Chapter 6
Application of HPLC–DAD–QTOF to the Analysis of Natural and Synthetic Organic Pigments in Paint Layers

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1 Introduction

Among the whole topics in the field of art materials, one of the most interesting and fascinating is that concerning with the study of dyestuffs and organic pigments because it revels the ancient connection between art and life science. If the analysis of inorganic pigments is nowadays an habitual practice thanks to well-established techniques such as SEM-EDX (Scanning Electron Microscopy—Energy dispersive X-ray spectroscopy), Raman and other spectroscopic methods, the characterization of organic lakes is near to be considered as a routine procedure. The main factors contributing to the difficulty of the task are the intrinsic chemical complexity of the matrix, the availability of very small fragments of pictorial film, the low concentration of the organic pigments in the paint layers and the chemical similarity of the analytes.

Lake pigments have yet been analyzed using RAMAN microspectrometry although total elucidation of unknowns and similar dyes still is far from the goal of characterizing a lot of cases in which chemical similarity and interference of the matrix don't allow it [1].

The analysis of pure layers of colored lakes (for example a thick layer of a red lake glaze) has been practiced and achieved since the last twenty years [2]. When the lake was altered or was not pure, e.g. when present in the form of isolated grains of organic pigment in a inorganic matrix, its identification was almost impossible due to the lack of sensitivity of the old equipments [3]. While the red lakes have been extensively studied since the beginning [4], yellow ones were barely investigated and rarely detected in paintings. Nowadays HPLC (high performance liquid chromatography) technique is living a revolution in this research field due to the

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coupling of the new high sensitivity ultra-HPLC separation systems with powerful and extra sensitive and precise mass detectors. One of the highest performing among them is the Quadrupole—Time of Flight Mass Spectrometer (QTOF) [5]. Using this detector, together with DAD it is possible to detect substances in the range of atto-mol, making feasible the detection of red, brown, yellow and blue dyes in paint micro—samples even if in the form of glazes or as minor colored compound of organic-inorganic pigment mixtures [6]. In addition, several other compounds, including lipids, fatty acids, glycerides and di- and tri-terpenes, can be detected in the same analysis with a unique sample injection.

2 Materials and Methods

2.1 Experimental

Optical polarized light microscopy (PLM) analysis was performed on embedded samples, polished to get a cross section. Embedding resin was poly—ethyl-metacrylate Technovit 4004 (Kulzer). Micro—photographs were obtained using a Olympus BX-51 polarized light microscope, with reflected light, using visible or ultraviolet (UV) light. In this last case, autofluorescence of organic materials was used to obtain images to detect and delimitate the areas or layers of the cross section where animal glue, terpene resins, red lakes were present. Fourier transform infra—red spectroscopy (FTIR) analysis was performed on micro—sample's accessible surfaces (varnish and background layer mainly), using a Universal Attenuated Total Reflectance (UATR) accessory, without further preparation, and were recorded between 400 and 4000 cm^{-1} in a Bruker Tensor 27 spectrophotometer.

Scanning electron microscopy (SEM) with energy dispersive X-ray (EDX) elementary analysis was conducted with a Hitachi S340N Scanning Electron Microscope working with a EDX analyzer equipped with a Bruker Quantax X Flash SDD, with a spectral resolution of 125 eV Images where obtained working with a retro—dispersed electrons.

HPLC–DAD–QTOF analysis was performed on a Agilent 1200 HPLC system with autoinjector, equipped with a DAD detector and a Agilent 6530 QTOF detector. The conditions for HPLC–DAD–MS analysis were fixed as follows. Hydrolysis of the microsamples was achieved in 1.5 mL Eppendorf vials, adding to less than 100 μg sample, 100 μL of a mixture of water/methanol/hydrofluoric acid 4 M 1:1:2 (volume) [6]. After heating 10 min to 60 °C, vial was evaporated to dryness at 100 °C with nitrogen flushing. Extraction of the soluble organic compounds was performing by adding 200 μL of a mixture methanol/dimethylformamide 1:1, sonicating the closed vial without temperature controlling (always less than 50 °C) for 15 min. Later the closed vial was heated 110 °C during 20 min, the it was allowed to cool to room temperature, opened and evaporated to 20–30 μL at 100 °C, with nitrogen flushing. 1–2 μL was injected.

