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Direct inlet mass spectrometry for a rapid characterization of indigo in lipidic and proteinaceous matrices

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ABSTRACT

Direct inlet mass spectrometry (DI-MS) was examined for its suitability to quickly identify indigo in mixtures with lipidic and proteinaceous binders, such as those characteristic of paintings and polychromy. The technique was tested on naturally aged reference mixtures of indigo with linseed oil, rabbit skin glue, and whole egg. DI-MS with electron ionization at 70 eV served as an efficient tool for screening indigo in complex matrices and provided, at the same time, a mass spectral fingerprint of the organic binder in just a few minutes. The technique was applied to the identification of indigo in a blue sample from a mural painting dated from the 18th century and housed in an Andean church in Bolivia. At the same time, the acquired data revealed for the first time the use of an egg tempera binding medium in an Andean mural painting.

1. Introduction

Natural indigo has been widely used as a textile dye and as an oil and watercolor pigment [1]. Until the end of the nineteenth century, indigo was exclusively prepared from the fermentation in water of leaves of plants of the families Papilionaceae, Brassicaceae, and Polygonaceae. The most important sources of indigo are *Indigofera tinctoria* L., *Indigofera suffructicosa* Mill., *Isatis tinctoria* L., and *Polygonum tinctorium* [2]. Indigotin (**1**) (Fig. 1), the compound responsible for the blue colour in indigo, is formed by enzymatic hydrolysis of the precursor glycosides indican and isatan B to give indoxyl, which is subsequently oxidized by atmospheric oxygen to the colorless *leuco* indigotin and then to **1** [1]. If indoxyl is oxidized to isatin during the processing of indigo plants, the combination of this molecule with one molecule of indoxyl leads to indirubin (**2**) (Fig. 1), a red minor isomer of **1**. The commercial production of synthetic indigo by BASF in 1897 rapidly displaced the natural sources of the dye [1,2].

Indigo is one of the oldest natural blue dyestuffs and it has been identified in textiles from different places in the world [2–6]. As a pigment, it is suitable for watercolors and tempera painting and it has found application in glue medium and oil paints [7–10]. Indigo and Prussian blue, the latter not before 1777, seem to have been the blue pigments most employed in the workshops of Cuzco in the 17th and 18th centuries. Indigo was used in mixtures with other pigments, most commonly with lead white to obtain different shades of blue [11–13], and with orpiment (As_2S_3) to produce green [14].

Several analytical techniques have been applied for the detection of indigoids in artworks and archaeological objects. High-performance liquid chromatography (HPLC) coupled with diode-array (DAD) and mass spectrometer detectors is by far the most well-established technique for the identification of dyestuffs in archaeological and historical textiles [15]. Prior to HPLC analysis, indigo has to be extracted from the fibres. As a *vat* dye, it does not require a mordant metal to attach to the fibres and extraction with dimethyl sulfoxide (DMSO) is generally used [3,16]. Mass spectrometry techniques were also applied to the identification of indigoids in textiles. DMSO and dimethylformamide (DMF) extracts

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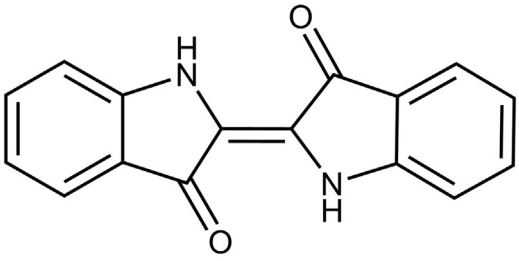

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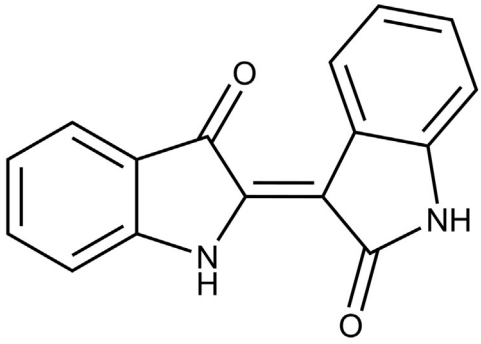

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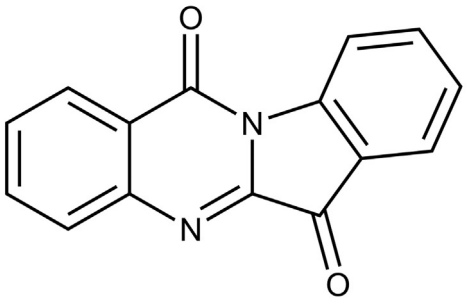

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Fig. 1. Chemical structures of indigotin (**1**), indirubin (**2**), and tryptanthrin (**3**).

of fibres dyed with moluscs containing indigotin, 6-bromoindigotin and 6,6′-dibromoindigotin were successfully analyzed by direct exposure mass spectrometry and negative ion pressure photoionization mass spectrometry [17–20]. Direct analysis of indigo dyed textiles was also assessed by time of flight secondary ion mass spectrometry (TOF-SIMS) [21,22], laser desorption mass spectrometry (LD-MS) [23] and fibre optics reflectance spectroscopy [24]. Recently, an analytical procedure based on pyridine extraction, silylation and further analysis by gas chromatography coupled to mass spectrometry (GC-MS) succeeded in the identification of indigo in two historical textiles [25].

