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CHIMICA ANALITICA

Elaborato del Tirocinio:

HPLC-DAD-MS and MALDI strategies for anthraquinoid lakes identification in paint samples

Relatore: Dott.ssa Ilaria Degano

Correlatore: Dott.ssa Anna Lluveras Tenorio

Controrelatore: Dott. Alessandro Mandoli

Candidato: Francesca Sabatini

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Contents

Introduction

Della natura di un rosso il quale vien chiamato lacca
Si fa lacca di cimatura di drappo, o ver di panno, ed è molto bella all'occhio.
Di questa ti guarda, però che ella ritiene sempre in sé grassezza, per cagione dell'allume, e non dura niente né con tempere né sanza tempere, e di subito perde suo colore.

Cennino Cennini [1]

The thesis aims at developing and optimizing analytical methods for the characterization of anthraquinoid lakes and proteinaceous materials used as paint materials by means of chromatographic and mass spectrometric techniques (High Performance Liquid Chromatograph with Diode Array and Mass Spectrometric detector, Gas Chromatography/ Mass Spectrometry, Matrix Assisted Laser Desorption Ionization and Laser Desorption Ionization - Mass Spectrometry). Anthraquinoid dyes are aromatic compounds derived from anthraquinone and they have been the most applied red organic colorants in the history of art. In order to be used for painting purposes, dyes have to be precipitated either as metal complexes or adsorbed on an inert substrate in an insoluble form called lake [2]. Without this preparation the dyestuff has no 'body' and it cannot be used mixed in the binding media like the other solid pigments. Lakes are characterized by a high coloring power given by the dye and translucency provided by substrate [3]. For these features and their low covering propriety they have been widely employed in glazing. This technique, exhibited by several European painters of XIV and XIX century, consisted in the overlapping of colored paint layers on already dried paint layers in order to achieve different nuances [4]. Red anthraquinoid lakes, as madder, kermes, and cochineal lakes and Indian lac, were mainly used to enrich the red tone of opaque inorganic layers [5]. These lakes were prepared from natural dyes of plant, as madder, and animal origin, as kermes, cochineal and lac dye. Most of these natural sources were available in Europe since ancient times, while some others have been imported since early times from India and South and Central America [3]. In Figure 1, the geographical locations of natural sources of red dyes are depicted.

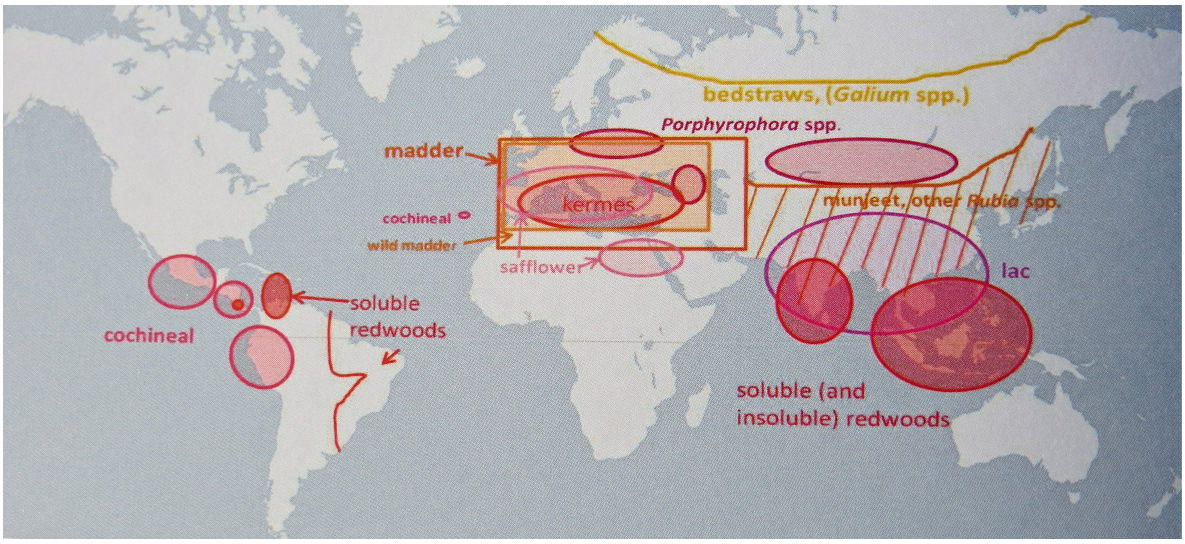


Figure 1: Map showing the geographical location of natural sources of red dyes [3].

The natural binding media used in painting are complex mixtures of organic species mainly constituted by glycerolipids, polysaccharides and proteinaceous materials. Binders were employed for the application of the organic and inorganic colorants on the substrate resulting in thin and cohesive paint layers with a heterogeneous composition [6].

The characterization of organic materials is challenging due to physical transformations, ageing processes and interactions among different materials that may possibly occur. In addition, the tiny amount of sample available has to be kept in mind for the optimizing of the procedures of analysis [7].

This thesis addresses one of the most important goals for the analytical chemist working in cultural heritage field: the development of promising strategies allowing to maximize the information achievable while minimizing the amount of sample needed. A multistep GC/MS procedure for the simultaneous characterization of proteinaceous and polysaccharide materials, glycerolipids, natural waxes, terpenoid resins, possibly present in a unique sample has been already published [7] and it perfectly fulfills the requirement explained above. Nevertheless, the potentialities of this combined procedure might be further improved if the analysis of the lakes could be included. In this way, it could be possible to characterize all organic materials possibly used in an unique micro-sample. The achievement of this goal is rather arduous because several factors should be taken into account, above all the fact that the materials to analyze are heterogeneous mixtures of organic compounds. Thus the influence of the glycerolipid, polysaccharide and proteinaceous contents of lakes as raw materials on the identification of the binder has to be evaluated. In addition, the modification in detectability of the lakes possibly caused by the presence of the binders has to be investigated. Therefore, the analysis of reference materials and paint model systems is of paramount importance for our studies.
The main goal of the thesis project, undertaken at the Chemical Science for the Safeguard of the Cultural Heritage (SCIBEC) workgroup (Dipartimento di Chimica e Chimica Industriale, Università di Pisa), and at the Department of Biochemistry and Microbiology of ICT in Prague, was to develop and optimize methods for the analysis of the anthaquinoid lakes, the most used and important for painting purposes, as well as to evaluate the role played by these materials in the detection and analysis of proteinaceous binders.
Particularly, Chapter 1 contains a review of the literature related to the main features and proprieties of dyes, lakes and binders involved in our study and the analytical techniques, procedures and sample treatments generally used for their detection and characterization. In Chapter 2 materials, instrumentation, detection and quantitation parameters as well as the model paint systems and the archeological samples analyzed are presented. In Chapter 3 the development and optimization of a HPLC-DAD and LC-MS/MS method for the analysis of anthraquinoid lakes is described. The different extraction solutions, pH conditions and solvents of injection used to optimize the procedure are reported. Extraction yields quantitatively evaluated from HPLC-DAD chromatograms are discussed, as well as LC/MS-MS mass spectra used for a better characterization of the unknown compounds formed. Chapter 4 reports on the investigation of the mutual influence of lake-binder. In detail, HPLC-DAD has been applied on naturally aged and freshly paint model systems to discuss the influence of the presence of the binder on the lake identification, while GC/MS analyses have been used to evaluate the contribution of the saccharide, lipid and proteinaouces fraction of lakes in the determination of binders. Chapter 5 describes the optimization of a MALDI-ToF-MS method, implying a trypsin cleavage, for the analyses of proteins,

and two LDI-ToF-MS methods, a direct one and another making use of hydrofluoric acid for dyes extraction, for identification of anthraquinoid lakes. Chapter 6 reports on the results of the analyses of selected archeological and historical paint samples carried out by applying the procedures and techniques described in the previous chapters.
On the basis of the results obtained, important considerations about the methods developed and the achievement of the goals are provided.

Bibliography

(1) C. Cennini, *Il Libro dell'arte o Trattato della Pittura di Cennino Cennini*, Felice Le Monnier Editore, Firenze, 1859.

(2) E. Martuscelli, *I Coloranti Naturali nella Tintura della Lana Arte, Storia, Tecnologia e 'Archeo-Materials Chemistry'*, Programma Nazionale di Ricerca Beni Culturali (MIUR) La Conservazione dei Tessuti Antichi, vol. II

(3) J. Kirby, M. Van Bommel, A. Verhecken, *Natural Colorants for Dyeing and Lake Pigments, Practical Recipes and their Historical Sources*, Archetype Publications, 2014.

(4) T. M. Simon*, Glazing*, The Art of Composition, 2008.

(5) M. Rosato, *Caratterizzazione delle lacche rosse in manufatti pittorici antichi*, tesi di laurea, Università degli studi di Pisa, Facoltà di Scienze Matematiche, Fisiche e Naturali, Dipartimento di Chimica e Chimica Industriale, 2000.

(6) L. Campanella, A. Casoli, M. P. Colombini, M. Marini Bettolo, M. Matteini, L.M. Migneco, A. Montenero, L. Nodari, C. Piccioli, M. Plossi Zappalà, G. Portalone, U. Russo, M.P. Sammartino, *Chimica per l'Arte*, ed. Zanichelli, 2007.

(7) A. Andreotti, I. Bonaduce, M. P. Colombini, G. Gautier, F. Modugno, E. Ribechini, *Combined GC/MS Analytical Procedure for the Characterization of Glycerolipid, Waxy, Resinous and Proteinaceous Materials in a Unique Paint Microsample*, Analytical Chemistry, no. 78, pp. 4490-4500, 2006.

Chapter 1

State of the art

1.1 The analysis of materials in paintings

Cultural heritage is one of the principal products of mankind to be preserved for future generations. Since the late 18th century, an analytical approach to art and archaeological objects was developed as a result of the progressive practical application of the ideas of art historians like Johann Winckelmann (1717–1768 A.D). He believed that the study of artworks should be based on their examination rather than based on texts [1, 2, 3]. Since then, the number of techniques and procedures applied and optimized for the chemical characterization of materials employed in artworks [4], such as paintings [5], textiles [6, 7], potteries [8], manuscripts [9], cosmetics [10] and archeological woods [11] has constantly grown.

From a physical-chemical point of view, paintings can be considered as tri-dimensional arrays of several layers [12] made of a heterogeneous mixture of organic and inorganic materials belonging to different chemical classes. These compounds are subjected to interactions, ageing and environmental effects which modify the original composition of the materials. Over time, paint layers may exhibit yellowing, cracking, darkening, loss of cohesion and of stability. Consequently, paintings can be defined as complex evolving systems [13, 14].

In particular, organic materials have been employed as binders, adhesives, waterproofing materials and colorants in paintings [15]. The characterization of organic painting materials is of a paramount importance because it allows to determine the artists' painting techniques and binders recipes, and to study the interaction and degradation phenomena due to ageing. This type of information is fundamental from the artistic point of view, as far as it may permit to date and locate a work of art and to know technological skills of civilizations. Moreover, it is fundamental to choose the most suitable procedures and materials during restoration campaigns [16, 17, 18, 19]. Nonetheless, the analysis of organic materials is particularly arduous for the chemist because they are subjected to ageing, physical transformations and oxidation processes [13]. In addition, the small amount of sample available and the mixture of materials present in unknown proportions increase the difficulty of their chemical characterization [20]. Consequently, nowadays research mainly aims at developing new promising strategies that allow maximizing the information achievable and minimizing the sample needed.

1.2 Organic paint materials

In antiquity organic compounds used as painting materials were obtained from natural materials derived from plant or animal sources. In particular, organic dyes can be classified as tannins, flavonoids, indigoids, anthraquinones according to the chemical classes of their chromophores containing molecules. Organic materials used as binders belong to different classes of biological molecules, such as glycerolipids, polysaccharides, proteins, natural waxes and natural resins [12].
The main goal of the following sections is to introduce dyes and binders used as painting materials showing their main features, properties and applications. This information is necessary to understand the different protocols and analytical techniques applied to paint samples.

1.2.1 Dyes

Colorants are characterized by their ability to absorb the visible part of the electromagnetic spectrum (380-780 nm). The term colorant is often used for both pigments and dyes. Pigments are prevalently inorganic and insoluble in water, oils and resins. They are dispersed in the medium and they are rather high light-fastness. On the contrary, dyes are soluble organic compounds and therefore easily soluble in water or in another binding medium. Due to their chemical structure, dyes are light vulnerable and labile [15]. Dyes have been historically used in artworks such as paintings, inks and archaeological textiles. In order to be used for painting purposes, dyes have to be either precipitated as salts, as metal complexes, or adsorbed on an inert substrate in an insoluble form called lake [27]. In particular, a lake is an artificial pigment obtained from one or more organic natural dyes fixed by absorption or complexation on an insoluble and inorganic material [17]. Lakes are generally less stable to heat and light than inorganic pigments [15].
One of the most relevant examples of lakes used in paintings is that obtained from anthraquinoid dyestuffs, exploited for the production of red lakes [19].

1.2.1.1 The chemistry of anthraquinoid dyes

Anthraquinoid dyes are mixtures of aromatic compounds derived from the molecule of anthraquinone (9,10-anthraquinone), which belongs to quinones. These are a class of organic compounds formally derived from aromatic rings by conversion of an even number of -CH= groups into –C (=O)– groups with any necessary rearrangement of double bonds, resulting in a fully conjugated cyclic dione structure [24]. The anthraquinone can be considered the building block of this class of colorants and substitutions on the two aromatic rings give the particular coloration to the dye. The structures of the components of anthraquinoid dyes, such as madder, kermes, cochineal, lac dye, and their precursors [17, 25, 26] are shown in Figures 1.1-1.2 and their peculiar substituents and molecular formulas are reported in Tables 1.1- 1.2.

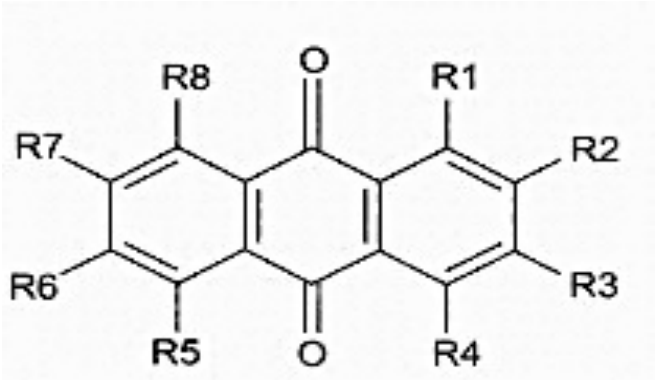


Figure 1.1: General structure of anthraquinones I.

Table 1.1: Names, abbreviations, substituents and molecular formulas of anthraquinones I and precursors (adapted from [26]).
(* Anthraquinoid chromophores containing molecules researched in this thesis are in bold.)

compound name	**abb**	**R_1**	**R_2**	**R_3**	**R_4**	**R_5**	**R_6**	**R_7**	**R_8**	**molecular formula**
alizarin	Ali	OH	OH	H	H	H	H	H	H	$C_{14}H_8O_4$
xanthopurpurin	Xpu	OH	H	OH	H	H	H	H	H	$C_{14}H_8O_4$
antragallol	Agl	OH	OH	OH	H	H	H	H	H	$C_{14}H_8O_5$
rubiadin	Rub	OH	CH_3	OH	H	H	H	H	H	$C_{15}H_{10}O_4$
morindone	Mor	OH	CH_3	H	H	OH	OH	H	H	$C_{15}H_{10}O_5$
munjistin	Mun	OH	COOH	OH	H	H	H	H	H	$C_{15}H_8O_6$
purpurin	Pur	OH	OH	H	OH	H	H	H	H	$C_{14}H_8O_5$
pseudopurpurin	Ps.pu	OH	OH	COOH	OH	H	H	H	H	$C_{15}H_8O_7$
emodin	Emo	OH	H	OH	H	H	CH_3	H	OH	$C_{15}H_{10}O_5$
flavokermesic acid (laccaic acidD)	Flk	CH_3	COOH	OH	H	OH	OH	H	OH	$C_{16}H_{10}O_7$
kermesic acid	Ker	CH_3	COOH	OH	H	OH	OH	H	OH	$C_{16}H_{10}O_8$
carminic acid	Car	OH	C-glucose	OH	OH	H	OH	COOH	CH_3	$C_{22}H_{20}O_{13}$
tectoquinone	Tec	H	CH_3	H	H	H	H	H	H	$C_{15}H_{10}O_2$
quinizarina	Qza	OH	H	H	OH	H	H	H	H	$C_{14}H_8O_4$
chysophanol	Chr	OH	H	CH_3	H	H	H	H	OH	$C_{15}H_{10}O_4$
aloe-emodin	Ale	OH	H	CH_2OH	H	H	H	H	OH	$C_{15}H_{10}O_5$
quinalizarin	Qlz	OH	OH	H	H	OH	H	H	OH	$C_{14}H_8O_6$
ruberythric acid	Rba	OH	O-primeverose	H	H	H	H	H	H	$C_{25}H_{26}O_{13}$

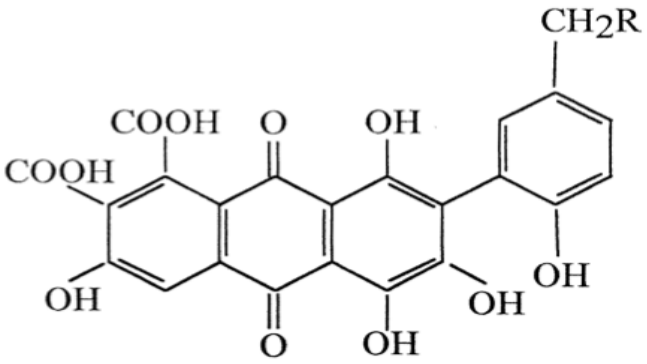


Figure 1.2: General structure of anthraquinones II.

Table 1.2: Names, abbreviations, substituents and molecular formulas of anthraquinones II (adapted from [26]). (*Anthraquinoid chromophore containing molecule researched in this thesis is in bold.)

compound name	abb	R	molecular formula
laccaic acid A	lac A	$CH_2CH_2NHCOCH_3$	$C_{26}H_{19}O_{12}N$
laccaic acid B	lac B	CH_2CH_2OH	$C_{24}H_{16}O_{12}$
laccaic acid C	lac C	$CH_2CH_2(NH)COOH$	$C_{25}H_{17}O_{13}N$
laccaic acid E	lac E	$CH_2CH_2NH_2$	$C_{24}H_{17}O_{11}N$

Anthraquinones have been the most applied organic colorants for red hues ranging from orange to pink shades before synthetic dyes appeared on the market. Their easy availability in nature and great resistance to photo-oxidation are plausible explanations for their employment since ancient times [17]. Ancient Egyptians and Persians already used alizarin extracting the rubierythric acid, alizarin glycoside, from madder. A detailed description of the main features of vegetal and animal anthraquinoid dyes is presented in appendix A.

1.2.1.2 Preparation of lakes

In order to prepare a lake, dyestuffs are firstly extracted in solution from their natural sources and the resulting coloring material is co-precipitated on an inert inorganic substrate. By this procedure, colored insoluble particles are formed (through complexation or absorption phenomena), and can be separated by precipitation and filtration, washed and dried to obtain a lake, which appears as a solid pigment [21]. In Figure 1.3 the main steps for the preparation of a lake from a dyestuff are outlined.

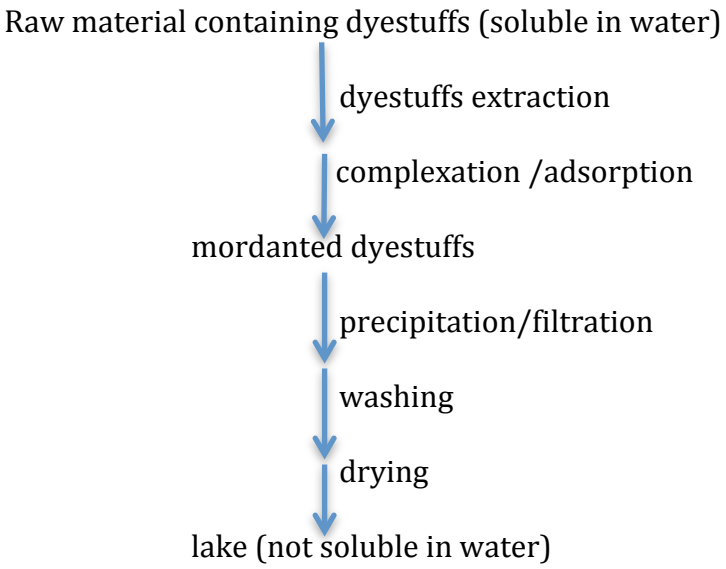


Figure 1.3: Scheme for the preparation of lakes

The preparation of a lake can be compared to the dying process of textiles consisting in two steps. In the first one, the fiber is plunged in a water solution containing the mordant, generally alum*; then the fiber is soaked in the water extract of the dye, which precipitates on the fiber by complexing the mordant ion [28]. Other salts used in lake production were chalk (calcium sulphate, $CaSO_4$) and gypsum (calcium suplhate dihydrate, $CaSO_4 \cdot 2H_2O$). The kind of metallic cation chosen may change and consequently various color shades could be achieved. A similar procedure was applied for lake preparation and the compound obtained is called *true lake* [22].

1.2.1.3 Anthraquinoid lakes

Over the centuries artists have applied lakes with different painting techniques and on several supports. Lakes have performed a less important role in painting compared to inorganic pigments because of their lower light-fastness and great sensitivity to atmospheric agents and to pH variations [29]. Other parameters affecting the scarce stability of their color are the preparation method and the kind of inorganic supports used [30, 31, 32]. As a consequence, lakes were applied in a great extent for miniatures in illuminated manuscripts, and in a less extent in mural paintings and in easel or canvas painting [22].

In miniatures (from 1000 b.C) lakes were often mixed with inorganic pigments to achieve brighter colorations. This practice was widely used because in manuscripts colors were protected from degradation processes induced by light and external agents [29, 33].

In mural paintings instead, several factors discouraged an extended use of lakes. First of all, *fresco* technique does not allow the painter to use lakes, due to the strong alkaline pH given by the medium. Therefore, lakes used to be applied on dried plaster in *secco* technique using mainly egg or animal glue as binders [34]. Secondly, mural paintings are highly exposed to environmental conditions and therefore subjected to strong degradation phenomena.

* The definition stands for double sulfate salts with formula $M^{I}M^{II}(SO_4)_2 \cdot 12\ H_2O$ (M^{I}: K^+, Na^+, NH^{4+}; M^{II}: Al^{3+}, Fe^{3+}, Cr^{3+}). In particular, the hydrated potassium aluminium sulfate $KAl(SO_4)_2 \cdot 12H_2O$ was used as a mordant

Thanks to the low covering but high coloring power of lakes, they were widely employed in glazing technique. Glazing consisted in the overlapping of colored paint layers on already dried paint layers, and it is usually applied to modify the final aesthetic of easel or canvas paintings. For this purpose, lakes were applied with a large amount of oil or egg as binder above inorganic pigments layers to achieve specific nuances and transparent colorations. The drying time of the glaze depended on the amount and the type of paint medium used. The low colorant/binder ratio used to produce glaze layers increased the risk of partial or total loss of the glaze layer and therefore of the final coloration of the painting.
Red lakes, prepared from anthraquinones dyes, were particularly employed for glazing on canvas. Several European paintings of XIV and XIX century exhibit examples of glazing technique [35]. Red anthraquinoid lakes, as madder lake, kermes lake, cochineal lake and Indian lac, were mainly used over a more opaque inorganic red layer to create a deep, rich red color [22]. A short description of main features of these anthraquinoid lakes and possible structure of alizarin lake are summarized in appendix A.

1.2.2 Binding media

Binding media are complex natural compounds used to provide cohesion to the paint layer, to enable the application of dyes and pigments on the substrate and to protect paintings from environmental factors. They have been used pure or mixed together in a great number of different techniques [36].
Through the centuries, the most used natural binding media have been glycerolipid, polysaccharide and proteinaceous materials while natural waxes and resins have been mainly used as protective coatings or varnishes [15].
An overview of the main proprieties of glycerolipid, polysaccharide and proteinaceous materials, the most used binders for glazing technique, are reported below.

1.2.2.1 Glycerolipids

Glycerolipids are esters of glycerol with fatty acids, containing little amounts of compounds such as sterols and vitamins. They are the most common class of medium-size molecules produced by living organisms and they can derive from vegetal or animal sources. The most important drying or semi-drying oils used in paintings, extracted from several plants, are: linseed oil, poppy seed oil, walnut oil, safflower oil and tung oil. These oils are able to create strong films in contact with air, being characterized by long chains of unsaturated fatty acids in which the number of conjugated double bonds is proportional to the siccative propriety of the oil. Unsaturated fatty acids undergo photoxidation phenomena degrading in lower molecular weight species. Notwithstanding the photoxidation can also cause polymerization and crosslinking processes during oil curing leading to the formation of a polymeric network and thus a solid paint film [15, 37].
In the painting technique, thin layers of oil paint with dispersed lakes with sporadic addition of natural resins, were applied over the top of an opaque dried layer [38]. Light traveled through the glaze and was reflected back out of the opaque layer below. Sometimes the artist chose to apply several glaze layers adding increasing amounts of oil to each subsequent one in order to minimize cracking phenomena [32].

1.2.2.2 Polysaccharide materials

Polysaccharides are polymers made of aldopentoses, aldohexose and uronic acids linked together by glycosidic bonds. Examples of polysaccharides used as binding media are: cellulose, starch, honey, vegetable mucilage and particularly plant gums. Gums were obtained from some specific plant exudates or from the endosperms of their seeds. In the Mediterranean basin the gums used were: Arabic gum (exuded by *Acacia senegal* or *Seyal*), tragacanth gum (exuded by *Astragalus*) and fruit tree gum (obtained from cherry, peach, plum, apricot trees). Other gums that are expected as being used in Indian area are locust bean (extracted from *Ceratonia siliqua* kernels), guar (extracted from *Cyanaposis tetragonolobu* endosperms), ghatti (exuded by *Anogeissus latifolia*), karaya gum (exuded *by Sterculia urens*) [15, 20].
Though plant gums have been employed as binders in tempera techniques, their widest application set has been in water-colors, glazing and restoration materials. The layers obtained were thinner and less permanent than oil ones, but suitable for rough textures [32, 38].

1.2.2.3 Proteinaceous materials

Proteins are macromolecules made up of one or more chains of amino acids linked by peptide bonds between the carboxyl and amino groups of two different adjacent amino acids. Aged proteins are rather labile and they can incur in denaturation with a consequent modification of their reactivity. Possible causes of denaturation are: temperature, presence of reducing substances, alkaline treatment and changes in pH. As a result peptide bonds are hydrolyzed and consequently molecular weight decreases. Moreover the interaction with other organic and inorganic materials present in the work of art can induce crosslinking reactions with glycerolipids, followed by reduction of proteins solubility, and to complexation to metallic cations respectively. Photo-oxidation can also incur and oxalate salts are formed. In the end proteins can also be attacked by microorganisms [15, 39].
The main proteinaceous binding media used in art are of animal origin and they are: milk or casein (obtained from filtration and heating of acidified milk precipitate), egg (whole, yolk or albumen) and animal glues (obtained from bones, skin or muscles) [40].
Egg has been the most used proteinaceous material for the execution of glazes with tempera technique [38].

1.3 Analytical techniques and procedures

Some critical factors need to be considered when a procedure for the analysis of organic materials in paintings is set up. The simultaneous presence of several organic materials in the same painting layer (both original or resulting from restoration processes), the presence of non-original compounds as consequence of ageing, the environmental contamination and the very low percentage of the organic compounds on the overall weight of the samples make the analyses of organic materials challenging [20].

Non-destructive and micro-destructive techniques have been applied to the analyses of organic materials in paintings. Micro-destructive analytical techniques are generally applied after screening. A great variety of instrumental techniques and procedures has progressively been developed and optimized in an attempt to enhance the detection limit, sensitivity, resolution, repeatability and accuracy of analytical results [2]. Dyes and binders identification can be performed by assessing the presence of some specific markers or by the semi-quantitative and quantitative detection of specific compounds. Moreover, the construction of adequate databases, composed by reference materials and paint model systems analyzed in the same working conditions, is fundamental for the data interpretation in the case of the analysis of historical or archaeological samples [41].

1.3.1 Lakes analyses

Until now the analytical techniques and procedures developed for the analyses of dyes have been mainly focused on textile artifacts [22]. Analyses of lakes in painting samples are challenging because of the low percentage of dyes used in traditional lakes (1-3 % w/w), the large amount of binders and the difficult extraction of dyes from the matrix [22, 31]. In addition the lakes do not have a defined composition because of the possible presence of degradation products and impurities and lack of detailed information about original recipes [17, 42].
Non-destructive and micro-destructive analytical techniques applied for anthraquinoid dyes in historical artifacts are listed below.

1.3.1.1 Non-destructive analytical techniques

The advantages of non-destructive analytical techniques are their rapidity and the possibility to leave the sample undamaged for further investigations. The main drawback is that the kind of information obtained is generally related to the sum of the compounds present in the sample. Thus, results are not exhaustive, especially for the exact identification of organic dyes [17]. The most useful and suitable techniques with their potentialities are listed below:

- Optical microscopy (OM) in visible (VIS) or ultraviolet (UV) light: they permit the observation of colored layers (VIS) and their fluorescence (UV), respectively. Faded painting layers are extremely fluorescent due to the presence of fluorescent auto-oxidized oil and resins. Analyses conducted on these kinds of artifacts may thus not be able to provide any information about lakes used, but only about conservation state [43];
- Fiber Optics Reflectance Spectroscopy (FORS): it entails the use of a portable instrumentation that allows performing measurements *in situ*. It provides a reflectance spectrum, which is transformed in an absorption one and thus can be used for dyes identification and for the analysis of color variations on paintings (colorimetry). The drawback of this technique is the strong dependence of the reflectance spectra on the characteristics of the sample matrix, the roughness of paint layers and the external conditions. Therefore the development of a suitable database of absorption spectra of artists' materials is essential to enable their identification through comparisons. Databases have to be built up using reference materials applied as closely as possible to artists' techniques [44];

- Fourier Transform Infrared Spectroscopy (FT-IR) also coupled to microscope (μ-FT-IR): the sizes of paintings samples are usually too small to be analyzed by conventional FT-IR spectrophotometry. The use of μ-FT-IR permits to overcome this limitation and analyses of cross sections can also be carried out [45]. Matrix signal interference must be taken into account because it may prevent dyes identification [23]. This technique was applied for dyes identification in medieval manuscripts [33];
- Raman Spectroscopy, Fourier Transform Raman Spectroscopy (FT-Raman), Surface Enhanced Resonance Raman Scattering (SERS): Raman Spectroscopy is a very powerful technique for the detection of molecular structures and studies of physical proprieties [23]. It is largely employed for the characterization of organic materials, but proteins and matrix strong fluorescence have to be taken into account. Fluorescence signal covers the Raman one, but the interference can sometimes be filtered out changing excitation wavelength of the incident radiation. FT-Raman and SERS techniques deeply increase the quality of results and, in certain condition, the normally weak Raman scattering is enhanced up to seven orders of magnitude. SERS is applicable *in situ* and it shows a high spatial and spectral resolution [17, 31]. In addition a minimally invasive and destructive version of SERS, capable of providing maximal information with minimal volume of sample, has been applied for identification of alizarin and lac dye. Colorants molecules are adsorbed on metal nanoparticles and fluorescence can be effectively quenched through electron-transfer from the excited molecule to the metal [46];
- 3D Fluorescence Reflectance Spectroscopy and Microspectrofluorimetry: 3D Fluorescence Reflectance Spectroscopy is an *in situ* technique, which allows the differentiation among species with similar fluorescence emission spectra. This technique is also able to reveal dyes mixtures in a non-destructive way. The main drawback is the high influence of matrix on the spectra, thus a collection of reference spectra have to be acquired. Many dyes used in manuscripts inks were examined and characteristic 3–D spectral fingerprints were obtained [9]. Microspectrofluorimetry is an *in situ* technique with wide sensitivity and high spatial resolution. It is appropriate for selective excitation of pigments in a painting film but it has the same disadvantages mentioned above. The technique has been applied to some cross-sections of Vincent van Gogh and Lucien Pissarro paintings [47] and to a glass unguentarium from Celsa (Roman colony) [10], revealing the presence of purpurin lake and madder lake respectively;
- Laser Desorption Mass Spectrometry *(LDMS):* it is a promising analytical tool for the study of painting materials because it puts together the advantages of laser micro-probing and mass spectrometric analysis. Probing in the micrometric range with a focused laser beam provides sufficient resolution to investigate individual layers in paint samples. Ultra Violet Laser Desorption and Ionization (UV-LDI) is particularly attractive because enables to sample the surface of cross-sections directly and rapidly without any sample preparation. In addition, when low power laser is employed, only a minimal amount of material is removed leaving the sample "intact" for other methods of analysis. Both Ion Trap (ITMS) and Time-of-Flight (TOF-MS) detectors can be used [21];
- Matrix Assisted Laser Desorption Mass Spectrometry (MALDI-MS): the use of a matrix combined with low power laser is appropriate for non-volatile and thermally labile species. Since desorption is not necessarily a resonant process, wavelengths ranging from the far UV to the far IR regions can be employed [21]. MALDI-MS provided to be an excellent

screening method for confirming the presence of carminic acid in mixtures like those used in easel paintings [48]. A cosmetic tool from Celsa (Roman colony) was analyzed revealing the presence of madder [10].

1.3.1.2 Micro-destructive analytical techniques

Micro-destructive analytical techniques are applied after screening techniques in order to identify dyes unequivocally. Liquid extraction of chromophores containing molecules from the matrix and their separation must be achieved before their analysis. Chromatographic techniques coupled with UV-Vis spectrophotometric or mass spectrometric detectors are the most suitable set-ups for dyes characterization [41]. The analyses are mainly qualitative and carried out by comparison with reference material. However quantitation can be useful to distinguish different dyes origins on the basis of ratios between some specific components [17]. A list of the most used techniques and their potentialities is reported below:

- UV-Vis spectroscopy, Absorption, Fluorescence or Reflectance Microspectrophotometry: it is not a separative technique but it requires extraction of dyes. Afterwards the solution can be analyzed and the achieved spectrum can be compared with that of reference materials and standard compounds treated with the same procedure. Fluorescence mode is more suitable than absorption one for the analyses of small painting samples, due to its higher sensitivity. Reflectance Microspectrophotometry is highly sensitive, but it does not allow to achieve an accurate distinction among different kind of lakes [31];
- Thin Layer Chromatography (TLC): before the development of HPLC methods, TLC was the method of election for anthraquinoid red lakes analyses because of its rapidity, simplicity, cheapness and suitability for small samples [22, 31, 49]. Retention factors (Rf) can be calculated and dyes identification is realized by comparison to standard Rf [50]. Nevertheless, the chromatographic separation of dyes from other kinds of compounds is not completely unambiguous. After the development of analytical HPLC, TLC technique became obsolete for this type of analysis [31];
- Gas Chromatography (GC): it has not been widely applied due to the high molecular weight and polarity of anthraquinoid dyes, which make them not volatile. Therefore identification and quantitation of *Rubia tinctorum* constituents has been carried out using derivatization procedures. *N, O*-bis (trimethylsilyl) trifluoroacetamide (BSTFA) and *N-tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) were tested as derivatizing agents [51];
- Pyrolisis-Gas Chromatography (Py-GC): it is usually coupled with a mass spectrometric detector. The potential of analytical pyrolysis in the field of cultural heritage has increased in recent years with the use of the derivatizing agent tetramethylammonium hydroxide (TMAH) in the so-called pyrolysis-methylation [52]. Also silylating derivatizing reagents, as hexamethyldisilazane (HDMS), have been used and an increased sensibility and detectability of anthraquinoid compounds, respect to TMAH, has been achieved [53]. This methodology is simple, rapid and provides preliminary information about the principal classes of organic materials such as colorants [52]. It has been applied for madder lake identification in a cosmetic tool from Celsa (Roman colony) [10];
- Capillary Electrophoresis (CE): it can be coupled with DAD and ESI-MS detector. It has not been widely used, but the identification of chromophores containing molecules of

cochineal, lac-dye and madder lake has been performed. ESI-MS was found to be much more appropriate for this purpose because of its higher sensitivity (detection limits in the range 0.1–0.5 μg/mL) and selectivity. The advantage of this technique is the possibility to get a fast separation of target compounds with a little amount of sample. The proposed method can also be very useful as a cheap and rapid screening technique for more hydrophobic anthraquinones, or applied as preliminary step before LC/ESI-MS analyses [54];

- High Performance Liquid Chromatography (HPLC): nowadays it is the most widely applied technique due to its rapidity, high sensitivity and great chromatographic efficiency. It has permitted to obtain quantitative identification of cochineal principal and secondary constituents and to assign vegetal taxonomy of different species of *Rubia* [25]. In literature some more recent studies based on liquid chromatography has been carried out for detection of minor compounds [31, 45, 56, 57]. Reverse Phase Liquid Chromatographic (RP-LC) carried out with octylsilane (C8) or octadecylsilane (C18) bound to silica particles columns are the best choices because of anthraquinoid dyes polarity and consequent water-solubility [49]. The use of microbore columns (i.d. microbore =2.1 mm, i.d. standard bore=4.1 mm) gives higher chromatographic resolution because the same volume of sample is injected in a column with a half internal diameter implying lower flow and quadruplicated sensibility. A lower flow enables reduction of solvent consumption and easier MS coupling. These columns have longer life and they are suitable for little samples [31]. The elution is conducted in gradient mode and the commonly used mobile phase solutions are water, methanol and acetonitrile. The last one is an aprotic solvent with low viscosity and absorbance in the range of maximum absorbance of anthraquinoid dyes maximizing their analytical response [25, 58]. The presence of an acid substance in the eluent system was found necessary for the separation of compounds as well as for their detection. Its use is useful to suppress hydrogen ions formation avoiding multiple peaks and tailing retrograding acid-base equilibrium of the solvated compounds. Another reason to use acid is to hinder unspecific interactions of analyzed compounds with silanol groups of the column stationary phase [25, 31, 58]. The most common mobile phase modifiers are phosphates and organic acids such as formic and trifluoracetic acid. Notwithstanding also acetic acid and methanesulfonic acid have been tested [25]. TFA is the most suitable acid thanks to its high volatility, which permits to interface the chromatographic systems with MS detection and promotes a better peaks resolution. Its optimal concentration is 0.1% (v/v) as higher or lower concentrations provide overlap between different anthraquinoid chromatographic peaks [25].

  Ultrahigh pressure liquid chromatography (UHPLC) has led to a more efficient and sensitive characterization of anthraquinoid dyes and minor compounds with an increased chromatographic resolution over HPLC. The possibility to use columns with smaller particles size and to reach higher pressures allows faster flow rates, shorter runtimes and lower solvent consumptions [57]. Moreover objects, from which sampling was previously not possible, may now be examined due to the greatly reduced requirements for sample amount and lower limit of detection. Identification of dyes can be carried out also in heavily degraded samples [59]. An increase of 41–61% resolution and a decrease of 91–422% limit of detection, depending on the dye compound, have been obtained for anthraquinoid dyes. Columns with different size of particles and compositions of the

stationary phase, different mobile phases, gradient elution programs, flow rates, temperatures and runtimes have been tested and a final method optimized. In addition several natural reference materials were analyzed in order to create a database for future characterization of dyes in cultural heritage objects [57]. In the end UPLC will be probably rapidly adopted as the method of choice for natural product dyestuff analysis in the field of heritage science [59].

The detection system can be a spectrophotometer or mass spectrometer. UV-Vis spectra recorded by spectrophotometer show high absorbance of anthraquinoid dyes and separation of possible interferences occurring during elution [22]. A diode array detector is the most suitable tool for quantitative analysis [42, 56]. Since compounds with similar structures have similar spectra, their unambiguous attribution remains difficult in the absence of reference materials. Also co-eluated compounds are problematic because not differentiable [25]. HPLC-DAD has been widely applied for anthraquinoid lakes analyses [58, 60, 61] and one application has been the identification of carminic acid contained in historical maps belonging to The Royal Chancellery Archives (Granada) [62].

Fluorescence detector is not widely used even if it is more selective, sensitive and with lower detention limits for some compounds respect to DAD [17, 63].

Mass spectrometric detector improves detention limits, selectivity and molecular identification efficiency even if it sometimes shows a lower repeatability than DAD [42]. Working in negative mode, electrospray ionization coupled with ion trap analyzer has shown the best results for anthraquinoid dyes. MS detector permits to reveal all compounds of interest and separates also the co-eluted ones. Nevertheless the possible presence of isobaric compounds does not allow the unambiguous identification of dyes only by information about molecular ion. Thus, the use of MS^n technique enables the analyst to attribute the structures through the interpretation of the observed fragmentations and comparison with reference materials. Mass spectrometric methods show a high sensitivity, resolution and low detection limits. In the end the combination of a separative technique (which provides retention time, elution order, peaks shape) with spectrophotometric and spectrometric ones (UV-Vis, MS/MS) is really powerful for anthraquinoid dyes identification [25].

1.3.1.3 Sample treatments

Before applying a chromatographic technique, such as HPLC, dyes must be extracted from artifacts. Extraction of the compounds of interest from the matrix is an important step in each analytical procedure [2]. The selection of the most appropriate extraction method is a challenge because of the uniqueness of samples and the tiny amount of colorant contained [64]. A wide range of extraction methods has been proposed in literature but most of them have been applied exclusively to textiles. Only few methods have been successfully applied to painting samples and they are discussed below:

- Boiling mixture of organic solvent and strong acid [51, 58]: the organic solvent is methanol and the acid usually is hydrochloric or sulfuric acid. Rather extreme acidic conditions (HCl 10% or 18%, H_2SO_4 10% at boiling temperature) followed by extraction into a suitable solvent are the most effective and used method [64]. Nevertheless some undesired effects limit its utilization: degradation of a series of labile chromophores (decarboxylation of

pseudopurpirin and munjistin respectively to purpurin and xanthopurpurin) [65], esterification of carboxylic compounds [66] and cleavage of glycosidic bonds (many species can be identified only on the basis of the presence and the position of glycosylation). In addiction HCl is corrosive and difficult to evaporate [67];

- Transmethylation with boron trifluoride and methanol (BF_3/MeOH): it is a suitable method for breaking up highly polymerized painting films and dissolving the dye out of the lake pigment. If BF_3 has a high concentration, some compounds might react. Moreover, the esterification of anthraquinones containing carboxyl groups, such as pseudopurpurin and munjistin, can occur [31];
- Formic acid: it is a weak acid which is used in an extracting solution of 5HCOOH: 95MeOH [67]. In this way a mild extraction of anthraquinones and flavonoids is performed also on small samples. Little or no decompositions of chromophores containing molecules occurs;
- Complexation agents: ethylenediaminetetracetic acid (EDTA) is used because it is a strong chelator of aluminium and of other ions releasing dyes molecules without decomposing them. An extracting solution of $2H_2$EDTA: 10ACN: 88MeOH was used for anthraquinones and flavonoids extraction and the method compared to HCl or HCOOH ones. The EDTA method worked better to extract flavonoids while anthraquinones gave higher yields firstly with formic acid and then with HCl [67]. Finally, in order to improve the extraction yields of complexation agents method, it is also possible to use an organic solvent as dimethylformamide (DMF) [36];
- Hydrofluoric acid: it is a weak acid and, in its dissociated form, also a strong aluminium-complexing agent as efficient as a strong acid [68]. The essential mechanism of extraction by HF is the formation of a strong aluminum-fluoride complex. The removal of a large fraction of the aluminum ions moves the thermodynamic equilibrium of aluminum-fluoride complex towards the protonated form of anthraquinoid compound. In this way it is possible to work at much milder pH (c.a 1.5) than with HCl. In fact because there is no more competition for the phenolate groups of anthraquinones; dyes may thus be freed from their aluminum complexes without hydrolyses reaction. A milder pH is also suggested for a higher efficiency of HF that is in its non-complexing protonated form. This method permits both quantitative and qualitative characterization of the extracts [69].