The chromatographic HPLC method is detailed now. Column Agilent Zorbax-C18 SB 1200 bar, 50 × 2.1 mm, particle size 1.8 mm, temperature 40 °C, flow 0.7 mL/min. Mobile phase was composed of (A) 0.1% (volume) aqueous formic acid and (B) pure acetonitrile, working with the following gradient: t = 0 min 90% A linear gradient to 100% acetonitrile at 25 min. This composition was held till 30 min. DAD detector swifts between 200 and 800 nm, monitorizing at λ = 275 nm, λ = 350 nm, λ = 550 nm and λ = 600 nm. QTOF detector worked in negative mode [Electrospray ESI (−)], gas temperature 300 °C, gas flow 8 L/min, nebulizer: 55 psi, sheath gas temp 400 °C, sheath gas flow 12 l/min, capilar voltage (−) 3500 V, fragmentor: 185 V. Acquisition was done in MS and MS/MS mode, with a mass range of 100–1700 m/z units. For indigoid dyestuffs MS detector worked also in positive mode [Electrospray ESI (+)] with the same conditions, but with a gas flow of 5 L/min.

2.2 Results and Discussion

All the chromatograms obtained with the above described methodology have been processed with the Agilent Mass Hunter software. Data processing involved the comparison of the UV–VIS and MS chromatograms in order to isolate the peaks related to coloured compounds, the extraction from the Total Ion Chromatograms (TIC) of all the single ion chromatograms (EIC—Extracted Ion Chromatogram) and finally the identification of compounds by comparison with a reference database and by study of the fragmentation pattern in the MS/MS spectra. The characterization of colored lakes started with the identification of those compounds notoriously assigned to the different species, i.e. carminic acid ($C_{22}H_{20}O_{13}$), kermesic acid ($C_{16}H_{10}O_8$) and flavokermesic acid ($C_{16}H_{10}O_7$) for the cochineal; brazilein ($C_{16}H_{12}O_5$), brazilin ($C_{16}H_{14}O_5$) and type C ($C_{13}H_8O_5$) for the brasilwood; alizarin ($C_{14}H_8O_4$), purpurin ($C_{14}H_8O_5$) and rubiadin ($C_{15}H_{10}O_4$) for the madder; laccaic acids (A–F) for Lac dye. In a second step the detailed study of the fragmentation patterns allowed the identification of the glycosides and other secondary compounds. All the masses detected and recognized as markers, but not yet identified, have been catalogued as unknown compounds. The identification of such minor compounds, not directly related to the previous ones, is being carried out.

The analyses of indigoid dyes revealed the limited efficiency of the developed method in the negative ionization of this specific class of compounds. In any case this not represented a big problem since the recognition of blue indigoid dyes (indigo and woad) is possible thanks to the characteristic UV visible spectra of their main compounds, indigotin and indirubin, which presents a typical absorption around 616 and 550 nm respectively. The more challenging results are been achieved with yellow and green lakes [7], with the full characterization of almost all the aglycons (mainly flavonoid dyes) and the related glycosides. In Fig. 1 are shown the UV–VIS and the Mass Chromatograms of a green lakes obtained from ripe buckthorn berries (*Rhamnus cathartica* L.), with the identifyed compounds on each of the peaks [8].

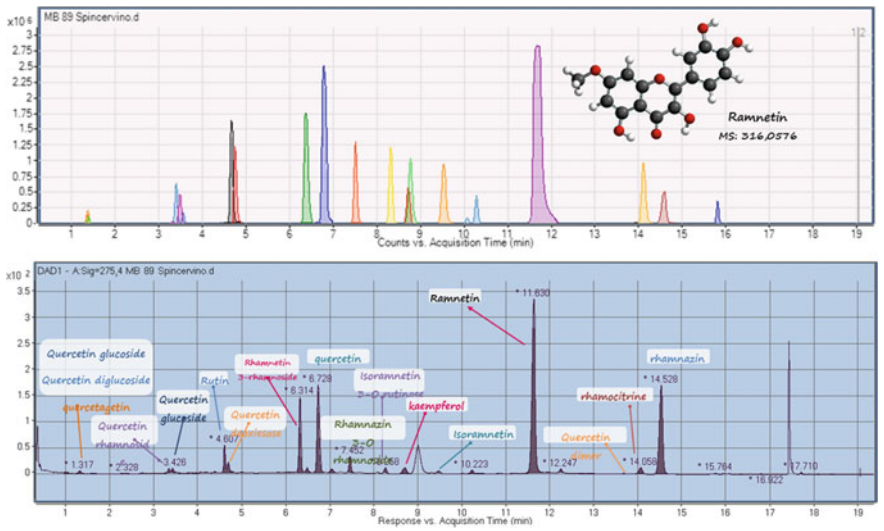

Fig. 1 LC–MS EIC (extracted ion current) and LC–UV–VIS (275 nm) chromatograms of the sample MB89 (*R. cathartica*)