Regarding the identification of indigo in paintings, micro-Raman spectroscopy is usually applied as a nondestructive technique, although it is often limited by the high fluorescence of aged binders in the complex painting matrix [10,26]. In the last decade, surface-enhanced Raman spectroscopy (SERS) has been increasingly applied to the identification of organic pigments in paintings and textiles [27]. Although this technique is very sensitive, SERS analysis of indigo in paintings requires a sample pretreatment, for example, with H_2SO_4 [28]. Recently, tip-enhanced Raman spectroscopy (TERS) has proved efficient for the identification of indigo on dyed rice paper references [29]. Finally, reactive pyrolysis under methylating or silylating conditions, in combination with mass spectrometry (Py-GC-MS), has been evaluated for the analysis of reference painting mixtures of indigo with linseed oil as a binder [7]. The main drawback of this methodology was the detection of various derivatives of indigo in low levels in comparison with the methyl esters of fatty acids from the binder.

With the aim of searching for a simple and rapid methodology for the identification of indigo in lipidic and proteinaceous matrices, such as those in paintings, the goal of the present study was to evaluate the application of DI-MS with electron ionization at 70 eV to the identification of indigo in naturally aged reference mixtures with linseed oil, rabbit skin glue and whole egg. At the same time, we wished to explore if the binder and its degradation products could be characterized in the same analysis. Finally, the technique was applied to the analysis of a blue sample from a mural painting dated from the 18th century and housed in an Andean church in Bolivia.

2. Experimental

2.1. Model samples

The model samples were prepared by applying synthetic indigo, mixed to a binder in a ratio binder/indigo = 1.0, on glass slides. Three different types of binder were used: linseed oil, rabbit skin glue, and whole egg. Each model sample had dimensions of 2.5 cm × 5.0 cm and a thickness of about 0.15 mm. The samples were naturally aged at room temperature for 13 years. Indigo was purchased from Allied Chemical & Dye Corporation, New York, USA. Linseed oil, rabbit glue, and egg were purchased from local suppliers.

2.2. Painting sample

One microsample (M6) was extracted with a scalpel from the blue area of the handle of a flower vase (Fig. 2) in a wall painting from the church of Copacabana de Andamarca in Bolivia before a restoration treatment. The church wall paintings, dated from the early 18th century, depict the escathological themes of Death, Judgment, Glory, and Hell. The pictures of the Last Four Things were vital for the Catholic belief and were part of the process of evangelization done under the Spanish domain [30]. The painting sample was analyzed by DI-MS in the same conditions as the model samples.

2.3. Instrumentation

Elemental chemical analyses of the blue layer and the ground layer of the mural painting microsample M6 were obtained by using a field environmental scanning electron microscope (FE-SEM) Zeiss:Supra 40 coupled with an energy-dispersive X-ray spectrometer (SEM-EDS) INCA X Sight (Oxford Instruments). DI-MS analyses were carried out on a ThermoScientific EM/DSQ II spectrometer with electron ionization at 70 eV in the positive ion mode. The samples (<0.5 mg) were placed in a small glass vial, which was inserted into the direct insertion probe and introduced into the ion source. The temperature was increased from 100 °C to 400 °C at a rate of 40 °C/min. The mass spectrometer was scanned over a m/z range of 40–1050. Mass spectral fingerprints were obtained by averaging the mass spectra in the indicated time range