  In brief, HF method is a mild extraction suitable for unstable colorants. It avoids conversion of glycosides into aglycones and achieves a high yield for all the colorants present. In addition it allows the analyst to investigate a wide range of colorants classes and mixtures applied on different supports and in various matrices or complexes [64]. It is efficient also with lakes containing different type of metal cations such as Ba, Sn, Fe, Cr, Cu, Pb, Zn, Ca, Si [69]. Moreover precious information about biological source and recipes is provided. The drawbacks of this method consist in the precautions for manipulation and treatment of HF. Its toxicity both for contact and for inhalation requires protections and its corrosive power, especially toward glass, requires the use of Teflon laboratory ware.

1.3.1.4 Binding media analyses

In situ techniques such as Fiber Optics Reflectance Spectroscopy (FORS) [70], Spectroscopy UV-Vis [71], Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFTS) [72] are usually used in paintings both for inorganic materials and for binding media analyses. These techniques have some limitations given by the features of the work of art analyzed as the irregularity of the surface, the low amount of binder used, the presence of inorganic and of degradation compounds. Their use is mainly indicative of the presence of a binder and, eventually, of the belonging family. However they are very useful to highlight the presence and localization of inorganics in the artwork, facilitating the sampling.

Paintings are multi-material and multilayer systems and available samples are very small. Unique identification of the materials used and their localization have to be provided in order to establish the painting technique and conservation state. Analytical methodologies able to maximize information and minimize sampling have to be set up [2, 16]. Several techniques are used for these purposes and all of them show their own advantages and drawbacks.

Fourier transform infrared (FTIR) microscopy has been widely applied to the investigation of painting layers because all organic and inorganic materials used show characteristic absorptions in the mid-IR range (4000–600 cm^{-1}). This technique can be applied to paint samples prepared as both thin and cross-sections and it is nowadays one of the few methods which allow the stratigraphic characterization of all organic materials. The development of micro-sampling accessories has become an essential tool in the destructive analysis of small samples. Attenuated Total Reflection–Fourier transform infrared (ATR) spectroscopy with the introduction of miniaturized ATR crystals allows the analysis of smaller quantities. Both FTIR mapping and imaging techniques can be used for this kind of analyses. While imaging mode has a higher spatial resolution and it takes only few minutes for scanning a surface, mapping mode takes some hours to produce spectra with higher signal-noise ratio (S/N) and thus more reliable results. A collection of a large number of FTIR spectra makes possible the mapping and the chemical characterization of different materials present in multi-layered paintings [73]. The main limitation of the technique is the inexact attribution of the organic media: it is not possible to distinguish among organic compounds belonging to a binding class because their functional groups are the same.

Separation techniques have been very successful in analyses of binders thanks to their ability of separating different organic components of the painting matrix. Thus, Gas Chromatography/Mass Spectrometry (GC/MS) [74, 75, 76, 77], Pyrolysis- Gas Chromatography/Mass Spectrometry (Py-GC/MS) [78, 79, 80, 81], Capillary Electrophoresis-UV-Vis spectroscopy (CE-UV-Vis) [82, 83] or with conductivity detector [84] and Liquid Chromatography/Mass Spectrometry (LC/MS) have been applied for the characterization of glycerolipidic, polysaccharide and proteinaceous materials. Chromatographic techniques hyphenated to mass spectrometry detector are superior to the spectroscopic ones since they can identify the type of compounds used and alteration products formed through the fragmentation pattern [2, 25].

Gas chromatography has been the method of election for binding media analysis because it is able to separate and detect them with high specificity and sensitivity. Its main drawback is the need to use volatile compounds, thus one or more pre- treatment steps are required before separation of the analytes in the chromatographic system. As far as painting binders originally used (triglycerides, proteins, polysaccharides) and polymerized or cross-linked products formed are macromolecules, they need to be reduced to low molecular weight compounds (respectively fatty

acids, amino acids, sugars) [20]. The fragmentation can be achieved by Py-GC/MS or by wet-chemical treatment of the samples before GC/MS. Pyrolysis degrades macromolecules in polar and low volatile compounds, which need thermally assisted in situ derivatization [78]. Silylating (i.e. hexamethyldisilazane, HDMS) [78, 81] or methylating (i.e. tetramethyl ammonium hydroxide, TMAH) [79] derivatizing agents can be chosen. Py-GC/MS is a fast, inexpensive and efficient technique to obtain the fingerprint of the organic materials avoiding sample loss and contamination. Nevertheless interpretation of pyrograms is difficult because original macromolecules are highly fragmented by pyrolysis and unspecified compounds are formed. Thus interpretation is based on molecular markers. On the contrary combination of chemical wet pretreatment of the sample and GC/MS is unequaled in its capacity to define the composition of the samples at a molecular level [78]. The wet chemical pretreatment often includes the chemolyses, useful to break macromolecules, and the derivatization, necessary to obtain volatile compounds. Derivatization can be carried out either by chloroformating (i.e. ethyl chloroformate, ECF) [71] or silylating (i.e. BSTFA or MTBSTFA) [75, 77, 78] agents.

Recent advances in chromatography have been focused not only on the development of alternative sample pre-treatments and analytical procedures but also in innovative instrumental devices and technologies [2].

Considering the heterogeneity of materials present in paint sample and the tiny amount normally available, the simultaneous characterization of more than one class of compounds could be useful to establish sample composition avoiding the risk to go under the limit of detection of the technique [16]. A multistep GC/MS procedure, using ammonia extraction as chemical pretreatment, has been developed for the simultaneous characterization of proteinaceous and polysaccharide materials, glycerolipids, natural waxes, terpenoid resins, eventually present in an unique sample. Amino acids are transformed in tert-butyldimethylsilyl (TBDMS) derivatives, fatty acids in trimethylsilyl (TMS) esters, monosaccharides in trimethylsilyl diethyl dithioacetals and uronic acids in trimethylsilyl diethyl dithioacetal lactones. The fractions are separated before derivatization and analyzed by GC/MS separately [13, 14]. The presence of a desalting clean up step (performed by C18 monolithic sorbent tip) makes the procedure reliable also in presence of high amounts of inorganic pigments. These compounds may catalyze cross-linking reactions and form complexes with amino acids and proteins decreasing the real detectable amount of proteinaceous material [39].

Some new and successful techniques such as capillary liquid chromatography coupled with mass spectrometry detection (usually with a nanoLC/nanoESI/Q-q-TOF MS/MS set up) has been developed and applied for glycerolipids [85] and proteins analysis. Focusing on proteins a hydrolyses step, able to break peptide bonds and to obtain peptides, is carried out by enzymes as trypsin [86]. This analytical approach is greatly powerful in comparison to GC/MS one. In fact GC-MS entails quantitative determination of the amino acidic fraction evaluating the single percentages of components and their comparison with a database through a statistical method (Principal Components Analyses-PCA) [87]. Instead LC/MS/MS analysis is able to determine the masses of peptide fragments obtained by enzymatic cleavage. Enzyme breaks the peptide bonds only between specific amino acids therefore information about amino acid sequences of proteins is provided. This leads to unambiguous identification of proteins even if only a small number of peptides are released by specific cleavage [86].

Another innovative method for protein identification is Matrix Assisted Laser Desorption Ionization- Time of-Flight/ Mass Spectrometry (MALDI-ToF/MS). This technique is also preceded by enzyme cleavage (trypsin) when dealing with proteins analyses. This pretreatment is much

more gentle and specific than acidic hydrolysis thus other components of color layers can be saved for further analysis. MALDI is a soft ionization technique and mass spectra obtained do not show high fragmentation of peptides. Therefore a comparison among molecular ions of real samples spectra with reference material ones, belonging to a database of commonly used binders, enables a reliable identification of proteins. Their comparison makes possible fast characterization of proteinaceous binders in color layers even in old and highly aged matrices [88, 89]. Besides the high specificity of the cleavage given by trypsin (between arginine and lysine of peptide chains) and the simple sample preparation, this method has the advantage to be fast due to the lack of preliminary chromatographic separation of peptides. Moreover it shows a low detection limit of single protein (in the range from picomoles to femtomoles), which allows extremely tiny amount of material necessary for such analysis [90]. The drawbacks consist in the presence of interferences and in the absence of structural information about proteins caused by the lack of fragmentation. An MS^n set up can overcome this latter problem.

It should be highlighted that, LC/MS/MS and MALDI-ToF/MS methods, allowing the univocal identification of proteins, solve the difficulty of characterize mixtures of proteinaceous binders, which is a typical limit of the other commonly used analytical methods [89].

Bibliography

(1) J.J. Winckelmann*, Opere di G.G Winckelmann. Prima Edizione Italiana Completa*, per i Fratelli Giachetti, Prato, 1830-1834.

(2) M.T. Doménech-Carbò, *Novel Analytical Methods for Characterizing Binding Media and Protective Coatings in Artworks*, Analytica Chimica Acta, vol. 621, pp. 109–139, 2008.

(3) M. Bacci, A. Casini, M. Picollo, B. Radicati, L. Stefani, *Integrated Non-Invasive Technologies for the Diagnosis and Conservation of the Cultural Heritage*, Journal of Neutron Research, vol. 14, pp. 11-16, 2006.

(4) A. M. Pollard, Carl Heron, *Archeological Chemistry*, Royal Society of Chemistry, Cambridge, 1996.

(5) J. Mills, R. White, *Organic Chemistry of Museum Objects (Conservation and Museology*), Butterworth-Heinemann, 1987.

(6) G.W. Taylor, *Natural Dyes in Textile Applications*, Review of Progress in Coloration and Related Topics, vol. 16, pp. 53-61, 1986.

(7) A.K. Samantaa, P. Agarwal, *Application of Natural Dyes on Textiles, Indian Journal of Fiber and Textile*, vol. 34, pp. 384-399, 2009.

(8) A.L. Wilson, *Elemental Analysis of Pottery in the Study of its Provenance: a review*, Journal of Archaeological Science, vol. 5, pp. 219–236, 1978.

(9) M. Clarke, *A new Technique for the non-destructive Identification of Organic Pigments, Dyes and Inks in situ on Early Mediaeval Manuscripts, using 3-D Fluorescence Reflectance Spectroscopy*, in 6th International Conference 'Non Destructive Testing and Microanalysis for the Diagnostics and Conservation of the Cultural and Environmental Heritage', pp. 1423-1435, 1999.

(10) J. Pèrez-Arantegui, E. Ribechini, G. Ceprià, I. Degano, M.P. Colombini, J. Paz-Peralta, E. Ortiz-Palomar, *Colorants and Oils in Roman Make-ups–an Eye Witness Account,* Trends in Analytical Chemistry, vol. 28, pp. 1019-1028, no. 8, 2009.

(11) J.J. Lucejko, *Wet Archeological Wood: Chemical Study of Degradation and Evaluation of Consolidation Treatments*, ph. D thesis in Chemical Science, Università degli studi di Pisa, Facoltà di Scienze Matematiche, Fisiche e Naturali, Dipartimento di Chimica e Chimica Industriale, 2010.

(12) L. Campanella, A. Casoli, M. P. Colombini, M. Marini Bettolo, M. Matteini, L.M. Migneco, A. Montenero, L. Nodari, C. Piccioli, M. Plossi Zappalà, G. Portalone, U. Russo, M.P. Sammartino, *Chimica per l'Arte*, ed. Zanichelli, 2007.

(13) A. Andreotti, I. Bonaduce, M. P. Colombini, G. Gautier, F. Modugno, E. Ribechini, *Combined GC/MS Analytical Procedure for the Characterization of Glycerolipid, Waxy, Resinous and Proteinaceous Materials in a Unique Paint Microsample*, Analytical Chemistry, no. 78, pp. 4490-4500, 2006.

(14) A. Lluveras, I. Bonaduce, A. Andreotti, M. P. Colombini, *GC/MS Analytical Procedure for the Characterization of Glycerolipids, Natural Waxes, Terpenoid Resins, Proteinaceous and Polysaccharide Materials in the Same Paint Microsample Avoiding Interferences from Inorganic Media*, Analytical Chemistry, no. 82, pp. 376-386, 2010.

(15) M.P. Colombini, F. Modugno, *Organic Materials in Art and Archeology,* Organic Mass Spectrometry in Art and Archaeology, edited by M. P. Colombini and F. Modugno, ed. Wiley, 2009.

(16) A. Andreotti, I. Bonaduce, M.P. Colombini, F. Modugno, E. Ribechini, *Characterisation of natural organic materials in paintings by GC-MS analytical procedures' in New Trends in Analytical, Environmental and Cultural Heritage Chemistry*, M. P. Colombini and L. Tassi editors, Research Signpost, pp. 389-424, 2008.

(17) I. Degano, *A Multiple-Analytical Approach for the Characterisation of Organic Dyes in Textiles*-ph. D thesis in Chemical Science, Università degli studi di Pisa, Facoltà di Scienze Matematiche, Fisiche e Naturali, Dipartimento di Chimica e Chimica Industriale, 2009.

(18) R. L. Feller, *Artist's Pigments-A Handbook of their History and Characteristics*, vol. 1, National Gallery of Art Washington, Cambridge University Press, 1986.

(19) I. Petroviciu, A. Medvedovici, F. Albu, I. Cretu, I. Vanden Berghe, *LC-MS as Analytical Technique for the identification of Natural Dyes in Historic Textiles, Romanian Reports in Physics*, vol. 64, pp. 507-515, 2012.

(20) M. P. Colombini, A. Andreotti, I. Bonaduce, F. Modugno, E. Ribechini, *Analytical Strategies for Characterizing Organic Paint Media Using Gas Chromatography/Mass Spectrometry,* Accounts of Chemical Research, vol. 43, no. 6, pp. 715-727, 2010.

(21) N. Wyplosz, Laser desorption mass spectrometric studies of artists' organic pigments, Molart, Amolf Fom, 2003.

(22) M. Rosato, *Caratterizzazione delle lacche rosse in manufatti pittorici antichi*, tesi di laurea, Università degli studi di Pisa, Facoltà di Scienze Matematiche, Fisiche e Naturali, Dipartimento di Chimica e Chimica Industriale, 2000.

(23) E. Martuscelli, *I Coloranti Naturali nella Tintura della Lana Arte, Storia, Tecnologia e 'Archeo-Materials Chemistry'*, Programma Nazionale di Ricerca Beni Culturali (MIUR) La Conservazione dei Tessuti Antichi, vol. II.

(24) IUPAC, Compendium of Chemical Terminology, 2nd ed. (the "Gold Book"), 1997.

(25) L. Rafaelly, S. Hèron, W. Nowik, A. Tchapla, *Optimisation of ESI-MS Detection for HPLC of Anthraquinone Dyes, Science Direct*, vol. 77, pp. 191-203, 2008.

(26) J. Hofenk de Graaf*, The Colorful Past: Origins, Chemistry and Identification of Natural Dyestuffs*, Abegg-Stiftung an Archetype Publications Ltd., 2004.

(27) G. T. Austin, Shreve's Chemical Process Industries, McGraw-Hill, New York, p. 357, 1984.

(28) J. Kirby, M. Spring, C. Higgit *The Technology of Red Lake Pigment Manufacture: Study of the Dyestuff Substrate*, National Gallery Technical Bulletin, pp. 71-87, 2005.

(29) F. Modugno, *Caratterizzazione dei leganti proteici in Dipinti murali mediante GC-MS in presenza di pigmenti Inorganici Interferenti*, Tesi di laurea, Università degli studi di Pisa, Facoltà di Scienze Matematiche, Fisiche e Naturali, Dipartimento di Chimica e Chimica Industriale, 1998.

(30) D. Saunders and J. Kirby, *Light-induced Colour Changes in Red and Yellow lake Pigments*, National Gallery Technical Bulletin, vol. 15, pp. 79-97, 1994.

(31) J. Kirby, R. White, *The Identification of Red Lake Pigment Dyestuffs and a Discussion on their Use*, National Gallery Technical Bulletin, pp. 56-80, 1996.

(32) T. M. Simon*, Glazing*, The Art of Composition, 2008.

(33) A. Casoli, Caratterizzazione Chimica delle Miniature, atti della III scuola nazionale di Chimica per i Beni Culturali, Palazzo Mansi (Lucca), pp. 97-102, 2000.

(34) J. Canaby, *Seminari d'arte: L'affresco,* ottavo quaderno, Unione tipografica Edizione torinese, pp. 10-15.

(35) S. Rinaldi, *La Fabbrica dei Colori: Pigmenti e Coloranti nella Pittura e nella Tintoria,* il Bagatto, 1986.

(36) I. Surowiec, *Application of High-Performance Separation Techniques in Archeometry*, Microchimica Acta, vol. 162, pp. 289–302, 2008.

(37) M. P. Colombini, F. Modugno, E. Ribechini, *GC/MS in the Characterization of Lipids,* Organic Mass Spectrometry in Art and Archaeology, edited by M. P. Colombini and F. Modugno, ed. Wiley, 2009.

(38) *Pigments through the Ages*, http://www.webexhibits.org/pigments/

(39) I. Bonaduce, M. Cito, M. P. Colombini, *The development of a Gas Chromatographic-Mass Spectrometric Analytical Procedure for the Determination of Lipids, Proteins and Resins in the Same Paint Micro-Sample Avoiding Interferences from Inorganic Media,* Journal of Chromatography A, no. 1216, pp. 5931-5939, 2009.

(40) M. P. Colombini, G. Gautier, *GC/MS in the Characterisation of Protein Paint Binders*, Organic Mass Spectrometry in Art and Archaeology, edited by M. P. Colombini and F. Modugno, ed. Wiley, 2009.

(41) I. Degano, Coloranti Organici e tessuti: dalla Chimica all'Archeologia, pp. 49-56, 2010.

(42) I. Surowiec, B. Szostek, M. Trojanowicz, *HPLC-MS of Anthraquinoids, Flavonoids, and their Degradation Products in Analysis of Natural Dyes in Archeological Objects,* Journal of Separation Science, vol. 30, pp. 2070–2079, 2007.

(43) E. Renè de la Rie, *Fluorescence of Paint and Varnish Layers*, part 1, Studies in Conservation, vol. 27, pp. 1-7, 1982.

(44) A. Aldrovandi, M.L. Altamura, M.T. Cianfanelli, P. Riitano, *I Materiali Pittorici: Tavolette Campione per la Caratterizzazione mediante Analisi Multispettrale,* OPD Restauro, vol. 8, pp. 191-210, 1996.

(45) R. D. Gillard, S. M. Hardman, *The Detection of Dyes by FTIR Microscopy*, Studies in Conservation, vol. 39, pp. 153-160, 1983.

(46) K. Chen, K. Vo-Dinh, F. Yan, M. B. Wabuyele, T. Vo-Dinh, *Direct Identification of Alizarin and Lac Dye on Painting Fragments using Surface-Enhanced Raman Scattering*, Analytical Chimica Acta, vol. 569, pp. 234–237, 2006.

(47) A. Claro, M. J. Melo, J. S. Seixas de Melo, K. Jan van den Berg, A. Burnstock, M. Montague, R. Newman, *Identification of Red Colorants in Van Gogh Paintings and Ancient Andean Textiles by Microspectrofluorimetry,* Journal of Cultural Heritage, vol. 11, pp. 27–34, 2010.

(48) M. S. Maier, S. D. Parera, A. M. Seldes, *Matrix-Assisted Laser Desorption and Electrospray Ionization Mass Spectrometry of Carminic Acid isolated from Cochineal*, International Journal of Mass Spectrometry, vol. 232, pp. 225–229, 2004.

(49) J. Wouters, *High Performance Liquid Chromatography of Antraquinones: Analysis of Plant and Insect Extracts and Dyed Textiles*, Studies Conservation, vol. 30, pp. 119-128, 1985.

(50) H. Schweppe, H Roosen-Runge, *Carmine- Cochineal Carmine and Kermes Carmine,* Artists' Pigment-A handbook of their Hystory and Characteristic, Cambridge University press, vol. 1, 1986.

(51) I. Boldizsàr, Z. Szùcs, Zs. Fuzfai, I. Molnàr-Perl, *Identification and Quantification of the Constituents of Madder Root by Gas Chromatography and High-Performance Liquid Chromatography*, Journal of Chromatography A, vol. 1133, pp. 259–274, 2006.

(52) D. Fabbri, G. Chiavari, H. Ling, *Analysis of Anthraquinoid and Indigoid Dyes used in Ancient Artistic Works by Thermally Assisted Hydrolysis and Methylation in the Presence of Tetramethylammonium Hydroxide*, Journal of Analytical and Applied Pyrolysis, vol. 56, pp. 167–178, 2000.

(53) M. J Casas-Catalan, M. T Domenech-Carbo, *Identification of Natural Dyes used in Works of Art by Pyrolysis-Gas Chromatography-Mass Spectrometry combined with in situ Trimethylsilylation,* Analytical Bioanalytichal Chemisty, vol. 382, pp. 259-268, 2005.

(54) M. Puchalska, M. Orlin´ska, M. A. Ackacha, K. Połec´Pawlak, M. Jarosz, *Identification of Anthraquinone Coloring Matters in Natural Red Dyes by Electrospray Mass Spectrometry coupled to Capillary Electrophoresis,* Journal of Mass Spectrometry*, vol. 38, pp.* 1252–1258, 2003.

(55) J. Wouters, *L'analyse de Colorants Organiques Naturels par Chromatographie Liquide haute performance et Traitement des Données par Ordinateur*, Pigments and Colorants, pp. 331-338, 1990.

(56) G. C. H. Derksena, H. A. G. Niederlanderb, T. A. van Beeka, *Analysis of Anthraquinones in Rubia Tinctorum Coupled with Diode-Array UV and Mass Spectrometric Detection*, Journal of Chromatography A, vol. 978, pp. 119 –127, 2002.

(57) A. Serrano, M. van Bommel, J. Hallett, *Evaluation between Ultrahigh Pressure Liquid Chromatography and High-Performance Liquid Chromatography Analytical Methods for Characterizing Natural Dyestuffs*, Journal of Chromatography A, vol. 1318, pp. 102–111, 2013.

(58) S. M. Halpine, *an Improved Dye Lake Pigment Analysis Method for High-Performance Liquid Chromatography and Diode-Array Detector*, Studies in Conservation, vol. 41, pp. 76-94, 1996.

(59) L. G. Troalen, A. S. Phillips, D. A. Peggie, P. E. Barran, A. N. Hulme, *Historical Textile Dyeing with Genista Tinctoria L.: a Comprehensive Study by UPLC-MS/MS Analysis,* Analytical Methods, vol. 6, pp. 8915–8923, 2014.

(60) J. Sanyova, *Mild extraction of Dyes by Hydrofluoric Acid in Routine analysis of Historical Paint Micro-samples,* Microchimica Acta, vol. 162, pp. 361-370, 2008.

(61) O. Deveoglu, E. Torgan, R. Karadag, *Characterization of Colouring Matters by HPLC-DAD and colour Measurements, Preparation of Lake Pigments with Aratrat Kermes (Porphyrophora Hameli Brand),* Jordan Journal of Chemistry, vol. 5, no. 3, pp. 307-315, 2010.

(62) R. Blanc, T. Espejo, A. Lòpez-Montes, D. Torres, G. Crovetto, A. Navalòn, J.L Vìlchez, *Sampling and Identification of Natural Dyes in Historical Maps and Drawings by Liquid Chromatography with Diode-Array Detection,* Journal of Chromatography A, vol. 1122, pp. 105–113, 2006.

(63) P. R. N. Carvalho, C. H. Collins, *HPLC Determination of Carminic Acid in Foodstuffs and Beverages Using Diode Array and Fluorescence Detection* Chromatographia vol. 45, pp. 63-66, 1997.

(64) J. Padfield*, Mild Extraction Methods for Organic Colorant Analysis, A Bibliographic Reference Database,* Charisma website, The National Gallery Scientific Department, http://research.ng-london.org.uk/scientific/colourant.

(65) H. Schweppe, H. der Naturfarbstoffe, Vorkommen, Verwenbdung, Nachweis, Nikol, Künstlerpigmente auf Basis von Krapplacken, Hamburg, pp. 242–245, 1993.

(66) E. S. B. Ferreira, A. Quye, H. McNab, A. N. Hulme, *Photo-oxidation Products of Quecetin and Morin as Markers for the Characterisation of Natural Flavonoid Yellow,* Dyes in History and Archeology, vol. 18, pp. 63–72, 2000.

(67) X. Zhang, R.A. Larsen*, Development of Mild Extraction Methods for the Analysis of Natural Dyes in Textiles of Historical Interest Using LC-Diode Array Detector-MS*, Analytical Chemistry, vol. 77, pp. 2022-2025, 2005.

(68) J. Sanyova, *Contribution a' L'étude de la Structure et des Propriétés des Laques de Garance,* Univerité libre de Bruxelles, Faculté des Sciences Appliquées, Service de Chimie Organique, pp. 200, 2000-2001.

(69) J. Sanyova, J. Reisse, *Development of a Mild Method for the Extraction of Anthraquinones from their Aluminum Complexes in Madder Lakes prior to HPLC Analysis*, Journal of Cultural Heritage, vol. 7, pp. 229–235, 2006.

(70) F. Rosi, A. Daveri, C. Miliani, G. Verri, P. Benedetti, F. Piqué, B. G. Brunetti, A. Sgamellotti, *Non-Invasive Identification of Organic Materials in Wall Paintings by Fiber Optic Reflectance Infrared Spectroscopy: a Statistical Multivariate Approach, Analytical Bioanalytical Chemistry,* vol. 395, pp. 2097–2106, 2009.

(71) J.J. Rorimer, *Ultraviolet, Rays and their Use in Examination of Works of Art*, Metropolitan Museum of Art, New York, 1931.

(72) F. Casadioa, L. Toniolo, *The Analysis of Polychrome Works of Art: 40 Years of Infrared Spectroscopic Investigations*, Journal of Cultural Heritage 2, pp. 71–78, 2001.

(73) R. Mazzeo, E. Josep, S. Prati, A. Millemaggi, *Attenuated Total Reflection–Fourier Transform Infrared Microspectroscopic Mapping for the Characterisation of Paint Cross-Sections*, Analytica Chimica Acta, vol. 599, pp. 107–117, 2007.

(74) R. Mateo-Castro, J. V. Gimeno-Adelantado, F. Bosch-Reig, A. Domenech-Carbo, M.J. Casas-Catalan, L. Osete-Cortina, J. De la Cruz-Canizares, M.T. Domenech-Carbo, *Identification by GC-FID and GC-MS of Amino Acids, Fatty and Bile Acids in Binding Media used in Works of Art*, Fresenious Journal of Analytical Chemistry, vol. 369, pp. 642-646, 2001.

(75) M. P. Colombini, F. Modugno, M. Giacomelli, S. Francesconi, *Characterisation of Proteinaceous Binders and Drying Oils in Wall Painting Samples by Gas Chromatography-Mass Spectrometry*, Journal of Chromatography A, vol. 846, pp. 113- 124, 1999.

(76) A. Lluveras Tenorio, J. Mazurek, A. Restivo, M. P. Colombini, I. Bonaduce, *The Development of a New Analytical Model for the Identification of Saccharide Binders in Paint Samples*, Chemistry Central Journal, vol. 7, pp. 1-17, 2012.

(77) M. P. Colombini, F. Modugno, *Characterisation of Proteinaceous Binders in Artistic Paintings by Chromatographic Techniques*, Journal of Separation Science, vol. 27, pp. 147–160, 2004.

(78) I. Bonaduce, A. Andreotti, *Py-GC/MS of Organic Paint Binders,* Organic Mass Spectrometry in Art and Archaeology, edited by M. P. Colombini and F. Modugno, ed. Wiley, 2009.

(79) J. D. J. Van den Berg, J.J. Boon, *Unwanted Alkylation during direct Methylation of Fatty (di) acids using Tetramethyl ammonium hydroxide reagent in a Curie-point Pyrolysis unit*, Journal of Analytical and Applied Pyrolysis, vol. 61, pp. 45-63, 2001.

(80) R. Stevanato, M. Rovea, M. Carbini, D. Favretto, P. Traldi, *Curie-point Pyrolysis Gas Chromatography Mass Spectrometry in the Art Field, Part 3, the Characterization of Some Non-proteinaceous Binders*, Rapid Communications in Mass Spectrometry, vol. 11, pp. 286-294, 1997.

(81) G. Chiavari, D. Fabbri, G. C. Galletti, S. Prati, N. Scianna, *Use of Pyrolysis-Gas Chromatography/Mass Spectrometry to Characterise Binding Media and Protectives from a Coronelli's Terrestrial Globe*, Journal of Cultural Heritage, vol. 7, pp. 67–70, 2006.

(82) M. A. L De Oliveira, V. E. S. Solis, L. A Gioielli, B. Polakiewicz, M. F. M. Tavares, *Method Development for the Analysis of Trans-Fatty acids in Hydrogenated Oils by Capillary Electrophoresis*, Electrophoresis, vol. 24, pp. 1641-1647, 2003.

(83) M. Groessl, S. Harrison, I. Kaml, E. Kenndler, *Characterisation of Natural Polysaccharides (Plant Gums) used as Binding Media for Artistic and Historic Works by Capillary Zone Electrophoresis*, Journal of Chromatography A, vol. 1077, pp. 80-89, 2005.

(84) S. M. Harrison, I. Kaml, V. Prokoratova, M. Mazanek, E. Kenndler, *Animal Glues in Mixtures of Natural Binding Media used in Artistic and Historic Objects: Identification by Capillary Zone Electrophoresis*, Analytical Bioanalytical Chemistry, vol. 382, pp. 1520–1526, 2005.

(85) J. La Nasa, E. Ghelardi, I. Degano, F. Modugno, M.P. Colombini, *Core Shell Stationary Phases for a Novel Separation of Triglycerides in Paint Oils by High Performance Liquid Chromatography with Electrospray-Quadrupole-Time of Fly mass Spectrometer,* Journal of Chromatography A, vol. 1308, pp. 114-124, 2013.

(86) M. Crhova Krizkova, S. Hrdlickova Kuckova, J. Santrucek, R. Hynek, *Peptide Mass Mapping as an Effective Tool for Historical Mortar Analysis,* Construction and Building Materials, vol. 50, pp. 219–225, 2014.

(87) F. Sabatini, *Caratterizzazione dei Materiali Organici utilizzati nelle Pitture Murali del Beiwusheng Huiguan, (Shaanxi, Cina) mediante un Approccio Multianalitico,* Università degli studi di Pisa, Facoltà di Scienze Matematiche, Fisiche e Naturali, Dipartimento di Chimica e Chimica Industriale, 2012.

(88) S. Kuckova, R. Hynek, M. Kodicek, *Identification of Proteinaceous Binders used in Artworks by MALDI-TOF Mass Spectrometry*, Analytical Bioanalytical Chemistry, vol. 338, pp. 201-206, 2007.

(89) R. Hynek, S. Kuckova, J. Hradilova, M. Kodicek, *Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry as a tool for Fast Identification of Protein Binders in Color Layers of Paintings*, Rapid Communication Mass Spectrometry, vol. 18, pp. 1896–1900, 2004.

(90) S. Kuckova, R. Hynek, M. Kodicek, *MALDI-MS Applied to the Analysis of Protein Paint Binders*, Organic Mass Spectrometry in Art and Archaeology, edited by M.P. Colombini and F. Modugno, ed. Wiley, 2009.

Chapter 2

Materials and methods

2.1 Reagents and materials

All solvents were Baker HPLC grade and were used without any further purification. For calibration of the analytical procedure and for GC/MS analysis, the following solvents and reagents were used: ammonia (NH_3), ethylenediaminetetraacetic acid disodium salt (Na_2EDTAH_20; Fluka, (USA)), dimethylformamide (DMF), hydrochloric acid (HCl), methanol (MeOH), trifluoroacetic acid (TFA; 99% purity, Fluka, (USA)), ethyl acetate (AcOEt; Anal R, BHD), pyridine (Py; 99% purity, Fluka, (USA)), *n*-hexane (pesticide analysis grade; Carlo Erba, (Italy)), diethyl ether (Et_2O; purity HPLC), triethylamine (TEA; 99.5% purity, Sigma-Aldrich, (USA)), *iso*-octane, ethanethiol (EtSH), acetonitrile (ACN), formic acid (FA). Bi-distilled water was used throughout.

The following solutions were prepared: NH_3 25% in water, EDTA 0.1% in water and H_2O : DMF with 0.1% EDTA for sample extraction; 6 M HCl in water for proteins extraction; 2 M TFA for sugars hydrolysis; 2EtSH: 1TFA (daily solution) for sugars mercaptalation; KOH in EtOH 10% for fatty acids saponification. The derivatizing agents used were: N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA; purity>97%, Sigma-Aldrich, (USA)), BSTFA with 1% trimethylchlorosilane (TMCS; purity>97%, Sigma-Aldrich, (USA)), N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA; purity>97%, Fluka, (USA)) with 1% TMCS, hexamethyldisilazane (HMDS; purity 99.9%, Sigma-Aldrich, (USA)).

For proteins and polysaccharides separation and purification steps, OMIX C4 tips (Agilent Technologies) of 100 µL capacity containing a solid extractive functionalized microphase made of C4 were used. PTFE filters of 4 mm of thickness and 0.45 µm pore diameter were chosen for saccharides clean-up double exchange resin Amberlite MB 6113 with colorimetric indicator (Fluka Analytical, (USA)) was used for the elimination of ions from hydrolyzed saccharide fraction.

For the analysis of the samples with MALDI-ToF and nano LC/MS-MS the following solutions and solvents were used: cleavage solution of ammonium hydrogen carbonate (NH_4HCO_3; Lachema (Brno, Czech Republic)) 50 mM in water and trypsin (TPCK, Promega Corporation, (USA)) 20µg/mL, wetting solution of acetonitrile (ACN; Sigma Aldrich, (USA)) and H_2O (ACN : H_2O), equilibration solution of trifluoroacetic acid (TFA; Sigma Aldrich, (USA)) 0.2% in H_2O, elution solution of 50% ACN in H_2O+ TFA 0.1% in H_2O, matrix solution of 2,5-dihydroxybenzoic acid (DHB; Sigma Aldrich, (USA)) consisting in 8 mg of DHB in 150 µL acetonitrile added with 250 µL of 0.2% TFA and 100 µL H_2O, hydrofluoric acid (HF; 38-40%, Merck, (Darmstadt, Germany)), formic acid (FA, Sigma Aldrich, (USA)), methanol (MeOH; LC/MS grade), LC/MS-MS solvent of injection of 97H_2O : 3 ACN with 0.1% of FA. The water used for the analyses was purified in a Milli-Q Direct Water system. The clean up and concentration steps were performed using a reverse phase C18 sorbent in microcolumn ZIP TIP (EMD, Millipore (USA)).

2.2 Standards and reference materials

The following solutions, except for that containing the amino acids, were prepared by weighing pure substances and were used as stock solutions. All the reagents were purchased from Sigma-Aldrich (USA) and the solutions prepared were stored at 4 °C. (i) Amino acids solution in 0.1 M HCl containing 12.5 μmol/mL of proline (Pro) and hydroxyproline (Hyp), 1.25 μmol/mL of cysteine, 2.50 μmol/mL of aspartic acid (Asp), glutamic acid (Glu), alanine (Ala), arginine, phenylalanine (Phe), glycine (Gly), hydroxylysine, isoleucine (Ile), histidine, leucine (Leu), lysine (Lys), methionine (Met), serine (Ser), tyrosine (Tyr), threonine and valine (Val). (ii) Mono and dicarboxylic acids solution in acetone containing 0.24 mg/g of lauric acid (Lau) and of suberic acid (Su), 0.28 mg/g of azelaic acid (A), 0.25 mg/g of myristic acid (My), 0.3 mg/g of sebacic acid (Se), 0.25 mg/g of palmitic acid (P), 0.51 mg/g of oleic acid (O) and 0.51 mg/g of stearic acid (S). (iii) Monosaccharides and uronic acids solution in bidistilled water containing 0.10 mg/g of D-(+)-galactose (Galact), L-(−)-fucose (Fuc), L-(+)-arabinose (Ara), L-(−)-rhamnose (Rhamn), L-(−)-mannose (man), D-(+)-xylose (Xyl), D-(+)-glucose (Glu), D-glucuronic acid (Gluc ac) and D-galacturonic acid monohydrate (Galact ac).

Stock solutions in methanol from commercially available chromophore containing molecules (c.a. 1 ppm and 100 ppm) were prepared and stored at 4 °C. Alizarin (97% purity) and purpurin (80.9% purity) were purchased from Fluka, (USA)), while carminic acid (90% purity) and laccaic acids (mixture of laccaic acid A and B) were purchased from Sigma Aldrich, (Milan, Italy).

For GC/MS procedure, the following solutions of internal standards were prepared: hexadecane (ED) in *iso*-octane (c.a. 80 ppm), norleucine (Nor) in bidistilled water (c.a. 138 ppm), tridecanoic acid (C13) in *iso*-octane (c.a. 135 ppm), mannitol (Man) in bidistilled water (0.10 mg/g). All the standards (purity 99%) were purchased from Sigma-Aldrich (Milan, Italy).

For MALDI-ToF analyses a calibration solution of seven peptides (Angiotensin II, Angiotensin I, Substance P, Bombesin, ACTH clip 1-17, ACTH clip 18-39, Somatostatin 28) named Pepmix (Bruker, (Germany)) was used. The following reference raw materials were used: madder lake (from *Rubia tinctorum*, 220 E, Zecchi, (Italia)), carmine (Alum lake of carminic acid- Cochineal, C-1022, Sigma (USA)), Indian lac (from *Coccus lacca*, Zecchi, (Italia)), Armenian cochineal (from dried insects), Kermes (from dried insects) and Lac dye were kindly provided by the American School of Classic studies (Athens, Greece). The following reference raw materials were used for paint model systems preparation: gesso di Bologna (Phase, (Italy)), boiled linseed oil (Talens, (Nederland)), linseed oil (650 Maimeri, (Italy)), Arabic gum (Sigma, (USA)), casein (caseina lattice extra, ABT 53171 BP05A, (Brescia, Italy)), albumin from chicken egg white lyophilized (Sigma-Aldrich, (USA)), egg (bought in an ordinary supermarket), rabbit glue (LeFranc, (France)).

2.3 Paint model systems

2.3.1 Naturally aged paint model systems

Nine paint model systems were prepared in the laboratory in 1999 by Marina Rosato during her thesis internship [1]. Three different lakes were used (Indian lac, carmine, madder lake) and applied directly on a glass slide (10 x 15 cm) or on a preparation layer on a glass slide (*gesso*/chalk and rabbit glue). The used binders were *tempera grassa* (3 parts of egg yolk, 1 part of linseed oil an 1 part of water) and boiled linseed oil. Three coats of paint were applied. The ratio between lake and binder was 1:2. Figures 2.1 a, b and c show a photograph of the paint model systems. Table 2.1 reports the composition and names of the paint model systems.

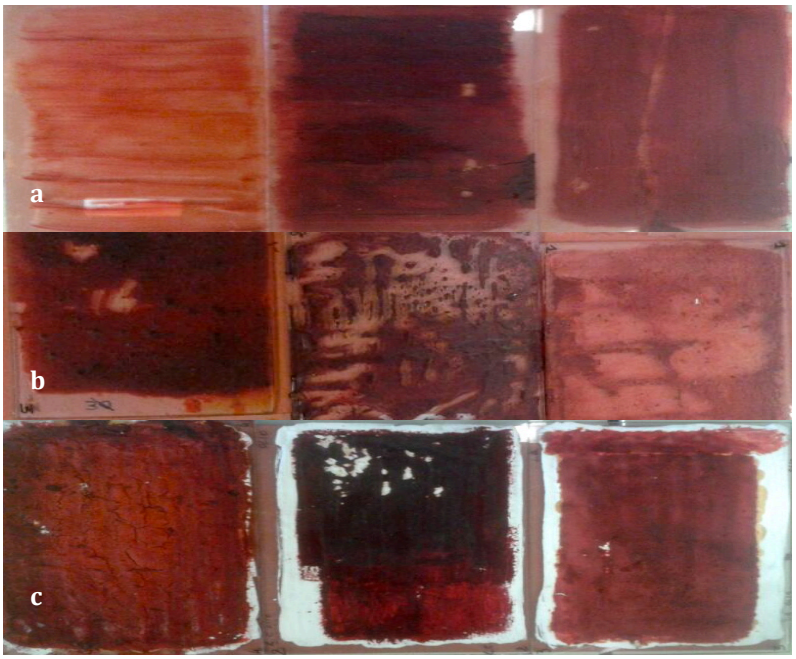


Figure 2.1: Paint model systems with **a)** boiled linseed oil; **b)** tempera grassa; **c)** preparation layer and boiled linseed oil.

Table 2.1: Composition and names of paint model systems.

binder	lake	preparation	name of sample
boiled linseed oil	Indian lac	-	ø 1
	cochineal		ø 2
	madder lake		ø 3
tempera grassa	Indian lac	-	tg 1
	cochineal		tg 2
	madder lake		tg 3
boiled linseed oil	Indian lac	*gesso*/chalk and rabbit glue	oil 1
	cochineal		oil 2
	madder lake		oil 3

2.3.2 Freshly prepared paint model systems

Seventy-one paint model systems were prepared using three different lakes (madder lake, carmine, Indian lac) and six different binders (casein, albumin, rabbit glue, egg, Arabic gum, linseed oil) pure or in solution. The binder/lake mixture was applied on glass slides (26 x 76 mm, 1 mm; Carlo Erba, (Italy)). Each lake was dispersed in each binder in different ratios in order to obtain different shades of color. All solutions were prepared in bidistilled water, except for casein prepared in ammonia (0.1% w/w). The solutions used were: casein (0.1% w/w), albumin (1% w/w and 5% w/w), rabbit glue (0.1% w/w, 1% w/w and 5% w/w) (the solution was heated in order to solubilize the glue), Arabic gum (1% w/w and 10% w/w). Whole egg, with possible addition of water, was used. Linseed oil was used undiluted. Figures 2.2 a, b and c show the preparation of the mixtures of linseed oil with the three different lakes. Figure 2.3 displays the paint model systems finished. Tables 2.2 and 2.3 illustrate the composition of the paint model systems in detail.

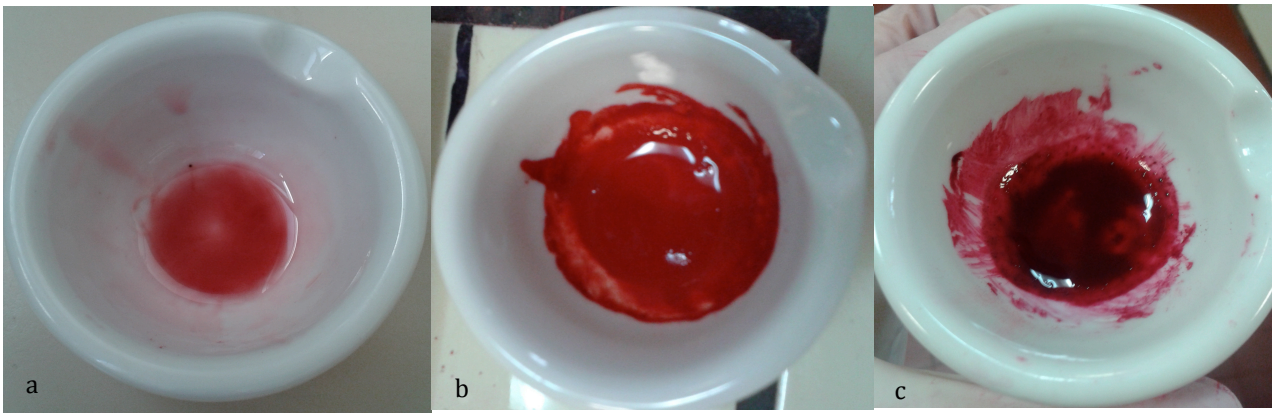


Figure 2.2: a) Madder lake with linseed oil; **b)** Indian lac with linseed oil and **c)** carmine with linseed oil.