The way of detecting and determine the source of a natural dyestuff lake pigment begins with the study of the cross section both by optical microscopy and SEM/EDX. The clues for selecting a sample for HPLC analysis are high transmission of polarized light, notorious visible fluorescence when lighted with UV light, and high carbon content in EDX, besides the occasional presence of mineral ions (i.e. aluminium, calcium, or sulphate, phosphate) being part of the inorganic support. In the case of synthetic dyes the method is applied in the same manner. For these case studies the database grows with every unknown elucidated. Here, the tools for the analysis of the unknown compound, once isolated by chromatography, are UV–VIS spectra, MS spectra with the value of accurate molecular mass (up to for decimal digits), and isotopic pattern of molecular ion. Both data allows obtaining the molecular formula within a short file of possibilities. These delimit the search, among all the possibilities of colour index [9], to a very limited group of molecules. At least, fragmentation in the MS/MS spectra may help to decide doubtful cases.

3 Natural Dyestuff Analysis. "San Francisco's Life" A Series of XVIII Paintings with Interesting Materials

This analytical technology was also applied to paint micro—samples taking out during the conservation process with the aim of characterize materials, mainly varnishes, dyestuffs and binders, over paints, and all the important data which affects the restoration procedures. The series of paintings represent San Francisco's

life (see as an example Fig. 2) are big size oil on canvas compositions (216 × 270 cm), done by Zacarías González Velázquez (Madrid, 1763—ibídem, 1834), for the decoration of S. Francisco el Grande Basel (Madrid). Pupil of Diego Salvador Maella, he was a Royal court's painter in 1755 and worked as Director of Painting at the Academy of Fine Arts of San Fernando since 1765. He is a recognized XVIII master painter working in wall paintings, some of the with fresco technique. He also painted big format oil paintings such as this set of canvas on the life and miracles of San Francisco. They were done to decorate the cloister of the convent of Madrid's Basilica at 1787. His style is fully inside the Neoclassicism.

Mineral or let us say, inorganic pigments, found in the original brushstrokes are the usual ones found at the end of XVIII century. It is the normal set of classical pigments added to several important contributions of XVIII century chemists to art materials palette. Talking first about the classic mineral pigments found, these are azurite, white lead, charcoal black, vermillion, copper green (verdigris or "*cardenillo*"), red and brown ochre. Some other, specific of XVIII century, are Prussian blue and Naples Yellow (lead antimoniate). The Prussian blue found with PLM was confirmed by IR spectroscopy, because of the presence of cyanide absorption at 2085 cm^{-1}. It is important to notice the observation, in PLM and SEM/EDX studies, of the presence of significant amounts of organic colored grains. This points to the possibility of the presence of organic lakes together with those mineral pigments described above. All these data made these samples so interesting for HPLC studies [10] (Figs. 3 and 4).

In order to check the performance and sensitivity of our chromatographic system, four micro—samples of different colors (blue, green, red and brown colors) were analyzed using the above described methodology in the experimental section. Samples were compared with a huge database created at this facility with a great variety of natural dyestuffs, in order to confirm the presence of coloring compounds, just to be able to assign the obtained result to a particular dyestuff, i.e. species of origin. Particularly, four coloring compounds were detected in the analysis of the selected samples. In dark red samples, carminic acid was detected (Fig. 5). It was not possible to detect and quantify other components of cochineal reds, like kermesic and flavokermesic acid. These last compounds can help us to distinguish among the different types of cochineal (i.e. Polish cochineal, American cochineal or the Armenian one) [11]. The low concentration of these kermesic acid derivative, always below the detection limit of the technique and the date of the paintings points to *Dactylopius coccus* or American cochineal. It was admixed with low quantities of vermillion, and whitish starch grains that can be identified in the cross section.