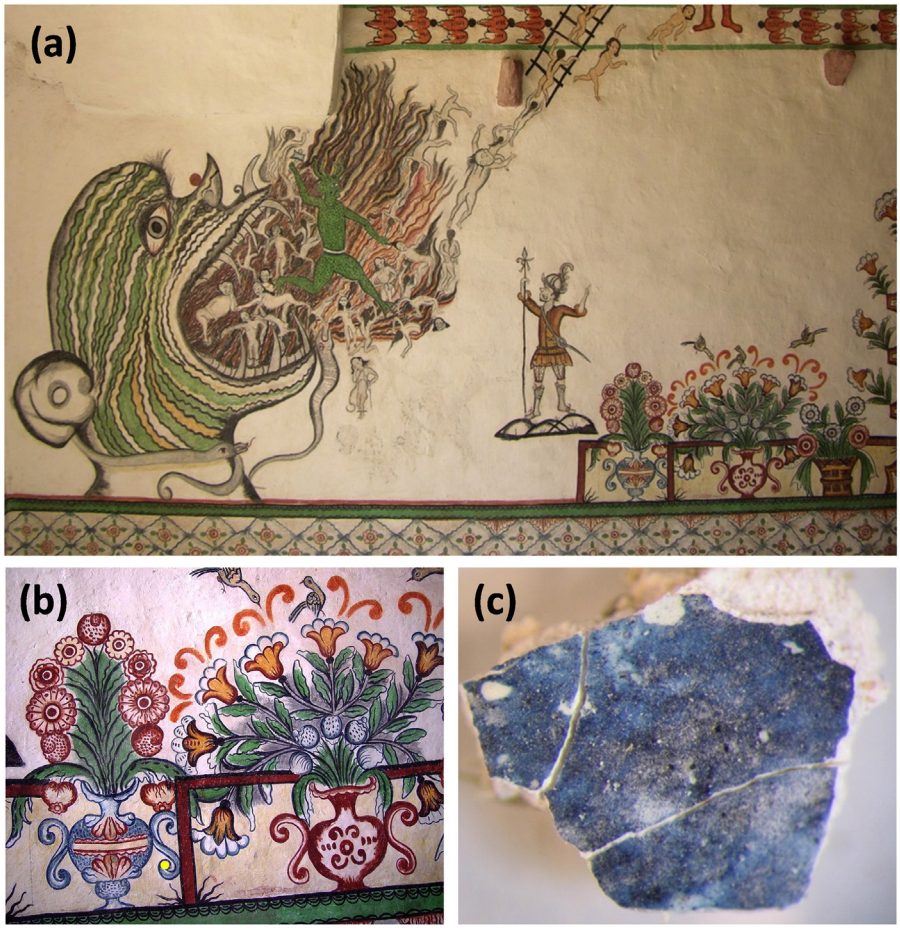

Fig. 2. (a) Detail of a wall painting from the church of Copacabana de Andamarca; (b) detail of the floral decoration in (a) and indication of the sample location by a yellow circle; (c) sample of the blue pigment (M6) taken from the handle of the blue flower vase.

of the total ion chromatogram (TIC). Analytical HPLC with diode array detection (HPLC-DAD) was carried out on a Gilson 506C HPLC system using a Phenomenex Gemini 5 μm column (25 cm × 4.6 mm internal diameter). Gradient elution was performed using mixtures of MeOH and 1% (v/v) aqueous orthophosphoric acid as solvents. The gradient started with 36% MeOH during 5 min and was raised to 90% MeOH within 10 min, followed by 20 min at this condition. Solvents utilized in the HPLC were filtered through a 0.2 μl filter prior to use. The flow rate was 0.8 ml/min and the detection wavelength was 284 nm.

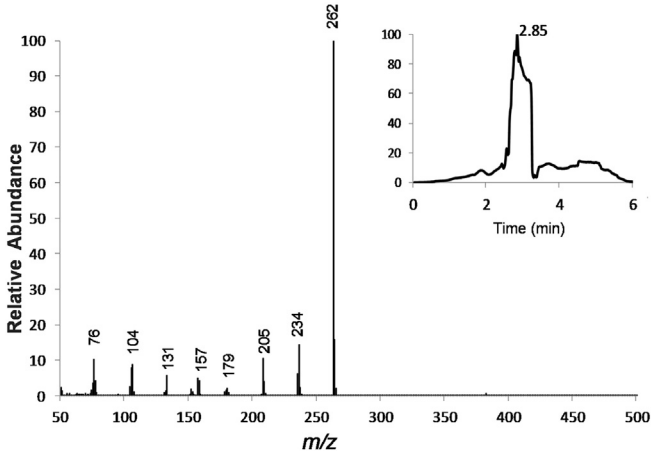

Fig. 3. Mass spectrum of synthetic indigo standard at 2.85 min of the TIC curve.

3. Results and discussion

3.1. Indigo

The mass spectrum of synthetic indigo at 2.85 min of the TIC (Fig. 3) obtained by DI-MS analysis in the positive ionization mode exhibits a base peak at *m/z* 262, which corresponds to the molecular ion of indigotin (**1**), together with peaks at *m/z* 234 $[M\text{-}CO]^+$ and 205 $[M\text{-}2CO\text{-}H]^+$. These three peaks are molecular markers for indigo that enable a rapid identification of the pigment by electron ionization mass spectrometry [19]. In order to evaluate if DI-MS could be applied for the identification of indigo in mixtures with proteinaceous and lipidic binders, microsamples of naturally aged reference mixtures of indigo with rabbit skin glue, linseed oil, and whole egg were analyzed in the same conditions as for the indigo reference.

