direct comparison (discontinuous trace at the top). Note the magnification factors applied to the detected ion signal for a better visualization of the chromatographic traces. See Table 1, Figs. 3 and 4, and the Supplementary Information for the identification of the complexes, a representation of the assigned molecular structures and the corresponding MS/MS fragmentation spectra.

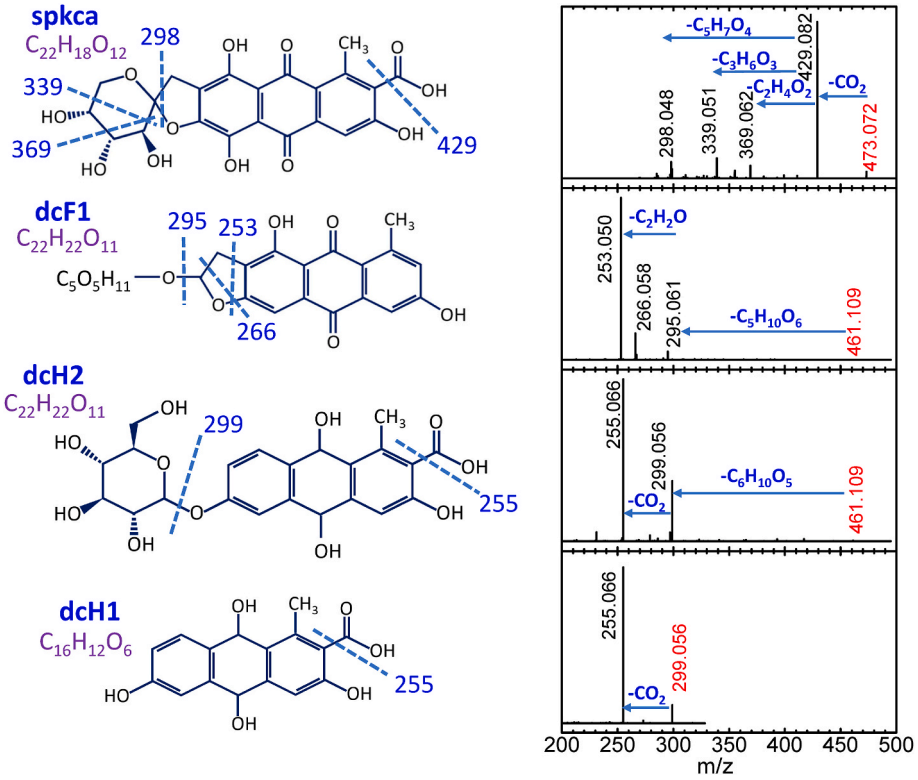

Fig. 3. Molecular structures (left) and MS/MS fragmentation patterns (right) of selected compounds identified in the Lanzarote cochineal extract. The main bond cleavages are indicated as dashed lines in the structures and as mass losses in the MS/MS spectra. The heavier mass indicated in each spectrum (in red) corresponds to the intact $[M - H]^-$ parent anion.

characterization was performed to expose the optical properties of the two samples in a more objective way. Fig. 1 shows that the extract of the Lanzarote cochineal displays a broad absorption band in the visible region (400–600 nm) with little absorbance in the red flank of the visible region. This observation is coincident with analogous bands reported for cochineal dyes in previous works [16]. Incidentally, we did not find any measurable difference in the UV–vis spectra of the methanol extracts with or without PTFE filtering, indicating that dye compounds are not retained in the filter.

The visible absorbance of the dye extract from the shipwreck cochineal (450–700 nm) is appreciably red-shifted with respect to its Lanzarote counterpart, which is consistent with its color tonality. Despite the fact that the Lanzarote and shipwreck specimens are American cochineal (*Dactylopius coccus*), the observed changes in the