In blue and green color, traces of indigotine are detected. In the case of green colors the mixture is quite complex. We have a first mineral fraction with copper green, Naples yellow and Prussian blue rich in alumina. In addition, HPLC analysis revealed the composition of the organic colored fraction as indigotine, chrysoeriol and trace amounts of indirubine were clearly identified (Fig. 6). The presence of indigotine was not a surprise, because in these paintings seem to be associated with Prussian blue and it was yet found in blue layers. On the other side, chrysoeriol is a

Fig. 2 "S. Francisco in the presence of sultan". Zacarías González Velázquez

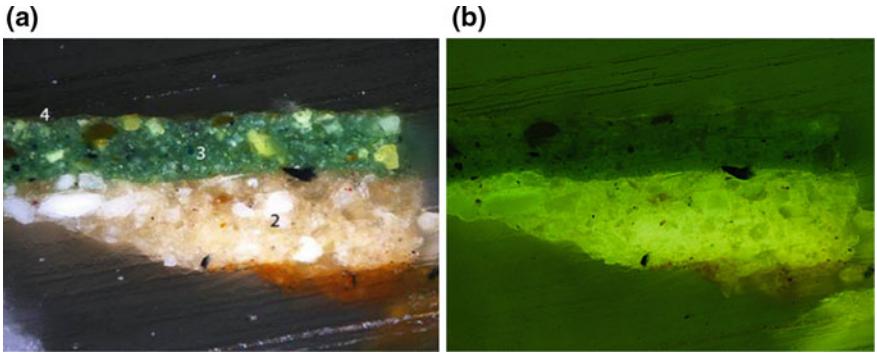

Fig. 3 **a** (left) Cross section of sample PFZ-P3, 300X. Layer 1 is a red imprimatur of red—ochre earths. Layer 2 is a second gray imprimatura. Green layer nr. 3, is a complex mixture of green earth, Naples yellow (lead antimoniate), Prussian blue and a yellow dyestuff. **b** (right) The same cross section under UV light (blue filter), 300X

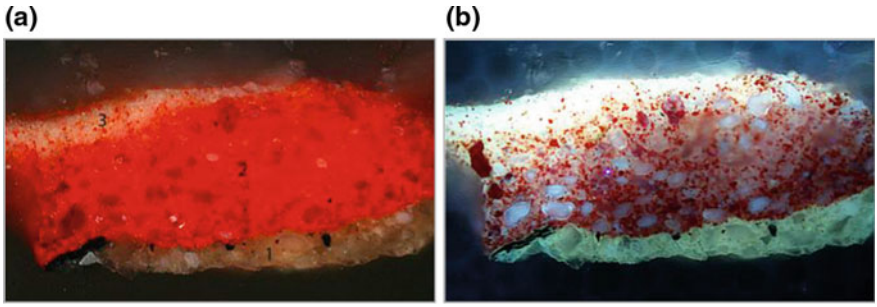

Fig. 4 **a** (left) Cross section of a intense red color mainly done with vermillion PFZ-P7, but also with a lot of starch grains accompanying. Visible reflected polarized light, 500X. Layer 1 is the grey imprimatura. Layer 2 a thick red layer, with vermillion (intense red mass) and starch (dark red areas). **b** (right) The same sample, under UV light (photography was taken with a visible filter), 500X Realize that vermilion is present in the form of intense red spots and grains, and that starch reveals as ovoid shaped white grains

natural methoxylated flavone (5, 7, 4′-trihydroxy-3′-methoxyflavone), present as a minor compound in several broom species. Only in *Cytisus scoparius,* a kind of broom coming from Scotland, chrysoeriol and its C-glycoside derivative are major compounds. In Spain, aliaga (*Genista hirsuta*) could have the same composition, it has been seldom used as dying material, but still is not fully investigated [9]. Broom, made mainly of available *Genista* sp., is a well known raw material used to the manufacture of green pigments, by mixing indigo or woad with a yellow lake made of broom's flowers [12]. Another possible explanation is that has to be checked is that it could by a minor component of local source for indigo *Isatis tinctoria*.