3.2. Indigo and rabbit skin glue

Rabbit skin glue, as well as other animal glues used as pigment binders and adhesives, is made up of collagen, the structural protein of connective tissue in skin, muscle, bone, and hide [31]. Analysis by DI-MS of the naturally aged mixture of indigo and rabbit skin glue

revealed the desorption of indigo in the 2.6–4.0 min range of the TIC curve, as indicated by the characteristic ions of the pigment at *m/z* 262, 234, 205, 179, 157, 131, 104, and 76 in the average mass spectrum in this range of time (Fig. 4a). In the 5.5–6.5 min range of the TIC, the average mass spectrum (Fig. 4b) showed the molecular marker peaks for indigo at *m/z* 262, 234, and 205 together with an intense peak at *m/z* 248 assigned to the molecular ion of tryptanthrin (**3**) (Fig. 1), a degradation product of indigotin (**1**), which has previously been identified in photodegraded indigo in solution [32]. The peak at *m/z* 219 may be derived from tryptanthrin and was attributed to $[M\text{-}CO\text{-}H]^+$. The intense peak at *m/z* 70, together with peaks at *m/z* 111, 124, and 154, were assigned to the thermal formation of 2,5-diketopiperazines (DKPs) from collagen [33,34]. These cyclic dipeptides have been reported as pyrolisis products of proteins and may be used as specific markers of proteins in complex matrices [33–35]. Recently, Fabbri et al. [33], using Py-GC-MS, studied the formation of DKPs during the pyrolysis of collagen. Proline-glycine DKP was the most abundant 2,5-diketopiperazine together with proline-proline DKP and proline-hydroxyproline DKP. These results are in accordance with the fact that glycine, proline, and hydroxyproline are the major constituents of collagen [31]. The peak at *m/z* 154 (Fig. 4b) is characteristic of the molecular ion of proline-glycine DKP, together with fragmentation peaks at *m/z* 70 and 111 [34]. The peak at *m/z* 70 is associated with the pyrrolidinium ion typical of proline fragmentation and has also been detected as the base peak in the mass spectra of proline-proline and proline-hydroxyproline DKPs, while the peak at *m/z* 124 is a characteristic ring fragmentation product of proline-hydroxyproline DKP [33].

3.3. Indigo and linseed oil

Linseed oil is a siccative oil with a very high content of C-18 unsaturated fatty acids, such as oleic (C18:1), linoleic (C18:2), and linolenic acids (18:3), which suffer oxidation and cross-linking reactions leading to the formation of oligomers and high molecular weight polymers of glycerolipids [31]. Analysis of the naturally aged mixture of indigo and linseed oil showed the molecular ions of palmitic (*m/z* 256) and stearic (*m/z* 284) acids in the average mass spectrum of the 2.0–2.3 min range of the TIC (Fig. 5a). Fragment ions formed by cleavage of the fatty acid backbones were observed at *m/z* 227, 213, 199, 185, 171, and 129 ($[C_nH_{2n}COOH]^+$), together with peaks at *m/z* 152, 97, and 83, indicative of dicarboxylic acids formed by oxidative cleavage of unsatured fatty acids moieties [36]. In the 2.4–5.5 min range of the TIC, the average mass spectrum (Fig. 5b) showed the molecular marker peaks for indigo at *m/z* 262, 234, and 205, as well as minor fragmentation peaks of fatty acids. No acylglycerides were detected indicating that they were depleted due to oxidation, hydrolysis, and cross-linking in the aged mixture. This is in accordance with the high content of C-18 di- and triunsaturated fatty acids in linseed oil, which undergo a rapid crosslinking during the drying process of the oil [31].

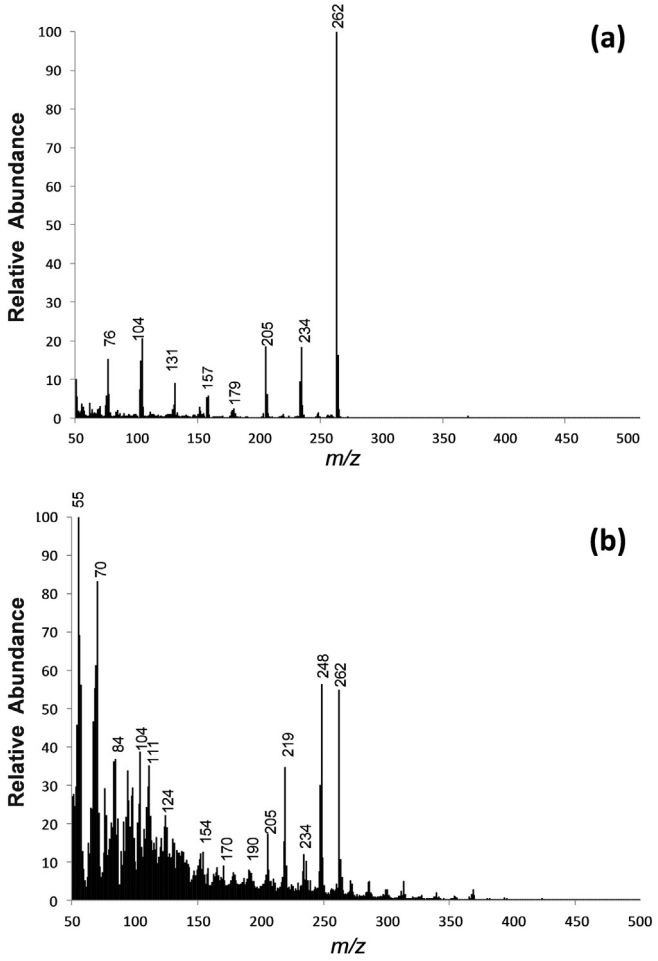

Fig. 4. Analysis by DI-MS of the naturally aged mixture of indigo and rabbit skin blue. Average mass spectra at (a) 2.6–4.0 min and (b) 5.5–6.5 min of the TIC.