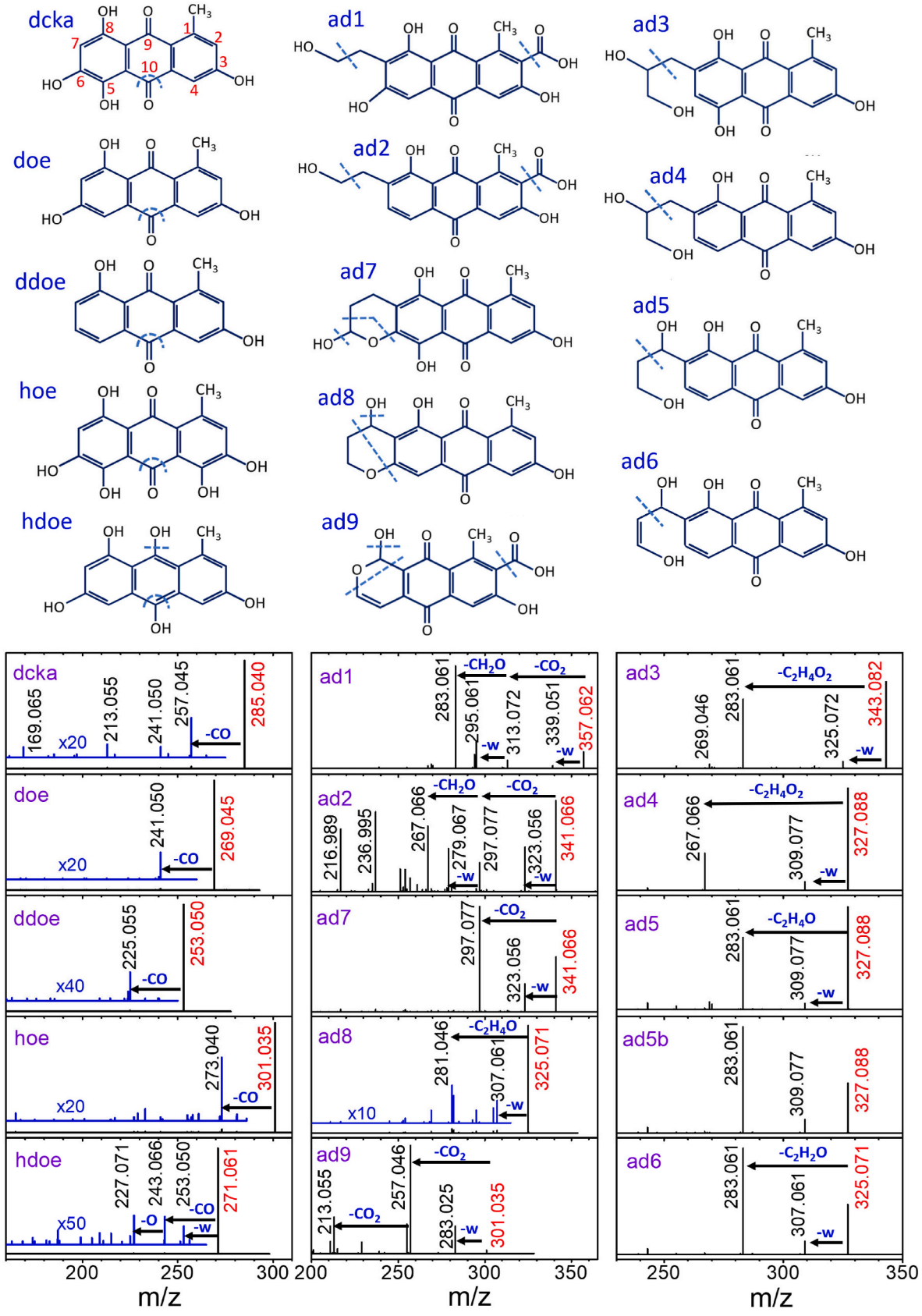

Fig. 4. Top: Molecular structures assigned to the aged dye compounds identified in the extract of the shipwreck cochineal powder. Bottom: MS/MS fragmentation pattern measured for each of the compounds. Note the magnification employed to show the weak fragment signals observed for some of the compounds with robust anthraquinone structures. The main bond cleavages are indicated as dashed lines in the structures and as mass losses in the MS/MS spectra (w denotes water). The heavier mass indicated in each spectrum (in red) corresponds to the intact $[M - H]^-$ parent anion.

spectrophotometric response points to different structural characteristics of their molecular components. As argued below, our mass spectrometric observations confirm the different molecular profiles of the two cochineal samples, indicating that aging in the shipwreck environment has resulted in extensive degradation of the original dyes produced by the insect.