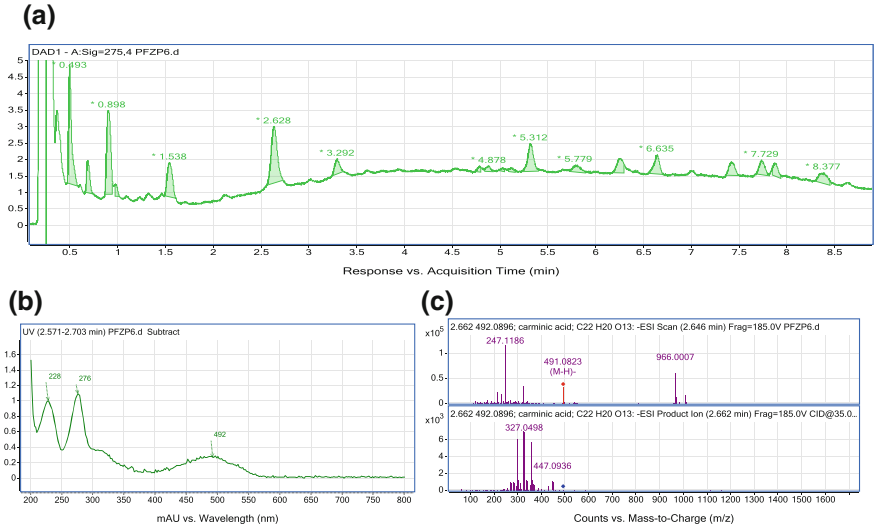

Fig. 5 HPLC analysis of a dark red sample, with vermilion and red lake, PFZ-P6. **a** (top) HPLC chromatogram, registered with the DAD detector at 275 nm. Peak at 2.6 min corresponds to carminic acid. **b** (bottom left) UV–VIS spectrum of carminic acid. **c** (bottom right) MS and MS/MS spectra of the scan at 2.6 min, which corresponds to carminic acid

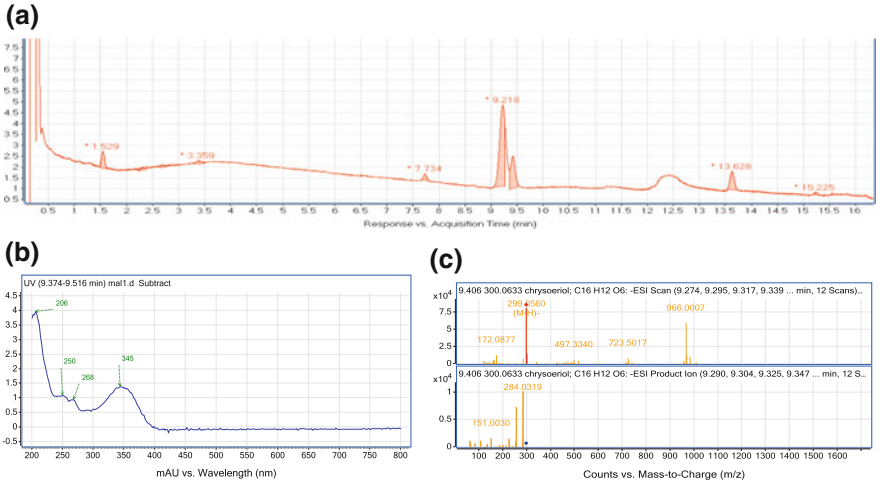

Fig. 6 HPLC analysis of green sample PFZ-P3. Compounds found are chysoeriol, indigotine and indirubine, **a** (top) HPLC chromatogram registered at 275 nm. Peak at 9.4 min corresponds to chrysoeriol, peak at 13.6 min to indigotine and at 15.2 to indirubine. **b** (bottom left) UV–VIS spectrum of chrysoeriol at 9.4 min. **c** (bottom right) MS and MS/MS spectra of scan at 9.4 min

4 Synthetic Dyestuff Analysis. Santa Lucía: A XVIIIth Century Sculpture Overpainted in XXth Century

The first case study is a XVIII century sculpture belonging to the Diocesan Museum of Córdoba's Collection. It is a polychromed wooden statue representing Saint Lucia with her iconografic features. The sample had been collected from a pale green layer in the back part of the saint's robe (see Fig. 7).

As shown in the cross section, the sample presents four superimposed layers: gypsum priming, a red earth layer, a lead white *imprimatura* and an opaque pale green layer. The green layer is composed of a mineral copper green pigment according to SEM imaging and EDX micro elementary analysis. It might be verdigris or altered blue verditer by means of SEM-EDX. Together with the Cu, elementary analysis detected also high contents of C, pointing to the presence of a considerable amount of organics. HPLC–DAD–MS analyses was done in order to characterize this organic compounds. The UV–VIS chromatogram revealed the presence of two blue organic compounds. Retention time, MS and MS/MS data revealed the presence of *methyl blue* (RT: 6.4 min; $C_{37}H_{29}N_3O_9S_3$), an early synthetic dye, and *indigotin* (RT: 13,9; $C_{16}H_{10}N_2O_2$), the main constituent on Indigo dye. As no traces of indirubin has been found in the sample, we concluded that it should be synthetic indigo, as indirubin is always present (as a minor compound) in natural blue dyestuffs derived from *Indigofera* or *Isatis* species. In the case of indigotin, the chromatogram has been recorded in positive polarity mode