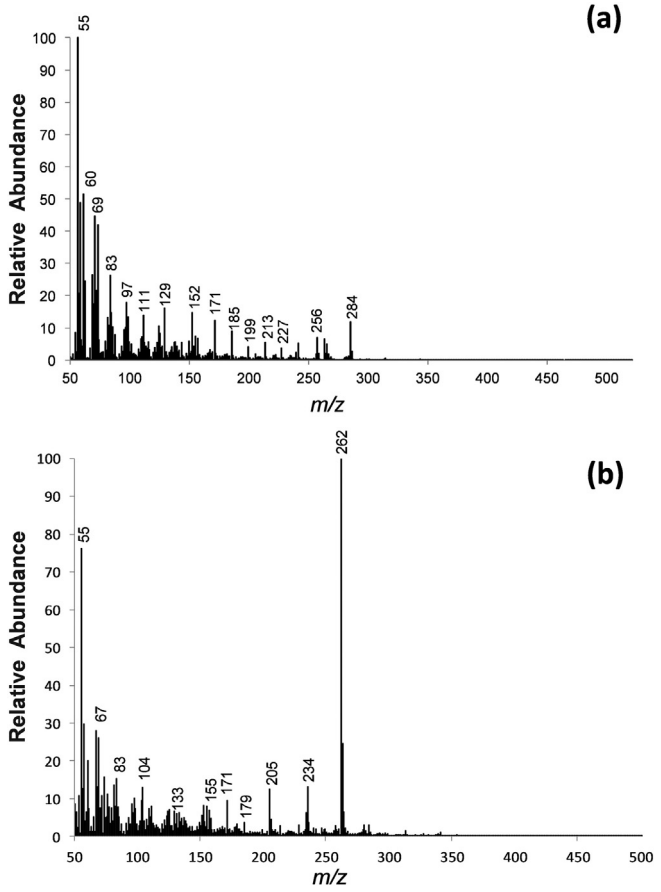

Fig. 5. Analysis by DI-MS of the naturally aged mixture of indigo and linseed oil. Average mass spectra at (a) 2.0–2.3 min and (b) 2.4–5.5 min of the TIC.

3.4. Indigo and whole egg

The main constituents of egg are water, lipids, and proteins. Egg yolk is rich in lipids made up of triglycerides (c. 65%) and phospholipids (c. 29%), with palmitic (16:0) and oleic (18:1) acids as the main fatty acids, and cholesterol (**4**; Fig. 7) (c. 5.2%). Egg white is composed of globular proteins called albumins, with ovalbumin accounting for 50% of total proteins [31]. Previously published work on unpigmented light-aged mock egg tempera paints by direct temperature-resolved mass spectrometry (DTMS) reported mainly changes in the glycerolipids composition and oxidation products of cholesterol [37]. DI-MS analysis of the mixture of indigo and whole egg revealed molecular ions of palmitic (*m/z* 256) and stearic (*m/z* 284) acids in the average mass spectrum of the 1.6–2.3 min range of the TIC (Fig. 6a), together with fragment ions formed by cleavage of the fatty acid backbones at *m/z* 227, 213, 199, 185, 171, 157, and 129 ($[C_nH_{2n}COOH]^+$) and peaks at *m/z* 97 and 83, due to dicarboxylic acids. The higher intensity of the molecular ion at *m/z* 256 in comparison to that of stearic acid was ascribed to the high content of palmitic acid in egg lipids [31].

The average mass spectrum (Fig. 6b) of the 2.8–4.0 min range of the TIC showed molecular marker peaks for indigo at *m/z* 262, 234, and 205, as well as fragmentation peaks of fatty acids and low-