Trace metal analysis was performed on the two cochineal samples to probe any potential chemical manufacture of the insects that could have contributed to their molecular composition or degradation. Historical sources describe the drying of the cochineal insects in the american plantations and their shipping to Europe as a granulate or powder material, without any chemical processing [10]. We corroborated this expectation with an elemental analysis of the samples, following the suggestion of one reviewer of this manuscript. One main concern was the potential chemical transformation of the cochineal material to produce carmine pigment lake, which would also lead to sizeable changes in the UV–vis spectrum. The result of the elemental analysis is displayed in Fig. S1, and shows no evidence for any processing of this kind. A carmine pigment lake features a characteristic high content of Al and Ca, as shown in Fig. S1 for a commercial reference material (CAS 1390-65-4). In contrast, the signals for these two metals in the two cochineal samples are weak and show an Al/Ca ratio inconsistent with a lake. The Lanzarote cochineal was positively delivered to us as dried insect; so that it can be safely concluded that the same is true for the cochineal collected from the shipwreck, as expected from the historic sources. Other than that, the wreck cochineal displays strong signals from Cl and Na (from marine salt) and the modern cochineal from Si and K (presumably from the soil and substrate where is was grown).

3.2. Identification of dyes extracted from modern cochineal specimens

The analysis of the modern Lanzarote cochineal led to the identification of a broad ensemble of anthraquinone dyes, the majority of which have been reported in previous works [15,16]. Table 1 lists the dye compounds assigned in this study, based on their elution times, exact masses and fragmentation mass spectra. Mass-selected chromatographic profiles recorded experimentally in negative ion mode are represented in Fig. 2.

Carminic acid (**ca**) is consistently the overwhelmingly dominant dye compound in the extract of the Lanzarote cochineal (*m/z* 491.083, elution time t = 10.4 min). Common minor *C*-glycoside anthraquinone compounds are as well neatly observed, namely **dcII** (*m/z* 475.089), **dcIII** (*m/z* 490.099), **dcIV** (*m/z* 491.083), **dcVII** (*m/z* 491.083), **dc7** (*m/z* 611.104), **dc8** (*m/z* 447.094) and **dc9** (*m/z* 611.104), along with kermesic acid (**ka**, *m/z* 329.030) and flavokermesic acid (**fa**, *m/z* 313.035) [15]. In addition, a set of *O*-glycosides, namely **dcOfka** and **pp6** (*m/z* 475.089), **pp9** (*m/z* 491.083), **pp10** and **pp12** (*m/z* 431.098) and diglycosides **pp3**, **pp5** (*m/z* 653.138) are identified. The assignment of the molecular structures of these compounds from the MS/MS fragmentation spectra is discussed in depth in previous works, where also the short-hand denominations of the products were introduced [15,16,21]. The fragmentation patterns recorded in our experiments and the postulated molecular structures are included as Supplementary Information (Figs. S2 and S3) for completeness, to aid in the rationalization of the present results for the less common compounds and the degradation products discussed in this work.

Concise guidelines for the justification of the structural assignments are reproduced in the following. The presence of the carboxylic groups (mostly linked to carbon C2, see numbering in Fig. S2) in a given compound is monitored by the loss of CO_2 (*m/z* 43.990) in the MS/MS spectrum; *O*-linked glucosides (on carbons C6 or C7) typically display a loss of $C_6H_{10}O_5$ (*m/z* 162.053), while *C*-linked glucosides produce fragments associated with the loss of $C_3H_6O_3$, $C_4H_8O_4$ and $C_5H_8O_5$ groups (*m/z* 90.032, 120.042 and 148.037, respectively); moreover, the anthraquinone core may fragment through the loss of C═O groups (*m/z* 27.995). Note that the use of high-resolution mass spectrometry is key to unambiguously assess the stoichiometry of the molecules and their fragments. For instance, we will see that resolved exact masses are relevant to discern the loss of CO_2 indicated above from the loss of a C_2H_4O group (*m/z* 44.027); other examples are of course possible, as isobaric nominal masses (but different exact masses) apply to O, CH_4 or NH_2 moities.