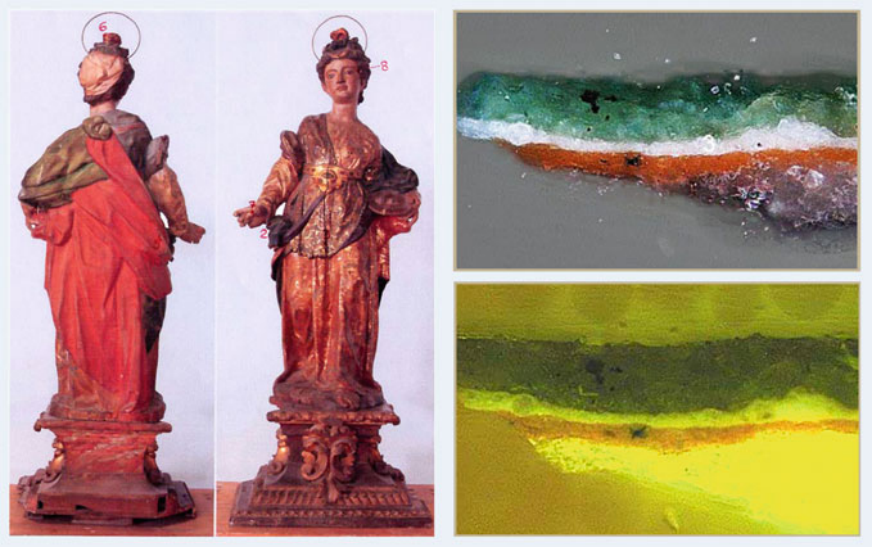

Fig. 7 (left) Saint Lucia, polychromed wood sculpture, XVI century; (right) cross section at 300X of sample SLC-1 (green color of the robe) in visible (UP) and UV light (down)

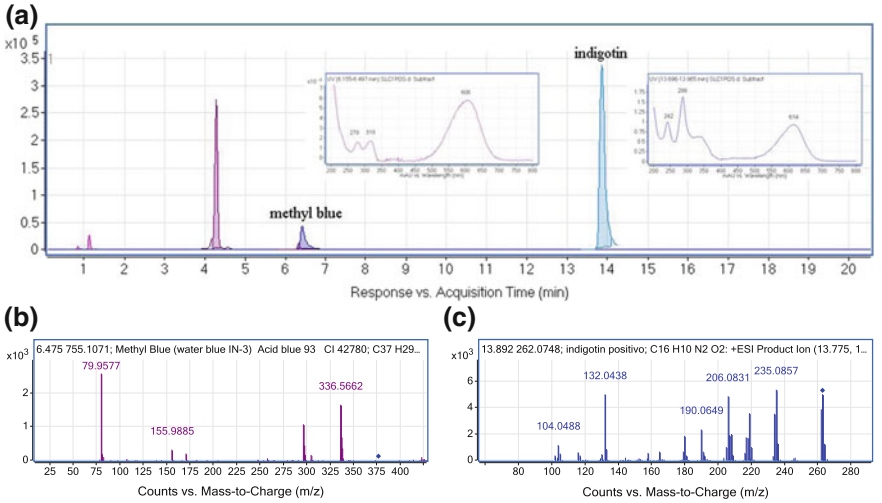

Fig. 8 HPLC–QTOF–MS chromatogram, UV–VIS spectra (up) and MS/MS spectra (down) of the compounds identified in sample SLC-1

of the mass detector, as indigoid dyes present a very low percentage of ionization in negative mode. Mass chromatogram, UV–VIS and MS/MS spectra of the two compounds are shown in Fig. 8. So, although the initial diagnostic for the bluish green was that it was fully original, based only on the elementary analysis EDX, organic analysis of the pigmentation leaded us to conclude that green layer was an overpaint or reintegration layer, done at the beginnings of XXth century or later. The presence of these two organic dyestuffs also explains the dusty and whitish aspect of the green color, as organic dyestuffs suffer a lot more of fading with visible and UV irradiation than minerals pigments do.

5 Synthetic Dyestuff Analysis. Contemporary Art. Paint on Canvas (Oil + Acrylic). Manuel Millares "Cuadro 63" (1959)

Contemporary art is one of the fields where dyestuff analysis can do important advances in the knowledge of organic components, essential for the work of art's appearance and conservation state. We bring here a case study in which a red synthetic organic pigment. "Cuadro 63" (Antonio Millares, 1959) is an abstract composition on canvas support. In it, oil technique coexists with acrylic and may be other synthetic binding media. Organic pigments were suspected to be present using SEM/EDX analysis, as carbon signal increase and no metal was detected during elementary analysis of cross sections. Also, as it may be seen in Fig. 9b the aspect