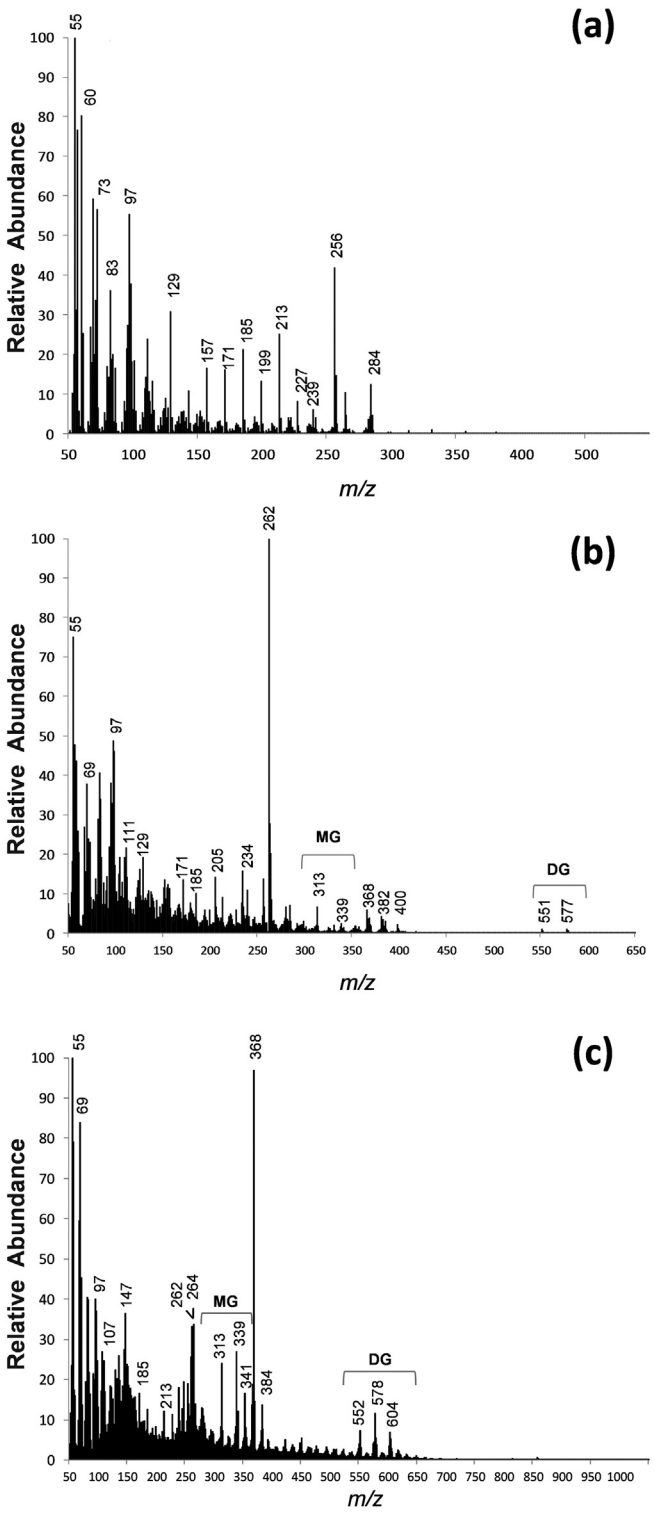

Fig. 6. Analysis by DI-MS of the naturally aged mixture of indigo and whole egg. Average mass spectra at (a) 1.6–2.3 min, (b) 2.8–4.0 min, and (c) 7.0–7.3 min of the TIC. MG, monoglycerides; DG, diglycerides.

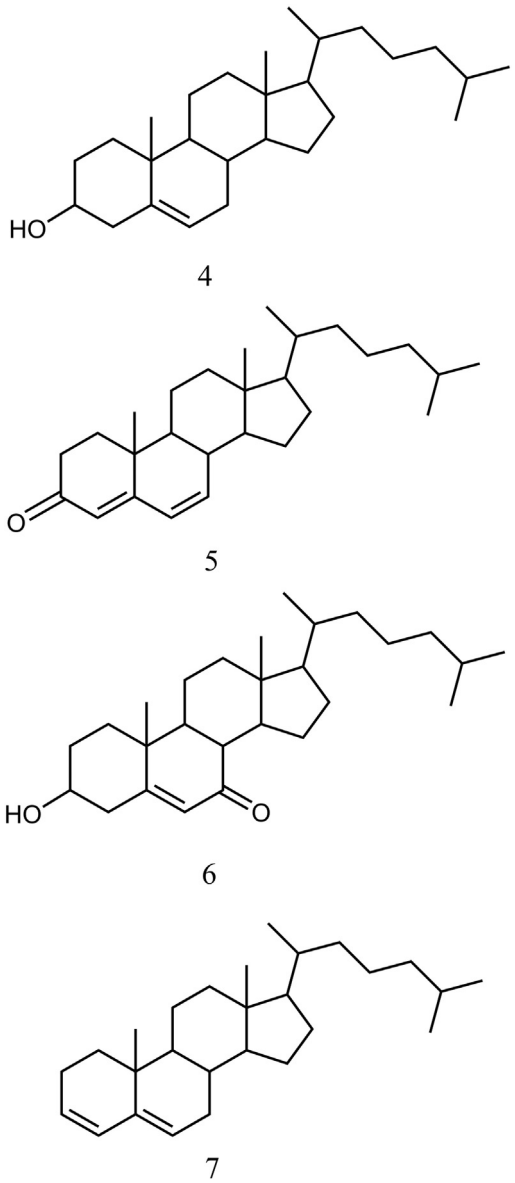

Fig. 7. Chemical structures of cholesterol (**4**), cholesta-4,6-dien-3-one (**5**), 3-hydroxycholest-5-en-7-one (**6**), and 3,5-cholestadiene (**7**).