Figure 2.3: Paint model systems ordered by color gradation.

Table 2.2: Composition of paint model systems with proteinaceous binders.
(* The ratios binder/lake are approximated to the unit. ** Addiction of a weighed amount of water.)

binder	lake	name of sample	composition* (lake/binder)
casein in ammonia (0.1% w/w)	carmine	C01C1	1:4
	Indian lac	C01IL1	1:4
	madder lake	C01ML1	1:6
albumin in bidistilled water (1% w/w)	carmine	A1C1	1:13
		A1C2	1:4
		A1C3	1:34
	Indian lac	A1IL1	1:11
		A1IL2	1:36
		A1L3	1:5
	madder lake	A1ML1	1:13
		A1ML2	1:3
		A1ML3	1:29
albumin in bidistilled water (5% w/w)	carmine	A5C1	1:3
		A5C2	1:4
	Indian lac	A5IL1	1:3
		A5IL4	1:4
	madder lake	A5ML2	1:2
		A5ML4	1:3
egg	carmine	EC1	1:7
		EC2	1:17
	Indian lac	EIL1	1:16
		EIL2	1:3**
		EIL3	1:7**
	madder lake	EML1	1:8
		EML2**	1:2
rabbit glue in bidistilled water (0.1% w/w)	carmine	RG01C1	1:5
		RG01C2	1:43
	Indian lac	RG01IL1	1:37
		RG01IL2	1:45
	madder lake	RG01ML1	1:5
		RG01ML2	1:24
rabbit glue in bidistilled water (1% w/w)	carmine	RG1C1	1:4
		RG1C2	1:24
	Indian lac	RG1IL1	1:5
		RG1IL2	1:14
	madder lake	RG1ML1	1:25
		RG1ML2	1:4
rabbit glue in in bidistilled water (5% w/w)	carmine	RG5C1	1:6
	Indian lac	RG5IL1	1:26
	madder lake	RG5ML1	1:5

Table 2.3: Composition of paint model systems with saccharide and lipid binders.
(* The ratios binder/lake are approximated to the unit.)

binder	lake	name of sample	composition* (binder/lake)
Arabic gum in bidistilled water (1% w/w)	carmine	AG1C1	1:4
		AG1C2	1:34
	Indian lac	AG1IL1	1:4
		AG1IL2	1:32
	madder lake	AG1ML1	1:1
Arabic gum in bidistilled water (10% w/w)	carmine	AG10C1	1:3
		AG10C4	1:6
		AG10C5	1:16
		AG10C6	1:20
		AG10C7	1:32
	Indian lac	AG10IL1	1:6
		AG10IL3	1:4
		AG10IL5	1:14
		AG10IL6	1:21
		AG10IL7	1:51
	madder lake	AG10ML4	1:2
		AG10ML5	1:11
		AG10ML6	1:20
		AG10ML7	1:52
Linseed oil	carmine	LOC1	1:6
		LOC4	1:7
		LOC5	1:10
		LOC6	1:23
		LOC7	1:48
	Indian lac	LOIL1	1:3
		LOIL5	1:10
		LOIL6	1:19
		LOIL7	1:50
	madder lake	LOML2	1:26
		LOML6	1:3
		LOML7	1:2

2.4 Instrumentation

2.4.1 Gas Chromatography/Mass Spectrometry (GC/MS)

A Gas Chromatographic 6890 Network GC system (Agilent Technologies, (USA)) equipped with a PTV injector was coupled with a 5975 (for proteins and saccharides) or with a 5973 (for lipids) Mass Selective Detector (Agilent Technologies, (USA)) single quadrupole mass spectrometer was used. For the gas chromatographic separation, a HP-5MS fused silica capillary column (5% diphenyl/95% dimethyl-polysiloxane, 30 m x 0.25 mm i.d., 0.25 μm film thickness, Agilent Technologies, (USA)) with deactivated silica precolumn (2 m x 0.32 mm i.d., J&W Scientific, Agilent Technologies, (USA)) was used. The precolumn was connected to the column with a quartz press-fit. The working conditions were: the carrier gas was used in the constant flow mode (He; purity 99.995%) with a flow of 1.2 mL/min for proteins and lipids, 1.0 mL/min for saccharides; the mass spectrometer operated in the EI positive mode (70 eV), the MS transfer line temperature was 280 °C, the MS ions source temperature was kept at 230 °C and the MS quadrupole temperature was at 180 °C. MS spectra were recorded both in total ions current (TIC) in the range 50-600 m/z and in single ion monitoring (SIM) mode. The PTV injector and the chromatographic oven were programmed as follows: (i) for amino acids the PTV injector was used in splitless mode at 220 °C, the oven programme was set at an initial temperature of 100 °C isothermal for 2 min, then 4 °C/min up to 180 °C, isothermal for 15 min; (ii) for lipids and resins the PTV injector was used in splitless mode at 280 °C and the oven programme was: 80 °C isothermal for 2 min, 10 °C/min up to 200 °C, isothermal for 3 min, 10 °C/min up to 280 °C, isothermal for 3 min, 20 °C/min up to 300 °C isothermal for 20 min; (iii) for saccharides the PTV injector was used in splitless mode at 250 °C, the oven programme was: 50 °C isothermal for 2 min, 5 °C/min up to 190 °C, isothermal for 20 min, 5 °C/min up to 280 °C, isothermal for 15 min [2, 3].

2.4.2 Microwave oven

A microwave oven MLS-1200 MEGA (Milestone Microwave Laboratory System, (USA)) MDR technology, High Performance Microwave Digestion, with a module for exhausted vapors evacuation Exhauste Module EM-45/A was used. The conditions for the hydrolysis of proteins were: power 250 W for 10 min, power 500 W for 30 min in the vapor phase with 30 mL of 6 M HCl at 160 °C. The conditions for the hydrolysis of lipids were: power 200 W with 300 μL of KOH_{EtOH} 10% at 60 °C for 120 min. The conditions for the hydrolysis of polysaccharides were: power 500 W with 0.5 mL of 2 M TFA (added directly into closed PTFE conic vials) and 45 mL of bidistilled water at 120 °C for 20 min.

2.4.3 High Performance Liquid Chromatography-Diode Array Detector (HPLC-DAD)

A HPLC consisting of a PU-2089 Quaternary Pump with degasser (Jasco International Co, (Japan)), equipped with an autosampler AS-950 (Jasco International Co, (Japan)) and coupled to a spectrophotometric diode array detector MD-2010 (Jasco International Co, (Japan)) was used. The data were processed with ChromNav software. The working conditions were: spectra acquisition in the range of 200-650 nm every 0.8 sec with a resolution of 1 nm; liquid chromatographic

separation at room temperature (30 °C) on an analytical reverse phase column TC-C18 (2) (4.6 x 150 mm, particle size 5 µm, Agilent) with a precolumn TC-C18 (2) (4.6 x 12.5 mm, particle size 5 µm, Agilent), flow rate of 1 mL/min and injection volume 20 µL. The adopted chromatographic conditions are reported in Table 2.4 where the two solutions are: eluent A: TFA (0.1% v/v) in bidistilled water, eluent B: TFA (0.1% v/v) in CH_3CN.

Table 2.4: HPLC-DAD elution program.

time (min)	A %	B%
0-5	85	15
30	50	50
32	85	15
45	85	15

2.4.4 Liquid Chromatography - Electrospray Ionization – Quadrupole - Time-of-Flight (LC-ESI-Q-ToF)

A HPLC 1200 Infinity, coupled with a Quadrupole-Time of Flight tandem mass spectrometer 6530 Infinity Q-ToF detector by a Jet Stream ESI interface (Agilent Technologies, (USA)), was used. The data were processed with Mass Hunter Qualitative Analysis software. The working conditions were: liquid chromatographic separation performed at 30 °C on an analytical reverse phase column Agilent Zorbax Extend-C18 (2.1 x 30 mm, particle size 1.8 µm, Agilent (USA)) with a precolumn Extended C18 (2.1 x 12.5 mm, particle size 1.8 µm, Agilent (USA)), injection volume of 2 µL. ESI conditions: drying gas (N_2; purity>98%), temperature 350 °C, flow 10 L/min; capillary voltage 4.5 KV; nebulizer gas pressure 35 psig; sheath gas (N_2; purity>98%) temperature 375 °C, flow 11 L/min. High resolution MS and MS/MS acquisition range was set from 100 to 1000 m/z in negative mode; nozzle, skimmer and octapole RF voltages were set at acquisition with an MS and MS/MS scan rate of 1.04 spectra/sec. Chromatographic conditions are set out in Table 2.5. The two solutions used are: eluent A: FA (0.1% v/v) in CH_3CN, eluent B: FA (0.1% v/v) in bidistilled water.

Table 2.5: LC gradient elution program.

time (min)	A %	B%
1.0	15	85
6.0	50	50
8.0	70	30
15,0	90	10
20.0	100	0
20.2	15	85
22.0	15	85

2.4.5 NanoLiquid Chromatography- Electrospray Ionization- Quadrupole-Time-of-Flight (LC-ESI-Q-ToF)

An HPLC Dionex Ultimate 3000 RSLC nano (Dionex, Germany) connected to a mass spectrometer ESI-Q-ToF. Maxis Impact (Bruker, Germany) was used. The trap column was an Acclaim PepMap 100 C18 (100 µm x 2 cm, size of reverse phase particles 5 µm, (Dionex, Germany)) and the analytical column was an Acclaim PepMap RSLC C18 (75 µm x 250 mm, size of reverse phase particles 2 µm). The sample solutions were loaded onto a trap column with a flow rate of 5 µL/min with a mobile phase of 0.1% formic acid in water (A) for 5 min (mobile phase B was a solution of

0.1% formic acid in acetonitrile). The peptides were eluted from the trap column to the analytical column with a flow rate of 0.3 μL/min. Peptides were eluted directly to the ESI source captive spray (Bruker (Daltonics, Germany)). The mass spectrometer was set up in positive ion mode with precursors selection in the range of 400–2200 Da; up to ten precursors from each MS spectrum were selected for fragmentation.
Raw data were evaluated using Data Analysis software (Bruker (Daltonics, Germany)). Proteins were identified using Mascot version 2.4.1 (Matrix Science, (UK)) by searching the protein database Uniprot version 20110-12 (parameters for database search: oxidation of methionine and hydroxylation of proline as variable modifications, tolerance 50 ppm in MS mode and 0.05 Da in MS/MS mode). Chromatographic conditions are presented in Table 2.6.

Table 2.6: LC gradient elution program.

time (min)	A%	B%
0	97	3
5	97	3
85	50	50
86	10	90
95	10	90
96	97	3
110	97	3

2.4.6 Matrix - Assisted Laser Desorption/Ionization Time-of-Flight - Mass Spectrometry (MALDI-ToF-MS)

A Bruker-Daltonics Biflex IV MALDI–ToF mass spectrometer instrument with a standard nitrogen laser (337 nm) in positive and negative reflector mode was used. The instrument was externally calibrated, before each measurement. A minimum of 1500 laser shots was collected for each spectrum; laser power was adjusted each time in order to get the best resolution. The acquisition range was set between 700 and 3500 Da (m/z) for proteins and between 0 and 1000 Da (m/z) for dyes, with an accuracy of 0.3 Da.
The resulting spectra were processed with XMASS software, mMass (Bruker, (Germany)) and proteins ones compared against a database of proteinaceous binders most frequently used in artwork developed in the laboratory.

2.5 Acquisition parameters

2.5.1 GC/MS acquisition parameters

The combined GC/MS analytical procedure applied permits the simultaneous characterization of glycerolipids, natural waxes, terpenoid resins, proteinaceous and polysaccharide materials eventually present in a unique sample avoiding interferences from inorganic media. This multistep procedure allows the transformation of fatty acids in trimethylsilyl (TMS) esters, monosaccharides in trimethylsilyl diethyl dithioacetals and uronic acids in trimethylsilyl diethyl dithioacetals lactones, amino acids in tert-butyldimethylsilyl (TBDMS) derivatives. The three fractions are generated and analyzed separately by GC/MS [2, 3].

Chromatograms of the different fractions were acquired both in total ions current (TIC) mode and in single ion monitoring (SIM) mode. Acquisition ranges and m/z values used for SIM acquisition are shown in Tables 2.7, 2.8 and 2.9.

Table 2.7: Acquisition time ranges and m/z values used for SIM acquisition of mono- and dicarboxylic acids. (*Alkane used as injection standard. **Fatty acid used as derivatization standard.)

analyte	acquisition range (min)	m/z
hexadecane *	11-13	71, 85
lauric acid	12-14	132, 257
suberic acid	12-14	187, 303
tridecanoic acid *	13-15	271
azelaic acid	14-16	201, 317
myristic acid	14-16	177, 132, 285
sebacic acid	15-17	215, 331
palmitic acid	17-19	313
oleic acid	19-21	145, 339, 354
stearic acid	20-22	132, 341, 356

Table 2.8: Acquisition time ranges and m/z values used for SIM acquisition of monosaccharides and uronic acids. (*Alditol used as internal standard.)

analyte	acquisition range (min)	m/z
mannitol *	37-39	129, 217, 319
xylose	40-42	129, 249, 319
arabinose	40-42	129, 249, 319
rhamnose	43-45	171, 333
fucose	45-47	129, 171,333
galacturonic acid	50- 52	135, 217, 305
glucuronic acid	52-54	135, 217, 257, 305
glucose	53-55	217, 249, 331, 405
mannose	53-55	217, 249, 331, 405
galactose	54-56	217, 249, 331, 405

Table 2.9: Acquisition time ranges and m/z values used for SIM acquisition of amino acids. (*Alkane used as injection standard. **Amino acid used as derivatization standard.)

analyte	acquisition range (min)	m/z
alanine	11-13	158, 232
glycine	12-14	147, 218, 246
hexadacane*	13-15	71, 85
valine	14-16	186, 260
leucine	15-17	200, 302
isoleucine	15-17	200, 302
norleucine**	15-17	200, 302
serine	19-21	362, 390
proline	20-22	258, 330
phenylalanine	21-23	302, 336
aspartic acid	22-24	302, 418
glutammic acid	24-26	330, 462
hydroxyproline	26-28	460,462

The quantitative determination of amino acids, mono- and dicarboxylic acids, aldoses and uronic acids is performed by using standard solutions, building calibration curves and evaluating daily recoveries [4].

Running blanks of the procedure highlighted a low level of contamination. The detection limit (LOD) and the quantitation limit (LOQ) of the analytical procedure for amino acids, mono-and dicarboxylic acids, aldoses and uronic acids were calculated. The LODs and LOQs obtained at a statistical significance level of P=0.05 are reported in Table 2.10.

Table 2.10: LODs and LOQs of mono- and dicarboxylic acids, aldoses and uronic acids and amino acids, standard compounds.

compound	LOD (µg)	LOQ (µg)
lauric acid	0.06	0.10
suberic acid	0.03	0.06
azelaic acid	0.01	0.01
myristic acid	0.06	0.09
sebacic acid	0.00	0.01
palmitic acid	0.09	0.14
oleic acid	0.27	0.47
stearic acid	0.49	0.52
xylose	0.05	0.10
arabinose	0.03	0.05
rhamnose	0.00	0.00
fucose	0.01	0.04
galacturonic acid	0.04	0.09
glucuronic acid	0.03	0.07
glucose	0.41	0.99
mannose	0.03	0.06
galactose	0.04	0.09
alanine	0.02	0.03
glycine	0.04	0.10
valine	0.02	0.03
leucine	0.03	0.05
isoleucine	0.01	0.03
serine	0.02	0.04
proline	0.02	0.03
phenylalanine	0.01	0.02
aspartic acid	0.04	0.06
glutammic acid	0.02	0.07
hydroxyproline	0.01	0.04

2.5.2 HPLC-DAD detection and quantitation parameters

Five operating wavelengths were chosen in agreement with maxima of absorbance of antraquinones: 254, 270, 275, 285 and 480 nm.
Retention times and maxima of absorbance wavelengths for the standard anthraquinoid chromophores containing molecules are reported in Table 2.11.

Table 2.11: HPLC-DAD retention times and maximum absorbance wavelengths for the anthraquinoid chromophores containing molecules (*sh=shoulder).

colorant	tr (min)	λ_{max} (UV)	λ_{max} (Vis)
carminic acid	10.5	248, 278, 311	490
laccaic acid A	14.7	290, 340 (sh)	485, 500
alizarin	25.0	247, 278 (sh), 330	434
purpurin	28.6	255, 290	485, 518 (sh)

The quantitative analyses of the anthraquinones were based on calibration curves built up on five standard solutions from 0.5 to 5.0 ppm, prepared as methanol dilutions of the stock standard solutions of the commercially available pure standards.
The commercial laccaic acids standard contains both laccaic acid A and B. Their quantitation was thus based on the integration of the peak due to laccaic acid A and was only used as semi-quantitative indication. Note that the simultaneous presence of laccaic acid A and B characterizes Indian lac over all other kinds of dye; thus, they can be used as molecular markers in spite of their quantitation [1].
Figure 2.4 shows the chromatogram of the standard solution of c.a. 5 ppm anthraquinones, while Table 2.12 reports the calibration curves parameters, limits of detection (LODs) and quantitation (LOQs) of the procedure for each anthraquinone. LOD was estimated as the concentration corresponding to an area of three times the signal of the analytical blank (methanol) while LOQ ten times, as recommended by IUPAC. Then, standard solutions of the four analytes at the estimated LOD concentrations were prepared and injected in order to verify the estimated LODs and LOQs.

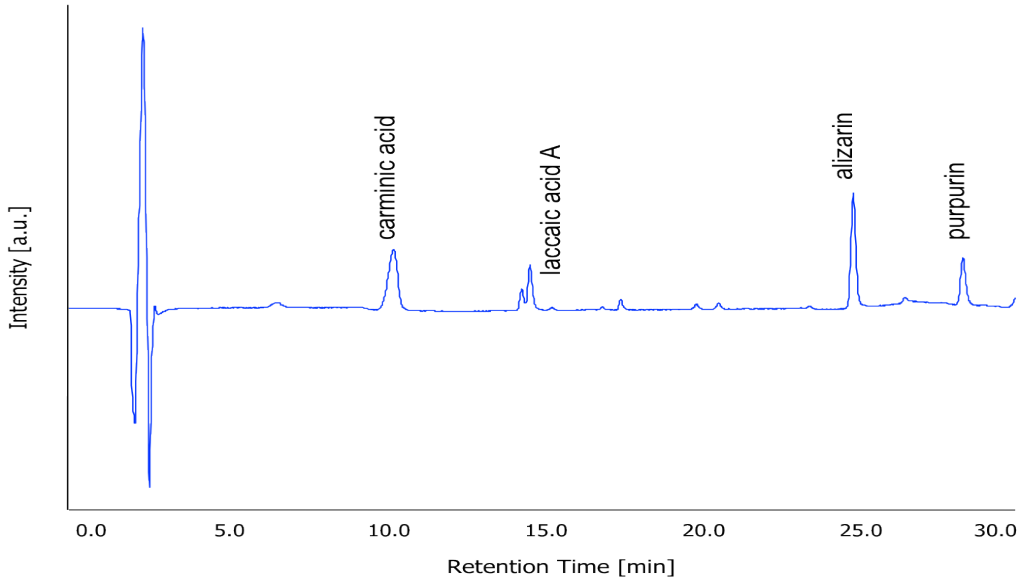


Figure 2.4: HPLC-DAD chromatogram of the standard solution of c.a. 5 ppm of carminic acid, laccaic acids, alizarin and purpurin acquired at 480 nm.

Table 2.12: HPLC-DAD calibration curves parameters (equation of the curve: y= px + q; p: slope; q: intercept; R^2: Pearson coefficient), limits of detection (LOD), limits of quantitation (LOQ) of the anthraquinoid chromophores containing molecules.

colorant	p	q	R^2	LOD (ppm)	LOQ (ppm)
alizarin	45233	-9580.3	0.99842	0.15	0.50
purpurin	85819	-4720.1	0.99982	0.20	0.67
carminic acid	46396	-9584.3	0.99842	0.25	0.83
laccaic acid A	21462	-4112.4	0.99691	0.17	0.57

2.5.3 LC-ESI-Q-ToF-MS/MS detection parameters

Acquisition mode selected was negative mode as this allowed to obtain a higher ratio signal-noise (S/N) [5]. Retention times of the selected analytes, with their molecular weights and pseudo-molecular ions in negative mode are shown in Table 2.13.

Table 2.13: LC-MS/MS retention times, molecular weights, molecular ions in negative mode of anthraquinoid chromophores containing molecules.

compound name	tr (min)	MW (g/mol)	$[M-H]^-$
carminic acid	1.3	492	491.090
laccaic acid A	2.5	537	536.091
alizarin	11.5	240	239.043
purpurin	12	256	255.037

Blanks were run periodically to rule out carry-over effects.

2.5.4 MALDI-ToF detection parameters

A solution of 2,5-dihydroxybenzoic acid (DHB) was used as matrix because of its suitability for organic compounds [6].
Both positive and negative modes were tested. Positive mode is the most common way in which spectra are acquired because protonation creates mono-charged species in MALDI. Negative mode produces both mono-charged and multiply charged ions, the number of negative ions obtained is smaller and the reproducibility of the spectra is lower in comparison to positive mode [7, 8]. Molecular markers of peptides were detected only in positive mode while molecular markers of dyes were detected in both modes.
Instrumental calibration for peptide analysis was performed by comparison with the peptide masses present in Pepmix solution and reported in Table 2.14.

Table 2.14: m/z of peptides present in Pepmix used for proteins internal calibration in positive mode. All peptides are mono-protonated.

peptides	m/z
angiotensin II	1046.54180
angiotensin I	1296.68480
substance P (SP)	1347.7354
bombesin	1619.82330
ACTH-clip (1-17)	2093.0820
ACTH-clip (18-39)	2465.19830
somatostatin (28)	3147.47100

Instrumental calibration for the analysis of chromophore containing molecules was performed on the known masses of DHB matrix and those of the molecular markers of alizarin, purpurin, carminic acid and laccaic acids. The masses used for positive and negative modes are given in Tables 2.15 and 2.16, respectively.

Table 2.15: m/z of ions obtained from ionization of anthraquinoid dyes mixture in DHB matrix used for lakes internal calibration in positive mode.
(*Carminic acid does not ionize in positive mode thus neither molecular ion nor protonated ion are detectable.)

ions	m/z
$[2DHB+H-2(H_2O)]^+$	273.03990
$[2DHB+H]^+$	309.06100
$[2DHB+Na]^+$	331.04300
$[2DHB-H_2O]^+$	290.04270
$[DHB+H]^+$	155.03440
$[DHB+Na]^+$	177.01640
$[DHB-OH]^+$	137.02660
$[DHB]^+$	154.02660
$[DHB+H+CO_2]^+$	199.02430
$[alizarin+H]^+$	241.04226
$[purpurin+H]^+$	257.03717
*[carminic acid]$^+$	492.09039
$[laccaic\ acid\ A+H]^+$	538.09073
$[laccaic\ acid\ B]^+$	496.09039

Table 2.16: m/z of ions obtained from the ionization of anthraquinoid dyes mixture in DHB matrix used for lakes internal calibration in negative mode.

ions	m/z
$[2DHB-H-2(H_2O)]^-$	271.03990
$[2DHB-H]^-$	307.06100
$[2DHB+Na]^-$	331.04300
$[2DHB-H_2O]^-$	290.04270
$[DHB-H]^-$	153.03440
$[DHB+Na]^-$	177.01640
$[DHB-OH]^-$	137.02660
$[DHB]^-$	154.02660
$[DHB-H+CO_2]^-$	197.02447
$[alizarin-H]^-$	239.03443
$[purpurin-H]^-$	255.02930
$[carminic\ acid]^-$	492.09039
$[laccaic\ acid\ A]^-$	538.09073
$[laccaic\ acid\ A+Na]^-$	558.06484
$[laccaic\ acid\ B]^-$	496.09039

2.6 Historical samples

Some historical samples of different nature, composition, provenience and dating were analyzed with MALDI-ToF-MS, nano LC-ESI-Q-TOF-MS/MS, LDI-ToF-MS and HPLC-DAD. A complete list of samples with their relative information is reported in Table 2.17.

Table 2.17: Names, descriptions, proveniences, dating and analyses performed on historical samples.

sample name	**sample description**	**provenience artwork**	**date**	**MALDI-ToF-MS**	**nano LC-ESI-Q-ToF**	**LDI-ToF-MS**	**HPLC-DAD**
Buddha	polychromy on clay preparation	Great Buddha statue of the Bamiyan Valley (Afghanistan)	7th century A.D	X	X	X	X
Delos	wall painting	(Delos, Greece)	b.C.	X	X	X	X
Kor 26	wall painting	(Corinth, Greece)	b.C.	X	X	X	X
FG 2	wall painting	apsidal ceiling of Freiberg cathedral (Germany)	16th century A.D.	X	X	X	X
Palmetta	painting on a funerary clay vessel	Chamber tomb, Taranto (Italy)	3rd century b.C.	-	-	X	-

Bibliography

(1) M. Rosato, *Caratterizzazione delle Lacche Rosse in Manufatti Pittorici Antichi*, tesi di laurea, Università degli studi di Pisa, Facoltà di Scienze Matematiche, Fisiche e Naturali, Dipartimento di Chimica e Chimica Industriale, 2000.

(2) I. Bonaduce, M. Cito, M. P. Colombini, *The development of a Gas Chromatographic-Mass Spectrometric Analytical Procedure for the Determination of Lipids, Proteins and Resins in the Same Paint Micro-Sample Avoiding Interferences from Inorganic Media,* Journal of Chromatography A, vol. 1216, pp. 5931-5939, 2009.

(3) A. Lluveras, I. Bonaduce, A. Andreotti, M.P. Colombini, *GC/MS Analytical Procedure for the Characterization of Glycerolipids, Natural Waxes, Terpenoid Resins, Proteinaceous and Polysaccharide Materials in the Same Paint Microsample Avoiding Interferences from Inorganic Media*, Analytical Chemistry, vol. 82, pp. 376-386, 2010.

(4) M.P. Colombini, A. Andreotti, I. Bonaduce, F. Modugno, E. Ribechini, *Analytical Strategies for Characterizing Organic Paint Media Using Gas Chromatography/Mass Spectrometry,* Accounts of Chemical Research, vol. 43, no. 6, pp. 715-727, 2010.

(5) S.M. Halpine, *An Improved Dye Lake Pigment Analysis Method for High-Performance Liquid Chromatography and Diode-Array Detector*, Studies in Conservation, vol. 41, pp. 76-94, 1996.

(6) E.D. Hoffmann, V. Stroobant, *Mass Spectrometry- Principles and Application*, ed. Wiley, 2007.

(7) L. Rafaelly, S. Hèron, W. Nowik, A. Tchapla, *Optimisation of ESI-MS detection for HPLC of Antraquinone dyes*, Science Direct, vol. 77, pp. 191-203, 2008.

(8) A.C. Ho, J.H. Bowie, A. Fry, *Electron Impact Studies. Part LVII. Negative-ion Mass Spectrometry of Functional Groups. Simple Esters*, Journal of Chemistry Society B, pp. 530-533, 1971.

Chapter 3

Optimization of the extraction procedure of anthraquinoid lakes

3.1 Introduction

An HPLC-DAD and LC-MS/MS method has been developed for the analysis of anthraquinoid lakes. First, a suitable analytical protocol for the quantification of anthraquinoid dyes was built up and defined; afterwards it was applied to anthraquinoid lakes.

For the extraction of anthraquinoid dyestuffs from solid samples, two aqueous solutions were tested: ammonia (NH_3) and ethylenediaminetetraacetic acid (EDTA). Then, liquid-liquid extractions in different conditions were carried out in order to disclose in which fraction they are collected (the extracts or the residues). The performances of the extractions in different pH conditions were tested, the path followed and the modifications undergone by the analytes have been established and the role played by the solvents of injection was evaluated on standards of alizarin, purpurin, carminic acid and laccaic acids. A comparison of UV-Vis spectra and retention times of extract of carminic acid with those of synthesized acid-stable carmine (dcIII) was carried out in order to explain the presence of unexpected peaks in the HPLC chromatograms and UV-Vis spectra of anthraquinoid dyes extracted by ammonia solution. A reaction with ammonia has been suggested and discussed. EDTA extraction has been proposed in order to avoid amination phenomena and three procedures using two different solvents of injection were tested. A final procedure was developed and then applied to lakes and paint model systems in Chapter 4.

Quantitative data, as recoveries and relative coefficients of variations reported in in this chapter, were calculated for all the dyes using the integrated areas of the HPLC-DAD chromatograms and the calibration curves reported in Table 2.12. LC/MS-MS was used to confirm the HPLC-DAD results and characterize the unknown compounds.

3.2 Ammonia extraction

An extraction with 2 M NH_3 was tested on standards of alizarin, purpurin, carminic acid and laccaic acids. Both extracts and residues were analyzed to evaluate the recovery of the dye. Successive extractions with ethyl acetate (AcOEt) at acid pH were performed in order to improve the dyes recoveries in the extracts and, possibly reverse the amination process. Comparison with acid-stable carmine (dcIII) UV-Vis spectrum, whose preparation [1] is reported in Appendix B, was carried out. Two solvents of injection have been tested: i) 85% H_2O : 15% ACN, corresponding to the mobile phase used for liquid chromatography analyses, and ii) DMF: MeOH, which is known to be suitable for solubilising dyes [2].

3.2.1 Procedure and discussion

Few micrograms of anthraquinones were submitted to extraction with 200 µL of 2 M NH_3 in an ultrasonic bath for 120 min at 60 °C. The procedure was repeated twice. The supernatant was dried under nitrogen flow and then re-dissolved in 500 µL of 85% H_2O : 15% ACN solution. 20 µL and 2 µL of both extract and residual were injected in HPLC-DAD and LC-MS/MS, respectively.
Results showed that the NH_3 solution used was efficient in the extraction of antrhaquinoid dyes. The HPLC-DAD and LC-MS/MS chromatograms of residues correspond to that of the analytical blank and therefore they will be neither presented nor discussed.
HPLC-DAD and LC-MS/MS chromatograms, UV-Vis and MS spectra of the ammonia extracts are shown and discussed below for each dye analyzed.

- **Alizarin:**
  In Figure 3.1 the HPLC-DAD chromatogram of the ammonia extract of alizarin and the UV-Vis spectrum of the highest peak (25.4 min), compared to that of standard alizarin, are shown.

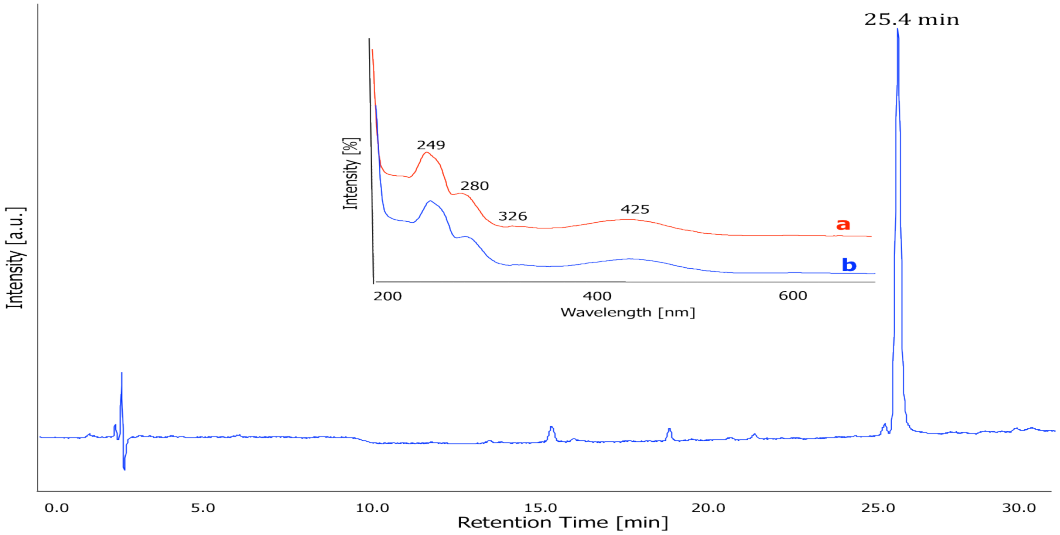


Figure 3.1: HPLC-DAD chromatogram acquired at 254 nm of ammonia extract of alizarin and comparison between UV-Vis spectrum of **a)** standard alizarin and **b)** peak at 25.4 min.

The correspondence between retention times and shapes of the UV-Vis spectra allows the unambiguous identification of the peak at 25.4 min as alizarin. A small peak immediately before alizarin (25.0 min) is also present in the chromatogram. The LC-MS/MS chromatogram highlights a couple of two close peaks (11.3 min and 11.5 min) corresponding to species with pseudomolecular ions at m/z= 238 and m/z= 239, respectively. The peak at 11.5 min clearly corresponds to alizarin, while that at 11.3 min corresponds to the unknown species in the HPLC-DAD chromatogram. In Figures 3.2 a and b the tandem MS spectra of the peaks at 11.3 min and 11.5 min are presented.

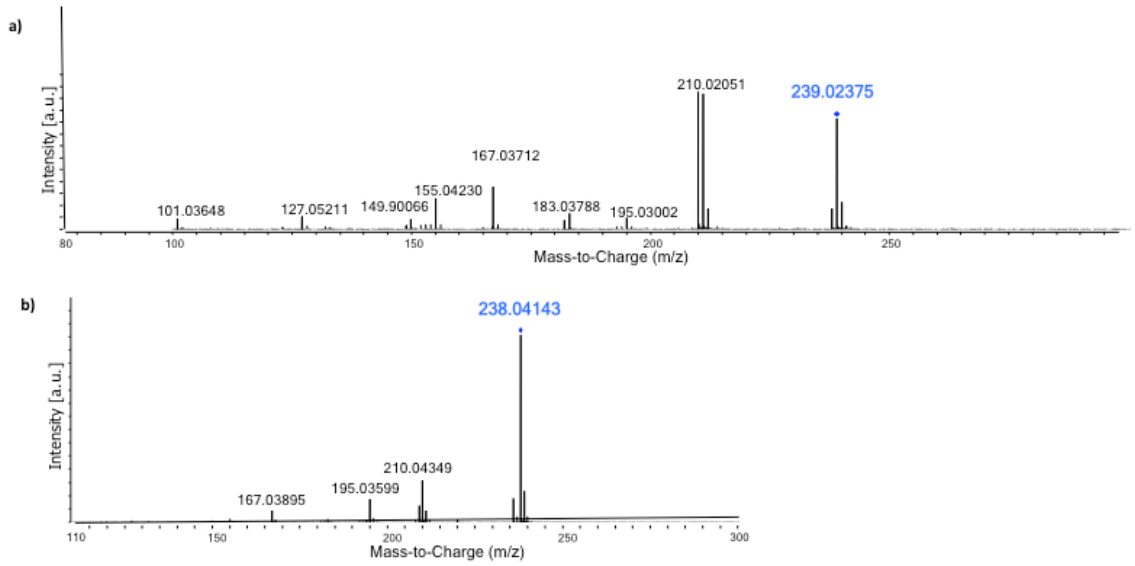


Figure 3.2: tandem MS spectra of peaks at **a)** 11.5 min and **b)** 11.3 min.

On the basis of the molecular formula calculated on the exact mass (239.2262 m/z), $C_{14}H_9O_3N$, we can propose that alizarin undergoes an amination process caused by the presence of NH_3: a hydroxyl group of alizarin is substituted in an amino one resulting in a compound with MW= 238. The transformation of the hydroxyl group in an imine one can be also suggested but data reported in literature confirm the first hypothesis [1]. Notwithstanding, alizarin recovery was around 99.52%, thus only a minor percentual is converted in the aminated form.

- **Purpurin:**

In Figure 3.3, the HPLC-DAD chromatogram of the ammonia extract of purpurin, and the UV-Vis spectrum of the highest peak (27.4 min), compared with that of standard purpurin, are shown.

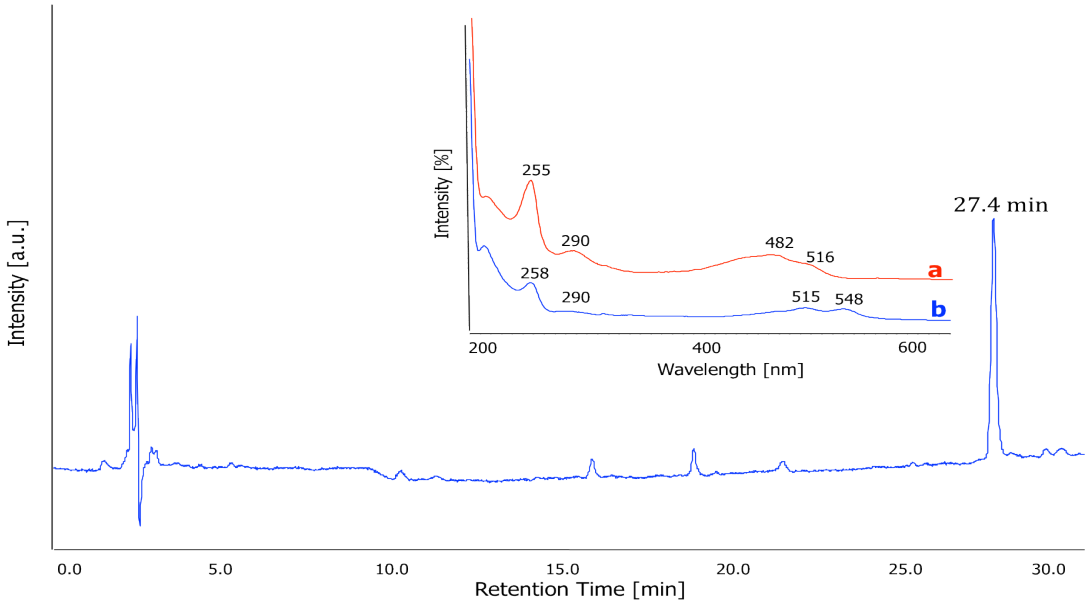


Figure 3.3: HPLC-DAD chromatogram acquired at 254 nm of ammonia extract of purpurin and comparison between UV-Vis spectrum of **a)** standard purpurin and **b)** peak at 27.4 min.

The chromatogram shows only one intense peak (27.4 min), but the different retention time and the UV-Vis spectrum in comparison to that of standard purpurin do not allow its identification as purpurin. The spectrum of the main peak in the chromatogram of the ammonia extract has two maxima around 500 nm absent in the reference spectrum. In the LC-MS/MS chromatogram only one peak is present. The mass spectrum associated shows a base peak at m/z=254 not in agreement with standard purpurin (pseudomolecular ion at m/z=255). In analogy with the discussion about alizarin, we hypothesize that, most likely, purpurin was completely converted to its aminated form.

- **Carminic acid:**

  Figure 3.4 displays the HPLC-DAD chromatogram of the ammonia extract of carminic acid.

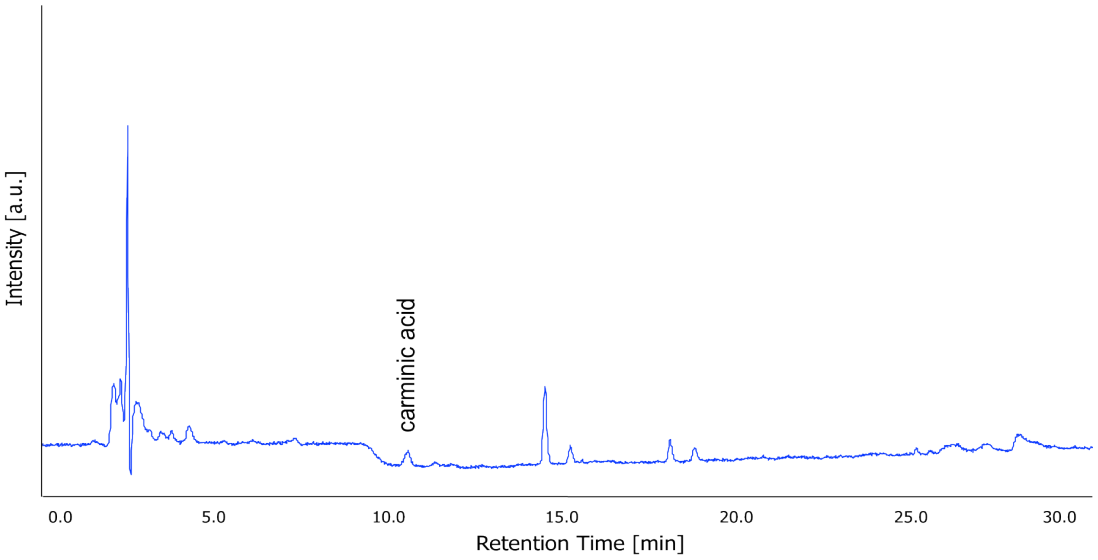


Figure 3.4: HPLC-DAD chromatogram acquired at 275 nm of ammonia extract of carminic acid.

The chromatogram closely resembles to that of the analytical blank and the small peak at 10.4 min may be assigned to carminic acid, but an amminated form cannot be excluded. Neither the UV-Vis spectrum nor the LC-MS/MS chromatogram give any useful information for peak identification, due to very low abundance of the compound found.

- **Laccaic acids:**

  In Figure 3.5, the HPLC-DAD chromatogram of the ammonia extract of laccaic acids is reported.

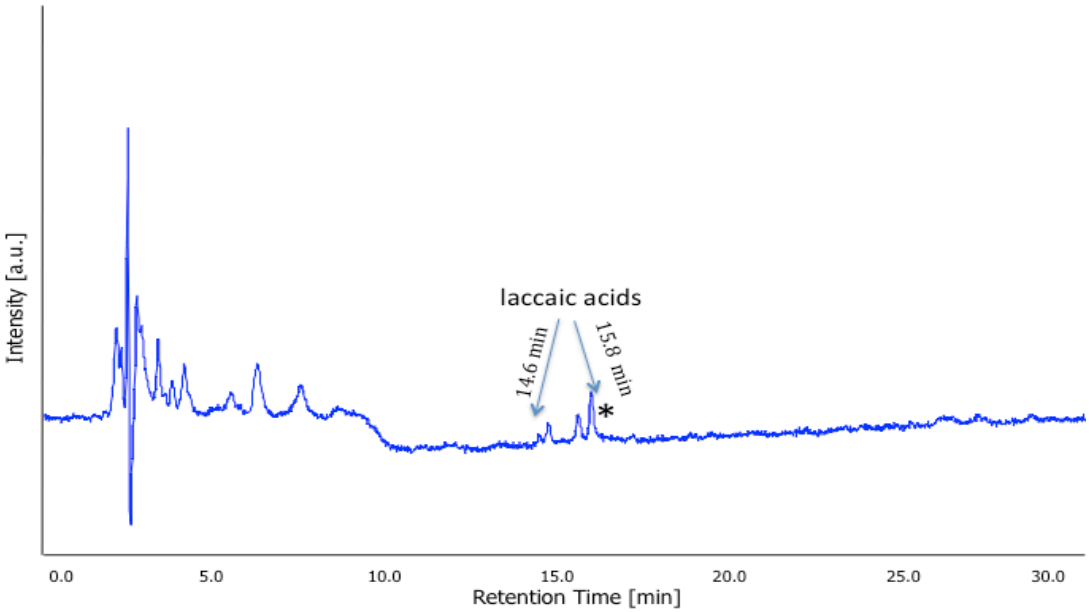


Figure 3.5: HPLC-DAD chromatogram acquired at 285 nm of the ammonia extract of laccaic acids (*= aminated laccaic acids).