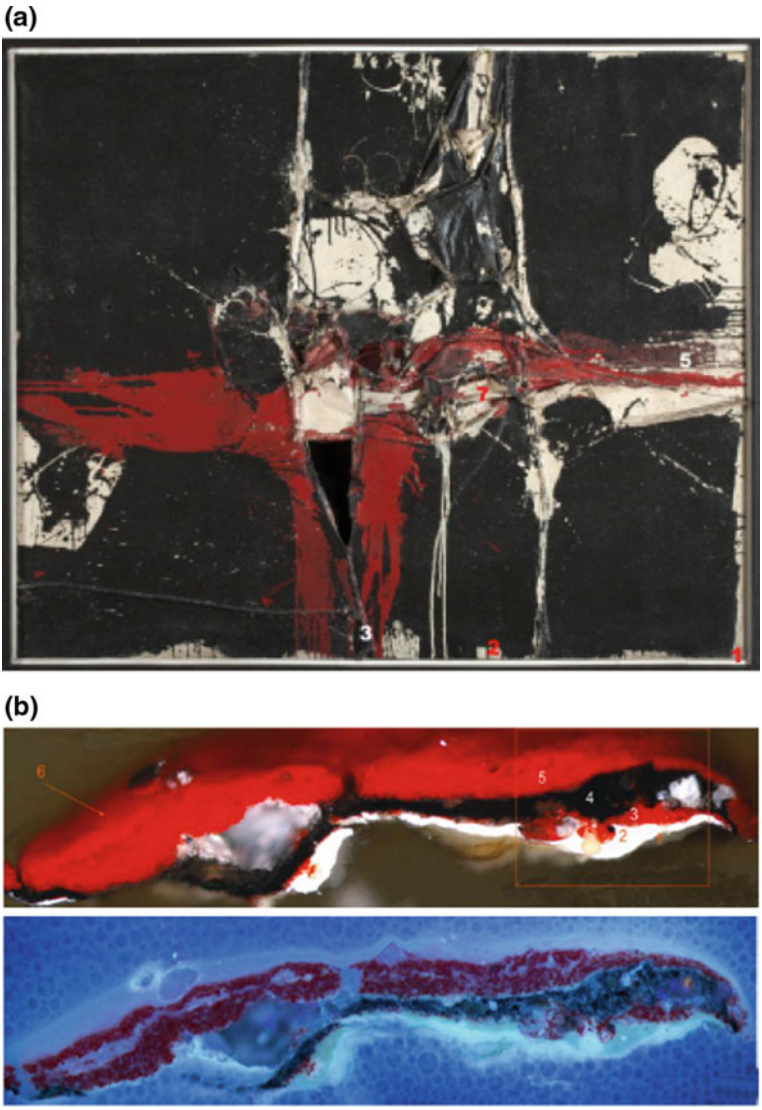

Fig. 9 **a** Up "Cuadro 63". Oil/acrylic on canvas. Manuel Millares (1959). **b** Middle: Cross section corresponding to the intense red color under visible light. Down: the same under UV light

of red color in the section is intense, rather continuous, with a high fluorescence under UV light, so that an organic pigment was suspected.

HPLC–DAD analysis yielded a solitary peak absorbing in the UV region, and also at nearly 500 nm (see Fig. 10a) in the visible region, identifying the dyestuff as a red compound. MS–MS analysis indicated a probable molecular mass of 308 u.m.a and a molecular formula C17H13N3O3 (see Fig. 10b). Not so many known