Table 1
Dyes identified in the extract of the modern Lanzarote cochineal. The chemical stoichiometry, the type of glucoside linkage of each compound and the mass of the detected $[M - H]^-$ anion (*m/z*) are indicated along with the observed chromatography elution times and relative ion signals (an intensity 1000 is arbitrarily assigned to the ion intensity detected for the most abundant compound, **ca**). See Fig. 2 for a representaton of mass-resolved chromatograms and Fig. 3 and the Supplementary Information to visualize the molecular structures and MS/MS fragmentation patterns.

dye molecule	composition *m/z*	glycoside linkage	elution time (min)	relative intensity
ca	$C_{22}H_{20}O_{13}$	*C*-	10.4	1000
pp9	491.083	*O*-	13.5	20
dcIV		*C*-	14.1	50
dcVII		*C*-	15.1	80
pp6	$C_{22}H_{20}O_{12}$	*O*-	9.5	25
dcII	475.089	*C*-	10.1	130
dcOfka		*O*-	13.2	25
dc7	$C_{29}H_{23}O_{15}$	*C*-	15.8	40
dc9	611.104	*C*-	18.1	3
dcF1	$C_{22}H_{22}O_{11}$	*O*-	13.8	40
dcH2	461.109	*O*-	22.5	10
spkca	$C_{22}H_{18}O_{12}$	spiroketal	16.1	9
isomer	473.072		16.7	4
isomer			17.0	4
isomer			17.4	15
pp10	$C_{21}H_{20}O_{10}$	*O*-	13.4	3
pp12	431.098	*O*-	19.3	7
isomer		*O*-	19.9	2
ka	$C_{16}H_{10}O_8$ 329.030	–	21.1	8
dcH1	$C_{16}H_{12}O_6$ 299.056	–	19.0	1.5
pp3	$C_{28}H_{30}O_{18}$	*diC*	9.2	1.1
pp5	653.138	*C– O*-	9.9	1.8
fka	$C_{16}H_{10}O_7$ 313.035	–	19.5	0.8
dcIII	$C_{22}H_{21}O_{12}N$ 490.099	*C*-	12.0	0.4
dc8	$C_{21}H_{20}O_{11}$ 447.094	*C*-	15.9	0.2

The observation of the **pp**# compounds (*O*-glucosides and diglucosides) in the *Dactylopius* cochineal samples deserves some consideration. These compounds were first assigned in a comprehensive investigation of *Porphyrophora* dyestuff profiles [16], and were initially considered potentially distinct biomarkers for that species. More recently, the presence of *O*-glycosides has been suggested from tandem MS/MS analysis of *Dactylopius* and lake dye samples, although in apparently smaller relative concentration than in the *Porphyrophora* samples [18]. Here, at least **pp6** and **pp9** show intensities comparable to that of **dcOfka** (glucopyranoside of flavokermesic acid, see Fig. S2) and only a factor 2 smaller than those of some of the *C*-glycosides, such as **dcIV** or **dc7**. In addition, we observe **pp10** and **pp12** (two isomers of **pp12** are detected) at lower relative concentrations. Moreover, we observe **pp3** and **pp5**, which is consistent with previous expectations around the presence of diglucosides in American cochineal [18].

Notably also, our analysis identifies the presence of spiroketalcarminic acid (**spkca**), a species reported more rarely in cochineal extracts. The structural features of **spkca** (shown in Fig. 3) and its stereochemical character were elucidated based on NMR signals, upon its first detection in cochineal [17]. The MS/MS spectrum reported here shows that **spkca** displays a fragmentation pattern qualitatively different from those of the *C*- and *O*-linked glucosides described above.