intensity molecular ions of mono- and diglycerides and cholesterol oxidation products, such as cholesta-4,6-dien-3-one (**5**) (*m/z* 382) and 3-hydroxycholest-5-en-7-one (**6**) (*m/z* 400) (Fig. 7). The peak at *m/z* 368 was assigned to 3,5-cholestadiene (**7**), a characteristic electron ionization fragment of cholesterol produced by the loss of a molecule of water. Nevertheless, this peak could also be ascribed to the loss of a neutral fatty acid from a cholesterol ester, as reported previously [37]. This was further supported by the intense peak at *m/z* 368 in the averaged mass spectrum (Fig. 6c) of the 7.0–7.3 min range of the TIC. This mass spectrum also showed molecular fragments due to monoglycerides, such as palmitic acid monoglyceride (*m/z* 313), oleic acid monoglyceride (*m/z* 339), and stearic acid monoglyceride (*m/z* 341), together with C_{35}, C_{37}, and C_{39} diglyceril ions at *m/z* 552, 578, and 604 [38]. The peaks at *m/z* 262 and 264 may be assigned to acylium ions formed as fragments of glycerolipids that contain linoleic and oleic residues, respectively [37].

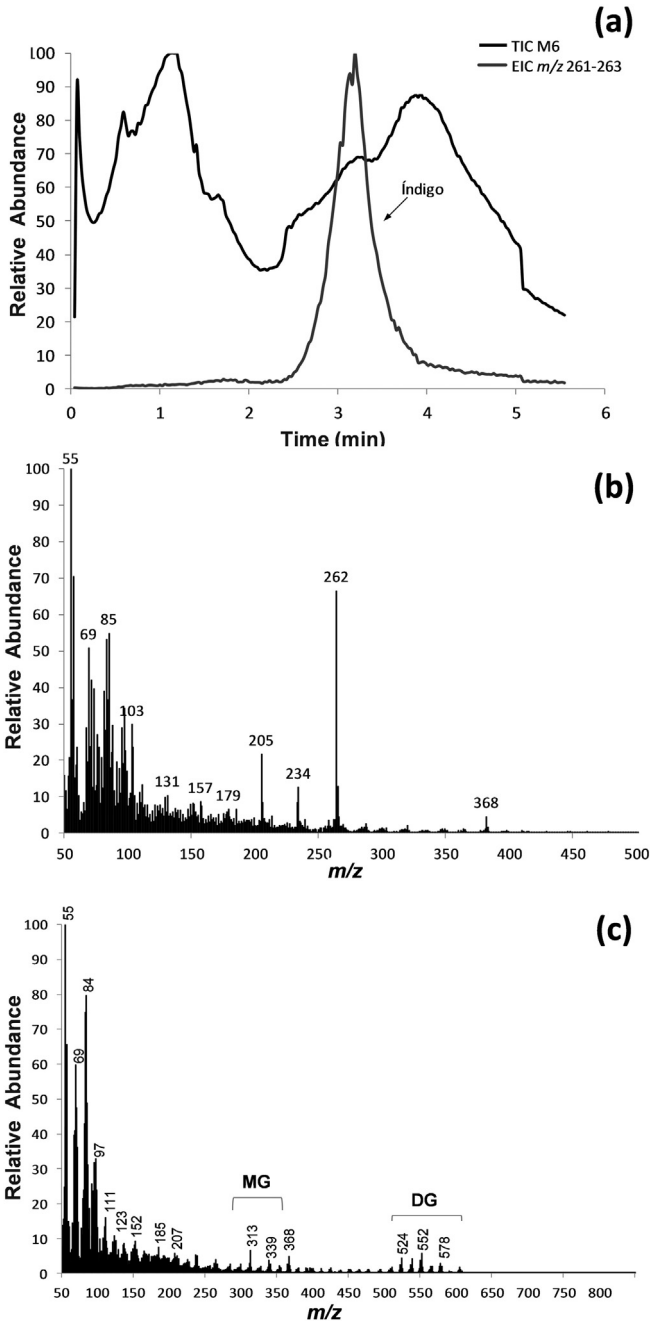

Fig. 8. (a) TIC curve of the blue sample (M6) from the mural painting of the church of Copacabana de Andamarca and extracted ion chromatogram in the range *m/z* 261–263. Average mass spectra at (b) 3.0–3.2 min and (c) 4.7–5.1 min of the TIC. MG, monoglycerides; DG, diglycerides.