In the chromatogram, two couple of small peaks around 14.6 min and 15.8 min are present, and they could belong to laccaic acids B and A and to their aminated forms respectively. Neither the UV-Vis spectra nor LC-MS/MS chromatogram give any useful information for peak identification due to the very low abundance of these species.

On the basis of the results obtained, the procedure was modified in order to improve the extraction yield and to reverse the amination process.
Dyestuffs solutions were submitted to ammonia extraction, as explained previously, then the dried ammonia extract was re-dissolved in 100 µL of TFA 1%. Afterwards, the colorants were extracted with 200 µL of ethyl acetate (AcOEt) for three times. The supernatant was dried under nitrogen flow, re-dissolved in 500 µL of 85% H_2O : 15% ACN solution and finally 20 µL and 2 µL of both extract and residual were injected in HPLC-DAD and LC-MS/MS respectively. The treatment was also repeated replacing the solvent of injection with DMF/MeOH. Both extracts and residuals were analyzed.

- **Alizarin and purpurin:**
  As concerns alizarin and purpurin, no significant differences were noticed with respect to the chromatograms obtained and discussed previously. Even when the solution for injection was modified, the chromatogram of alizarin extract looked like the one obtained with the 85% H_2O : 15% ACN solution, but the recovery of dyestuff was lower. The chromatogram of purpurin shows less peaks, but a low one corresponding to aminated purpurin is still present.

- **Carminic acid:**
  Better results were achieved for carminic acid. The comparison between the HPLC-DAD chromatograms of the extract and residual of carminic acid after the modified ammonia extraction is shown in Figure 3.6.

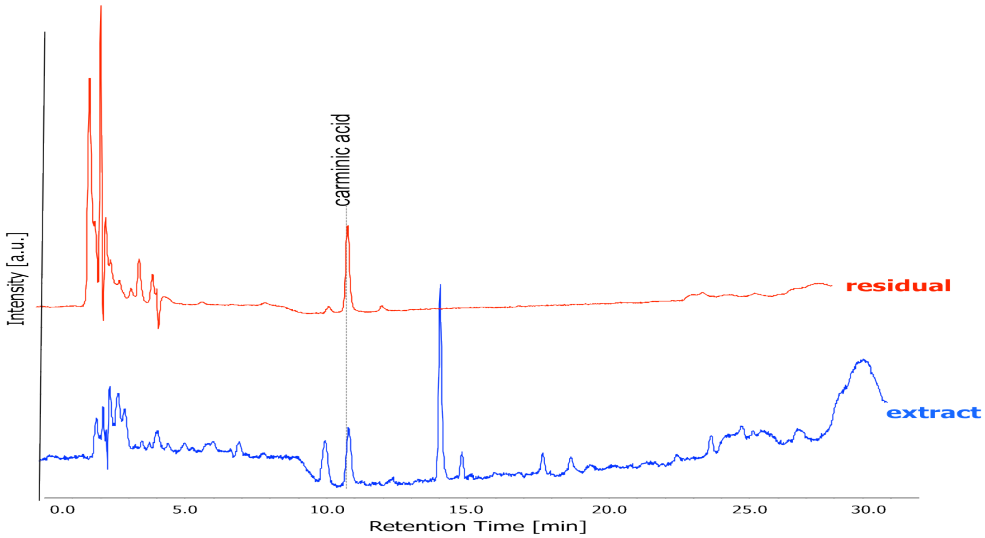


Figure 3.6: HPLC-DAD chromatogram acquired at 275 nm of ethyl acetate extract and residual of carminic acid after dissolution in 85% H_2O : 15% ACN.

The extract shows more chromatographic peaks than the residual, but in both chromatograms a peak can be assigned to carminic acid. The peak in the residual is higher. In Figure 3.7, the UV-Vis spectra of the main peak detected in the ethyl acetate residual, of carminic acid and of dcIII, whose synthesis and characterization is reported in Appendix B, are compared.

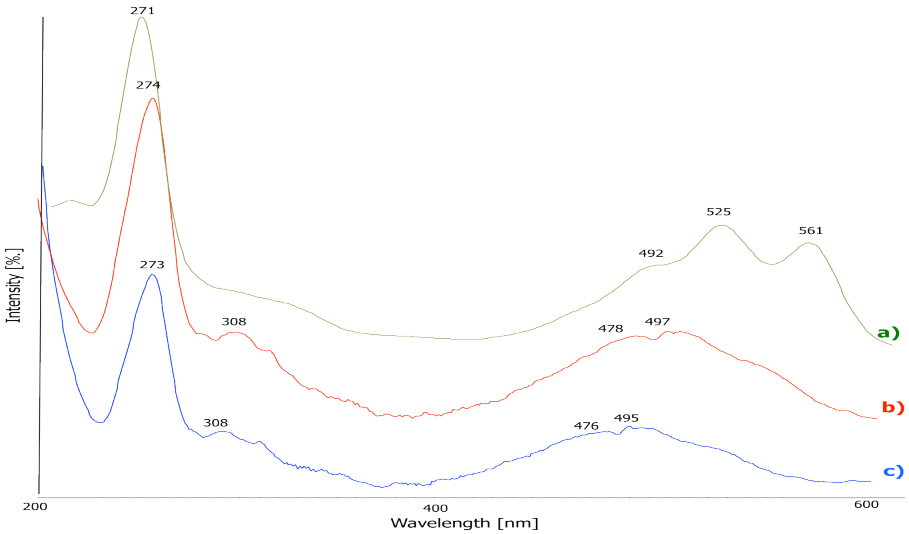


Figure 3.7: UV-Vis spectra at 275 nm of **a)** synthesized dc III; **b)** standard carminic acid; **c)** ethyl acetate residual of carminic acid.

The comparison allows us to identify the peak at 10.9 min with carminic acid and not with the aminated form because it has not the two characteristic maxima of dcIII around 480 nm. This identification is confirmed by LC-MS/MS chromatogram, which shows a peak with a mass spectrum dominated by m/z=491, pseudomolecolar ion of carminic acid, in the acid extract.

If the solvent of injection was replaced with DMF: MeOH, the chromatograms did not differ from those of the analytical blanks.

- **Laccaic acids:**

  In Figure 3.8, the HPLC-DAD chromatogram of laccaic acids residual and UV-Vis spectrum of peak at 16.4 min compared to that of acid laccaic A is shown.

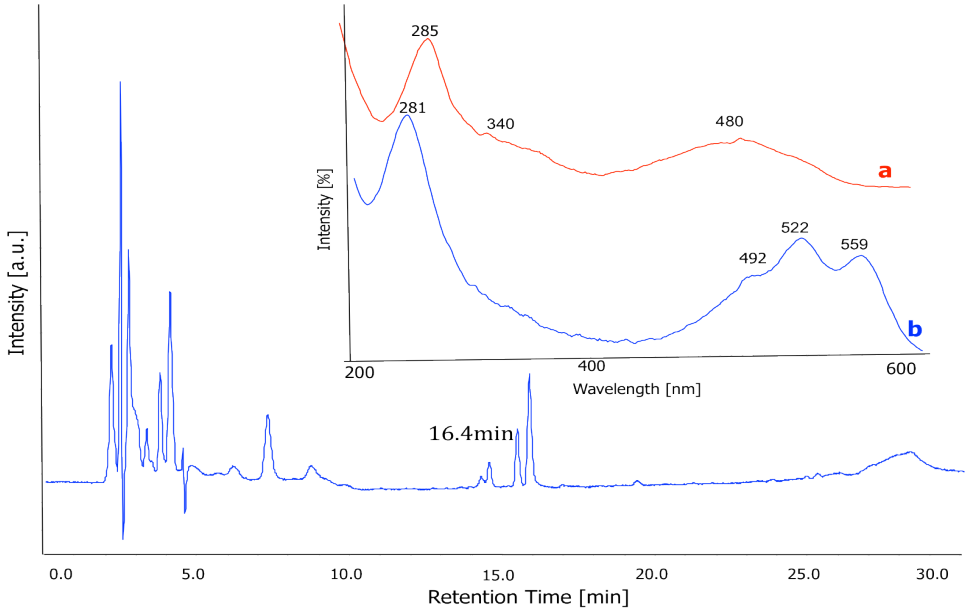


Figure 3.8: HPLC-DAD chromatogram acquired at 275 nm of ethyl acetate residual of laccaic acid and comparison between UV-Vis spectrum of **a)** standard laccaic acid and **b)** peak at 16.4 min.

The chromatographic profile does not present significant qualitative differences in comparison to the chromatogram obtained previously with the simple ammonia extraction. The intensities of the two couples of peaks are higher than those of the peaks in Figure 3.5. Thus, it was possible to acquire the UV-Vis spectrum of the peak at 15.8 nm and compare it with that of laccaic acid. Two maxima around 600 nm are visible and on the basis of the shape of the spectrum, the amination of laccaic acid can be suggested. The LC-MS/MS chromatograms do not give any confirmation about this hypothesis.
If the solvent of injection was replaced with DMF: MeOH, the chromatogram did not differ from that of the analytical blank.

The data collected allow us to highlight the main results obtained and problems encountered in this section:

i) ammonia extraction coverts alizarin, in a little percentage, and purpurin, totally, in their aminated forms. For carminic acid and laccaic acids no confirmed results were obtained;
ii) ammonia extraction at acid pH: amination reaction still occurred. Carminic acid is distributed between extract and residual and its comparison with dcIII allows to exclude the conversion in the aminated form. Laccaic acids were partially subjected to amination.
iii) replace of solvent of injection: no improvement in the yields of extraction has been noticed for any dyes.

3.3 EDTA extraction

Three different procedures using an EDTA solution as extraction agent were tested on standards of alizarin, purpurin, carminic acid and laccaic acids in order to define the best protocol for their analysis. Two different extraction solutions were tested on the basis of the literature [3]: i) EDTA 0.1% in water and ii) H_2O : DMF with 0.1% EDTA. Moreover another procedure, with a further extraction with ethyl acetate (AcOEt) and using EDTA 0.1% instead of NH3 solution, was also tested. Therefore, the three procedures were carried out using either 85% H_2O : 15% ACN or DMF: MeOH as solvent of injection and the recoveries evaluated were discussed for all the dyes. The procedure using ethylacetate, able to separate the dyes from organic binders when a mixture of them is analyzed, was applied also to reference materials of commercial madder lake, carmine and Indian lac. Alizarin lake was synthetized, as reported in Appendix C, and analyzed as well in order to find a justification for the low recovery obtained for commercial madder lake.

3.3.1 Procedure and discussion

3.3.1.1 Analyses of standard antraquinoid dyes

- **EDTA procedure**
  Few micrograms of anthraquinones were submitted to extraction with 200 µL of EDTA 0.1% in water in an ultrasonic bath for 120 min at 60 °C. The procedure was repeated twice. The supernatant was dried under nitrogen flow and then re-dissolved in 500 µL of 85% H_2O : 15% ACN or DMF : MeOH solution. 20 µL and 2 µL of extracts were injected in HPLC-DAD and LC-MS/MS, respectively.

- **EDTA/DMF procedure**
  Few micrograms of anthraquinones were submitted to EDTA procedure replacing EDTA 0.1% in water with of H_2O : DMF with 0.1% EDTA solution.

- **EDTA- AcOEt procedure**
  Few micrograms of anthraquinones were submitted to EDTA procedure, then the dried extract was re-dissolved in 100 µL of TFA 1%. Afterwards, the colorants were extracted with 200 µL of AcOEt for three times. The supernatant was dried under nitrogen flow, re-dissolved in 500 µL of 85% H_2O : 15% ACN or DMF : MeOH solution and in the end 20 µL and 2 µL of extracts were injected in HPLC-DAD and LC-MS/MS respectively.

In order to make a quantitative evaluation of the efficiency of the three procedures using the two different solvents of injection, the percentage recoveries of standard of alizarin, purpurin, carminic acid and laccaic acids with their coefficients of variation are presented in Table 3.1.

Table 3.1: Percentage recoveries and coefficients of variation (CV %) of anthraquinoid standard analyzed in triplicate with EDTA, EDTA/DMF, EDTA-AcOEt procedures using either 85% H_2O : 15% ACN or DMF : MeOH as solvent of injection.

		EDTA		EDTA/DMF		EDTA- AcOEt	
dyes	**solvent of injection**	**recovery %**	**CV%**	**recovery %**	**CV%**	**recovery %**	**CV%**
alizarin	85% H_2O : 15% ACN	9.8	3.3	61.6	6.7	27.3	8.6
	DMF : MeOH	64.0	9.6	96.7	0.6	86.2	1.9
purpurin	85% H_2O : 15% ACN	8.8	3.3	27.3	5.2	16.4	8.1
	DMF : MeOH	32.8	5.0	80.5	2.1	19.9	3.9
carminic acid	85% H_2O : 15% ACN	110.3	7.4	101.0	10.3	36.6	3.8
	DMF : MeOH	98.0	1.0	100.7	1.4	86.8	5.0
laccaic acid A	85% H_2O : 15% ACN	94.4	4.1	80.1	3.3	54.3	12.6
	DMF : MeOH	78.6	2.9	75.8	9.7	66.0	5.9

The HPLC-DAD chromatograms obtained with the three procedures using the two different solvents of injection have been compared for each dye analyzed[2]. The LC-MS/MS chromatograms and UV-Vis spectra are also discussed.

- **Alizarin:**

  In Figure 3.9 the HPLC-DAD chromatograms of the different procedures tested on alizarin are shown.

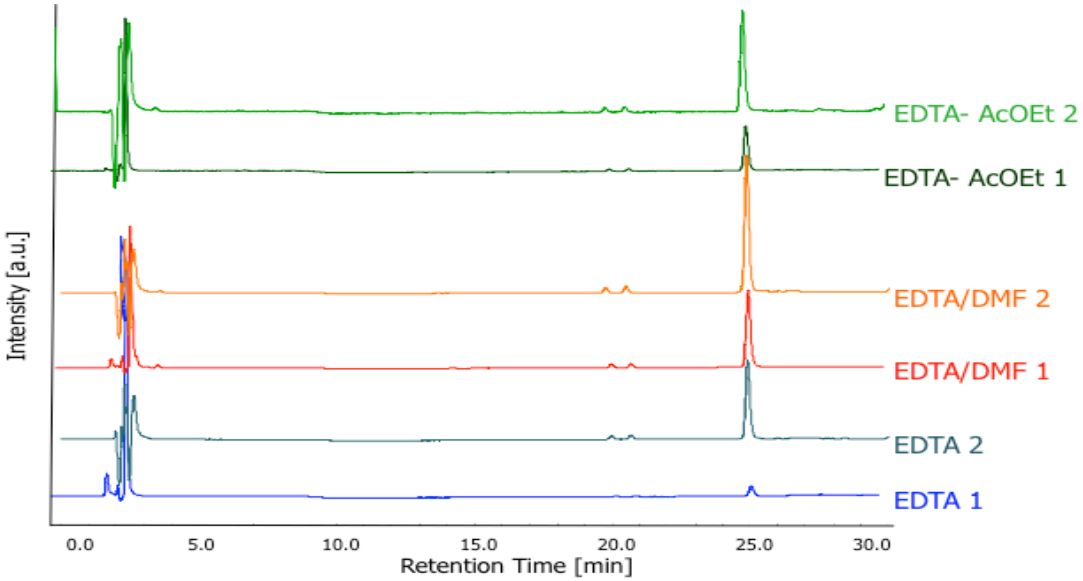


Figure 3.9: HPLC-DAD chromatograms acquired at 275 nm of alizarin submitted to EDTA, EDTA/DMF and EDTA-AcOEt procedures. The injection solvent is specified as follows: (1) 85% H_2O : 15% ACN ; (2) DMF : MeOH.

[2] The HPLC-DAD chromatograms of the three procedures are labelled as EDTA, EDTA/DMF, EDTA-AcOEt and the solvent of injection used is labelled with the number 1= H_2O : 15% ACN and 2= DMF : MeOH. The intensity scale is the same for all the chromatograms, which are merely shifted to improve clarity.

For all the procedures the correspondence between retention times and UV-Vis spectra allows the unambiguous identification of the peak at c.a. 25.0 min as alizarin. This identification is confirmed by LC-MS/MS chromatograms, which show a peak with a mass spectrum dominated by m/z= 239, the pseudomolecular ion of alizarin. The intensity of alizarin peak is the only difference among the HPLC-DAD chromatograms. The procedure using DMF : MeOH as solvent of injection gave higher recoveries for alizarin. The best procedure at all for alizarin analysis entails EDTA/DMF extraction with DMF : MeOH as solvent of injection.

- **Purpurin:**
  Figure 3.10 displays the HPLC-DAD chromatograms of the different procedures tested on purpurin.

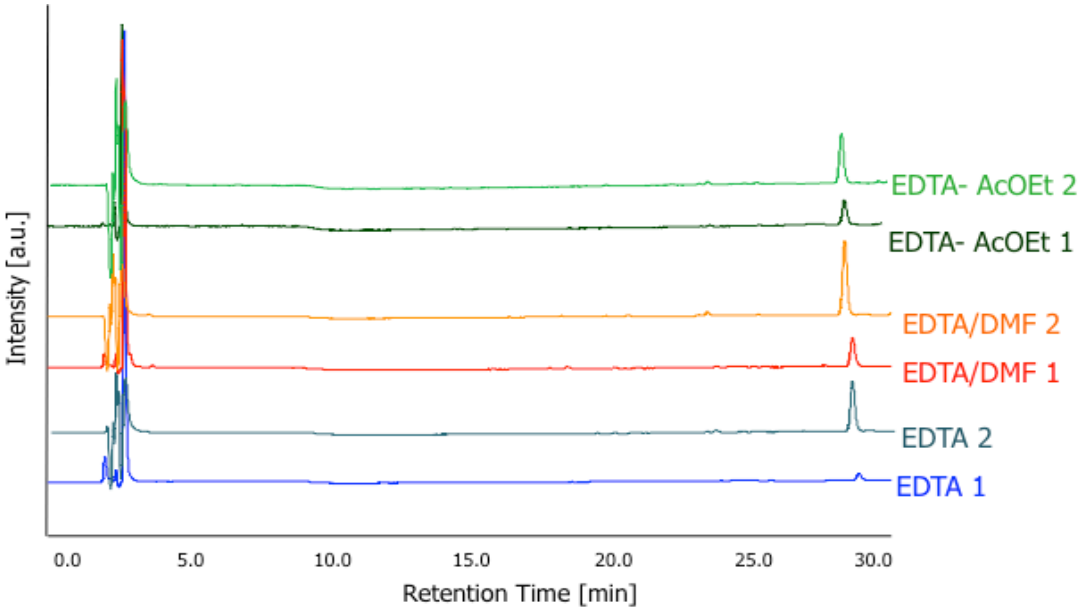


Figure 3.10: HPLC-DAD chromatograms acquired at 275 nm of purpurin submitted to EDTA, EDTA/DMF and EDTA-AcOEt procedures. The injection solvent is specified as follows: (1) 85% H_2O : 15% ACN ; (2) DMF : MeOH.

For all the procedures, the peak at c.a. 29.0 min in the HPLC-DAD chromatograms can be ascribed to purpurin on the base of retention time and the UV-Vis spectra. This identification is confirmed by the peak at m/z= 255 in the LC-MS/MS chromatograms, corresponding to the pseudomolecular ion of purpurin. The highest recovery for purpurin has been obtained with EDTA/DMF procedure using DMF : MeOH as solvent of injection.

- **Carminic acid:**
  In Figure 3.11 the HPLC-DAD chromatograms of the different procedures tested on carminic acid are reported.

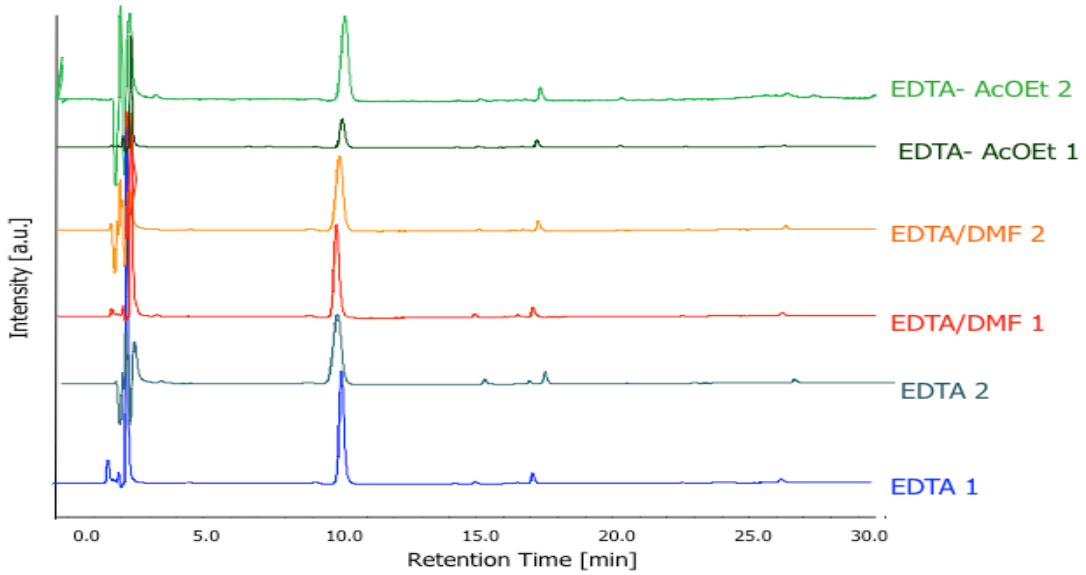


Figure 3.11: HPLC-DAD chromatograms acquired at 275 nm of carminic acid submitted to EDTA, EDTA/DMF and EDTA-AcOEt procedures. The injection solvent is specified as follows: (1) 85% H_2O : 15% ACN ; (2) DMF : MeOH.

All procedures allow for the unambiguous identification of carminic acid with the peak at c.a. 10.0 min in the HPLC-DAD chromatograms. This is confirmed by the UV-Vis spectrum and m/z= 491 pseudomolecular ion of carminic acid in the LC-MS/MS chromatogram. The peak of carminic acid in the HPLC-DAD chromatograms is very intense in all the procedures especially in EDTA and EDTA/DMF ones using H_2O : 15% ACN as solvent of injection. These two methods are the best for detection of carminic acid.

- **Laccaic acids:**

  In Figure 3.12 the HPLC-DAD chromatograms of the different procedures tested on laccaic acids are shown.

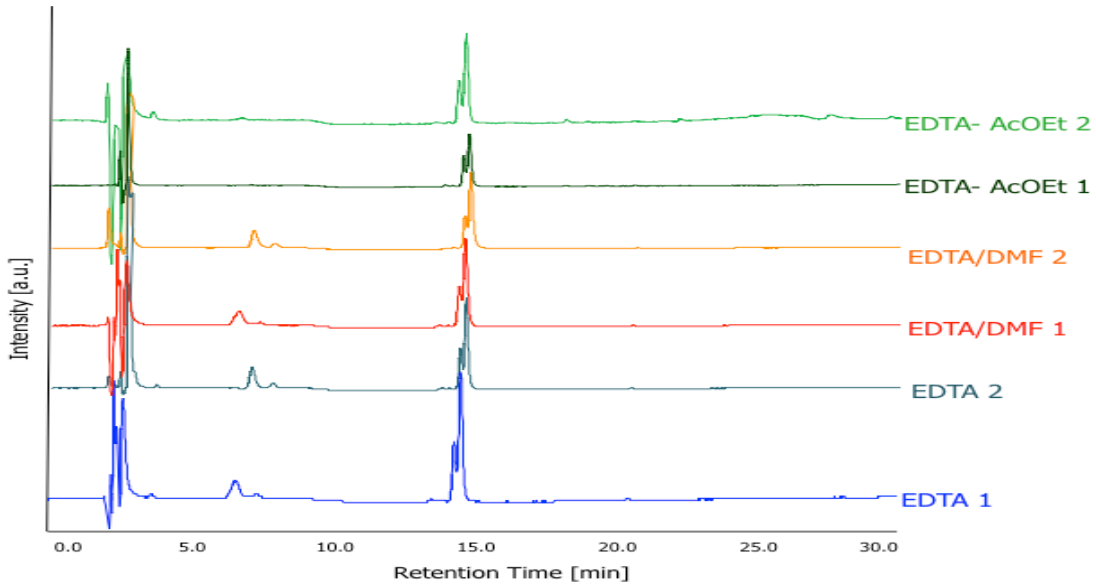


Figure 3.12: HPLC-DAD chromatograms acquired at 275 nm of laccaic acids submitted to EDTA, EDTA/DMF and EDTA-AcOEt procedures. The injection solvent is specified as follows: (1) 85% H_2O : 15% ACN ; (2) DMF : MeOH.

All procedures allow us to detect both laccaic acid A and B at c.a. 15.0 min in the HPLC-DAD chromatograms and their identification is confirmed by their UV-Vis spectra and pseudomolecular ions in the LC-MS/MS chromatograms, m/z= 495 and m/z= 536 respectively. In the HPLC-DAD chromatograms the peaks of laccaic acids A and B show a high intensity with all the procedures but especially with EDTA and EDTA/DMF ones using H_2O : 15% ACN as solvent of injection. In the procedures without AcOEt two more peaks at c.a. 7.0 min with UV-Vis spectra most probably corresponding to laccaic acids C and E are present. In Figure 3.13 the HPLC-DAD chromatogram of laccaic acids standard solution, submitted to EDTA/DMF procedure and using DMF : MeOH as solvent of injection, and UV-Vis spectra of peak at 7.2, 7.4, 14.6, 14.8 min are shown.

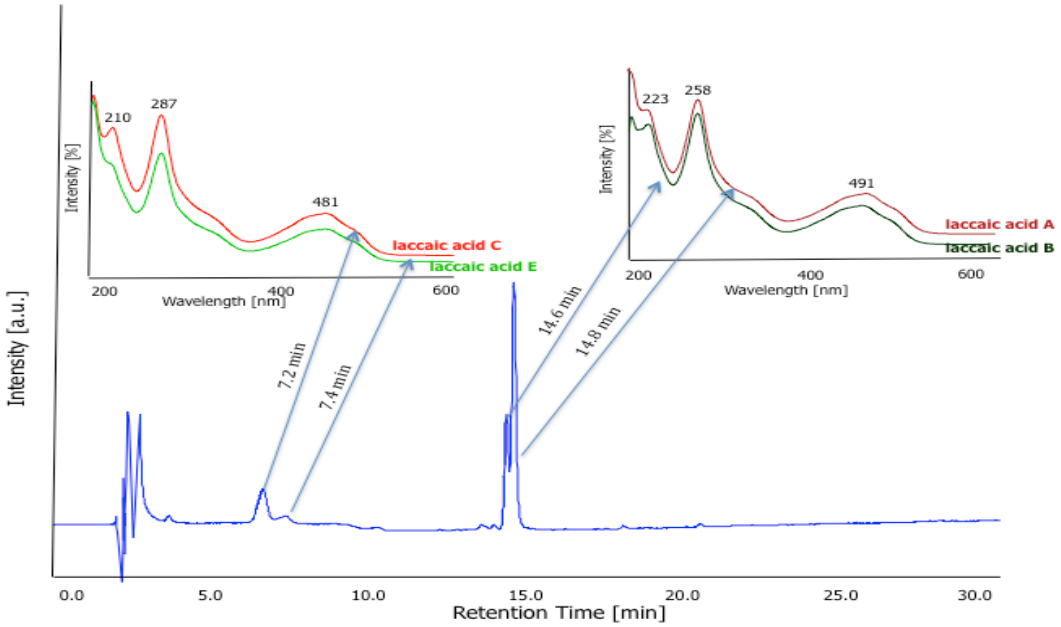


Figure 3.13: HPLC-DAD chromatogram acquired at 275 nm of laccaic acids submitted to EDTA/DMF procedure using DMF : MeOH as solvent of injection and UV-Vis spectra of peak at 7.2, 7.4, 14.6, 14.8 min.

The peaks around 7.2 min and 7.4 min can be assigned to laccaic acid C and laccaic acid E respectively, typical of Indian lac. It could be suggested that commercial standard of laccaic acids used was prepared purifying the raw Indian lac. Laccaic acids C and E are not present in AcOEt extract due to their high polarity and thus greater affinity to the aqueous phase with respect to A and B. The best recovery for laccaic acid A has been obtained with EDTA procedure using H_2O : 15% ACN.

Quantitative results highlight:

i) the use of EDTA solutions as extraction agents allows to get good recoveries for all the dyes tested;

ii) alizarin and purpurin have a different behaviour respect to carminic and laccaic acids. The first couple shows greater affinity to an organic solvent as DMF, thus the best procedure on terms of higher recoveries corresponds to H_2O : DMF with 0.1% EDTA as extraction solution and DMF : MeOH as solvent of injection. On the contrary the other two, due to their acidity,

prefer the aqueous EDTA 0.1% extraction solution and the more polar 85% H_2O : 15% ACN solvent of injection;

iii) the procedure using EDTA-AcOEt as extraction solution does not show any improvement of the recoveries in any case. On the contrary, the lower efficiency to extract the acid compounds due to their low affinity to the non polar AcOEt, is clearly highlighted by the data.

Even if AcOEt using DMF : MeOH as solvent of injection is not the method of election for any investigated compound, it allows to get rather high recovery for all of them and it is a good compromise especially if we aim at separating the dyes from possible organic binders in the residue. This procedure was applied to the analysis of lakes and, in the following chapter, for model systems (paint replicas). In Figure 3.14 the procedure selected for the further analyses is schematized.

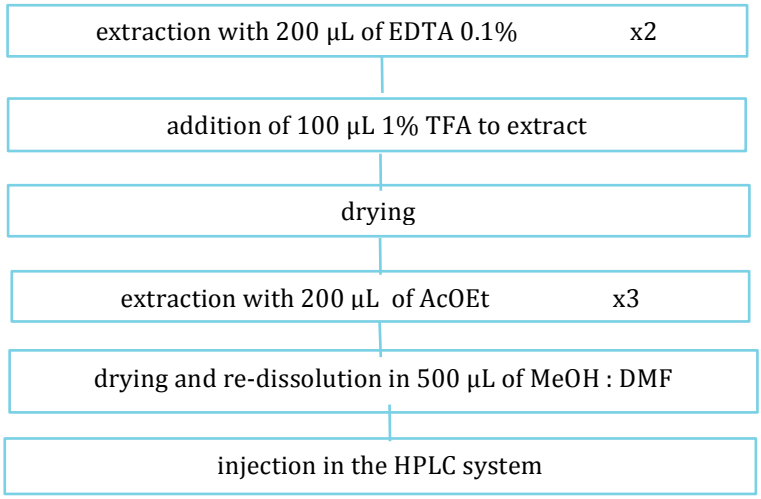


Figure 3.14: Overall procedure for the analysis of anthraquinoid dyes.

3.3.1.2 Analyses of reference anthraquinoid lakes

In order to evaluate the performance of the procedure on painting samples, reference lakes were tested. An amount of c.a. 0.2 mg of commercial lakes as carmine, Indian lac and madder lake and a laboratory synthesized one as alizarin lake (see Appendix C for the procedure of synthesis [4]) were submitted to the procedure reported in Figure 3.14.

In Table 3.2 percentage recoveries and coefficients of variation of colorant biomarkers contained in madder lake, carmine and Indian lac are presented. The recoveries of the lakes analyzed were calculated considering the percentages of metallic cation (aluminum) of the mordant used for their preparation. This information has been provided by Laser Induced Plasma Spectroscopy (LIPS) and Electro-Thermal Atomic Absorption Spectroscopy (ET-AAS) analyses conducted previously on these reference materials [5].

Table 3.2: Percentage recoveries and coefficients of variation (CV %) of dyes contained in anthraquinoid lakes calculated on three replicates.

lake	dye	recovery (%)	CV%
Indian lac	laccaic acid A	67.3	6.7
carmine	carminic acid	14.2	7.5
madder lake	alizarin	2.1	31
alizarin lake	alizarin	33.0	7.5

The HPLC-DAD chromatograms obtained for the four lakes analyzed are reported below.

- **Carmine:**
  In Figure 3.15 the HPLC-DAD chromatogram of carmine is shown.

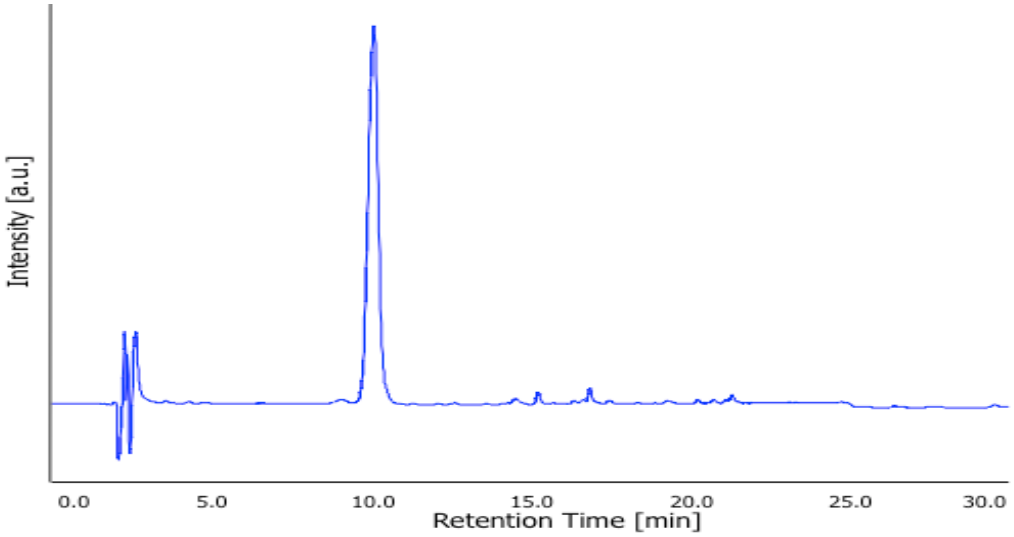


Figure 3.15: HPLC-DAD chromatogram acquired at 275 nm of carmine obtained with the procedure schematized in Figure 3.14.

The peak at c.a. 10 min is ascribable to carminic acid and it is the only intense peak present in the HPLC-DAD chromatogram. The other peaks at higher retention times correspond to the known minor components of cochineal, e.g. dcIV, dcVII, kermesic and flavokermesic acids.

- **Indian lac:**

  In Figure 3.16 the HPLC-DAD chromatogram of Indian lac is reported.

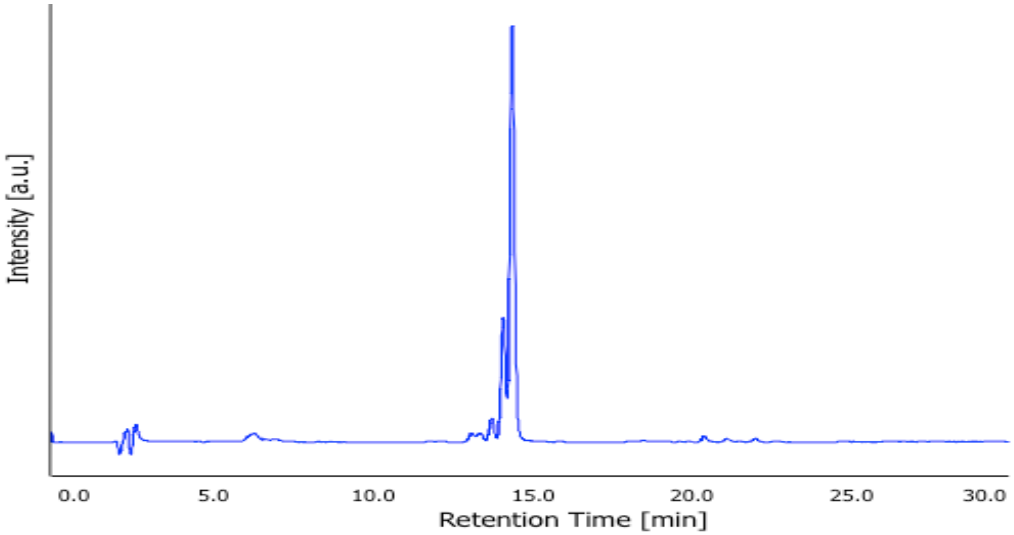


Figure 3.16: HPLC-DAD chromatogram acquired at 275 nm of Indian lac obtained with the procedure schematized in Figure 3.14.

Laccaic acid A and B are detected at c.a. 14.9 min and 14.7 min respectively in the HPLC-DAD chromatogram. Laccaic acid C is at c.a. 7.0 min while laccaic acid E is absent.

- **Madder lake:**

  In Figure 3.17 the HPLC-DAD chromatogram of commercial madder lake is presented.

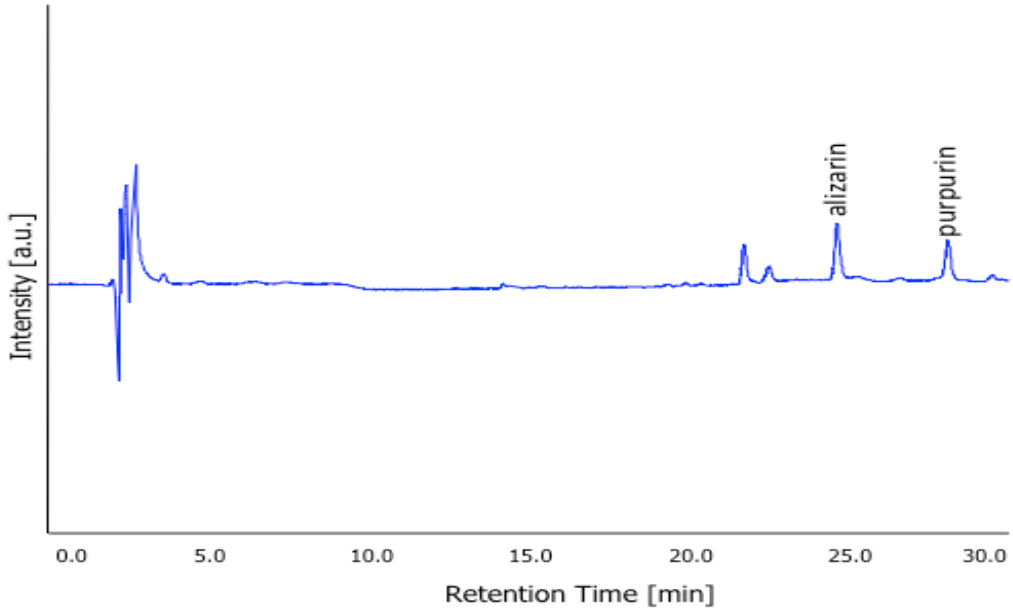


Figure 3.17: HPLC-DAD chromatogram acquired at 275 nm of madder lake obtained with the procedure schematized in Figure 3.14.

The peaks at c.a. 24.8 min and 28.8 min can be assigned to alizarin and purpurin respectively.

The intensities of the peaks are extremely low, and the calculated amount of the compounds is less that expected from sample weight. This unexpected low recovery was further investigated.

- **Alizarin lake:**
  Alizarin lake was synthesizd, as reported in Appendix C, and analyzed with the same procedure applied to madder lake in order to investigate if the low amounts of anthraquinones, and especially of alizarin, could be ascribed to the composition of the commercial madder lake (Zecchi) used. The HPLC-DAD chromatogram of alizarin lake is reported in Figure 3.18.

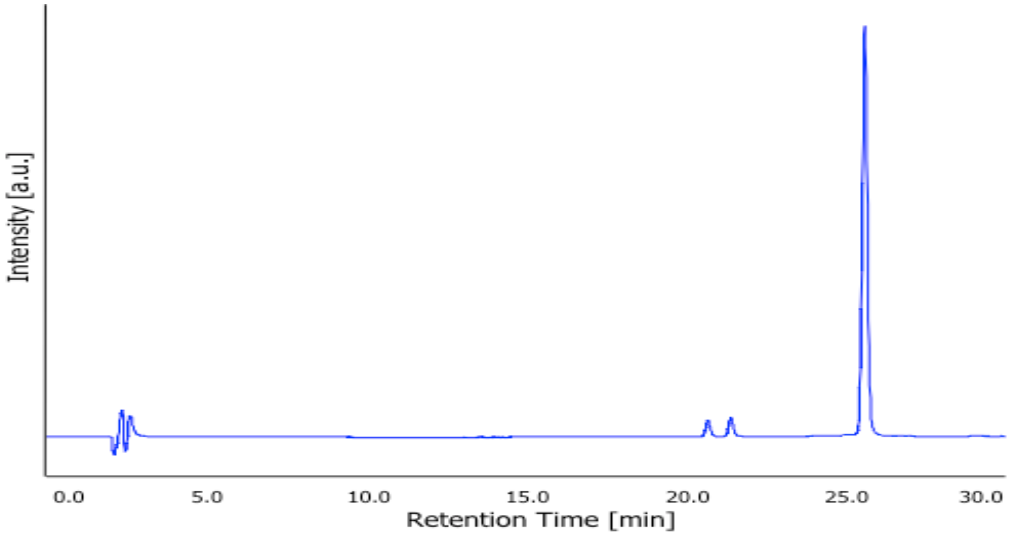


Figure 3.18: HPLC-DAD chromatogram acquired at 275 nm of alizarin lake obtained with the procedure schematized in Figure 3.14.

A high intense peak of alizarin is present in the HPLC-DAD chromatogram. The chromatographic profile is the same obtained for standard alizarin submitted to the analogous procedure. Therefore the low recovery of madder lake has to be attributed to the specific composition or method of preparation of the commercial madder lake (Zecchi).

The considerations based on the HPLC-DAD chromatograms can be discussed in relation to the information provided by LIPS and ET-AAS. These spectroscopic techniques revealed the lack of aluminium in Indian lac, thus it has to be considered an organic dye rather than a lake (Table 3.1 and 3.2). In fact, the recovery obtained for laccaic acid A in Indian lac is the same as the one in standard laccaic acids. Carmine has a composition carminic acid/Al around 77:1, therefore the dye is in large excess with respect to the metal and most probably carminic acid is also present in a not complexed form in the lake. For madder lake the ratio colorant material/Al was 0.2:1, thus there is no free colorant material in the lake. As regards alizarin lake, its composition was known by the procedure of synthesis [4] and the ratio alizarin/Al was 1:10.
Summarizing, AcOEt procedure allows to detect colorant biomarkers contained in all the anthraquinoid lakes analyzed.

3.3 Conclusions

Two strategies, using NH_3 or EDTA as extraction solutions, have been proposed and developed for the optimization of an HPLC-DAD and LC-MS/MS suitable method for the analysis of anthraquinoid dyes used as lakes in paint samples.

In the ammonia extraction, an amination reaction has been suggested for alizarin, purpurin, carminic acid and laccaic acids on the basis of their HPLC-DAD and LC-MS/MS chromatograms, UV-Vis and MS spectra. The presence of NH_3 could cause the transformation of a hydroxyl group of the dye in an amino one. Purpurin turned out to be completely converted, laccaic acids partially and alizarin only in very little percentage. Carminic acid, instead, was not converted at all by ammonia and this was confirmed by comparison between UV-Vis spectra of carminic acid and dcIII. Even if the procedure was slightly modified, neither the yields of extraction improved nor the amination process were reversed, thus extraction solution was replaced with EDTA.