Table 2
Anthraquinone dye degradation products identified in the extract of the shipwreck cochineal sample. The chemical stoichiometry and the $[M - H]^-$ anion masses (*m/z*) observed experimentally for each compound are indicated along with its chromatography elution time, and relative intensity (an intensity 1000 is arbitrarily assigned to the ion intensity detected for the most abundant compound, **dcka**). See Fig. 2 for a representaton of mass-resolved chromatograms and Fig. 4 to visualize the corresponding molecular structures and MS/MS fragmentation patterns.

dye	composition	elution	relative
molecule	*m/z*	time (min)	intensity
ca	$C_{22}H_{20}O_{13}$ 491.083	10.4	10
dcka	$C_{15}H_{10}O_6$ 285.040	27.4	1000
doe	$C_{15}H_{10}O_5$ 269.045	34.3	205
ddoe	$C_{15}H_{10}O_4$ 253.050	32.7	88
ad9	$C_{15}H_{10}O_7$	27.4	97
hoe	301.035	26.5	15
ad1	$C_{18}H_{14}O_8$ 357.062	21.3	46
ad3	$C_{18}H_{16}O_7$	20.8	33
ad3b	343.082	21.1	24
hdoe	$C_{15}H_{12}O_5$ 271.061	28.9	16
ad2	$C_{18}H_{14}O_7$	21.9	9
ad7	341.066	25.8	11
ad8	$C_{18}H_{14}O_6$	26.3	10
ad6	325.071	28.4	5
ad4	$C_{18}H_{16}O_6$	21.0	11
ad5	327.088	26.7	5
ad5b		27.7	5

After the cleavage of the carboxylic group on carbon C2, further dissociative decay proceeds through the loss of carbohydrate $C_xH_yO_z$ fragments consistent with the spiroketal moiety fused to the anthraquinone carbons C6/C7, as indicated in Fig. 3. The chromatographic profile suggests the presence of at least four spkca isomers (see Fig. 2) at elution times within 16–18 min, plausibly associated with different positions of the OH side groups. The MS/MS spectra of the four isomers are similar, as shown in the Supplementary Information (Fig. S4) for direct reference.

A novel compound is detected with *m/z* 461.109 and t = 13.8 min, denoted **dcF1**, that is consistent with the presence of a dihydrofuran group fused to the athraquinone. This compact structure shares some of the structural features of **spkca** discussed above. The heaviest fragment MS/MS spectrum has *m/z* 295.061; the lack of heavier fragments points to the presence of a side group that is readily lost as a whole in the fragmentation process. We tentatively assign this side group to a $C_5H_{11}O_6$ carbohydrate chain, *O*-linked to the hydrofuran group, taking into account the excess of H atoms with respect to any potential cyclic glucosidic structure. As shown in Fig. 3, the cleavage of the hydrofuran bonds would then produce the observed fragments with *m/z* 266.058 and 253.050. The latter peak corresponds to the anthraquinone core (in deprotonated form $C_{15}H_9O_4^-$) and provides the lightest and most intense fragment detected in the MS/MS spectrum. Possible mechanisms for collision induced ring opening and dissociation of hydrofuran groups fused to polyaromatic moieties have been outlined in previous works [24].

Interestingly, we also detect two further novel compounds with *m/z* 299.056 and t = 19.0 min (**dcH1**) and *m/z* 461.109 and t = 22.5 min (**dcH2**) that we could only assign to the anthrahydroquinone structures depicted in Fig. 3. Both of these compounds dissociate to the same reduced $C_{15}H_{12}O_4^-$ anthrahydroquinone core (which is observed in the mass spectrum in anionic form $C_{15}H_{11}O_4^-$ at *m/z* 255.066). In the case of **dcH1**, a single fragmentation process is detected involving the loss of a carboxylic acid side group, whereas for **dcH2** the additional cleavage of an hexose moiety is observed. Hence, **dcH2** is identified as an *O*-linked glucoside of **dcH1**. Incidentally, note in Fig. 3 the qualitatively different fragmentation patterns of **dcH2** and **dcF1**, despite the fact that they share the same mass. To the best of our knowledge, the presence of anthrahydroquinone dyes has not been documented in previous analyses of cochineal dye extracts.