3.5. Identification of indigo in an Andean mural painting sample

Analysis of the blue sample (M6) from the mural painting of the Andean church of Copacabana de Andamarca by optical microscopy and scanning electron microscopy coupled to an energy-dispersive X-ray spectrometer (SEM-EDS) revealed a blue pigment layer on a ground layer of calcium sulfate hydrate (gypsum) (data not shown). The absence of copper and iron in the blue layer discarded the use of azurite (a basic copper carbonate) or Prussian blue ($Fe_4[Fe(CN)_6]_3 \cdot \times H_2O$) as pigments and suggested the presence of indigo. Analysis by DI-MS of the solid microsample, directly introduced in the mass spectrometer, gave the TIC depicted in Fig. 8a.

In the 3.0–3.2 min range of the TIC, the average mass spectrum (Fig. 8b) revealed the molecular marker peaks for indigo at *m/z* 262, 234, and 205, together with fragmentation peaks of fatty acids and a minor peak at *m/z* 368 indicative of 3,5-cholestadiene (**7**) (Fig. 7). Further analysis of the average mass spectrum at 4.7–5.1 min of the TIC showed molecular fragments due to monoglycerides (*m/z* 313, 339), diglycerides (*m/z* 520–610), and 3,5-cholestadiene (**7**) (*m/z* 368). Cholesterol and its oxidation products were not identified in the wall painting sample, possibly because they are in very low amounts or because they did not survive in the painting over the centuries. Nevertheless, the presence of the peak at *m/z* 368 was in accordance with the results obtained for the naturally aged mixture of indigo with whole egg, and suggested the use of egg tempera paint.

The presence of indigo was further confirmed by HPLC-DAD analysis of the chloroform extract of the blue pigment of the sample M6. The UV spectrum of the peak in the chromatogram (Fig. 9) showed bands at 244, 284, 340, and 608 nm in accordance with those of the indigo standard and reported data [39].

4. Conclusions

Analysis by DI-MS using electron ionization at 70 eV has been successfully applied to the identification of indigo in proteinaceous and lipidic matrices. Indigo was easily characterized by marker peaks at *m/z* 262, 234, and 205 in the presence of various binders, which did not interfere in the identification of the pigment in the aged mixtures. The results presented showed that indigo degraded partially to trypthanthrin in the mixture with rabbit skin blue while it remained stable in the mixtures with linseed oil and whole egg. Interpretation of the mass spectral fingerprints as a function of time allowed chemical structure assignments of the binders and their degradation products. In particular, the peak at *m/z* 368 ascribed to 3,5-cholestadiene (**7**) may be considered a good marker for the presence of cholesterol as it can be produced either by the loss of a molecule of water from cholesterol or a neutral fatty acid from a cholesterol ester. In this way, DI-MS can serve as an efficient tool for the identification of indigo and its degradation product trypthanthrin in paintings and polychrome sculptures microsamples, offering (at the same time) information on the nature of the binder. Analysis by DI-MS of the blue microsample from the mural painting of the Andean church of Copacabana de Andamarca allowed the rapid identification of indigo and an egg tempera binder in just a few minutes. These results may be complemented with GC-MS analyses in order to obtain more information on the degradation stage of the binder.

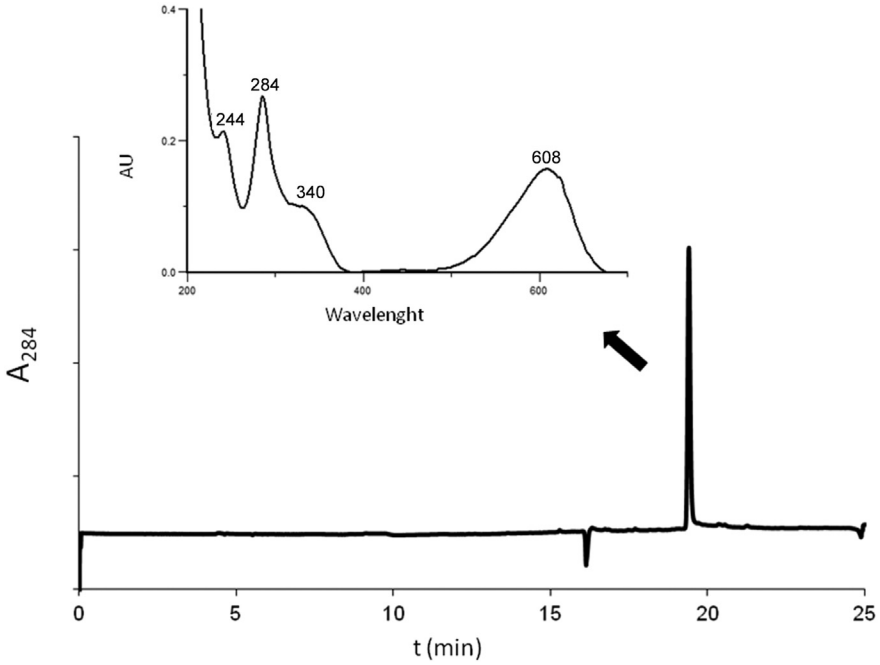

Fig. 9. HPLC chromatogram of the chloroform extract of sample M6 at 284 nm with UV spectrum of the peak at 19.41 min.

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