With regard to EDTA extraction, three different procedures (EDTA, EDTA/DMF and EDTA-AcOEt) with two different solvents of injection (85% H_2O : 15% ACN or DMF : MeOH) were tested on standards of alizarin, purpurin, carminic acid and laccaic acids. The HPLC-DAD chromatograms obtained with the different procedures and the respective recoveries were compared for each dye. Chromatographic profiles were not influenced by the analytical procedure applied. The trend of the recoveries was discussed in relation to the nature of the compound and the method of election was set for each dye. Alizarin and Purpurin showed greater affinity to an organic solvent as DMF, thus higher recoveries for these dyes were obtained with EDTA/DMF procedure using DMF : MeOH as solvent of injection. On the contrary, carminic acid and laccaic acid A, due to their acidity, gave higher yields with EDTA procedure with 85% H_2O : 15% ACN as solvent of injection. Although the EDTA-AcOEt procedure was not the best method for any dye, good recoveries were obtained using DMF : MeOH as solvent of injection. This method can be used as a fair compromise, if the analysis of binders left in the residual aqueous phase is needed. This method was applied for the analysis of three commercial lakes as carmine, Indian lac, madder lake, and a synthesized one as alizarin lake. The recoveries were discussed in relation to the composition colorant/Al of the lakes. The highest recovery was obtained for Indian lac and carmine was easy detectable too, although with much lower yields. Instead the HPLC-DAD chromatogram of madder lake showed only small peaks for alizarin and purpurin. The analysis of alizarin lake allowed to suggest that the low recovery of madder lake could be ascribed to its composition and preparation.

In the end an HPLC-DAD and LC-MS/MS method making use of an aqueous EDTA 0.1% extraction solution and AcOEt were developed and successfully applied for the analyses of standard anthraquinoid dyes and lakes.

Bibliography

(1) N. Sugimoto, Y. Kawasaki, K. Sato, H. Aoki, T. Ichi, T. Koda, T. Yamazaki, T. Maitani, *Structure of Acid-Stable Carmine*, Journal Food Hygiene Society Japan, vol. 43, no. 1, pp. 18-23, 2002.

(2) Degano, *A Multiple-Analytical Approach for the Characterisation of Organic Dyes in Textiles*-ph. D thesis in Chemical Science, Università degli studi di Pisa, Facoltà di Scienze Matematiche, Fisiche e Naturali, Dipartimento di Chimica e Chimica Industriale, 2009.

(3) X. Zhang, R.A. Larsen, *Development of Mild Extraction Methods for the Analysis of Natural Dyes in Textiles of Historical Interest Using LC-Diode Array Detector-MS*, Analytical Chemistry, vol. 77, pp. 2022-2025, 2005.

(4) J. Sanyova, *Contribution a' L'étude de la Structure et des Propriétés des Laques de Garance,* Université libre de Bruxelles, Faculté des Sciences Appliquées, Service de Chimie Organique, pp. 200, 2001.

(5) M. Rosato, *Caratterizzazione delle lacche rosse in manufatti pittorici antichi*, tesi di laurea, Università degli studi di Pisa, Facoltà di Scienze Matematiche, Fisiche e Naturali, Dipartimento di Chimica e Chimica Industriale, 2000.

Chapter 4

Evaluation of the mutual influence of lakes and binders in paint model systems

4.1 Introduction

This chapter is divided in two sections: the first one reports on the results achieved by HPLC-DAD and it aims at evaluating the influence of the binder on lake identification, the second one contains GC/MS results and discusses about he influence of the presence of the lake on binder identification.
In the first part, the HPLC-DAD procedure, developed in the previous chapter and reported in Figure 3.14, has been applied on naturally aged and freshly prepared paint model systems to verify if the characterization of the lake was possible also in the presence of a binder. The chromatographic profiles of model systems constituted by the same lake but different binders were compared and discussed.
In the second part, reference madder lake, kermes, Armenian cochineal and lac dye were analyzed by a multistep GC-MS procedure [1] and their lipid, saccharide and amino acid fractions have been evaluated. Theoretical profiles of model systems made by 1 : 1 mixture of one of the colorants with Arabic gum, whole egg, milk or animal glue were calculated to estimate the possible influences on binders identification.

4.2 HPLC-DAD results: influence of the binder on the lake identification

In order to test the influence of the binder on the identification of the lake, the procedure reported in Figure 3.14 was applied to 0.2 mg of selected paint model systems. The AcOEt procedure was chosen among those described in Section 3.3.1.1 and applied to standard anthraquinoid dyes, because it is a good compromise especially if we aim at separating the dyes from possible organic binders in the same micro-sample. In principle, if paint model systems were submitted to AcOEt extraction, the organic and aqueous fractions collected would contain dyes and proteinaceous or saccharides binders respectively. This separation can be useful for treating and analyzing the two fractions following different strategies, thus maximizing the information obtained from an unique micro sample.
The naturally aged and freshly prepared paint model systems, described in Section 2.3.1 and 2.3.2, were analyzed. The first sample set was constituted by the same carmine, Indian lac and madder lake, already analyzed in Section 3.3.1.2, applied with a lipid binder, i.e. boiled linseed oil used pure or mixed with egg. The ratio between lake and binder was 1:2. The second set of paint model systems was made up by a mixture of one of the three lakes mentioned above and a proteinaceous

binder i.e. casein, albumin, egg or rabbit glue. Comments about chromatographic profiles of paint model systems containing the same lake, but applied with different binders are reported below.

4.2.1 Analyses of naturally aged paint model systems

The HPLC-DAD chromatograms of the nine paint model systems analyzed[3], in which the ratio between lake and binder was 1:2, are compared in accordance to the kind of lake used, and shown in Figures 4.1, 4.2, 4.3.

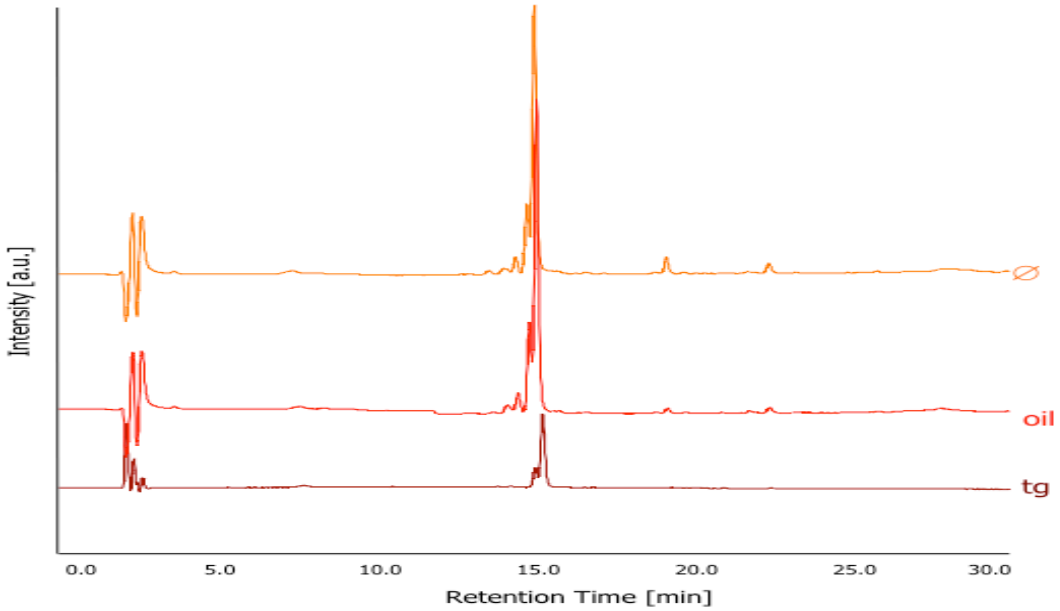


Figure 4.1: HPLC-DAD chromatograms acquired at 275 nm of natural aged paint model systems ø 1, oil 1, tg 1 prepared with Indian lac, obtained with the procedure schematized in Figure 3.14.

[3] The meaning of the labels of the samples is as follows: ø= boiled linseed oil without any preparation layer, oil= boiled linseed oil with a preparation layer, tg= *tempera grassa* without any preparation layer.

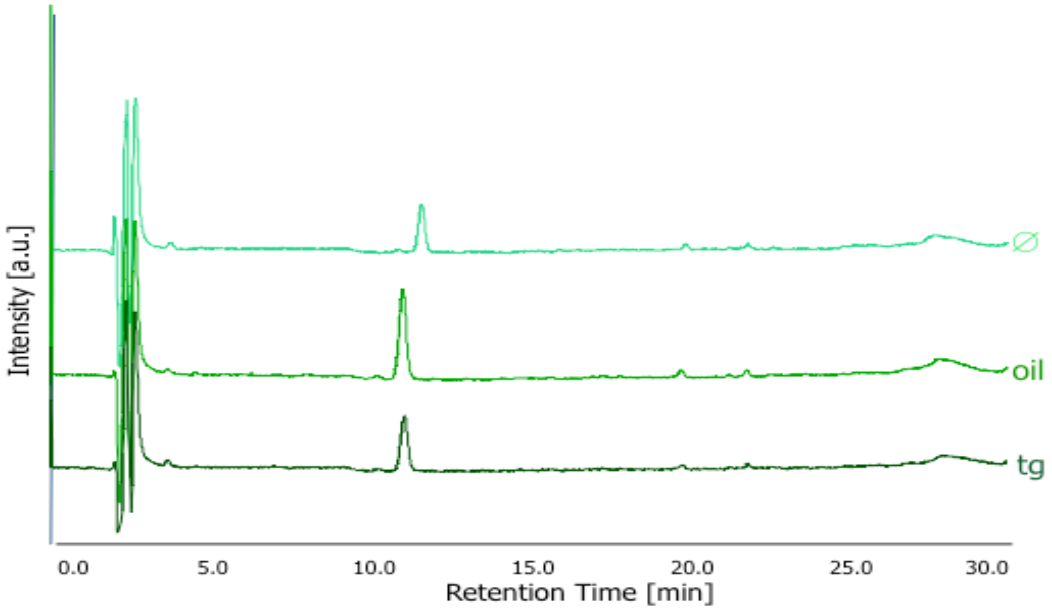


Figure 4.2: HPLC-DAD chromatograms acquired at 275 nm of natural aged paint model systems ø 2, oil 2, tg 2, prepared with carmine, obtained with the procedure schematized in Figure 3.14.

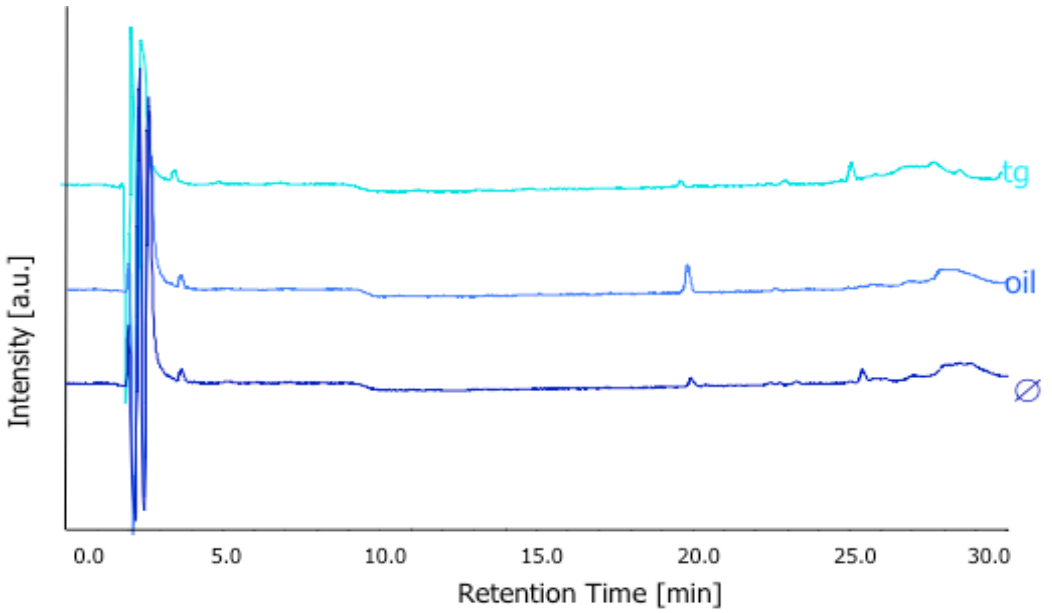


Figure 4.3: HPLC-DAD chromatograms acquired at 275 nm of natural aged paint model systems ø 3, oil 3, tg 3 prepared with madder lake, obtained with the procedure schematized in Figure 3.14.

In all model systems 1, prepared with Indian lac, the identification of laccaic acids A and B was successful especially for ø 1 and oil 1. Laccaic acids C and D were not detected.

Carminic acid was detected in all model systems 2.

Madder lake identification instead resulted being problematic because the peak of alizarin is very little and visible only in ø 3 and tg 3, while the purpurin one is not detectable due to a broad band in proximity of its retention time. Notwithstanding, taking into account the considerations reported in Section 3.3.1.2, the qualitative detection of the dyes could be satisfying for madder lake identification.

To summarize, the determination of the kind of lake used in natural aged paint model systems was possible in all the nine samples except for oil 3. The most intense peaks of colorant biomarkers were obtained for Indian lac. Madder lake, instead, was relatively difficult to characterize. These observations are in agreement with the recoveries reported for reference lakes in Table 3.2. In the HPLC-DAD chromatograms of all model systems no peaks ascribable to binders are present and the profiles of ø, oil and tg of the same lake do not differ from each other. Therefore the kind of binding medium used does not influence the determination of the lake.

4.2.2 Analyses of freshly prepared paint model systems

Twelve freshly prepared paint model systems, shown in Figures 4.4 a, b and c, were analyzed. One sample for each kind of lake combined with all types of proteinaceous binders and highest lake/binder ratio were selected as representative model systems.
The HPLC-DAD chromatograms of the twelve freshly prepared paint model systems analyzed[4] are presented in Figures 4.5, 4.6, 4.7.

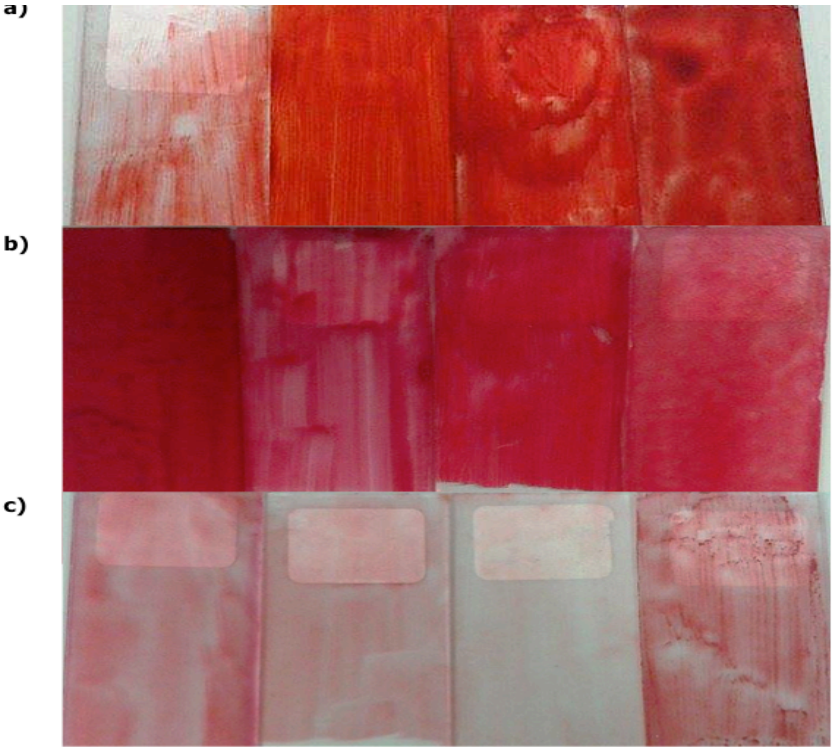


Figure 4.4: Freshly prepared paint model systems (from left to right): **a)** RG5IL, C01IL, EIL3, A5IL, **b)** RG5C, C01C, EC1, A5C1, **c)** RG5ML, C01ML, EML2, A5ML2. The samples codes correspond to RG5= rabbit glue 5% w/w, C01= casein 0.1% w/w, E= whole egg, A5= albumin 5% w/w; IL= Indian lac, C = carmine, ML = madder lake.

[4] The meaning of the labels of the samples is reminded: RG5= rabbit glue 5% w/w, C01= casein 0.1% w/w, E= whole egg, A5= albumin 5% w/w.

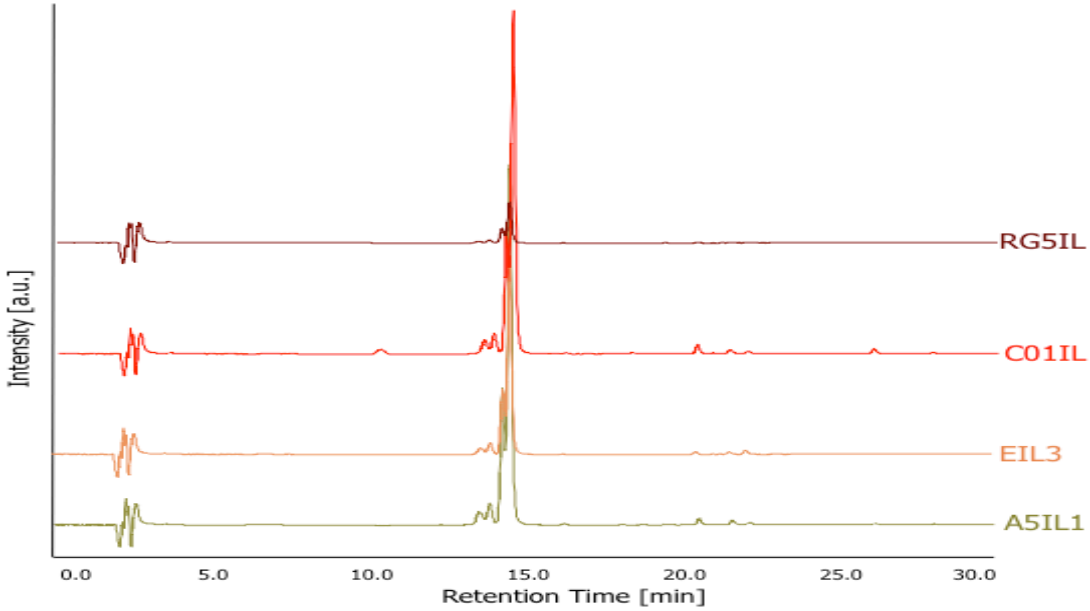


Figure 4.5: HPLC-DAD chromatograms acquired at 275 nm of freshly prepared paint model systems RG5IL, C01IL, EIL3 and A5IL obtained with the procedure schematized in Figure 3.14.

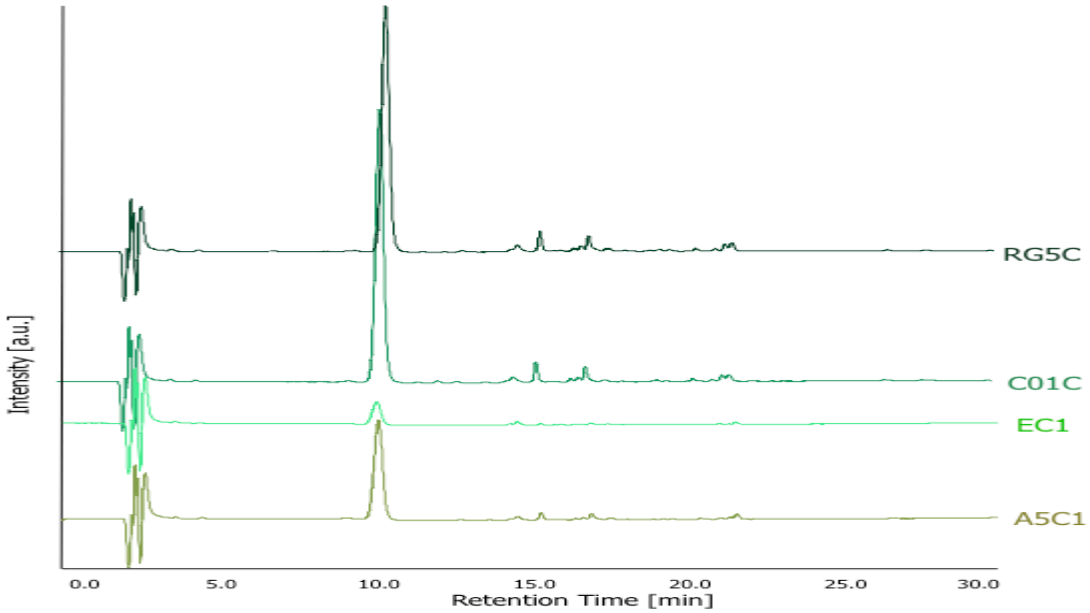


Figure 4.5: HPLC-DAD chromatograms acquired at 275 nm of freshly prepared paint model systems RG5C, C01C, EC1 and A5C1 obtained with the procedure schematized in Figure 3.14.

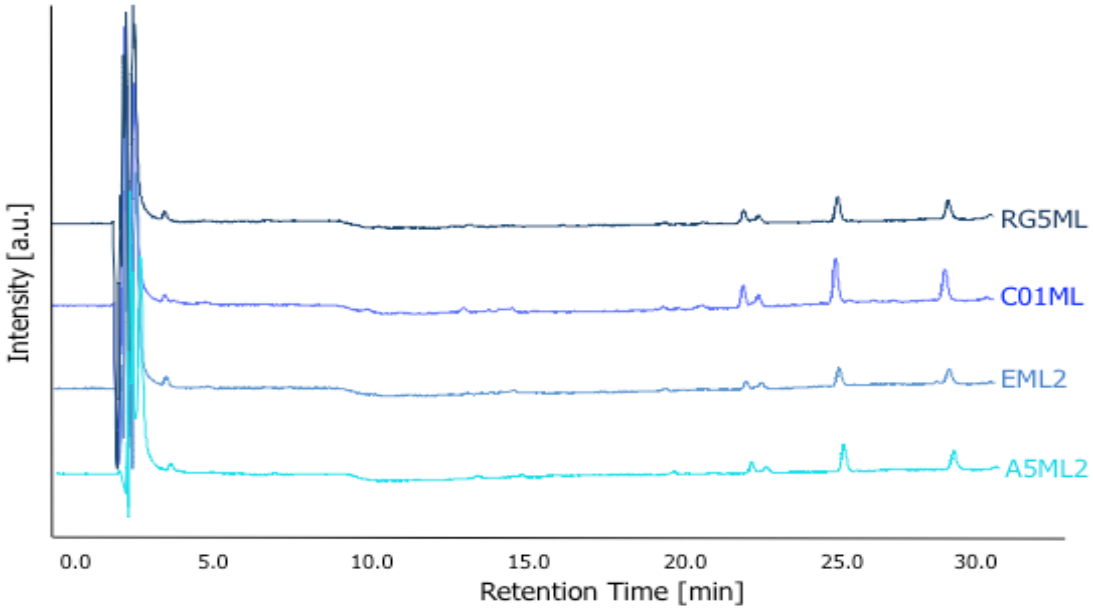


Figure 4.6: HPLC-DAD chromatograms acquired at 275 nm of freshly prepared paint model systems RG5ML, C01ML, EML2 and A5ML2 obtained with the procedure schematized in Figure 3.14.

In all model systems, the peaks belonging to laccaic acids A and B are very high with the exception of RG5IL in which they are less intense, but still easily detectable. Laccaic acids C and D were not present.
In the model systems prepared with carmine, the identification of carminic acid is unambiguous. The lowest peak of dyes is observed in EC1.
Alizarin and purpurin were detectable in all the model systems without any considerable difference due to the kind of binder used. The recoveries of alizarin and purpurin are definitely lower than those for laccaic acids and carminic acid, but the considerations reported in Section 3.3.1.2 have to be kept in mind. Nevertheless the identification of madder lake is unambiguous in all the investigated paint model systems.

Summarizing, the lake used in freshly prepared paint model systems was identified in all the samples analyzed. The same considerations reported for naturally aged paint model systems can be confirmed. The chromatograms did not show any peculiar feature that may allow us to differentiate between different kinds of binders. Moreover, all freshly prepared paint model systems show higher peaks of the colorant biomarkers than the aged ones, even if the lake/binder ratio was almost the same. A possible explanation might be the more difficult extraction of dyes from the aged paint layers due to the aged and possibly polymerized binding media.

4.3 GC/MS results: influence of lake on binder identification

In order to investigate the influence of the lake on the identification of the binder by the already published procedure based on GC/MS analyses, 0.4 mg of reference raw materials as kermes, Armenian cochineal and lac dye, and a commercial lake as madder lake were analyzed by the combined procedure [1]. This multistep procedure, depicted in Figure 4.7, allows the simultaneous characterization of glycerolipids, natural waxes, terpenoid resins, polysaccharides and

proteinaceous materials possibly present in a unique sample. The detailed steps and the strategies to interpreter the results are described in the literature [1, 2, 3, 4].

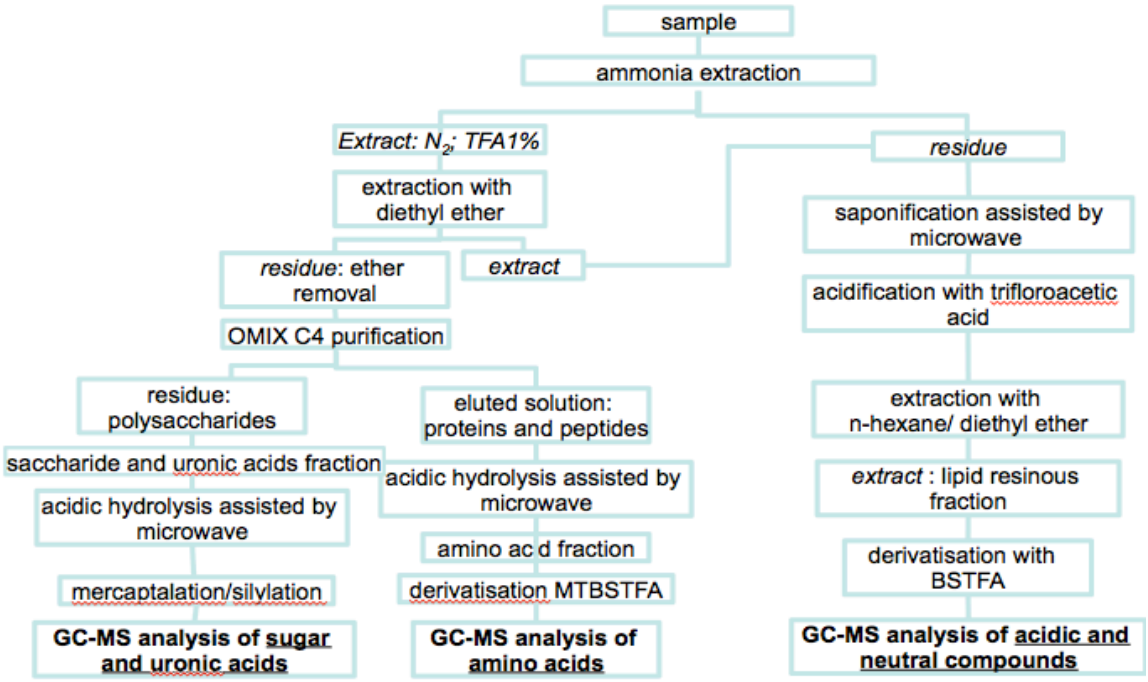


Figure 4.7: The combined GC/MS analytical procedure [1].

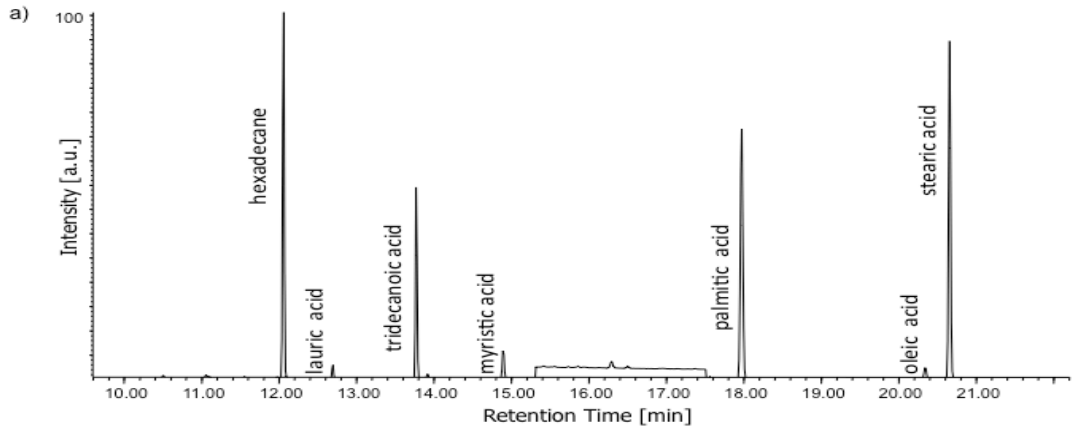
The relative percentage contents of lipids, saccharides and amino acids of the reference materials were evaluated and discussed. Once evaluated the lipid, saccharidic and protein content of the dyes/lakes, it was possible to simulate the GC/MS profiles that would be obtained when an anthraquinoid lake and a saccharide or proteinaceous binder are simultaneously present in the same sample. Theoretical values for 1:1 mixtures of one of the raw materials analyzed and a binder as Arabic gum, whole egg, milk and rabbit glue were calculated and commented. This strategy was already successfully applied for the evaluation of the sugar profile of a 1:1 mixture of the saccharide and proteinaceous binders as well as to sugar profiles of gums contaminated with 10% of the saccharide content of softwood and hardwood [5].

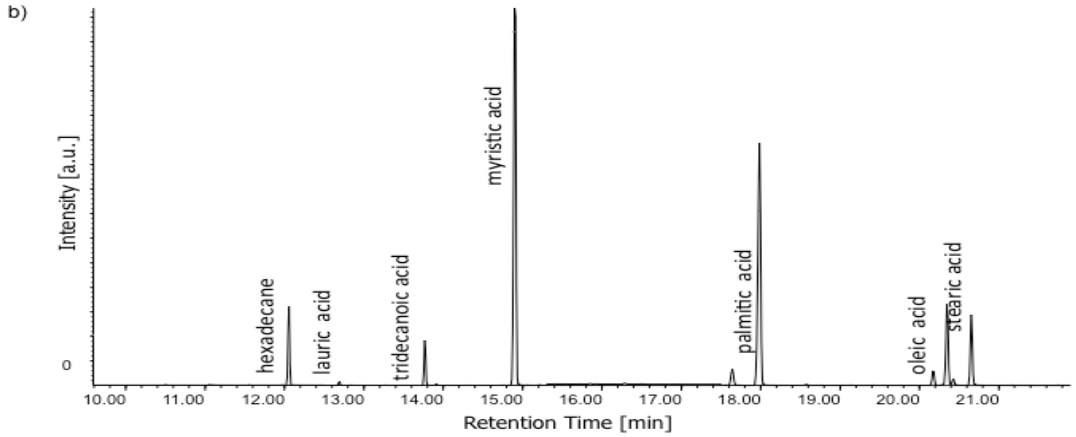
4.3.1 Evaluation of the contribution of the reference materials

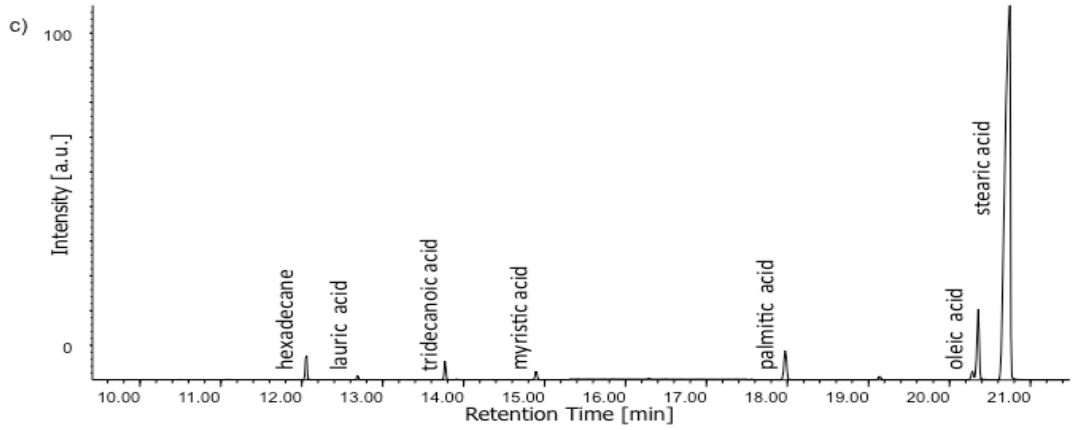
The analytical procedure reported in Figure 4.7 allows to collect three fractions (lipid resinous, saccharide and amino acidic one) and to analyze them separately by GC/MS. The results are presented for each fraction. The GC-MS chromatograms of the samples corresponding to those of the analytical blanks are not shown. For raw materials, whose amount of saccharide or proteinaceous material was below the limit of detection, relative percentage content of saccharides and amino acids were not reported.

- **Lipid resinous fraction:**

  All samples contain an amount of lipid material above the quantitation limit (LOQ= 1.4 µg). The Selected Ion Monitoring (SIM) of madder lake, kermes, Armenian cochineal and lac dye are shown in Figures 4.8 a, b, c, and d respectively. The relative percentage contents of lipids of the reference materials and their characteristic parameters values generally used for the determination of lipid material are reported in Table 4.1.







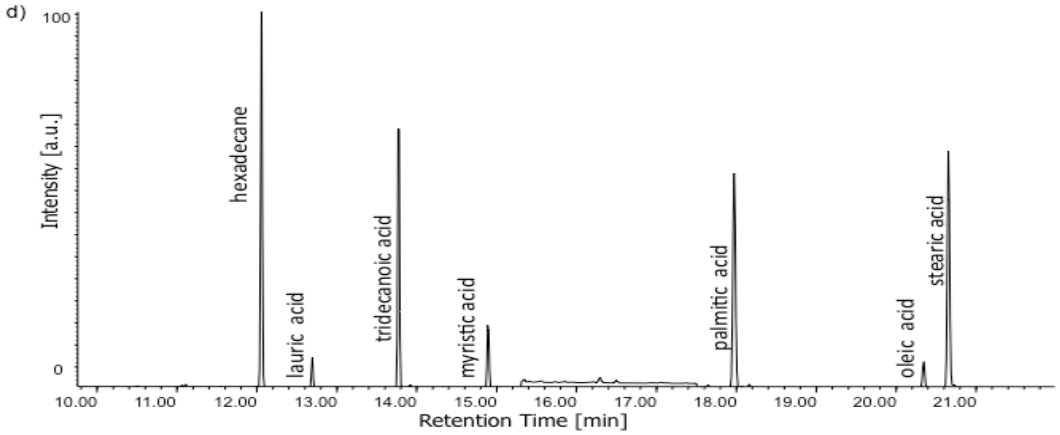


Figure 4.8: SIM chromatograms of lipid resinous fraction of **a)** madder lake, **b)** kermes**, c)** Armenian cochineal and **d)** lac dye.

Table 4.1: Relative percentage content of lipids of reference materials and characteristic parameters for the determination of lipid material (A/P= azelaic acid/palmitic acid, P/S= palmitic acid/ stearic acid, Σ D%= sum of dicarboxylic acids (sebacic, azelaic and suberic acids)).

	lau	sub	azel	myr	seb	palm	oleic	stear	µg tot	%AG	A/P	P/S	ΣD %
madder lake	1.1	0.1	0.2	3.1	0.0	46.4	6.6	42.6	2.1	4.7	0.0	1.1	0.3
kermes	0.2	0.0	0.0	30.2	0.1	30.6	24.7	14.2	13.9	34.6	0.0	2.2	0.1
Armenian cochineal	0.1	0.0	0.0	0.3	0.0	1.6	9.2	88.9	75.7	164.9	0.1	0.0	0.3
lac dye	1.5	0.1	0.8	4.8	0.0	29.0	9.1	54.6	1.6	3.6	0.03	0.5	0.9

All the reference materials show the presence of monocarboxylic acids, being stearic and palmitic acids the most abundant, and the complete absence of dicarboxylic acids. Kermes and Armenian cochineal show high percentages of lipid materials. The profiles and the average of the characteristic parameters (A/P= 0, P/S=1, Σ D%= 0.3) are not compatible with those of siccative oils, *tempera grassa* or egg [4], as expected. Moreover, the matching with egg profile has to be excluded because of the lack of cholesterol.
In lac dye, the molecular markers of shellac (aleuritic and butolic acids [6]), which might be present due to a scarce purification of the dye from the resin, were not found.

- **Saccharide fraction:**
  All the samples show an amount of saccharide material under the detection limit (LOD= 0.6 µg) with the exception of madder lake, whose saccharide content is above the quantitation limit (LOQ= 1.0 µg). The SIM chromatogram of madder lake is displayed in Figure 4.9 and the relative percentage content of saccharides of madder lake are reported in Table 4.2.

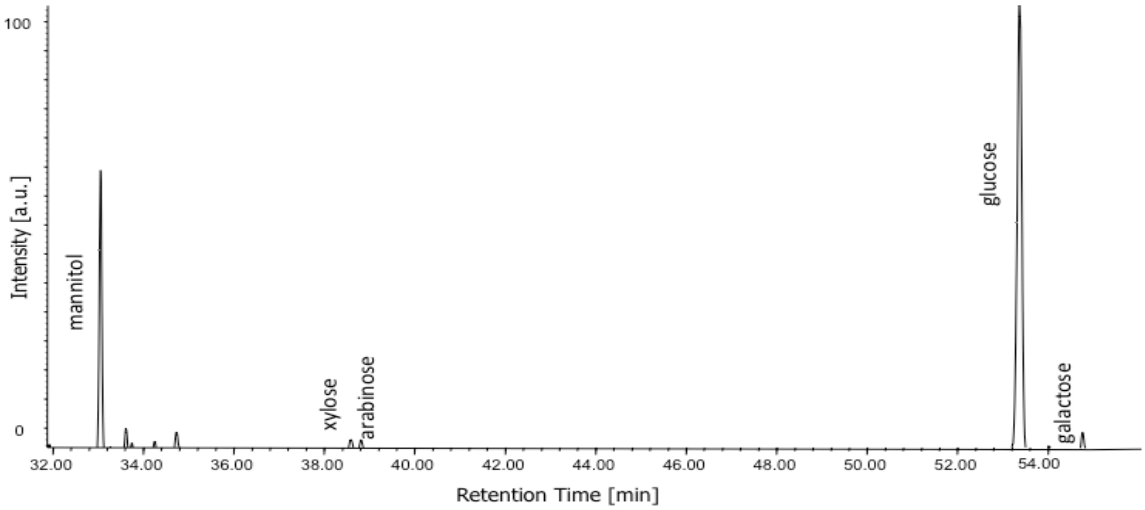


Figure 4.9 TIC chromatogram of saccharide fraction of madder lake

Table 4.2: Relative percentage content of lipids of madder lake

	xyl	ara	ramn	fuc	gal ac	glu ac	glu	mann	galact	µg tot	%Z
madder lake	2.0	1.8	0.0	0.1	0.0	0.0	95.8	0.0	0.4	2.1	0.5

The profile shows a high percentage of glucose and a low content of galactose. Moreover, the xylose/arabinose ratio is higher than one unlike any exudated gum. Therefore, the profile can not be confused with that of a polysaccharide gum one.

- **Amino acid fraction:**
  With the exception of lac dye, all samples contain an amount of amino acid material above the detection limit (LOD= 0.2 µg); Kermes and Armenian cochineal belong to the same family of *Coccus* type insects and they are the only ones with an amino acid content above the quantitation limit. (LOQ= 0.5 µg). It has to be highlighted that hydroxyproline, marker of animal glue [4], is absent in all the samples therefore an incorrect matching with glue profile has to be excluded.
  The SIM chromatogram of the proteianceous fraction of madder lake, kermes and Armenian cochineal are displayed in Figures 4.10 a, b and c and the relative percentage content of amino acid of the reference materials analyzed are listed in Table 4.3.

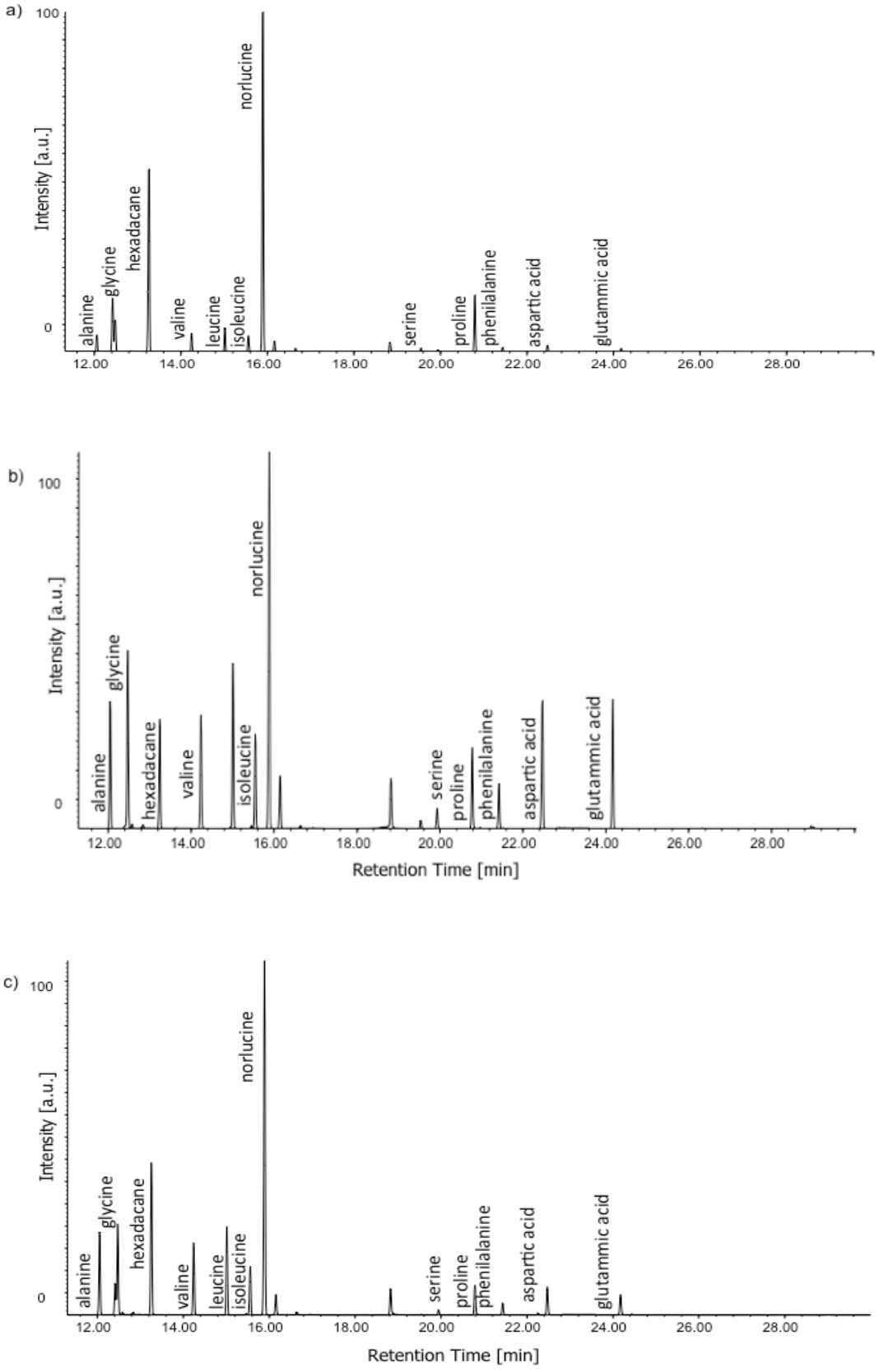


Figure 4.10: TIC chromatograms of amino acid fraction of **a)** madder lake, **b)** kermes and **c)**Armenian cochineal.