3.3. Identification of dyes and degradation products extracted from shipwreck cochineal specimens

The molecular profile of the dye extract from the cochineal collected from the shipwreck cargo is qualitatively different from that of the modern cochineal described above. A hint for molecular alteration was obtained from the colorimetry measurements described above. We now discuss the mass spectrometric evidence for the molecular structures of the degradation products.

Mass-selected chromatograms recorded for the shipwreck cochineal extracts are laid out in Fig. 2. Molecular structures and the experimental MS/MS spectra employed in their assignment are depicted in Fig. 4. Table 2 compiles the exact masses and stoichiometries, retention times and relative abundances (taken directly from ion signals in the MS spectra) of the different compounds.

Remarkably, only a residual signal from carminic acid is detected in the extract of the aged cochineal, with an ion intensity more than two orders of magnitude lower than in the modern cochineal (see Fig. 2). In fact, except for **ca**, no traces of any of the dye compounds present in the modern Lanzarote cochineal could be observed in the shipwreck cochineal. The composition of the aged cochineal extract is instead dominated by a degradation product with *m/z* 285.040, labelled dcka (for decarboxylated kermesic acid), with a relative ion signal 100 times stronger than carminic acid. Such product can be consistently assigned to the anthraquinone core of the carminic and kermesic acids, plausibly resulting from the chemical cleavage of the glucosidic and carboxylic side groups. The UV–visible absorbance of this compound measured with a Diode-array detector is represented in Fig. S5 for reference.

Analogous bare anthaquinone cores are identified at masses *m/z* 269.045 (**doe**), *m/z* 253.050 (**ddoe**) and *m/z* 301.035 (**hoe**), each of them only differing from each other and from **dcka** in the number of peripheral OH groups (see Fig. 4). Our analysis also identifies one compound (denoted hdoe) consistent with a bare anthrahydroquinone core at *m/z* 271.067, hence a mass differing in two H atoms (+2.016 Da) with respect to doe. Note that **doe** is commonly employed as shorthand notation for deoxyerythrolaccin. Consistently, we employ here **ddoe** for dideoxyerythrolaccin, **hoe** for hydroxyerythrolaccin and **hdoe** for the hydroquinone counterpart of **doe**. Interestingly, **doe** is the second most intense species in the extract, with an integrated ion signal roughly a factor 3.5 smaller than that of **dcka**. Whereas **doe** has been reported as a minor constituent of cochineal dye extracts [16], its apparently predominant presence in the aged cochineal from the ship cargo indicates that it largely results from the degradation of flavokermesic acid (through the loss of the acid group in C2) and/or plausibly also through the dehydroxylation of **dcka**.

The collisional fragmentation of the anthraquinone core compounds is typically little efficient due to the lack of side groups. The main dissociation route involves the cleavage of one quinone carbonyl C═O group (mass loss of 27.995) from positions C9 or C10, leading to fragments with an intensity one to two orders of magnitude weaker than that of the parent molecule (note the magnification factors in the MS/MS spectra of Fig. 4). The anthrahydroquinone hdoe undergoes as well a weak fragmentation, but it displays signatures of water loss (−18.011 Da) and of O loss (−15.995 Da), a presumable consequence of the more active reactivity of the COH group with respect to C═O.

It can be noted from Table 2 that the joint ion signals of these anthraquinone and anthrahydroquinone cores (**dcka** + **doe** + **ddoe** + **hoe** + **hdoe**) amount to roughly 85% of the total signal detected, with **dcka** alone providing 65% of the total ion signal. Hence, it can be

forseen that they may constitute more abundant biomarkers of cochineal dyes in aged materials than the major carminic, kermesic and flavokermesic acid constituents originally produced by the insects and typically detected in the extracts from well-preserved sources. Worth remarking is the fact that the unadverted presence of degraded anthraquinone compounds in historic materials may mislead the rationalization of observed dye profiles. For instance, **dcka**, **doe** and **ddoe** are isomeric to erythrolaccin, emodin and rubiadin, respectively. These latter dyes are of vegetal origin and are occasionally co-detected with cochineal dyes in historic materials with mixed plant/insect dying [13]. Identification of these dyes, only differing in the positions of the OH peripheral groups, then demands a careful identification of chromatographic elution times to avoid misassignments.