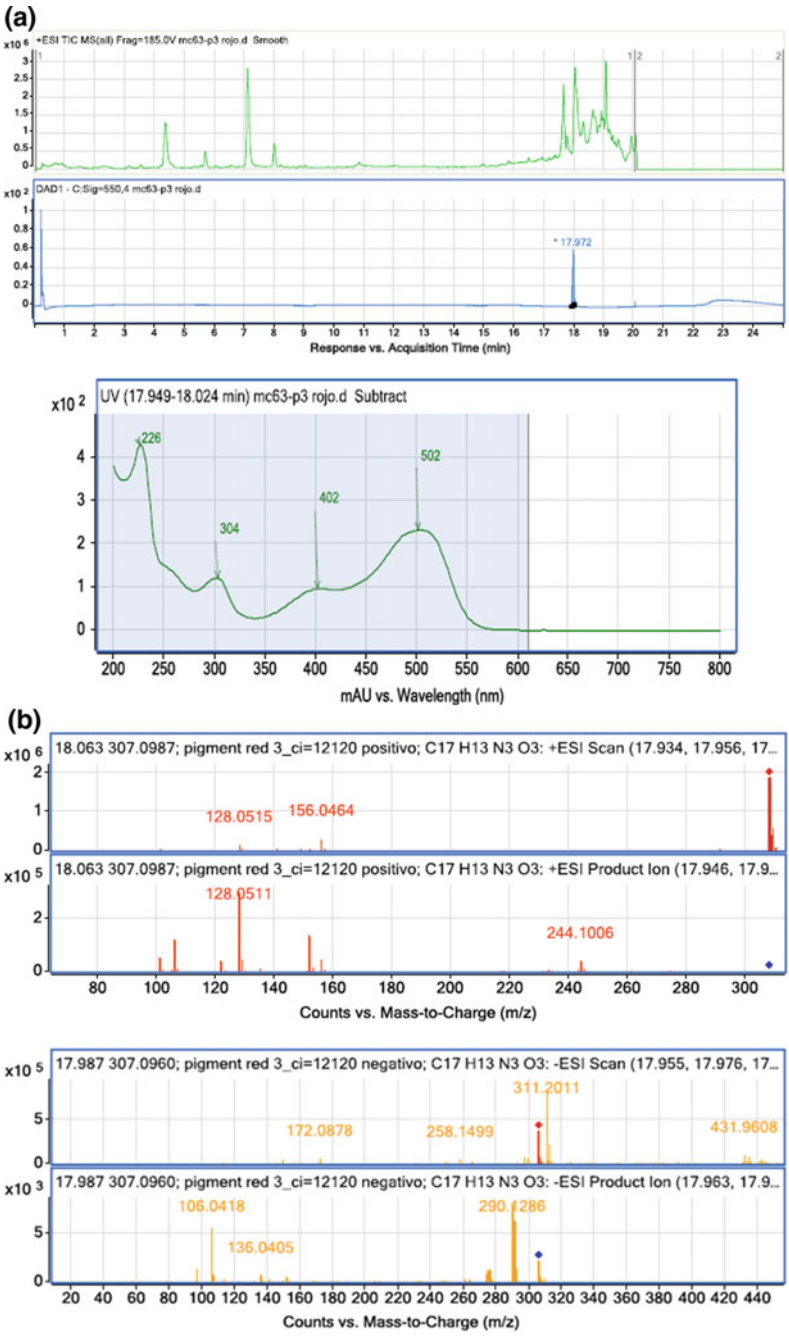

Fig. 10 **a** Up: chromatograms (TIC and Visible at 550 nm) of red paint sample from Millares' painting. Down: UV–VIS absorption spectra of the red dyestuff. **b** Up: Mass-mass spectra acquired with negative polarity of mass detector. Down: Acquired in positive polarity. **c** Chemical structure of Pigment Red 3 (CI 12120) or (1Z)-1-[(4-methyl-2-nitrophenyl) hydrazinylidene] naphthalen-2-one

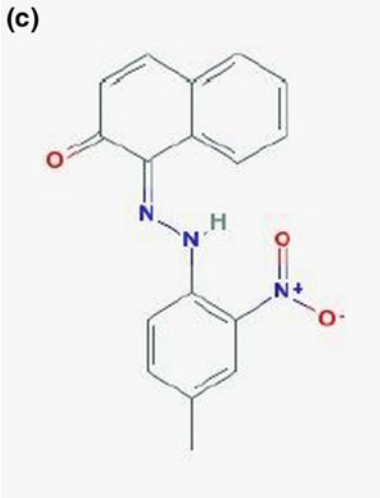

Fig. 10 (continued)

synthetic dyestuff has that molecular mass, and just one matches the molecular formula. It was identified so as Pigment red 3 (Colour index 12120), and its formula can be seen in Fig. 10c.

6 Conclusions

The analytic survey carried out in this research activity shown that there is still a lot of work to do to achieve a complete knowledge and understanding of the world of natural and synthetic organic pigments, its composition and its role in painting film degradation. Although many of the dyes traditionally used by artists are known and well documented, there is a variety of dyeing species not identified yet. With synthetic organic pigments, it is possible to make extraction and identification of soluble or hydrolizable synthetic dyestuffs inside paint matrixes and separate mixtures of them, which allows the source identification, chronological studies and diagnostic analysis of alteration or fading of these organic compounds.

Thanks to its versatility, sensibility and high resolution, the HPLC–Q–TOF–MS proved to be an efficient and powerful tool to the analyses of paint samples and in general for the characterization of Cultural Heritage micro—samples, and to do alteration studies regarding to its chemical stability. Also to the elucidation of unknown dye compounds, using its feasibilities regarding to molecular information, i.e. molecular formulas and fragmentations.

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