Table 4.3: Relative percentage content of amino acid of reference materials.

	ala	gly	val	leu	ile	ser	pro	phe	asp	glu	hyp	µg tot	%AA
madder lake	7.0	7.6	7.9	10.2	7.3	4.9	34.0	5.3	11.1	4.6	0.0	0.3	0.06
kermes	8.2	6.4	8.4	12.7	7.7	4.2	1.9	8.0	17.4	24.7	0.7	1.6	0.4
Armenian cochineal	13.0	9.0	12.2	14.5	8.6	3.9	7.4	4.7	22.6	4.2	0.0	0.7	0.14

4.3.2 Theoretical evaluation of the contribution of the reference materials on model systems

In paint layers, one or more binding media and anthraquinoid lakes can be simultaneously present, thus the possible modification in the profiles of the binders due to the presence of the anthraquinoid lakes need to be investigated in order to prevent misleading assignations. Theoretical values calculated for 1:1 mixtures of one of the reference materials analyzed and a binder as Arabic gum, whole egg, milk and animal glue are reported in Tables 4.4, 4.5, 4.6, 4.7 respectively. Although all reference materials analyzed show a rather high lipid content, the characteristic parameters, already presented in Table 4.1, exclude modifications in the lipid profile of a mixture with a siccative oil. Thus theoretical values for 1:1 mixtures of anthraquinoid colorant and siccative oil were not calculated. The sugar and amino acid profiles with their saccharide and amino acid contents of the gums and proteinaceous materials have been published elsewhere [6, 7, 2].

Table 4.4: Theoretical saccharide profile of Arabic gum and of paint model systems containing a mixture (1 : 1) of Arabic gum and madder lake.

	xyl	ara	ramn	fuc	gal ac	glu ac	glu	mann	galact
Arabic gum	0.0	37.2	11.2	0.0	0.0	7.0	0.0	0.0	44.7
madder lake : Arabic gum	0.0	36.9	11.1	0.0	0.0	6.9	0.6	0.0	44.4

Table 4.5: Theoretical saccharide profile of whole egg and of paint model systems containing a mixture (1 : 1) of whole egg and madder lake or kermes or Armenian cochineal.

	ala	gly	val	leu	ile	ser	pro	phe	asp	glu	hyp
egg	10.5	7.4	10.0	12.9	8.5	3.7	3.5	9.4	15.3	18.7	0.0
madder lake : egg	10.5	7.4	10.0	12.9	8.5	3.7	3.5	9.4	15.3	18.7	0.0
kermes : egg	10.5	7.4	10.0	12.9	8.5	3.7	3.5	9.4	15.3	18.8	0.0
Armenian cochineal : egg	10.5	7.4	10.0	12.9	8.5	3.7	3.5	9.4	15.3	18.7	0.0

Table 4.6: Theoretical saccharide profile of milk and of paint model systems containing a mixture (1 : 1) of milk and madder lake or kermes or Armenian cochineal.

	ala	gly	val	leu	ile	ser	pro	phe	asp	glu	hyp
milk	4.0	3.4	8.5	12.0	7.8	2.4	9.90	6.5	12.5	32.9	0.0
madder lake : milk	4.0	3.4	8.5	12.0	7.8	2.4	10.0	6.5	12.5	32.9	0.0
kermes :milk	4.1	3.4	8.5	12.0	7.8	2.4	9.8	6.5	12.6	32.8	0.0
Armenian cochineal :milk	4.1	3.4	8.5	12.0	7.8	2.4	9.8	6.5	12.6	32.8	0.0

Table 4.7: Theoretical saccharide profile of animal glue and of paint model systems containing a mixture (1 : 1) of animal glue and madder lake or kermes or Armenian cochineal.

	ala	gly	val	leu	ile	ser	pro	phe	asp	glu	hyp
animal glue	12.8	24.2	4.7	6.6	3.2	2.8	6.9	3.7	8.0	14.3	13.0
madder lake : glue	12.8	24.1	4.7	6.6	3.2	2.8	6.9	3.7	8.0	14.3	13.0
kermes : glue	12.8	24.1	4.7	6.6	3.2	2.8	6.9	3.7	8.0	14.3	12.9
Armenian cochineal :glue	12.8	24.1	4.7	6.6	3.2	2.8	6.9	3.7	8.0	14.3	13.0

The data reported in the tables show that the profiles of Arabic gum, egg, milk and animal glue are not significantly modified when an anthraquinoid colorant is present. The contents of saccharides and amino acids in the colorants analyzed, even if in some cases higher than the limit of quantitation, are too low in comparison to those of the binders. Nevertheless the theoretical data obtained have to be cross-validated with experimental ones in order to rule out the effective influence of dyes on binders identification.

4.4 Conclusions

In this chapter the mutual influence of lakes and binders in paint model systems has been investigated by HPLC-DAD and GC/MS techniques.

The analyses of naturally aged and freshly prepared paint model systems with the HPLC-DAD procedure, optimized in the previous chapter, allowed the identification of the antraquinoid lakes whatever was the binding media used. The better detectability of Indian lac, at second instance of carmine and the less easy characterization of madder lake were in agreement with the recoveries already reported for reference lakes. No peaks ascribable to any binder are present in any of the paint model systems. Thus, the influence of the nature and of the presence of the binder on the identification of the antraquinoid lake by this protocol has to be excluded. Nevertheless, the lower recoveries obtained for naturally aged painted replicas in comparison with the freshly prepared ones can suggest that the analyses of dyes in historical samples may be conveyed by the presence of aged binding media.

The evaluation of the relative percentage content of lipids, saccharides and amino acids in madder lake, kermes, Armenian cochineal and lac dye highlights a composition mostly consisting in monocarboxylic acids. Only madder lake, prepared from roots, has a saccharide component. Kermes and Armenian cochineal have a similar composition, rich in lipids and with proteinaceous material above the limit of detection. The common animal source from where they are extracted to

prepare kermes and carmine could be the explanation of these similarities. Lac dye contains only lipid material and the molecular markers of shellac are missing. The theoretical profiles calculated for 1:1 mixtures of the reference materials analyzed and Arabic gum or whole egg or milk or animal glue do not appear to be different to those of the reference binders. Notwithstanding, the application of the GC/MS procedure to the paint model systems, already analyzed by HPLC-DAD, will be performed in future, being necessary in order to rule out the effective influence of dyes on binder identification.

Bibliography

(1) A. Lluveras, I. Bonaduce, A. Andreotti, M. P. Colombini, *GC/MS Analytical Procedure for the Characterization of Glycerolipids, Natural Waxes, Terpenoid Resins, Proteinaceous and Polysaccharide Materials in the Same Paint Microsample Avoiding Interferences from Inorganic Media*, Analytical Chemistry, no. 82, pp. 376-386, 2010.

(2) I. Bonaduce, M. Cito, M. P. Colombini, *The development of a Gas Chromatographic-Mass Spectrometric Analytical Procedure for the Determination of Lipids, Proteins and Resins in the Same Paint Micro-Sample Avoiding Interferences from Inorganic Media,* Journal of Chromatography A, no. 1216, pp. 5931-5939, 2009.

(3) A. Andreotti, I. Bonaduce, M. P. Colombini, G. Gautier, F. Modugno, E. Ribechini, *Combined GC/MS Analytical Procedure for the Characterization of Glycerolipid, Waxy, Resinous and Proteinaceous Materials in a Unique Paint Microsample*, Analytical Chemistry, no. 78, pp. 4490-4500, 2006.

(4) M. P. Colombini, A. Andreotti, I. Bonaduce, F. Modugno, E. Ribechini, *Analytical Strategies for Characterizing Organic Paint Media Using Gas Chromatography/Mass Spectrometry,* Accounts of Chemical Research, vol. 43, no. 6, pp. 715-727, 2010.

(5) A. Lluveras Tenorio, J. Mazurek, A. Restivo, M.P. Colombini, I. Bonaduce, *The development of a New Analytical Mode for the Identification of Saccharide Binders in Paint Sample*, Plos One, vol. 7, no. 11, pp. 1-17, 2012.

(6) M.P. Colombini, I. Bonaduce, G. Gautier, *Molecular Pattern Recognition of Fresh and Aged Shellac,* Chromatographia, vol. 58, pp. 357-364, 2003.

(7) I. Bonaduce, H. Brecoulaki, M.P. Colombini, A. Lluveras, V. Restivo, E. Ribechini, *Gas Chromatographic–Mass Spectrometric Characterization of Plant Gums in Samples from Painted Works of Art,* Journal of Chromatography A, no. 1175, pp. 275–282, 2007.

Chapter 5

Development and optimization of Matrix - Assisted Laser Desorption/Ionization Time-of-Flight – Mass Spectrometry (MALDI-ToF-MS) and (LDI-ToF-MS) procedures

5.1 Introduction

Analyses of proteinaceous binders and anthraquinoid dyes have been performed respectively with Matrix- Assisted Laser Desorption/Ionization Time-of-Flight - Mass Spectrometry (MALDI-ToF-MS) and Laser Desorption/Ionization Time-of-Flight - Mass Spectrometry (LDI-ToF-MS) at the Department of Biochemistry and Microbiology of ICT in Prague.
With regard to the characterization of proteinaceous binding media, a procedure based on the cleavage of proteins in peptides and their concentration and purification, developed at the Department of Biochemistry and Microbiology of ICT in Prague [1], was applied. Selected parameters were also optimized for freshly prepared paint model samples in the presence of an organic lake. The analyses were carried out in positive mode. Reference commercial lakes were also analyzed to assess the possible presence of a proteinaceous content in the raw materials.
With regard to anthraquinoid dyes, LDI was applied both directly on the solid reference materials of dyes and lakes and on freshly prepared paint model samples, and after treatment with hydrofluoric acid. All the analyses were carried out both in positive and in negative mode.
The cross-influence of the kind of lake on proteins analysis and of proteinaceous binders on lakes analysis has been evaluated and discussed.

5.2 Analysis of proteinaceous binding media

Matrix- assisted laser desorption ionization (MALDI) is a widespread and powerful ion source for the production of gas-phase ions from large, non-volatile and thermally labile compounds such as proteins. Coupling with time-of-flight (TOF) analyzer allows to produce ions intermittently, and to quickly separate close masses in a short time and detect ions over a wide mass range [2]. Therefore a MALDI-ToF-MS procedure developed at the Department of Biochemistry and Microbiology of ICT in Prague has been applied for proteinaceous binders analysis. Different strategies and steps were tested in order to reduce the interference of anthraquinoid dyes, optimizing the procedure for proteinaceous binding media analysis in freshly prepared paint model systems. Investigated binding media were rabbit glue, egg, albumin and casein, prepared as solutions of different concentrations and applied in mixture with madder lake, carmine and Indian lac as described in Section 2.3.2.

Analyses were carried out in positive mode, the most common mode to acquire peptides spectra [3]. Results were compared with a database of the most frequently proteinaceous binders used in artworks and built up at the Department of Biochemistry and Microbiology of ICT in Prague[5] or processed by Mascot software [5].
Considerations about the influence of the different kinds of lakes on binders detection, comparison of profiles of model systems with different lake/binder ratios and concentrations of proteinaceous binder were undertaken.

5.2.1 Routine procedure

The procedure, developed in the Department of Biochemistry and Microbiology of ICT- Prague [1], consisted in addition of a 50 mM NH_4HCO_3 solution in water and 20 μg/mL trypsin and digestion for 2 h at 37 °C. In order to stop cleavage action of trypsin the sample was stored at -6 °C. Purification was performed with reverse phase C18 sorbent in micro-columns using three different solutions described in Section 2.1 (wetting solution for tip conditioning; equilibration solution for tip cleaning before and after sample loading; 10 μL of solution for elution of peptides). A mixture of 0.5 μL of sample with 0.5 μL of DHB matrix was spotted on steel plate, left to crystallize and analyzed by MALDI-ToF-MS.
The procedure was applied on c.a. 45 mg of model systems and thus a volume of 20 μL of cleavage solution was chosen. The presence of lake enabled the crystallization of the matrix preventing detection of proteins. Consequently, steps of purification from dyes had to be introduced in order to promote crystallization and improve the quality of mass spectra.

5.2.2 Optimization of the procedure

MALDI is relatively less sensitive to contamination from other species in comparison with other ionization techniques; nonetheless, high concentrations of contaminants can interfere with desorption and ionization process of samples [1]. In this work, different strategies have been undertaken to decrease the interferences of organic dyes.
First, 2 μL of TFA 10% were added to the sample after 2 h of digestion in order to stop cleavage action of trypsin and to increase acidity. Afterwards the digested sample was mixed and centrifuged. The supernatant was separated from the colored solid part and submitted to purification with C18 micro-columns. A mixture of 0.5 μL of sample and 0.5 μL of DHB matrix was spotted on the MALDI steel plate, left to crystallize and analyzed by MALDI-ToF-MS. This purification turned out to be not sufficient, especially for model systems containing high concentration of lakes or prepared with egg as binder. Whole egg is composed by albumen and yolk and it contains about 45% protein, 41% lipid and 2% cholesterol [1]. A high amount of lipids interferes with matrix crystallization preventing the analysis, thus elimination of lipids is necessary prior the analysis. Egg samples were submitted to a purification using two C18 sorbents in micro-columns. This double purification on the one hand further reduces the interference of dyes, but on

[5] The library contains the m/z values of the most often occurring peptides in reference binders, obtained after 2 h of trypsin digestion at 37 °C. The number of masses aquire experimentally that match with the theoretical masses in the database is called "score". Score percentage (%) represents the ratio between the number of the peaks corresponding to a specific binder and the number of all peaks found in the interval from 900 to 2000 Da in the acquired spectrum [4].

the other hand decreases the amount of peptides revealed because a fraction of them remains bonded to the lakes as a results, identification of egg was not possible with this procedure.
In order to set up a procedure able to detect peptides from binders in the model systems, a further step of purification had to be introduced. A homogeneous liquid–liquid extraction based on phase separation in a ternary solvent system (chloroform/water/methanol) was performed. A mixture of $1CHCl_3$: $5H_2O$: 4MeOH (15 µL: 60 µL: 75 µL) was added to an amount of c.a. 0.45 mg of model system, mixed and centrifuged. The procedure was first applied to model systems prepared with egg as binder and the separation of organic materials occurred in three phases. The upper and the lower ones were discarded and the middle one was left in the Eppendorf vial and let dried. After this step, the sample was treated as explained above. The procedure was applied to all the other model systems allowing to reduce the interference of not proteinaceous organic components of the samples.
Eventually, a multi-step procedure for the analysis of proteinaceous binders in the presence of anthraquinoid lakes was optimized and it is schematized in Figure 5.1.

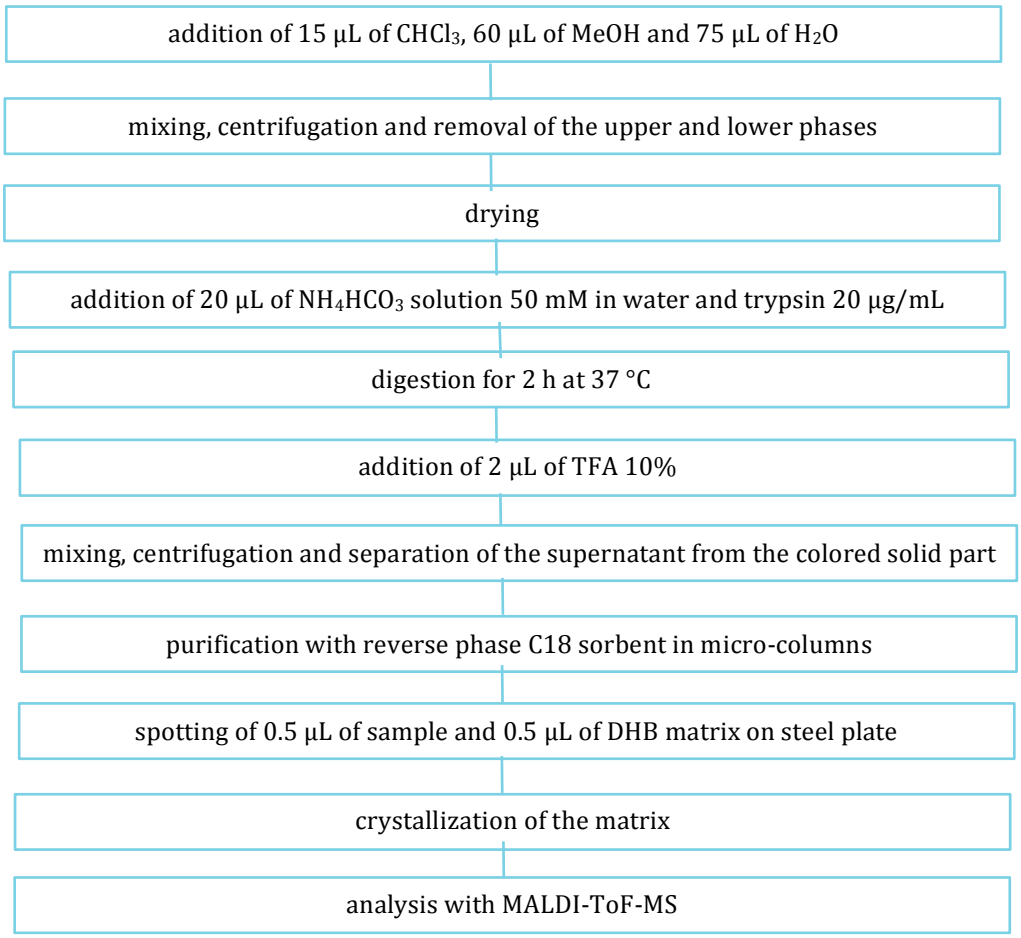


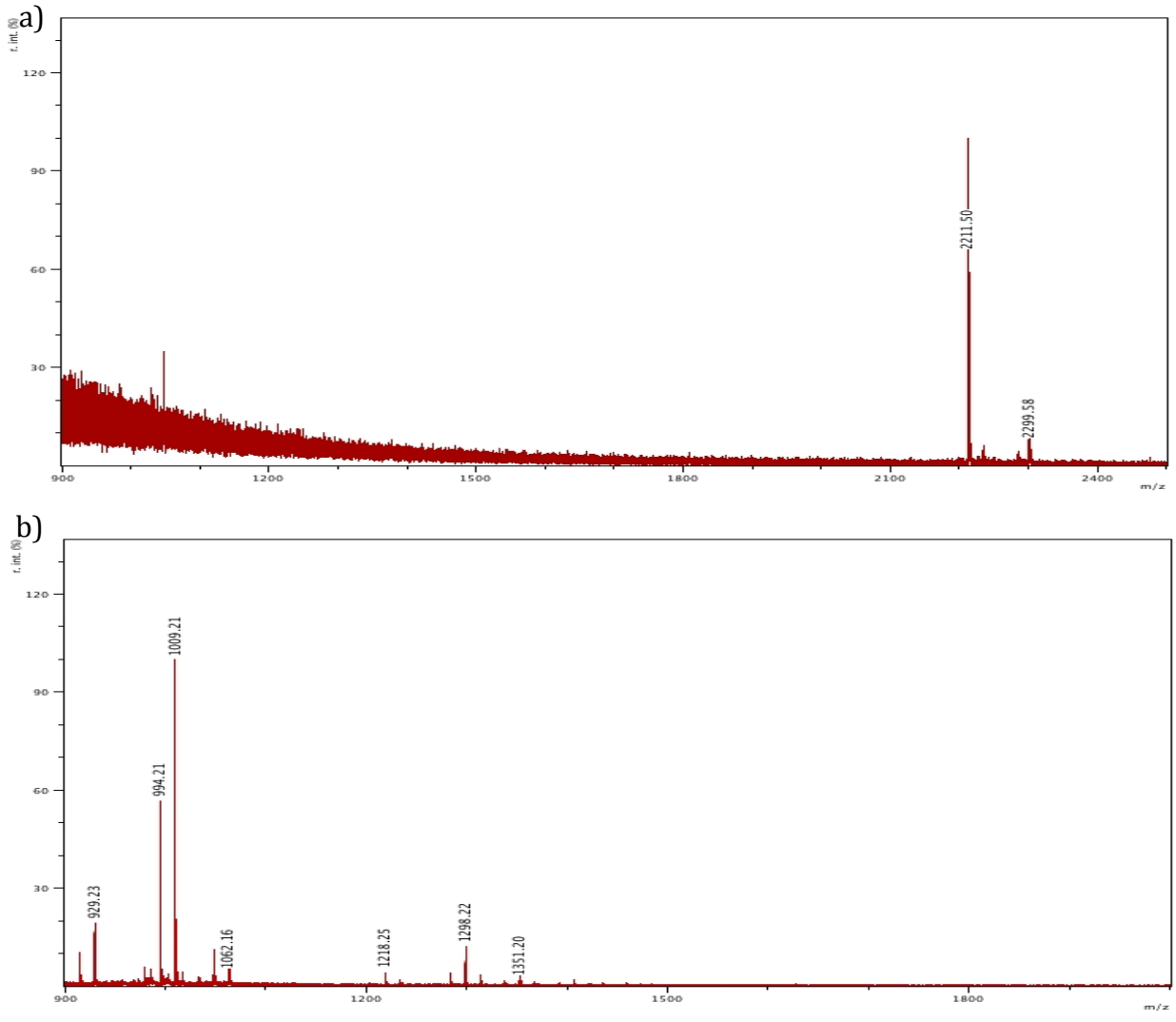
Figure 5.1: MALDI-ToF-MS optimized procedure for analysis of proteinaceous binding media in presence of anthraquinoid lakes.

5.2.3 Results

All the MALDI-ToF-MS spectra shown were obtained from the samples analyzed by the procedure reported in Figure 5.1. Firstly, the spectra of reference materials are shown and discussed. Secondly, the results of freshly prepared paint model systems are presented according to the kind of proteinaceous binder. Model systems with different lake/binder composition, kind of lakes and concentration of proteinaceous material have been analyzed and discussed. In the spectra, only the most intense m/z fragments are highlighted and grey circles label the molecular markers of the proteinaceous material analyzed.

5.2.3.1 Lakes reference materials

Reference materials of madder lake, carmine and Indian lac were analyzed in order to evaluate their proteinaceous content and better interpret MALDI-ToF-MS spectra of model systems. Moreover, a cross-validation with GC-MS results can be achieved. In Figures 5.2 a, b and c the MALDI-ToF mass spectra of madder lake, Indian lac and carmine are shown, respectively.



c)

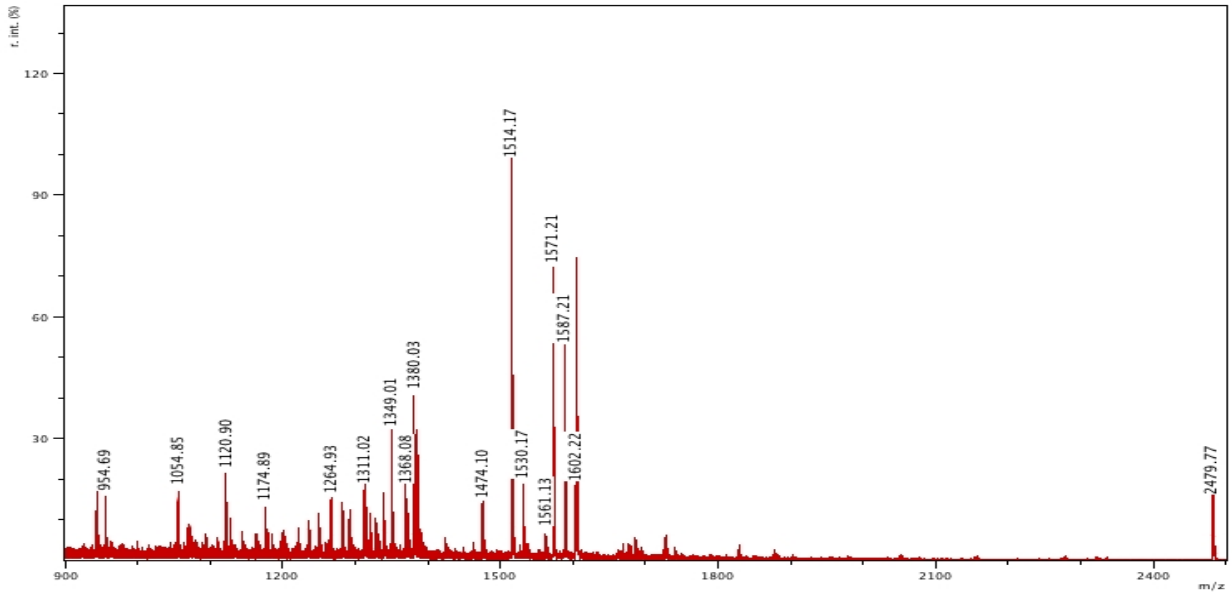


Figure 5.2: MALDI-ToF mass spectra of **a)** madder lake; **b)** Indian lac; **c)** carmine.

The mass spectrum of madder lake corresponds to that of the analytical blank runs before each analysis. The spectrum of Indian lac shows only few peaks, which do not correspond to any of the known molecular markers of protein binders. Carmine, instead, presents a significant number of peptides with some m/z values that in some cases are in agreement with those mentioned in [6], but not assigned to specific peptides. As expected, animal anthraquinoid dyes have a higher proteinaceous content in comparison with vegetal ones, which are richer in polysaccharides [6].
These results are in agreement with those obtained by GC/MS in Chapter 4, in which kermes and Armenian cochineal, natural sources for carmine preparation, show an higher proteinaceous content in comparison with the other raw materials analyzed.

5.2.3.2 Binding media

The analysis of paint model systems containing a proteinaceous binder and the different anthraquinoid lakes allowed to establish that:

i) in some cases peaks from the lakes can be observed in the mass spectra though identification of the binder is always possible;
ii) S/N ratio is highly dependent on the composition lake/ binder of the paint model system;
iii) rabbit glue and whole egg can always be identified on the basis of characteristic peptides;
iv) albumine and casein needed to apply strategies based on the submission of reference characteristic proteins (ovoalbumin from *Gallus gallus*, α-S1-casein and α-S2-casein) to *in silico* trypsin digestion taking into account post-translational modifications to find out specific m/z for their identification.

Below a detailed description of the results by type of preotinaceous binder analyzed is summarized.

- **Rabbit glue:**

  In Figures 5.3 a and b the MALDI-ToF mass spectra of the model systems prepared with carmine RG01C1 and RG01C2 are shown, respectively. The two spectra are compared with that of carmine, reference lake used for the preparation of the model system.

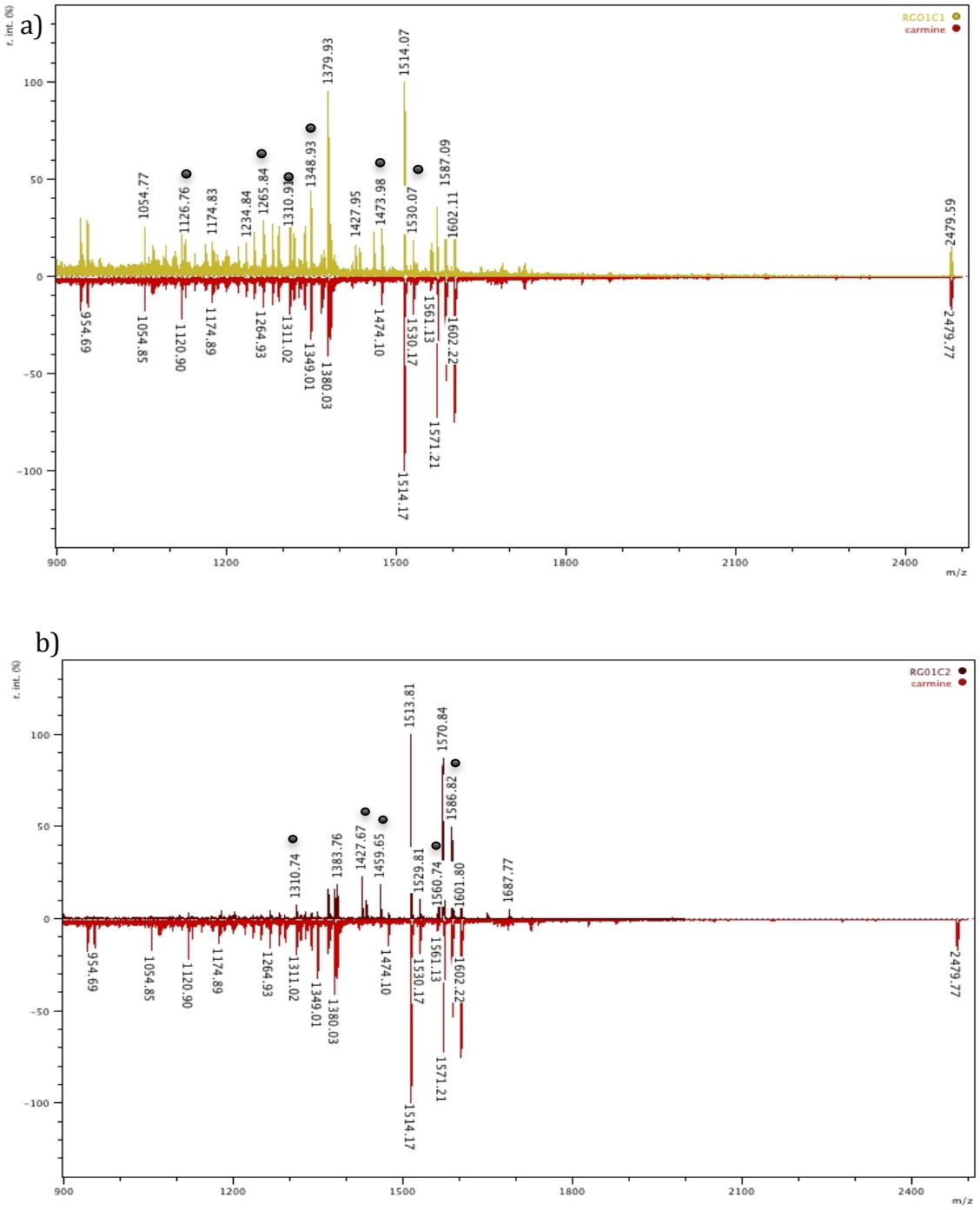
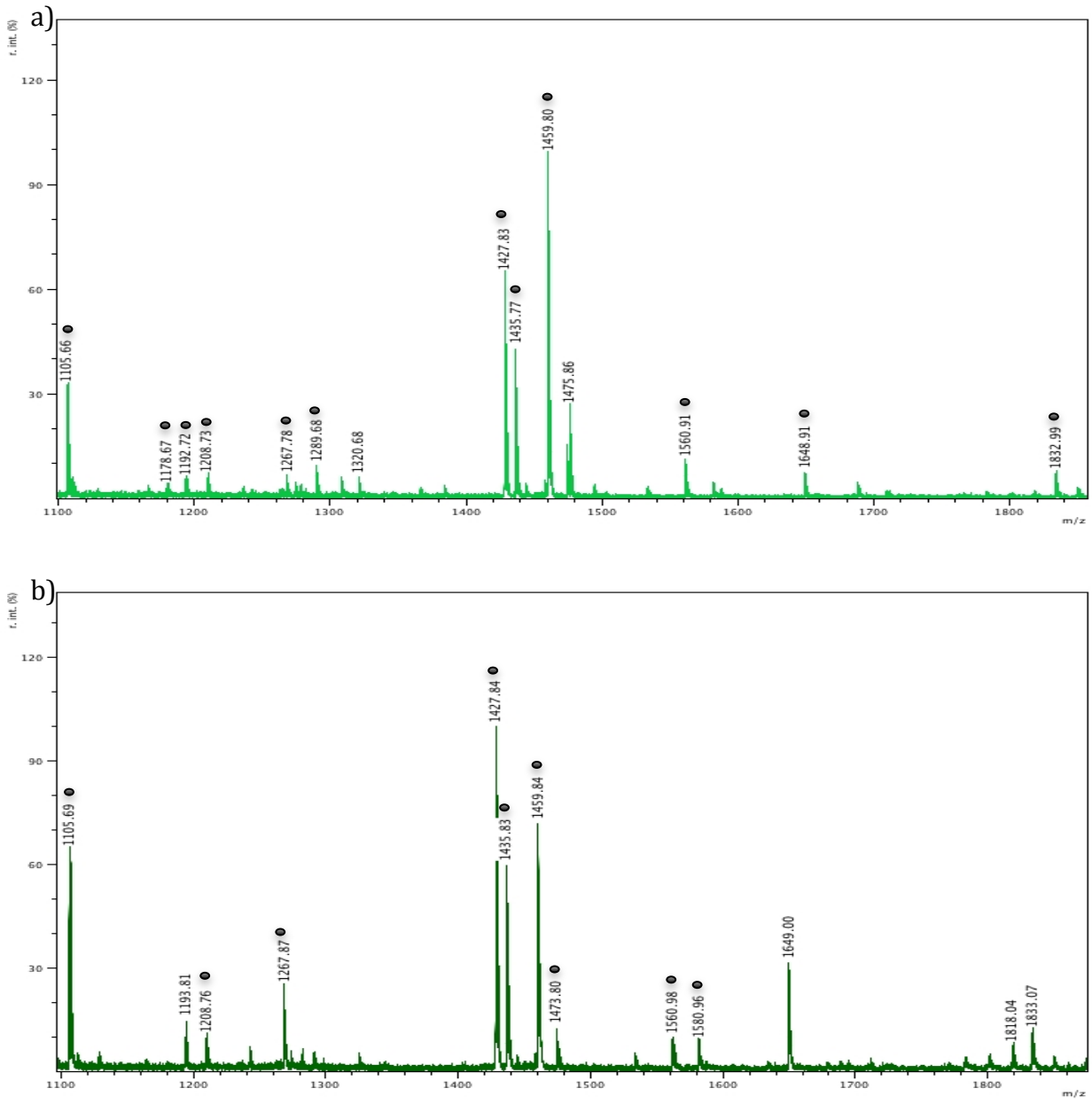


Figure 5.3: MALDI-ToF mass spectra of carmine compared to **a)** RG01C1 and **b)** RG01C2.

Both RG01C1 and RG01C2 were prepared with carmine and a solution of rabbit glue 0.1% w/w but the composition lake/binder was different, being 1:5 and 1:43 respectively. The mass spectrum of RG01C1 shows a lower S/N ratio than RG01C2 and the comparison with carmine suggests that noise is given by the interference of the lake. In RG01C2, which

contains a lower amount of lake, the ion fragments due to carmine are nearly absent and the S/N ratio is higher. Notwithstanding, in both cases the identification of rabbit glue is possible thanks to the high score percentage of its marker peptides.

In Figures 5.4 a, b and c the MALDI-ToF mass spectra of the model systems prepared with madder RG01ML1, RG1ML1, and RG5ML are shown, respectively.



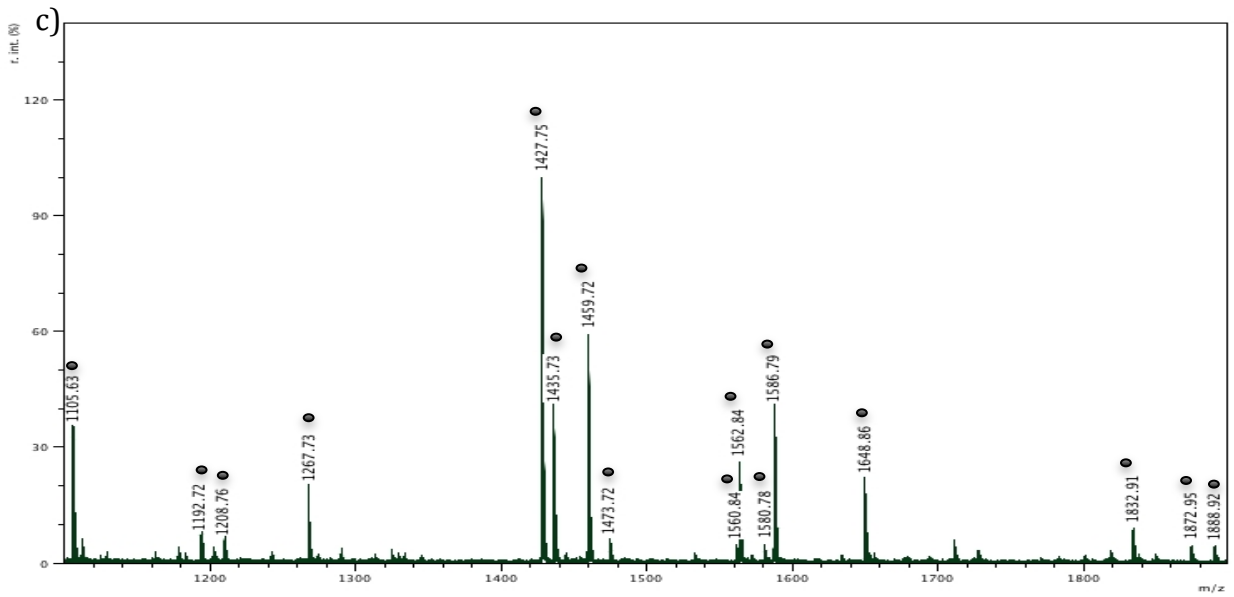
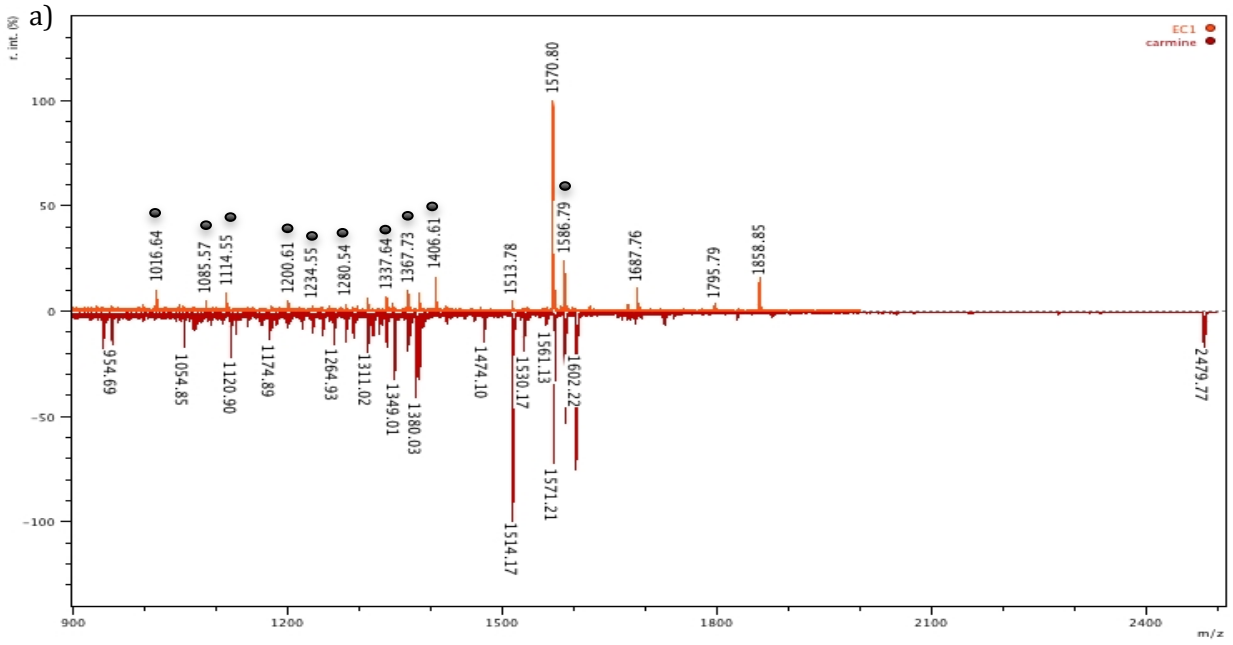


Figure 5.4: MALDI-ToF mass spectrum of **a)** RG01ML1; **b)** RG1ML; **c)** RG5ML.

RG01ML1 and RG1ML1 and RG5ML were prepared all with madder lake and they have a composition lake/binder around 1:5 while binding solutions used were rabbit glue 0.1% w/w, 1% w/w and 5% w/w, respectively. The spectra do not show any fragment given by the dye. As expected, increasing the concentration of the solution of rabbit glue, S/N ratio increased as well as score percentages. The same considerations stand for RG01IL1, RG1IL2 and RG5IL.

- **Egg:**

In Figures 5.5 a, b and c the MALDI-ToF mass spectra of EC1 compared to that of carmine, EML1 and EIL2 are shown respectively.



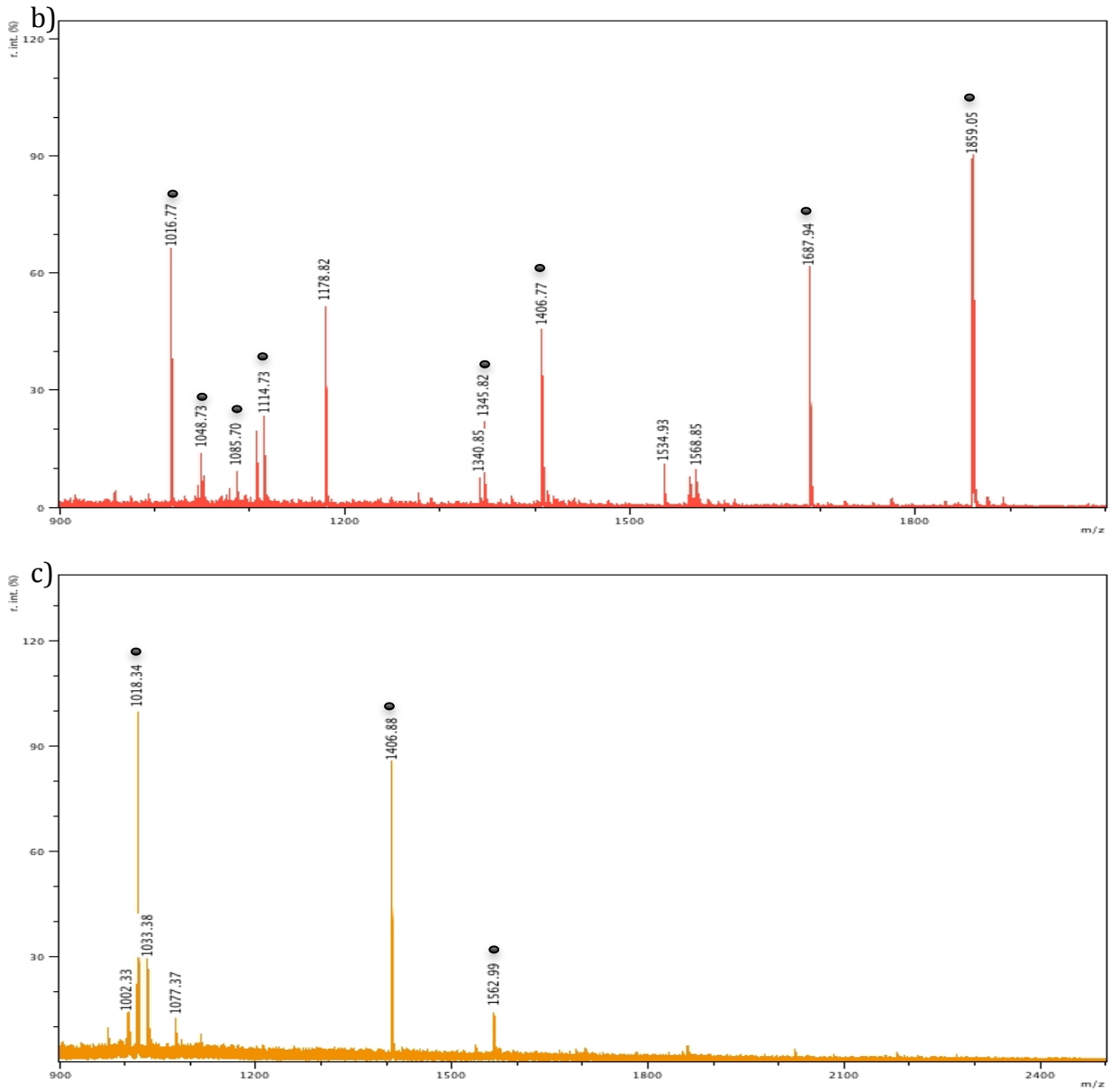


Figure 5.5: MALDI-ToF mass spectrum of **a)** EC1 compared to that of carmine; **b)** EML1; **c)** EIL2.