Along with the compact core compounds, nine additional degradation products are identified, none of which was present in the Lanzarote sample. We will refer to them as **ad1**-**ad9**, with 'ad' standing for 'aged dye'. Fig. 4 shows that most of them display intense fragment signals in the MS/MS spectrum, suggesting the presence of labile groups. A putative assignment of the structural features of these compounds from their fragmentation signatures and stoichiometry (derived from the exact mass) is outlined here under the assumption that the anthraquinone core is preserved in the aging process. Each of the proposed structures admits several isomers associated with different position of the OH side groups on the anthraquinone moiety.

Compounds **ad1** (m/z 357.062, t = 21.3) and **ad2** (m/z 341.066, t = 21.9) differ in one OH group, but they otherwise share identical structural features. Fig. 4 shows that the MS/MS spectra of these two compounds show signatures of the loss of COO (−43.990 Da), and also of loss of water (−18.011 Da) and of COH_2 (−30.010 Da). This is rationalized in terms of the presence of a carboxylic acid group in position C2 and of a CH_2CH_2OH side chain attached to the anthraquinone core. This latter side chain would plausibly be reminiscent of a glucosidic moiety in the original dye compound and would therefore be linked to position C7. Water loss appears as a distinct signal of the presence of a reactive OH groups in the side chain, hence not directly bound to the anthraquinone rings, as also found for the compounds discussed in the following.

Compounds **ad3** (m/z 343.082, t = 20.8) and **ad4** (m/z 327.088, t = 21.0) similarly differ from each other in one OH group while sharing identical overall structures. In this case, the carboxylic acid group is lacking and the stoichiometries indicate that the side group attached to the anthraquinone core (presumably at the position C7 of the original glucoside) is a $CH_2CH_2OHCH_2OH$ chain. The MS/MS spectra of both of these compounds display fragments associated with the loss of water (−18.011 Da) and of a CH_2OHCH_2OH group (−60.020 Da), consistent with cleavages in the side chain, as indicated in Fig. 4.

Compounds **ad5** (m/z 327.088, t = 26.7 and its isomer **ad5b** at t = 27.7) and **ad6** (m/z 325.071, t = 28.4) are structurally similar to **ad4**, but feature a different position of the OH groups in the side chain, with **ad6** displaying in addition an unsaturated C═C moiety (see Fig. 4). These assignments follow from the analysis of the corresponding fragmentation spectra, which display loss of a $C_2H_4O_2$ (−60.021 Da) group for **ad4**, of a C_2H_4O (−44.027 Da) group for **ad5** and of a C_2H_2O (−42.010 Da) group for **ad6**. Isomer **ad5b** is plausibly related to a structure similar to **ad5**, with a different position of the OH groups in the anthraquinone ring (*e.g.*, in C5 instead of C8).

Compounds **ad7** (m/z 341.066, t = 25.8) and **ad8** (m/z 325.071, t = 26.3) are identified as anthraquinones with an oxane (tetrahydropyran) end group fused to carbons C6/C7. Such assignment is based on the weak fragmentation pattern of **ad8** (suggesting a compact structure), and on the limited number of fragments observed for **ad7**. For **ad8**, collisional fragmentation is inefficient, with a main fragment associated with the loss of a CH_2CH_2O group (−44.026 Da) from the oxane moiety (see Fig. 4). Both **ad7** and **ad8** incorporate a side OH group to the oxane structure whose detachment proceeds through water loss (−18.011 Da). The position of the OH group in **ad7** favors as well a more efficient oxolane fragmentation through the net loss of CHO_2 (−44.998 Da). Note that the stabilization of the oxane end group could result from hexose degradation eventually leading to the cyclisation of side chains such as those present in **ad3**, **ad4** or **ad5**.

Compound **ad9** (m/z 301.035, t = 27.4) closes the catalog of dyes structurally identified for the shipwreck cochineal in this study. The combination of composition (exact mass) and fragmentation pattern for **ad9** is somewhat puzzling. While having the same molecular weight as **hoe**, its MS/MS spectrum shows abundant fragments, hence suggesting the presence of labile groups in its structure. Remarkably, the most intense fragment ions at m/z 257.046 and 213.055 indicate a sequential loss of two moieties with CHO_2 stoichiometry. A carboxylic acid COOH functionality seems an obvious choice as a side group. Moreover, the presence of a prominent fragment associated with water loss (m/z 283.025) also suggests a consistent assignment of an OC(OH) moiety, including a reactive end OH group. These evidences lead us to tentatively postulate an alteration of the anthraquinone core that keeps a carboxylic acid side group on its usual C2 position next to the methyl group but that incorporates one O heteroatom in the opposite end ring (see Fig. 4). This latter feature would have followed from a chemical aging pathway inducing ring opening and C atom loss in the ahtraquinone, followed by recombination to a cyclic *O*-linked arrangement.

4. Summary and conclusions

A combination of liquid chromatography and high resolution tandem mass spectrometry has been applied to the elucidation of the degradation products of cochineal dyes in historic samples exposed to harsh environmental conditions. A cochineal load collected from the cargo of a sunken 16th century wreck has been employed as case study and its dye composition has been contrasted to that of modern cochineal of the same species, namely *Dactylopius coccus*.

The analysis of the modern cochineal sample identifies a broad ensemble of carminic and (flavo)kermesic acid dye compounds reported in previous studies, along with *O*-linked glucosidic, spiroketal anthraquinone and anthrahydroquinone structures, that are novel or had been scarcely observed in American cochineal.

In the shipwreck cochineal, the original dye composition is almost completely replaced by degraded products. Aging of the dye molecules typically leads to cleavage of the carboxylic acid and glucoside groups. Partially degraded compounds are also identified with side chains reminiscent of the glucoside moiety, eventually also with an intact carboxylic group. Nevertheless, the dye composition of the shipwreck sample is amply dominated by compact anthraquinone cores (to a lesser extent, also anthrahydroquinone cores) without side groups. These structures are resilient to degradation and constitute potential targets for the identification of aged cochineal dye compounds.

In general terms, biomolecular analysis of dyestuff in historic and archeological materials should take into account that a varying concentration of natural dyes and of their degradation products may be present in the samples, depending on the track of preservation conditions and exposure to external agents. For instance, some of the degraded cochineal anthraquinones may be unadvertedly assigned to isomeric dyes of different origin. Also importantly, given the typically low concentration of dyes recovered from ancient substrates, analytical sensitivity may crucially benefit from the inclusion of degradation products into the ensemble of molecular biomarkers. As an illustration, for the extremely aged cochineal powder collected from the shipwreck in this study, only traces of carminic acid were detected with an abundance two orders of magnitude lower than its degraded anthraquinone counterpart.

As a final remark, dye degradation products loose important features of the original dye molecules that are currently employed for speciation, *e.g.* those related to the position of the hexose or carboxylic moieties. In order to support the identification of cochineal in heavily aged samples, it would be desirable that future studies characterize biomarker profiles of degraded dye compounds in further cochineal species.

Author contribution statement

- All authors have contributed equally to the preparation of the manuscript.

- Milagros Alzaga-García, Mercedes Gallardo-Abárzuza and José Manuel Higueras-Milena conducted the archeological campaign, collected and conserved the historic cochineal samples.

- Bruno Martínez-Haya coordinated the multidisciplinary analytical work, which was perfomed jointly by Carmen Domínguez-Castillo, María Jiménez-Hidalgo, José López-Gámez, Ana Rodríguez-Hortal, Auxiliadora Gómez-Morón, Esteban García-Viñas and Eloísa Bernáldez-Sánchez.

- Carmen Domínguez-Castillo, Auxiliadora Gómez-Morón and Bruno Martínez-Haya played the leading role in the data analysis.

Declarations

- There are no conflicts of interests or competing interests related to this work.

- This work does not involve issues requiring ethics approval and has had no impact on animal welfare. Biological materials (dried cochineal) were obtained from a farm and from an archeological excavation.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dyepig.2023.111313.

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