The comparison between EC1 and carmine highlights the presence of peaks that are generated by the lake. However, peaks from the egg peptides were present in both cases and the identification of egg was possible with all the kinds of lake. EML1 spectrum presents a higher S/N ratio in comparison to EIL2 one because of the different lake/binder ratio.

- **Albumin:**
  The mass spectra of model systems prepared with albumin do not present a high number of fragments and these m/z are not molecular markers specific of albumin but common to some of the other analyzed materials such as whole egg [1].
  In order to find out some molecular markers specific of albumin, ovalbumin (*Gallus gallus*) protein sequence was submitted to *in silico* trypsin digestion taking into account post-translational modifications. Protein sequence was acquired from Mascot and simulation performed by XMASS software. Masses of peptides were compared with the peak list of the

sample analyzed within a specified tolerance to see if there is any match. *In silico* fragmentation of the peptides, which were in common between simulated digestion and the spectrum of the sample analyzed, was performed and compared to their tandem MS experimental spectra. Using this strategy it was possible to define m/z=1345, m/z=1687 and m/z=1859 as markers and specific peptides of albumin. The identification of the albumin was, thus, carried out on the basis of the presence of these three fragments. In Figure 5.6, MALDI-ToF mass spectrum of A1IL3 with tandem MS spectra of m/z=1345, m/z=1687 and m/z=1859 is presented as representative of all the model systems prepared with albumin.

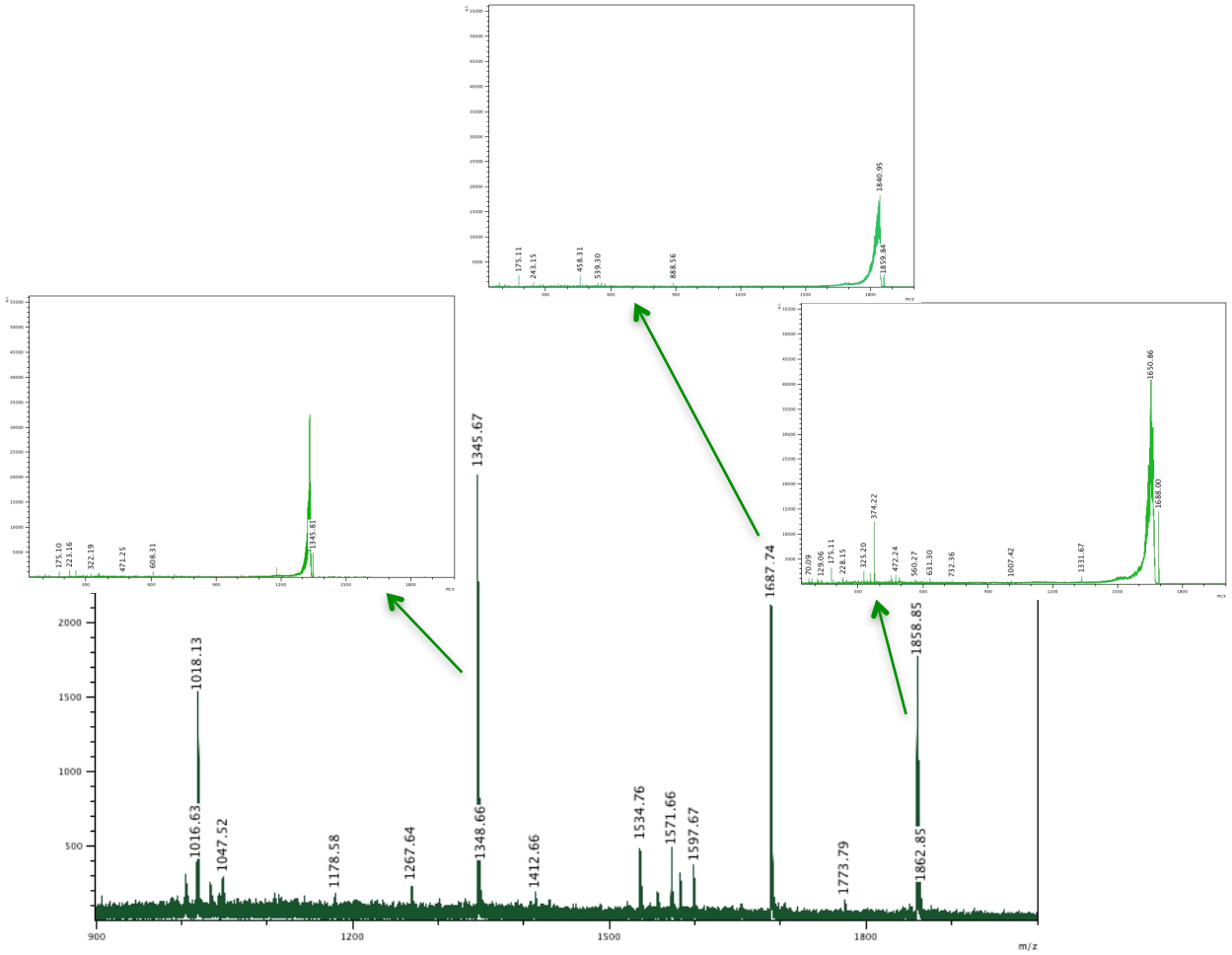


Figure 5.6: MALDI-ToF mass spectrum of A1IL3 and tandem MS spectra of m/z=1345, m/z=1687 and m/z=1859.

- **Casein:**

  The MALDI-ToF mass spectra of model systems prepared with casein contain several fragments. These m/z values are not molecular markers specific of casein but common to some of the other analyzed proteinaceous materials. The same procedure of fragmentation of albumin was applied to α-S1-casein and α-S2-casein sequences and in the end the m/z=1105, m/z=1267 and m/z=1759 were defined to be identifier for casein.

  In Figure 5.7, the MALDI-ToF mass spectrum of C01IL is shown as example. The masses marked by arrows correspond to peptides ascribed to casein.

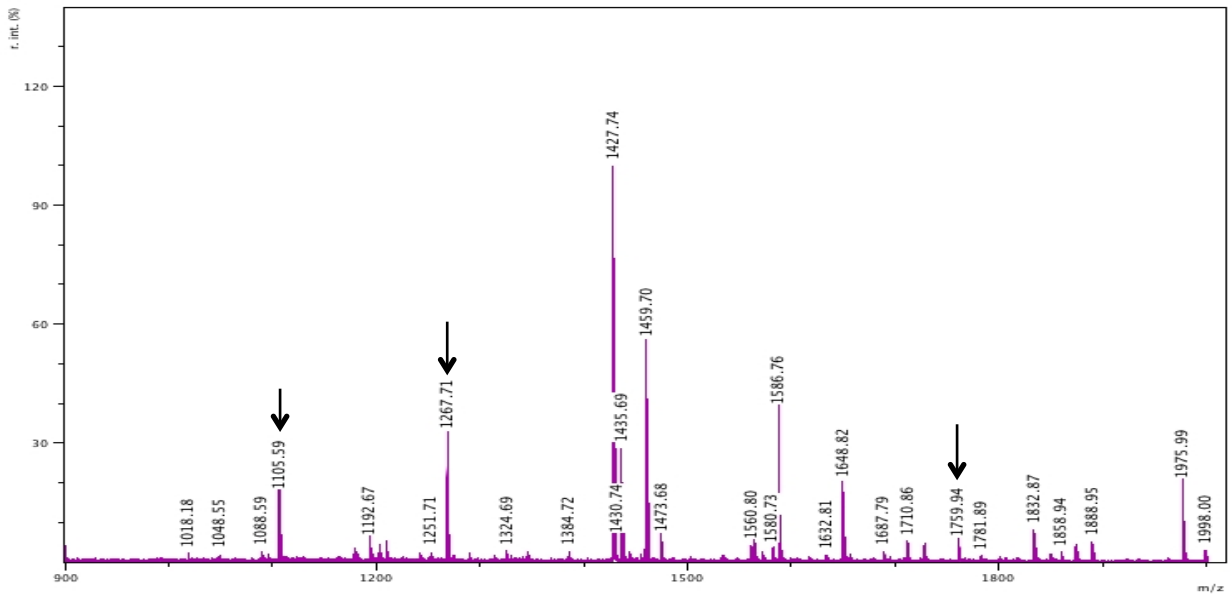


Figure 5.7: MALDI-ToF mass spectrum of C01IL with peptides ascribed to casein indicated by the arrows.

5.3 Analyses of anthraquinoid dyes

Laser Desorption/Ionization Time-of-Flight - Mass Spectrometry (LDI-ToF-MS) has also been applied for the detection of anthraquinoid dyes. In this case, the matrix was not used mainly because it was not necessary to obtain suitable ionization yields, and also in order to avoid possible ions adduct between matrix molecules and dyes [7]. In addition, the presence of the peaks due to the matrix obscures possible diagnostic peaks at m/z lower than approximately 700 Da [6].
Two different methods were applied on reference materials of dyes and lakes and on the freshly prepared paint model systems described in Section 2.3.2. The same proteinaceous model systems, characterized with MALDI-ToF-MS method and discussed above, were analyzed by LDI-ToF. Moreover, paint model systems prepared with saccharide and lipid binders were analyzed. The first method does not require any samples pre-treatment (direct method) [8], while the second one employs hydrofluoric acid and requires some preparative steps (HF method) [9]. Both positive and negative modes were tested. The best method of preparation and analysis mode has been evaluated for each dye. Moreover, the influence of the different kind of binding media on dyes analyses was evaluated and discussed below.

5.3.1 Direct method

Less than 1 mg of sample was dissolved in 1 mL of water and directly spotted on the steel plate. Spots were left to dry and then analyzed by LDI-ToF-MS [8]. In Figure 5.8, the steel plate with some sample spots is shown.

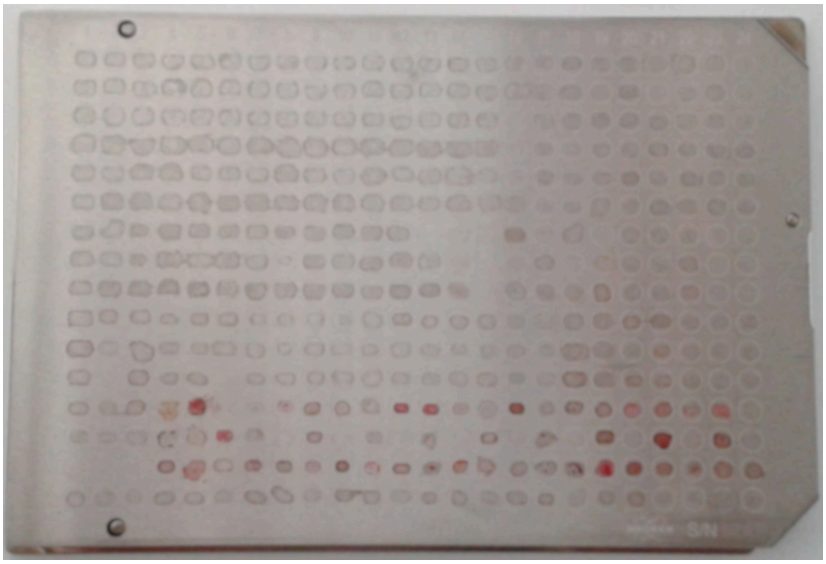


Figure 5.8: Steel plate with samples spots (colored dots).

5.3.2 HF method

A mixture of 10 µL of ACN and 10 µL of MeOH was added to c.a. 0.45 mg of sample and sonicated for 20 min until the swelling of the binding medium. The extraction of the dyes was carried out adding 20 µL of 4 M HF and the extract was left at room temperature until sample discoloration. The elimination of excess fluoride and other ionic and polar substances was performed with reverse phase C18 sorbent in micro-columns using ACN : MeOH solution and H_2O for the preconditioning, 0.01% TFA water solution after sample loading, and ACN : MeOH + TFA 0.01% solution for the elution of dyes. 1µL of eluted solution was spotted on the MALDI steel plate, left to dry and analyzed by LDI-ToF-MS [9]. In Figure 5.9 the steps of the procedure are schematized.

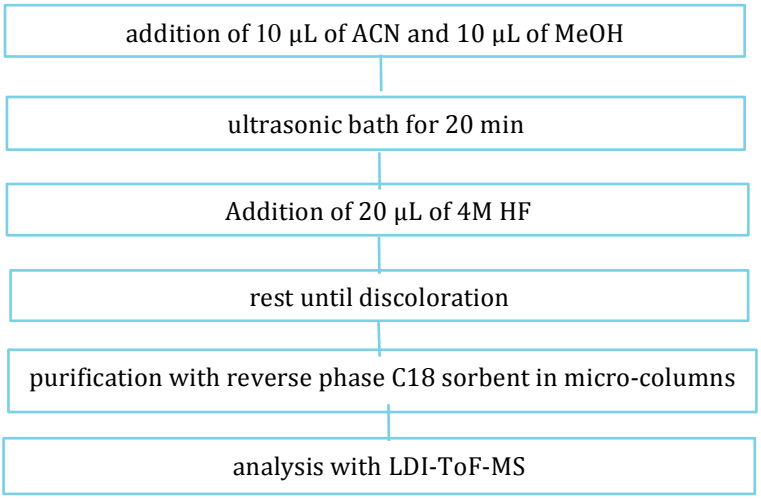


Figure 5.9: LDI-ToF-MS procedure using HF for analysis of anthraquinoid lakes in presence of binding media.

5.3.3 Results

The interpretation of the data is organized in three paragraphs according to the kind of lake. Each paragraph is divided in positive and negative mode. LDI-ToF mass spectra of reference dyes and lakes and paint model systems analyzed both with direct and HF methods are compared and discussed.

5.3.3.1 Analytical blank

In Figures 5.10 a and b the analytical blank obtained with HF extraction acquired in positive and negative mode respectively is shown in order to establish which peaks are not diagnostic for the samples analyzed.

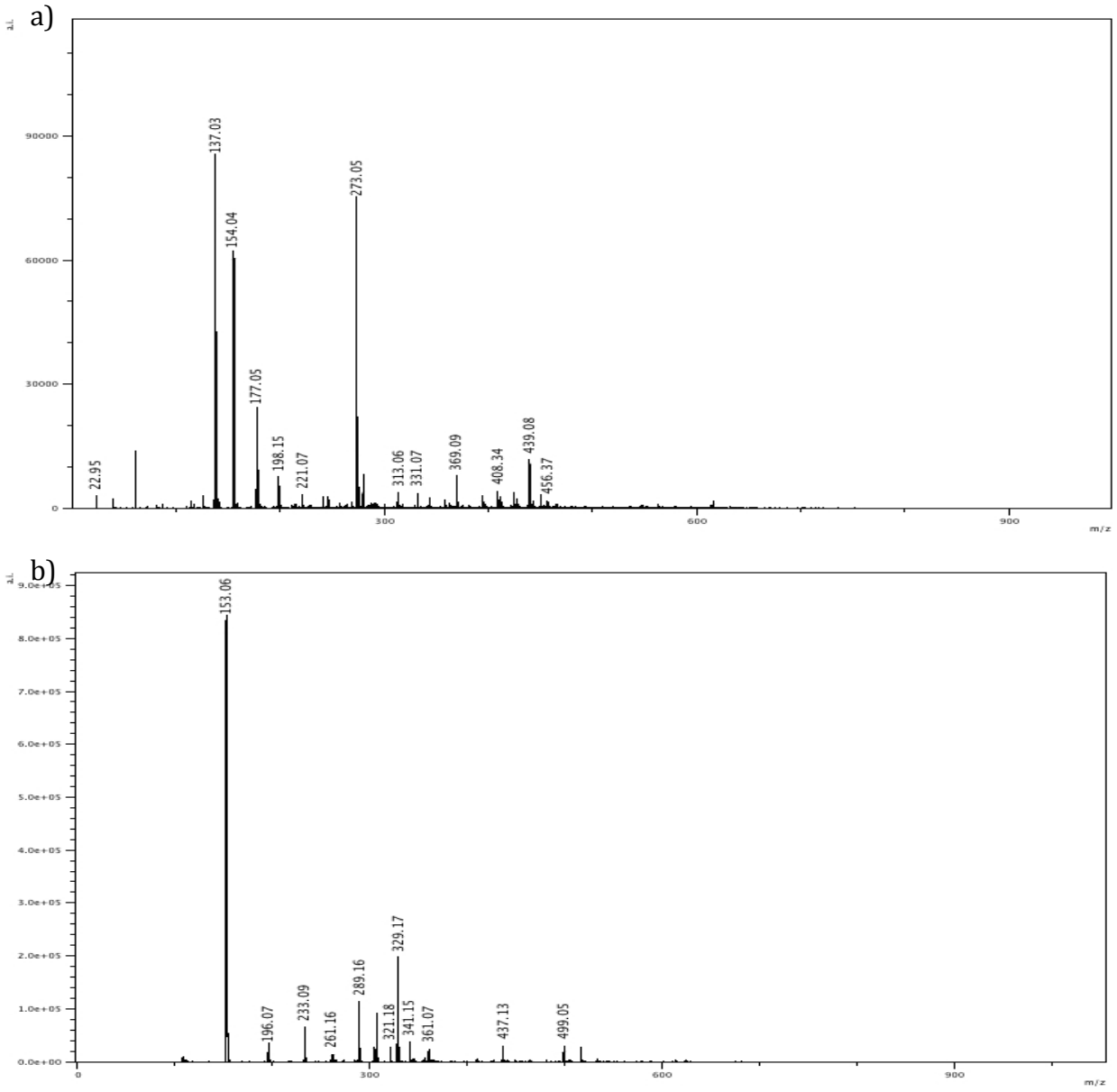


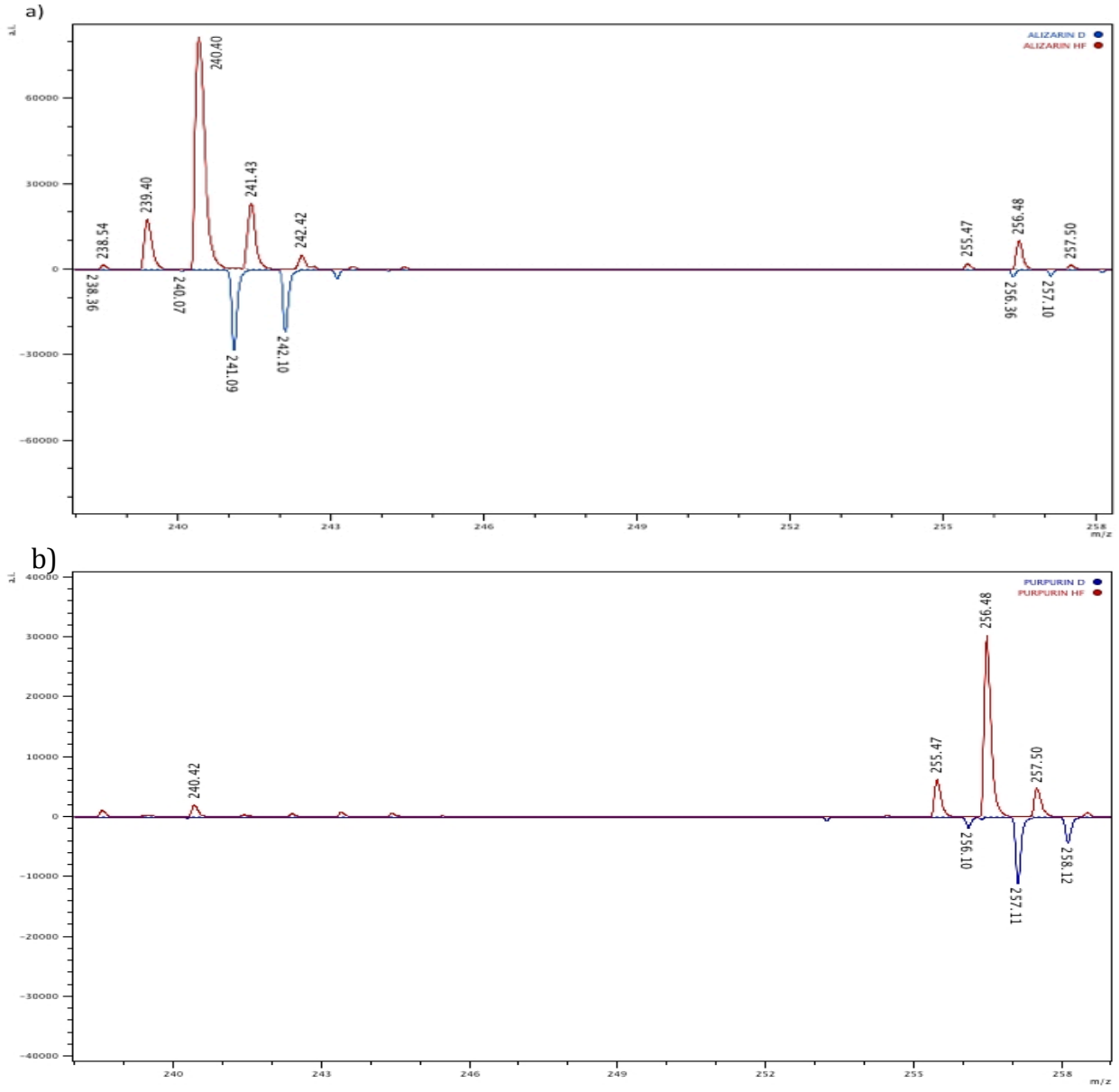
Figure 5.10: LDI-ToF-MS of analytical blank obtained with HF method in **a)** positive and **b)** negative mode.

5.3.3.2 Madder lake

LDI-ToF mass spectra presented for alizarin, purpurin and madder lake are zoomed in the 238-258 m/z range in order to visualize the peaks due to alizarin and purpurin. No other peaks of interest are present in the omitted part.

- **Positive mode**

  In Figures 5.11 a and b, the LDI-ToF mass spectra acquired in positive mode of reference alizarin and purpurin, dyes contained in madder lake, are shown respectively. In each figure, the mass spectra obtained by analyzing the standard anthraquinone directly or with HF extraction are compared. Figure 5.11 c shows the LDI-ToF mass spectrum of madder lake analyzed with HF method.



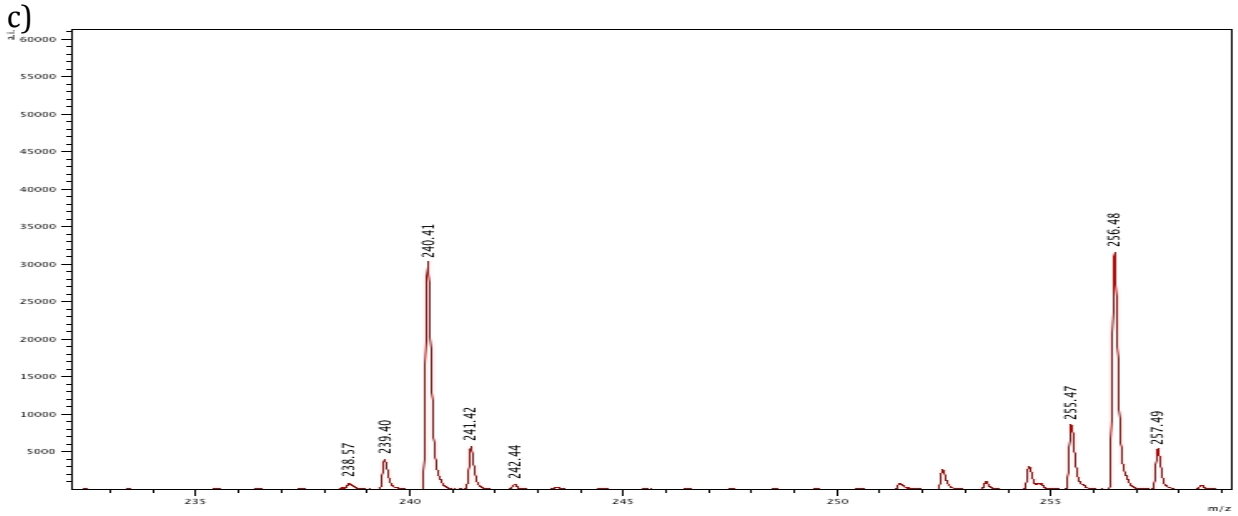
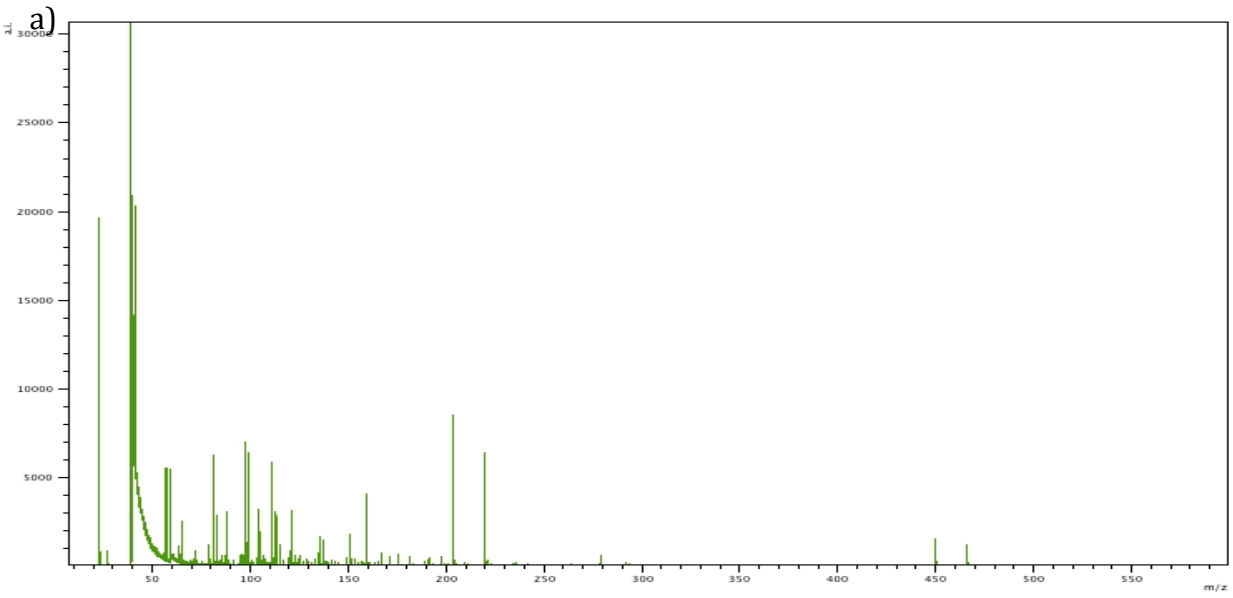


Figure 5.11: Comparison of LDI-ToF mass spectra obtained with HF and direct method of **a)** alizarin, **b)** purpurin and **c)** LDI-ToF mass spectrum of madder lake analyzed with HF method.

In all spectra, the isotopic clusters are centered at $[M]^+$ (m/z= 240 and m/z= 256 for alizarin and purpurin, respectively). Alizarin gives higher signals with both methods than purpurin, in the same conditions. The mass spectra of dyes submitted to HF procedure show higher intensity of the peaks. The detection of the colored molecules by direct method was not possible.

In Figure 5.11 c, no other peaks characteristic of madder lake and reported in literature are present [7]. In Figures 5.12 a and b, the LDI-ToF mass spectra of A1ML2 model system analyzed with direct method and a comparison between A1ML2 analyzed with HF method and madder lake spectrum are shown.



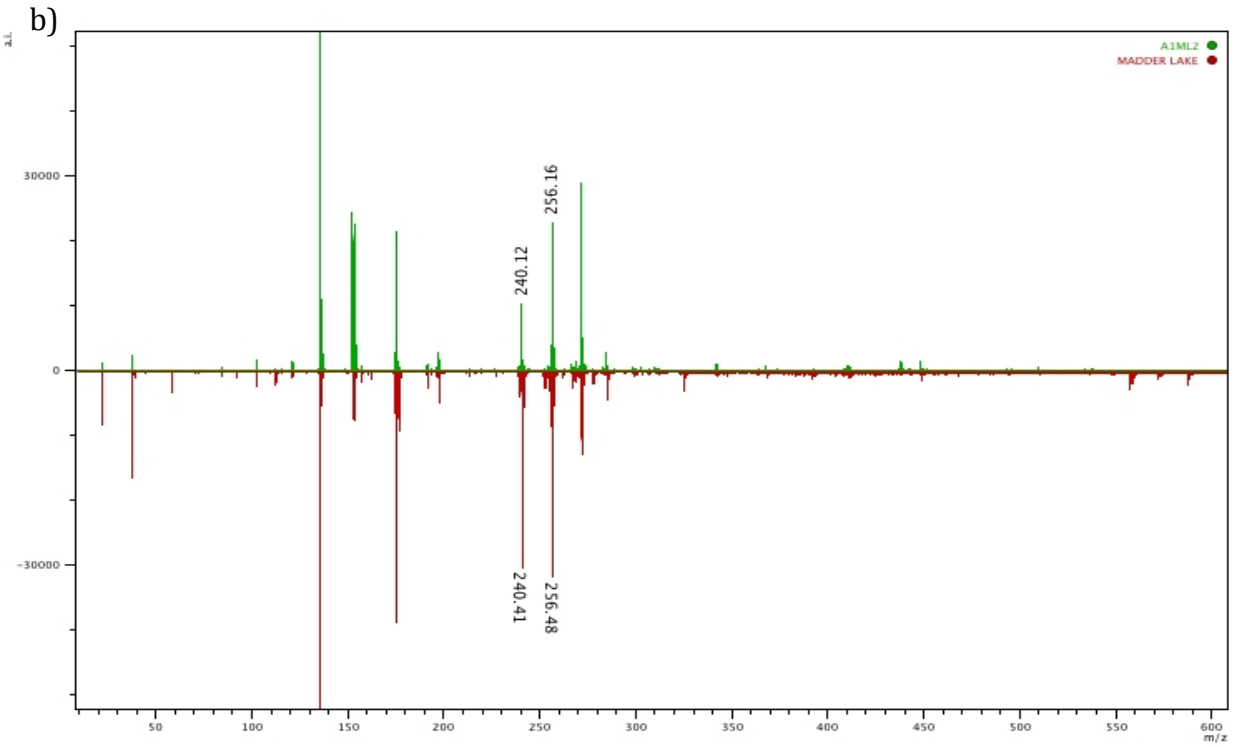


Figure 5.12: LDI-ToF mass spectrum of A1ML2 analyzed with **a)** direct method and **b)** HF method and compared to the spectrum of madder lake obtained by the same strategy.

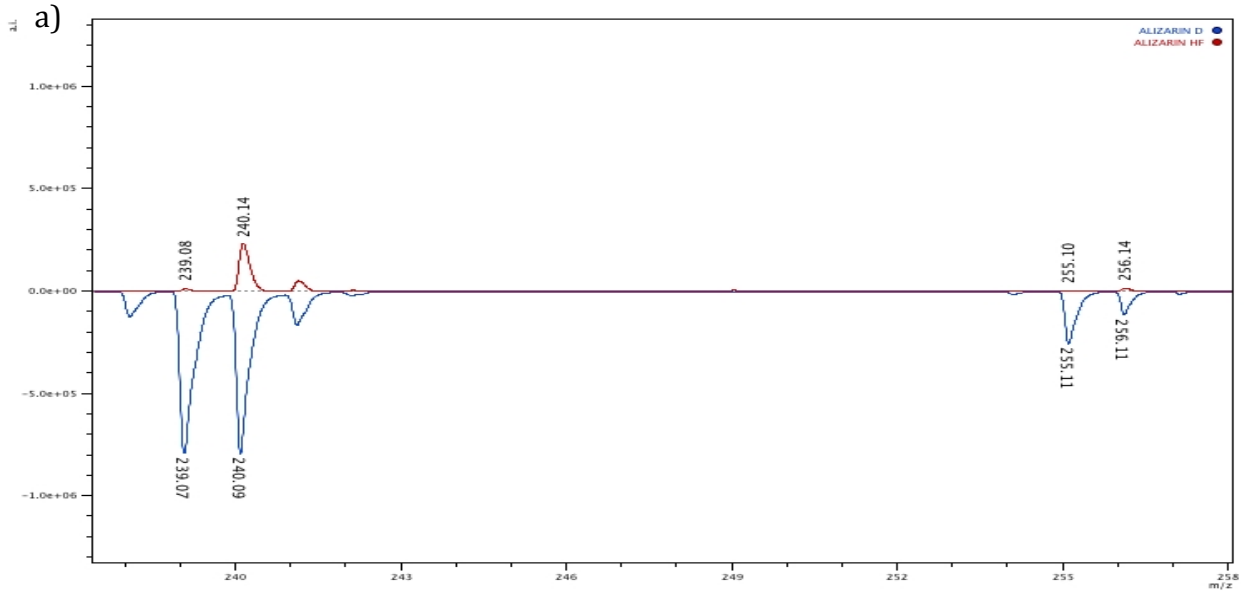
The model sample prepared with 1% w/w albumin was chosen as representative above all. The mass spectrum obtained with the direct method does not show the peaks due to alizarin and purpurin, but only several peaks due to the analytical blank, plus some unknown signals related to the binder. This finding is in agreement with the lack of detectability of madder lake by direct analysis. Figure 5.12 b shows how HF method allows to eliminate the interferences, by releasing only ions of madder lake. Therefore its determination in the model system is possible.

The spectra did not show any peculiar feature that may allow us to differentiate between different kinds of binders or lake/binder ratio.

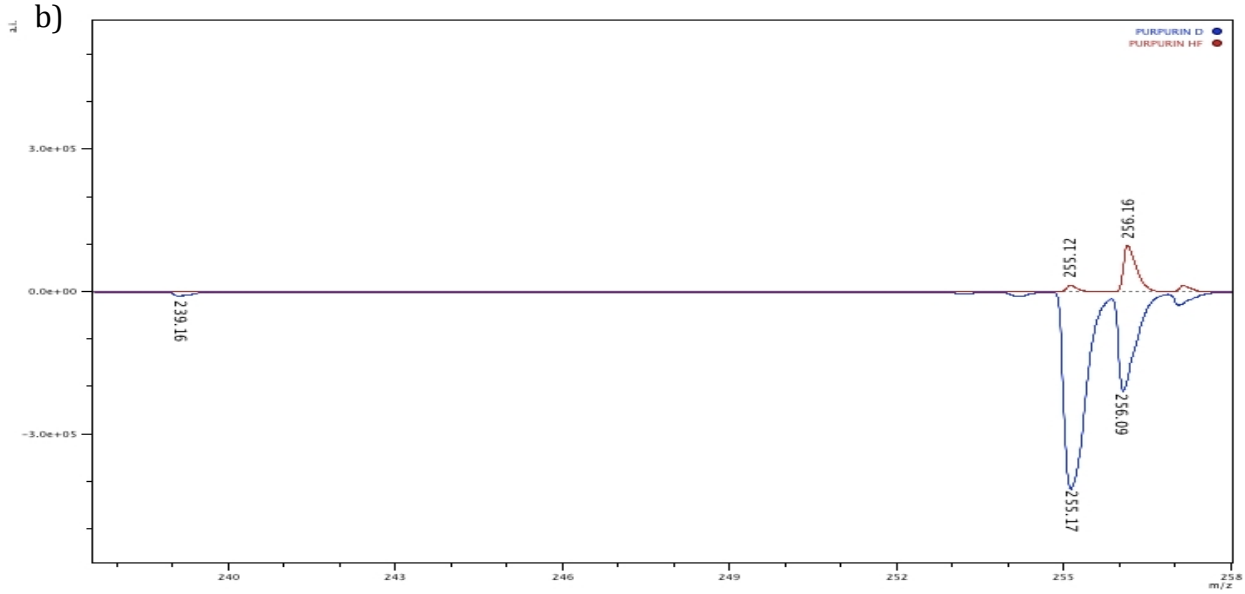
- **Negative mode**

  In Figures 5.13 a, b and c, the LDI-ToF mass spectra of reference alizarin and purpurin, and of madder lake, analyzed both with direct and HF methods are shown, respectively.

a)



b)



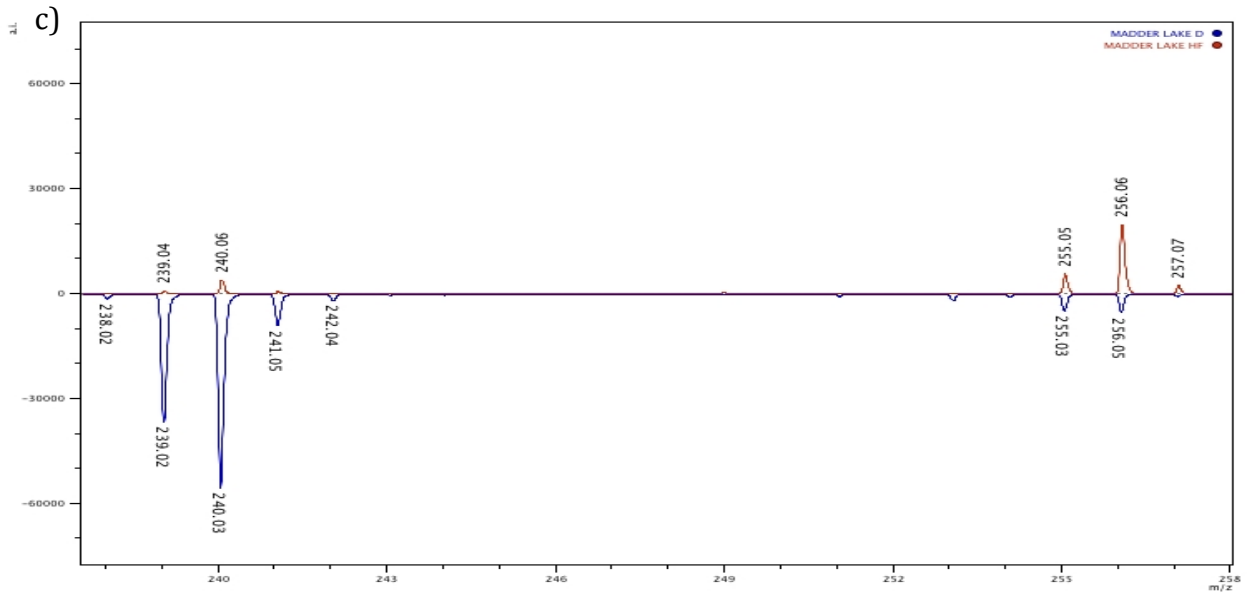
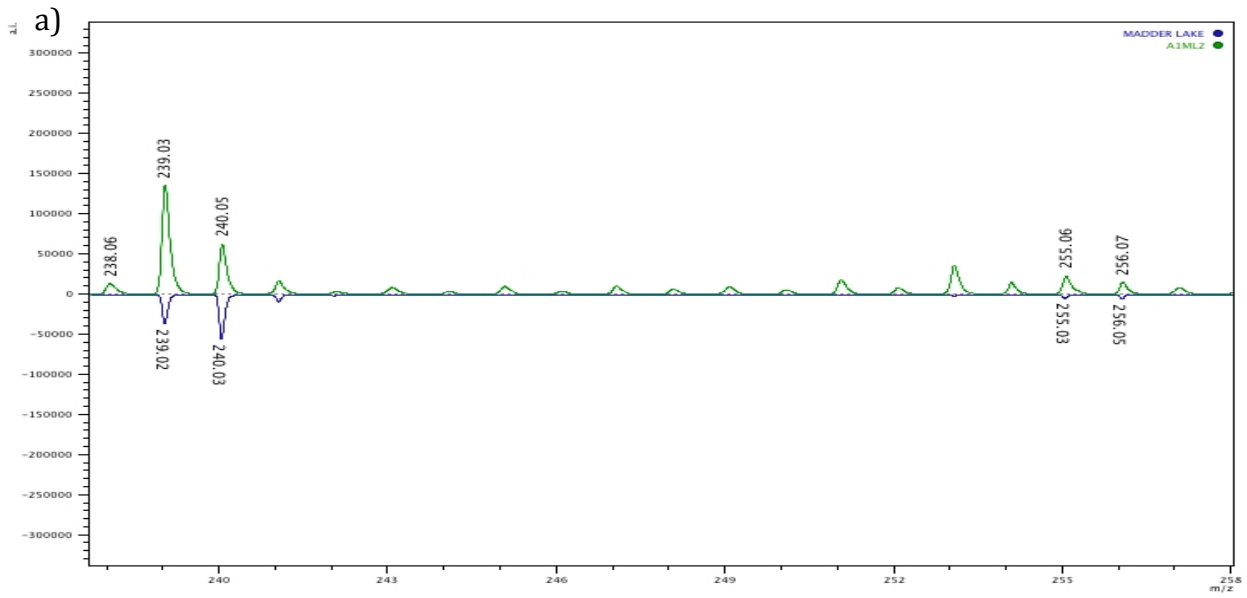


Figure 5.13: comparison of LDI-ToF mass spectra obtained with HF and direct method for **a)** alizarin, **b)** purpurin and **c)** madder lake.

In all the acquired spectra, the isotopic clusters are centered at m/z= 240 and m/z= 256, for alizarin and purpurin, respectively, and peaks at m/z= 239 and m/z= 255 are relatively intense. In all cases mass spectra of dyes submitted to direct method show higher intensity of the peaks. In Figures 5.14 a and b, the LDI-ToF mass spectra of A1ML2 model system analyzed with direct method and HF one, compared to that of madder lake, are shown.



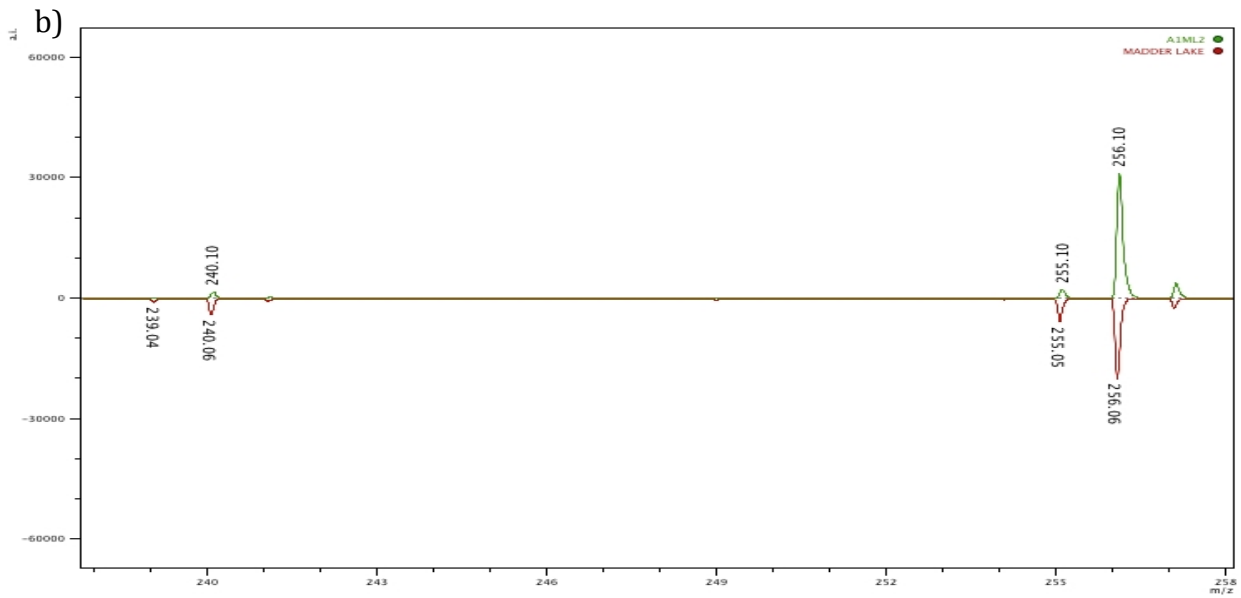


Figure 5.14: Comparison of LDI-MS spectra of madder lake with A1ML2 ones analyzed with **a)** direct method and **b)** HF method.

Madder lake is better detectable in the mass spectrum obtained for A1ML2 with the direct method than with HF method (the signal due to alizarin is higher than that of purpurin as happened in the spectrum of reference madder lake). Nonetheless, the presence of peaks, probably, due to the binder does not allow to obtain a good S/N ratio for purpurin.

Summarizing, either the analysis of samples prepared in the direct method and acquired in negative mode, or and that of samples extracted with HF and acquired in positive mode allow the identification of alizarin and purpurin in all the reference paint replicas. Nonetheless, HF extraction and acquisition in positive mode have to be preferred because they allow obtaining cleaner mass spectra and thus S/N ratio can be increased.

5.3.3.3 Carmine

Only negative mode is discussed for carmine because carminic acid did not ionize in positive mode in the adopted conditions.
LDI-ToF mass spectra presented for carminic acid and carmine are zoomed in the 450-520 m/z and 487-498 m/z range respectively, in order to visualize the molecular peak due to carminic acid.

- **Negative mode**

  In Figures 5.15 a and b LDI-ToF, the mass spectra of reference materials of carminic acid analyzed both with direct and HF method and carmine analyzed only after HF extraction are shown, respectively.

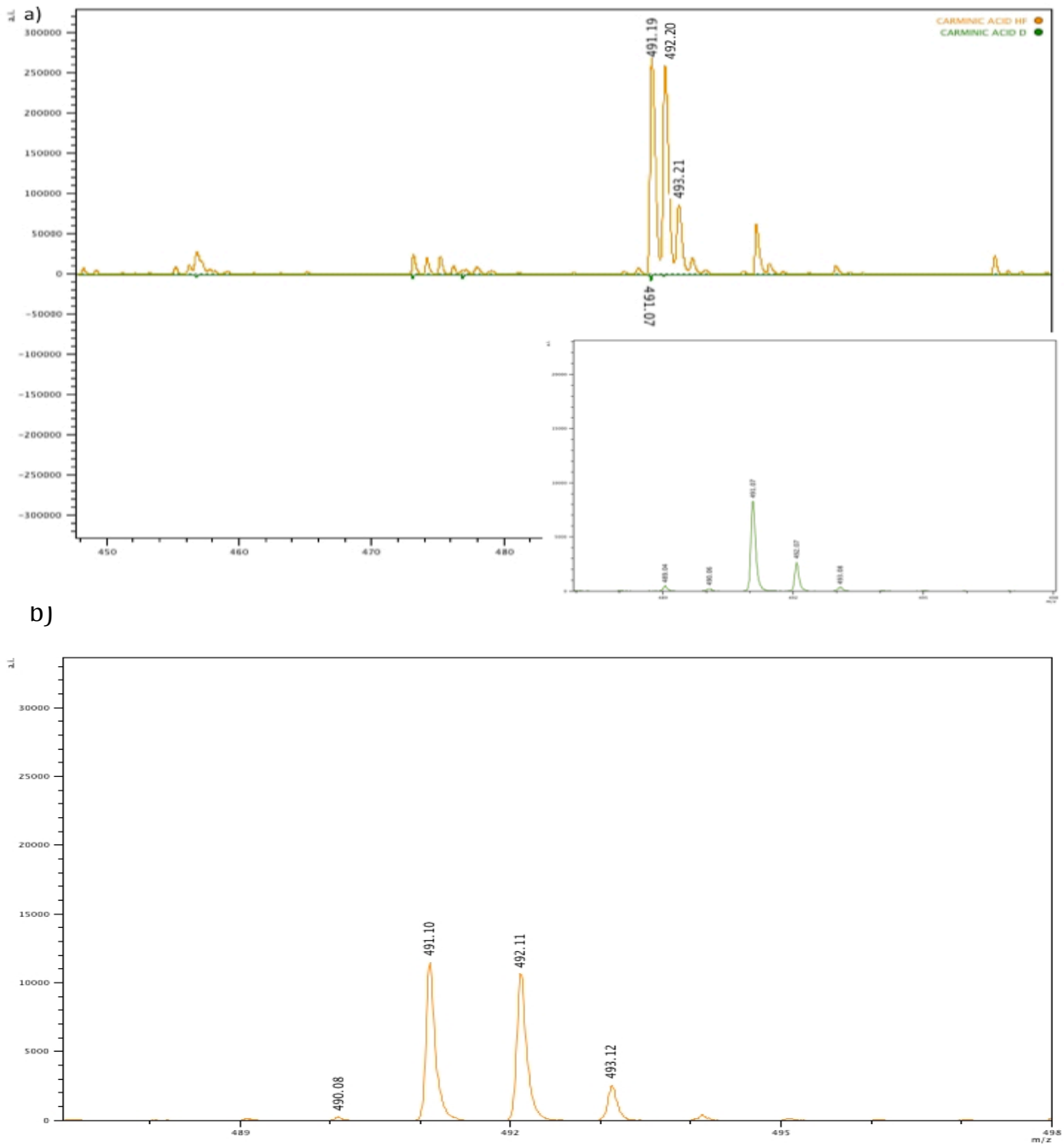


Figure 5.15: LDI-MS spectra of **a)** carminic acid analyzed with direct method and HF method and detail of direct method and **b)** carmine analyzed with HF method.

HF is the method of election for the analysis of carminic acid and carmine. The direct method allows to detect only the dye, but not carminic acid in the lake (as it happened to madder lake, see Section 5.3.3.2). In all the cases, the most intense peaks are m/z= 491,m/z= 492 and m/z= 493, corresponding to the pseudomolecular ($[M-H]^-$) of carminic acid, and its isotopic cluster. In Figure 5.16, the LDI-ToF mass spectrum of C01C model system, extracted with HF method, is shown as an example and compared to that of carmine.

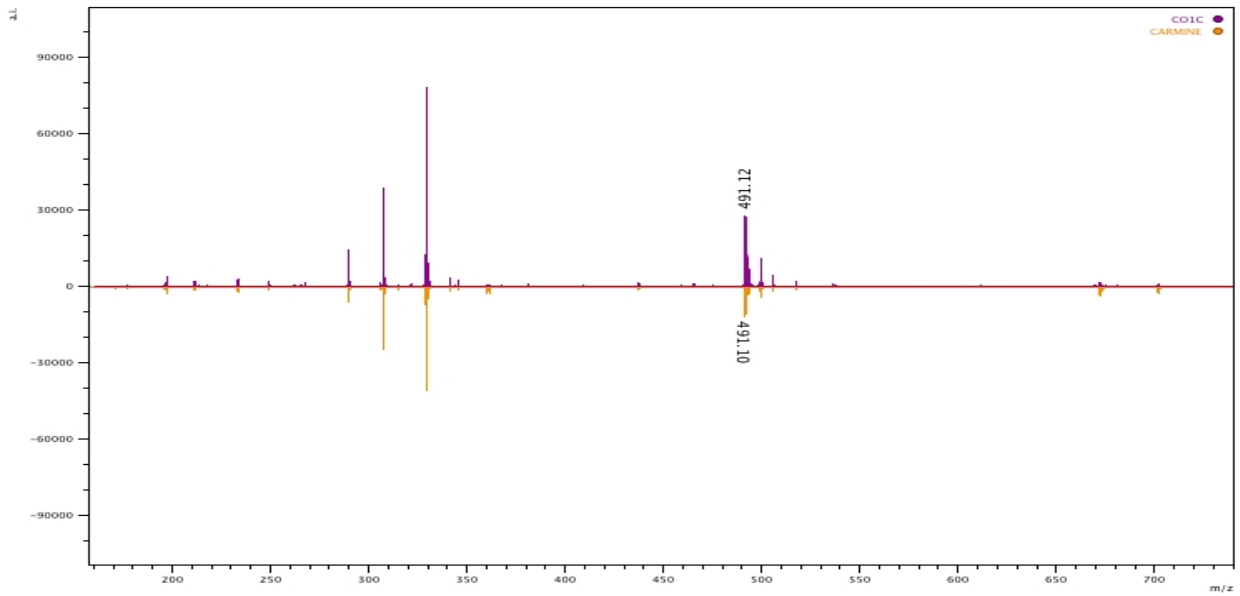


Figure 5.16: Comparison of LDI-MS spectra of C01C and carmine analyzed with HF method.

The identification of carminic acid and thus of carmine in model systems is possible only with HF method. The spectra do not show any peculiar feature that may allow us to differentiate between different kinds of binders or lake/binder ratio.

5.3.3.4 Indian lac

Only the mass spectra obtained with HF extraction method are discussed for Indian lac, because those obtained with the direct analysis correspond to that of the analytical blank.
LDI-ToF mass spectra presented for laccaic acids and Indian lac are zoomed in 493-545 m/z range in order to visualize laccaic acid A. Laccaic acid B ionization is not discussed.

- **Positive mode**

  In Figures 5.17 a and b, the LDI-MS spectra of reference materials of laccaic acids and Indian lac extracted with HF method are shown. No peak due to laccaic acids was detected in Indian lac if the direct method was applied.

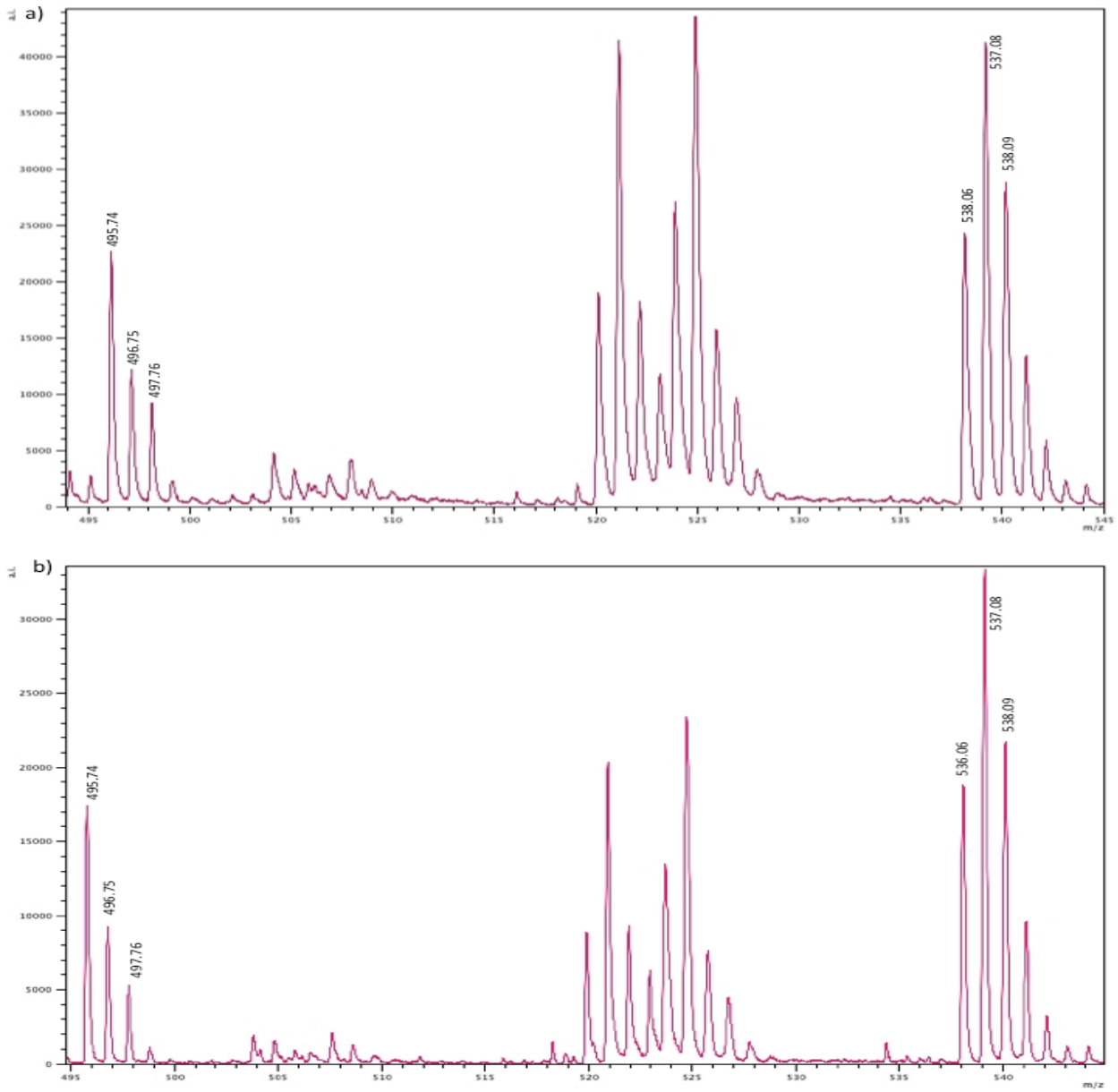


Figure 5.17: LDI-MS spectra obtained with HF method of **a)** laccaic acids and **b)** Indian lac.

The mass spectra of the pure dyes and of the lake are very similar, and in both of them the isotopic cluster of laccaic acid A is centered at m/z 537. In Figure 5.18, the LDI-ToF mass spectrum of C01IL model system after extraction with HF method is given as an example, and compared to that of Indian lac.

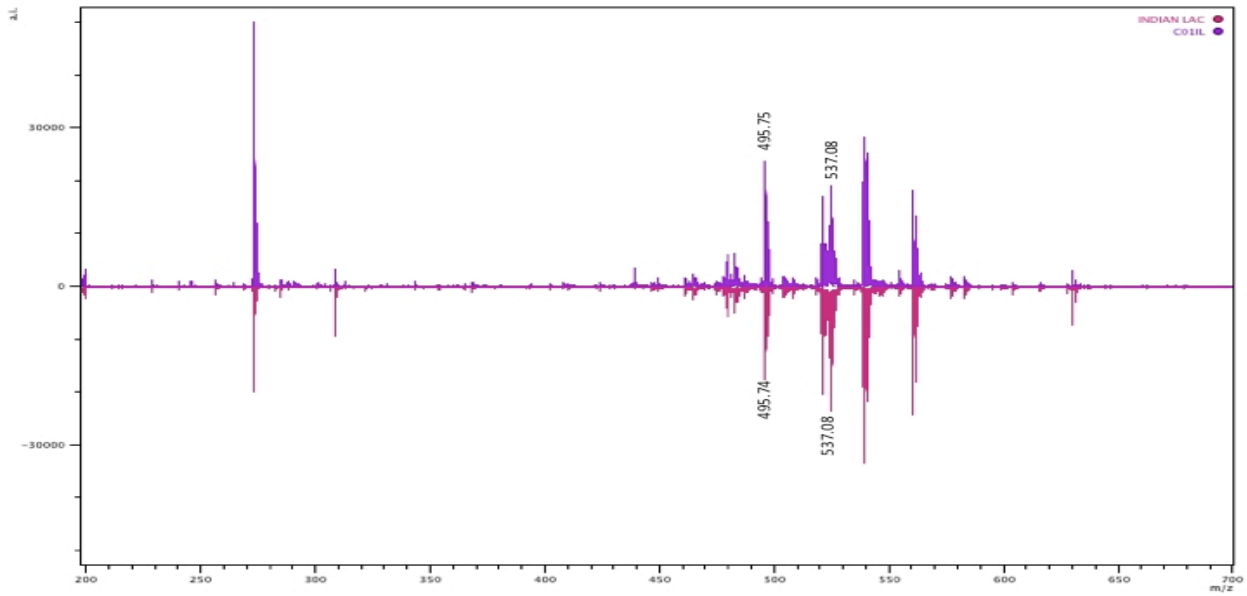
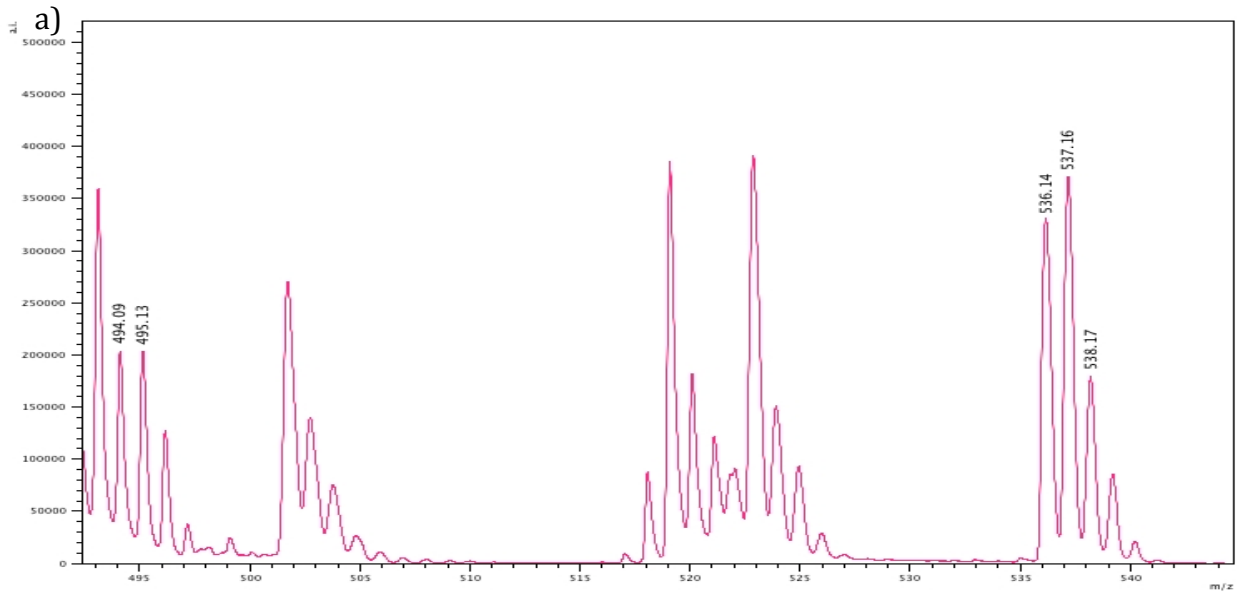


Figure 5.18: Comparison of LDI-MS spectra of C01IL and Indian lac analyzed with HF method.

- **Negative mode**

  In Figures 5.19 a and b, the LDI-MS spectra of reference materials of laccaic acids and Indian lac analyzed by HF method are shown. No peak due to laccaic acids was detected in Indian lac if the direct method was applied.



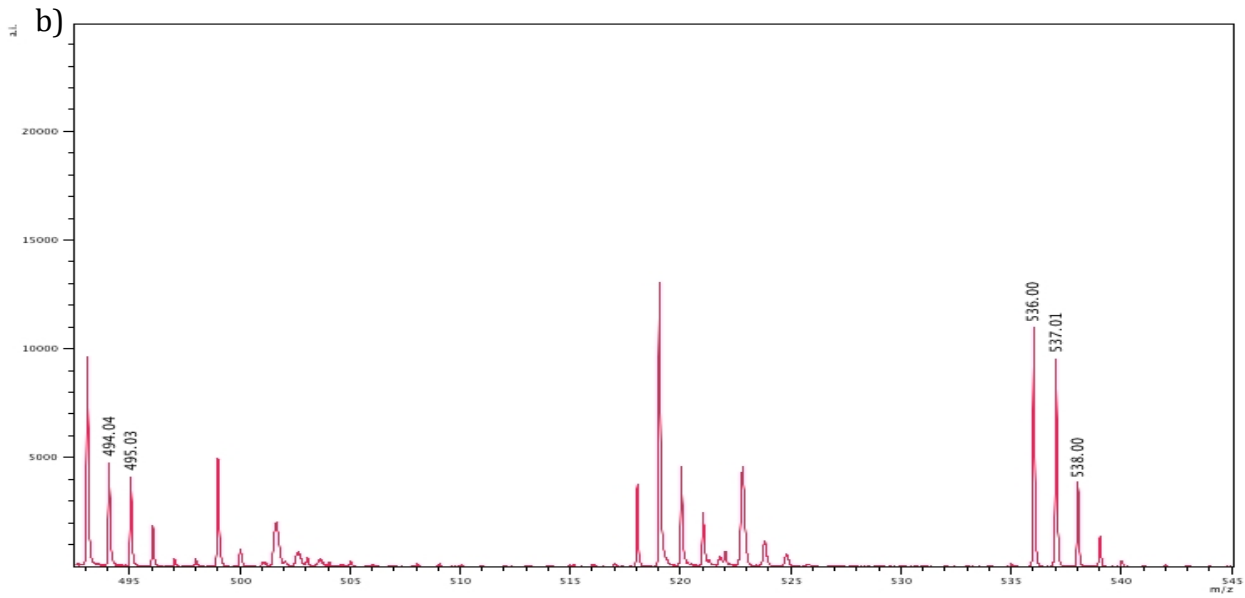


Figure 5.19: LDI-MS spectra obtained with HF method of **a)** laccaic acids and **b)** Indian lac.

The mass spectra of the dyes and those of the lake are very similar and in both of them the isotopic cluster of laccaic acid A is centered at m/z=537, which is the dye molecular ion. In Figure 5.20, the LDI-ToF mass spectrum of C01IL model system treated with HF method is presented as an example and compared to that of Indian lac.

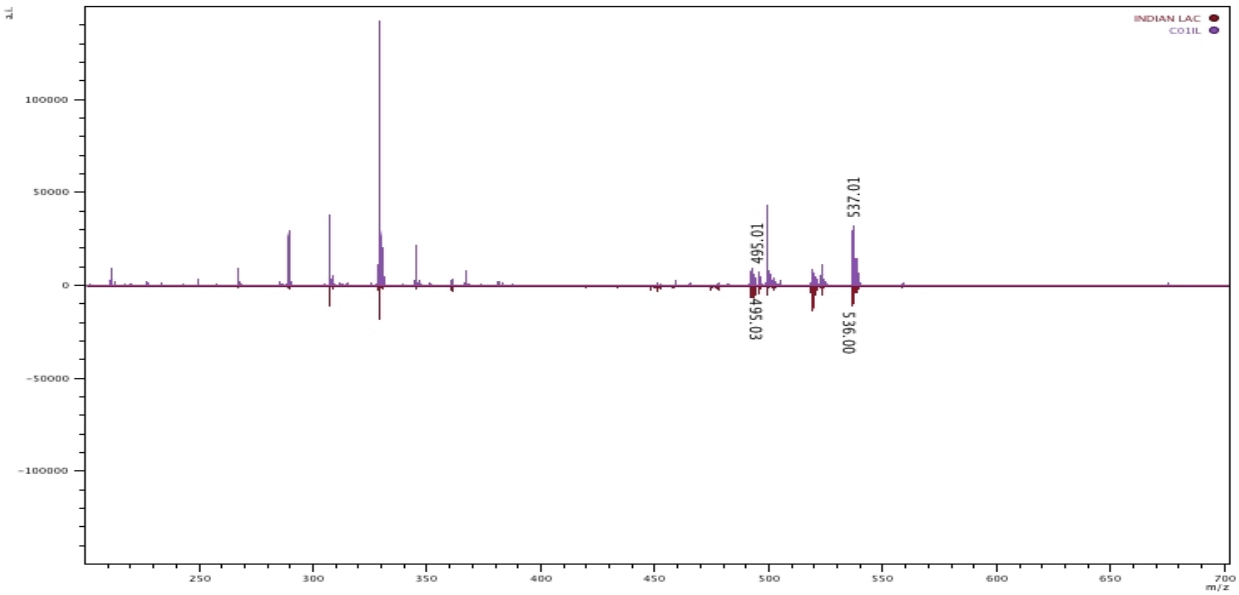


Figure 5.20: Comparison of LDI-MS spectra of C01IL and Indian lac analyzed with HF method.

The identification of Indian lac in CO1IL is possible thanks to the detection of the molecular ion of laccaic acid A.

In both modes the direct method does not enable to detect any peak due to laccaic acids in paint model systems. Therefore the method of choice for detection of Indian lac is HF treatment and analysis in positive mode increased S/N ratio respect to negative mode. No relevant considerations about the different kinds of binders used and lake/binder ratio chosen can be done.

Summarizing, the best strategy to detect Indian lac in a paint sample is HF treatment and positive detection mode.

5.4 Discussion and conclusions

With regard to the analysis of proteinaceous materials, all kinds of binding media have been successfully identified in the reference paint replicas by MALDI-ToF and MALDI-ToF-ToF detection. Rabbit glue and egg were characterized thanks to the comparison with a database of the most frequently proteinaceous binders used in artworks, while albumin and casein by comparing their tandem mass spectra to *in silico* trypsin digestion and fragmentation of peptides processed by Mascot server. The concentration of the proteinaceous binder in the sample directly influenced the S/N ratio in the spectra, as well as the score percentage in the identification. With all kinds of lakes, our optimization of the procedure in terms of purification steps was useful in order to improve the quality of mass spectra.
As regards dyes and lakes, their identification was successful with at least one of the methods of preparation and of analyses modes (positive or negative). The direct method was successful for characterizing reference materials of alizarin and purpurin in both modes, and carminic acid and madder lake in negative mode only. The analysis of model systems prepared with madder lake and treated with the direct method in negative mode allowed the identification of the dyes, but the presence of the peaks due to the binder caused decrease in the S/N ratio. The direct mode did not yield any fragment of the dyes for any of the other prepared paint replicas. Thus the direct method can not be used for the identification of lakes in freshly prepared paint model systems. The impossibility to detect lakes with the direct method can be attributed to the presence of complexes between aluminum ion and the dyes, which prevent the ionization of the molecular markers. If the HF method was applied, the complexation between fluoride and aluminum allowed the release of dye. Therefore, for all dyes and lakes HF method is the method of choice. Moreover, a better identification is achieved in positive mode for madder lake and Indian lac, while in negative mode for carmine. The lake/binder ratio and the kind of binding media influence neither the identification of m/z peaks of dyes nor their intensity. The mass spectra of model systems prepared with different kinds of binders, proteinaceous or lipid or polysaccharidic, do not show any relevant differences, and no peak peculiar of binding media seems to be present.

Summarizing, two methods for analyses of proteins and dyes in freshly prepared paint model systems have been developed, and the influence of the presence of lakes on the first and of the binder on the latter has been evaluated. In particular, we highlighted that the possible presence of a lake has to be taken into account for protein identification, while the nature of the binder do not significantly influence the identification of the organic lake. In Table 5.1 reference dyes and lakes and freshly prepared paint model systems analyzed with MALDI-ToF-MS and LDI-ToF-MS are listed.

Table 5.1: List of reference dyes and lakes and freshly prepared paint model systems analyzed with MALDI-ToF-MS and LDI-ToF-MS (+= positive mode, -= negative mode, n.a.= not analyzed, n.d.=not detected, x=detected, xx=best method).

samples	proteins	dyes			
		direct method		HF method	
	+	+	-	+	-
alizarin	n.a.	x	x	xx	x
purpurin	n.a.	x	xx	x	x
carminic acid	n.a.	n.d.	x	n.d.	xx
laccaic acids	n.a.	n.d.	n.d.	xx	x
madder lake	n.d.	n.d.	x	xx	x
carmine	x	n.d.	n.d.	n.d.	xx
Indian lac	n.d.	n.d.	n.d.	xx	x
C01C1	x	n.a.	n.d.	n.a.	xx
C01IL1	x	n.d.	n.d.	xx	x
C01ML1	x	n.d.	x	xx	x
A1C1	x	n.a.	n.d.	n.a.	n.a.
A1C2	x	n.a.	n.d.	n.a.	xx
A1C3	x	n.a.	n.d.	n.a.	xx
A1IL1	x	n.d.	n.d.	n.a.	n.a.
A1IL2	x	n.d.	n.d.	xx	x
A1IL3	x	n.d.	n.d.	xx	x
A1ML1	x	n.d.	x	n.a.	n.a.
A1ML2	x	n.d.	x	xx	x
A1ML3	x	n.d.	x	xx	x
A5C1	x	n.a.	n.d.	n.a.	xx
A5C2	x	n.a.	n.d.	n.a.	xx
A5IL1	x	n.d.	n.d.	n.a.	n.a.
A5IL4	x	n.d.	n.d.	xx	x
A5ML2	x	n.d.	x	xx	x
A5ML4	x	n.d.	n.d.	n.a.	n.a.
EC1	x	n.a.	n.d.	n.a.	xx
EC2	x	n.a.	n.d.	n.a.	xx
EIL1	x	n.d.	n.d.	xx	x
EIL2	x	n.d.	n.d.	n.a.	n.a.
EIL3	x	n.d.	n.d.	n.a.	n.a.
EML1	x	n.d.	x	xx	x
EML2	x	n.d.	x	xx	x
RG01C1	x	n.a.	n.d.	n.a.	xx
RG01C2	x	n.a.	n.d.	n.a.	xx
RG01IL1	x	n.d.	n.d.	xx	x
RG01IL2	x	n.d.	n.d.	xx	x
RG01ML1	x	n.d.	x	n.a.	n.a.
RG01ML2	x	n.d.	x	xx	x
RG1C1	x	n.a.	n.d.	n.a.	n.a.
RG1C2	x	n.a.	n.d.	n.a.	xx
RG1IL1	x	n.d.	n.d.	n.a.	n.a.
RG1IL2	x	n.d.	n.d.	xx	x
RG1ML1	x	n.d.	x	xx	x
RG1ML2	x	n.d.	x	n.a.	n.a.
RG5C1	x	n.a.	n.d.	n.a.	n.a.

RG5IL1	x	n.d.	n.d.	n.a.	n.a.
RG5ML1	x	n.d.	x	n.a.	n.a.
AG1C1	n.a.	n.a.	n.d.	n.a.	xx
AG1IL1	n.a.	n.d.	n.d.	xx	x
AG1ML1	n.a.	n.d.	x	xx	x
AG10C1	n.a.	n.a.	n.d.	n.a.	xx
AG10C7	n.a.	n.a.	n.d.	n.a.	xx
AG10IL7	n.a.	n.d.	n.d.	xx	x
AG10ML7	n.a.	n.d.	x	xx	x
LOC1	n.a.	n.a.	n.d.	n.a.	xx
LOC7	n.a.	n.a.	n.d.	n.a.	xx
LOIL7	n.a.	n.d.	n.d.	xx	x
LOML2	n.a.	n.d.	x	xx	x
LOML7	n.a.	n.d.	x	xx	x

Bibliography

(1) S. Kuckova, R. Hynek, M. Kodicek, *MALDI-MS Applied to the Analysis of Protein Paint Binders*, Organic Mass Spectrometry in Art and Archaeology, edited by M.P. Colombini and F. Modugno, ed. Wiley, 2009.

(2) E.D. Hoffmann, V. Stroobant, *Mass Spectrometry- Principles and Application*, ed. Wiley, 2007.

(3) L. Rafaelly, S. Hèron, W. Nowik, A. Tchapla, *Optimisation of ESI-MS detection for HPLC of Anthraquinone dyes, Science Direct*, vol. 77, pp. 191-203, 2008.

(4) S. Kuckova, R. Hynek, M. Kodicek, *Identification of Proteinaceous Binders used in Artworks by MALDI-TOF Mass Spectrometry*, Analytical Bioanalytical Chemistry, vol. 338, pp. 201-206, 2007.

(5) Matrix Science, http:// www.matrixscience.com.

(6) S. Kuckova, I. Nemec, R. Hynek, J. Hradilova, T. Grygar, *Analysis of Organic Colouring and Binding Components in Colour Layer of Artworks*, Analytical Bioanalytical Chemistry, vol. 382, pp. 275–282, 2005.

(7) N. Wyplosz, *Laser desorption Mass Spectrometric Studies of Artists' Organic pigments*, Molart, Amolf Fom, 2003.

(8) M. Stýblova, *Analýza Přírodních Organických Barviv a Pigmentů Pomocí Hmotnostní Spektrometrie*, Diplomovà Pràce, 2012.

(9) J. Sanyova, *Mild extraction of Dyes by Hydrofluoridic Acid in Routine analysis of Historical Paint Micro-samples*, Microchimica Acta, vol. 162, pp. 361-370, 2008.

Chapter 6

Analyses of historical samples

6.1 Introduction

The procedures developed and optimized in Chapter 3 and 5 have been applied to five paint samples from different archeological sites as described in Section 2.6 and shown in Figure 6.1. For the identification of proteinaceous binding media, MALDI-ToF-MS and nano LC–MS/MS techniques were applied. For the analyses of anthraquinoid dyes, both LDI-ToF-MS (performed with direct and HF method in positive and negative mode) and HPLC-DAD techniques have been employed. The results collected with the different techniques and obtained from previous analyses have been cross-checked and discussed.

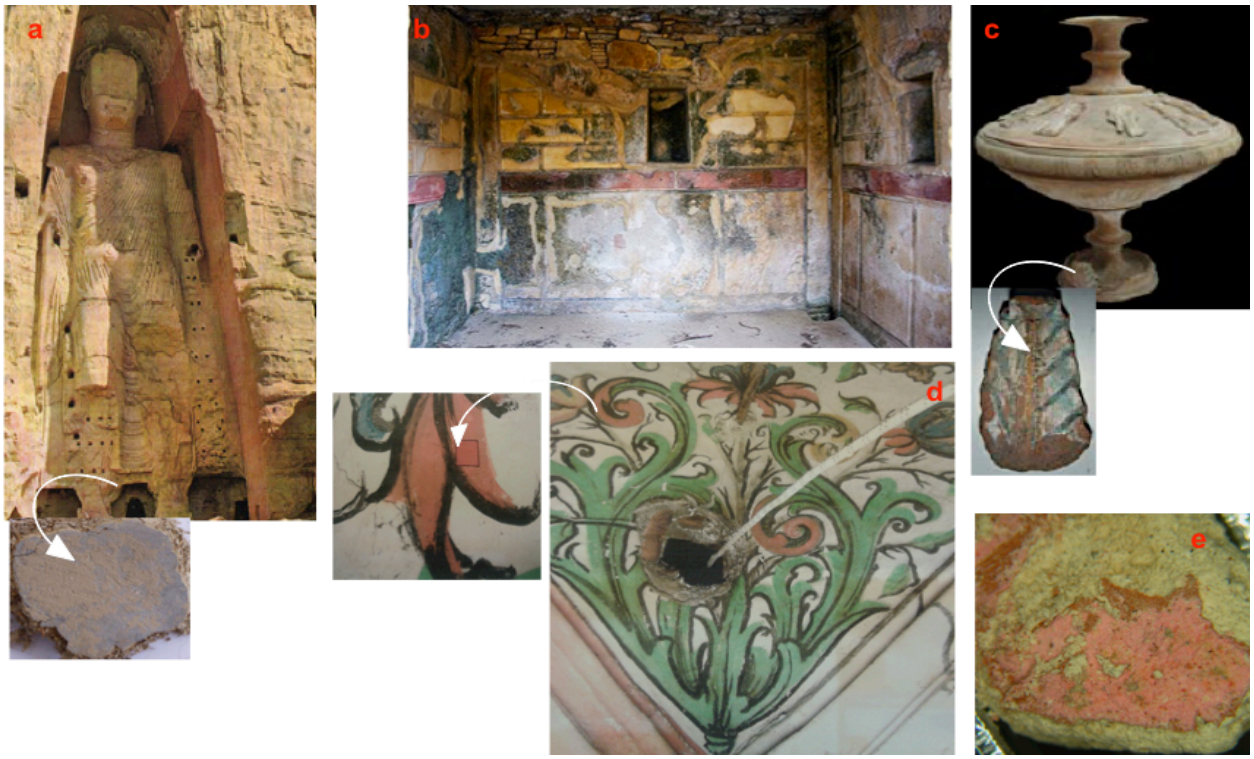


Figure 6.1: Archeological samples analyzed: **a)** polychrome Buddha statue (Afghanistan, 6th century)**; b)** wall paintings in Delos (Greece, b.C); **c)** Palmetta; **d)** FG 2 and **e)** Kor 26.
(For a, c and d the place of taking sample and the sample analyzed are displayed.)

6.2 Analyses of proteins

The MALDI-ToF-MS procedure, displayed in Figure 5.1, was applied to the historical samples named “Buddha”, “Delos”, “Kor 26” and “FG 2” and their mass spectra are reported in Figures 6.2 a, b, c and d. Palmetta was not analyzed due to low amount of sample available.

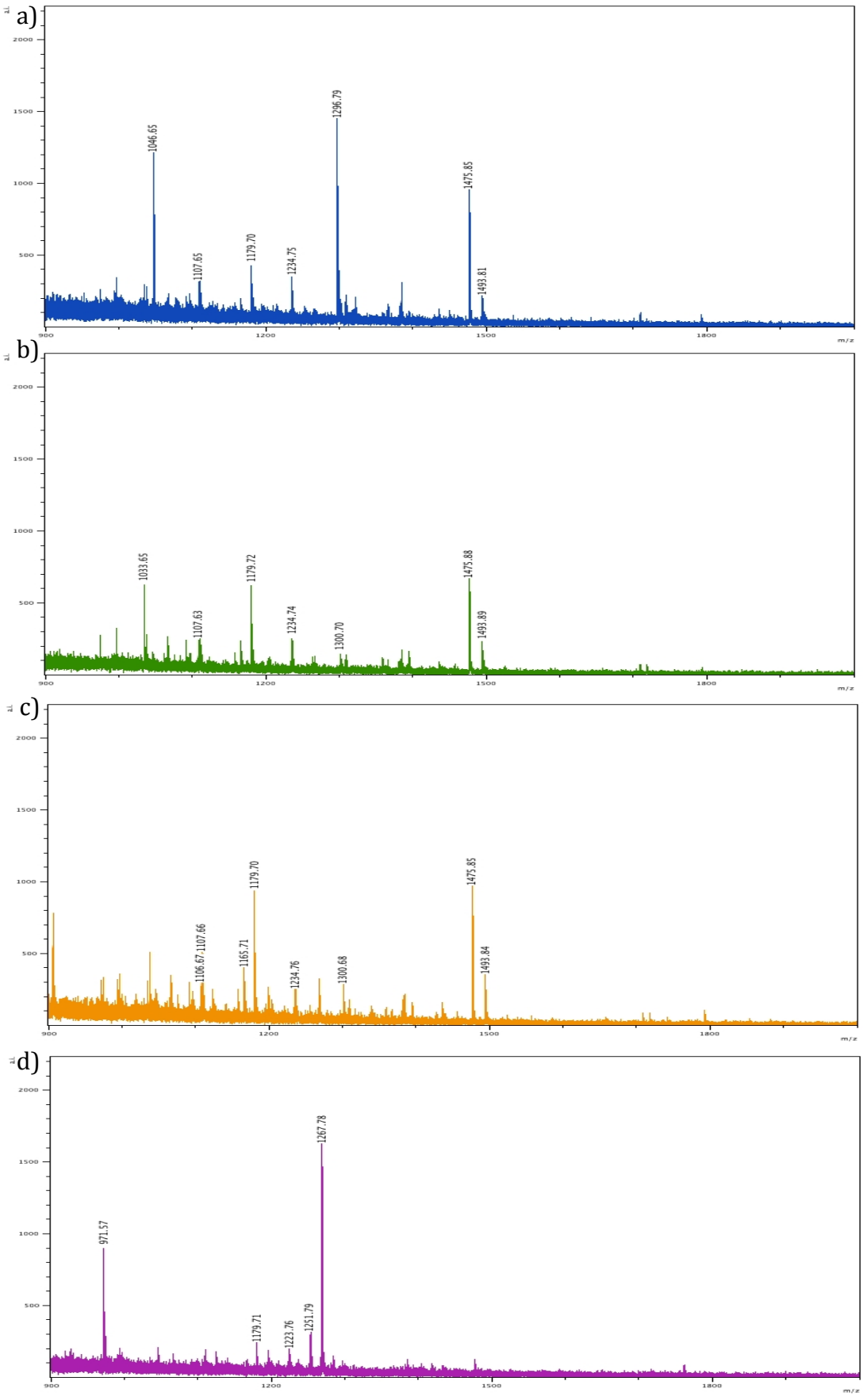


Figure 6.2: MALDI-ToF mass spectra in positive mode of **a)** "Buddha"; **b)** "Delos"; **c) "**Kor 26" and **d)** "FG 2".

The mass spectra shown in the previous figures do not differ from that of the analytical blanks. The few peaks present are not diagnostic for any binder with the exception of the m/z= 1108 ascribable to egg and detected in "Buddha", "Delos" and "FG2". Nevertheless previous studies highlighted the presence of egg in "Buddha" [1, 2, 3, 4] and "FG2" [5], thus LC–MS/MS analyses were carried out to confirm these results. The analytical procedure applied was the same as the one applied for MALDI-ToF-MS but, obviously, without the addition of matrix. The purified solution of peptides, obtained after the clean up step, were left to dry up in laboratory conditions, redissolved in a mixture of 97 H_2O : 3 ACN with 0.1% of FA and then injected in LC–MS/MS. This technique leads to the unambiguous identification of proteins thanks to the determination of amino acid sequences even if only a small number of peptides is released by trypsin cleavage. Two proteins, listed in Table 6.1, belonging to *Gallus gallus,* were found in the sample collected from the painted "Buddha". Thus the binding media used in this sample is egg.

Table 6.1: Amino acid sequences and molecular weight of proteins belonging to *Gallus gallus* and found in the sample collected from the painted "Buddha".

protein	sequence	MW (kDa)
apoliprotein A	K.LTPVAEEAR.D	30.7
uncharacterized protein	R.APKAPPGQHR.H	78.9

6.3 Analyses of anthraquinoid dyes

With regard to the identification of anthraquinoid dyes, "Buddha", "Delos", "Kor 26", "FG 2" and "Palmetta" samples were analyzed with LDI-ToF-MS. The procedure using HF, displayed in Figure 5.9, was chosen and both positive and negative modes were tested.
In positive mode all the samples show a small peak at m/z= 240, which is the molecular ion of alizarin. In the "Palmetta" sample the molecular ion of purpurin is present at m/z= 256.
In the mass spectra acquired in negative mode for "Delos", "Kor 26" and "Palmetta" samples, alizarin is not detectable while the peak corresponding with purpurin is present and higher than in positive mode. Comparison between LDI-ToF-MS mass spectra of madder lake with all the samples analyzed with HF method and acquired in positive and negative mode are shown in Figures 6.3 a and b respectively.

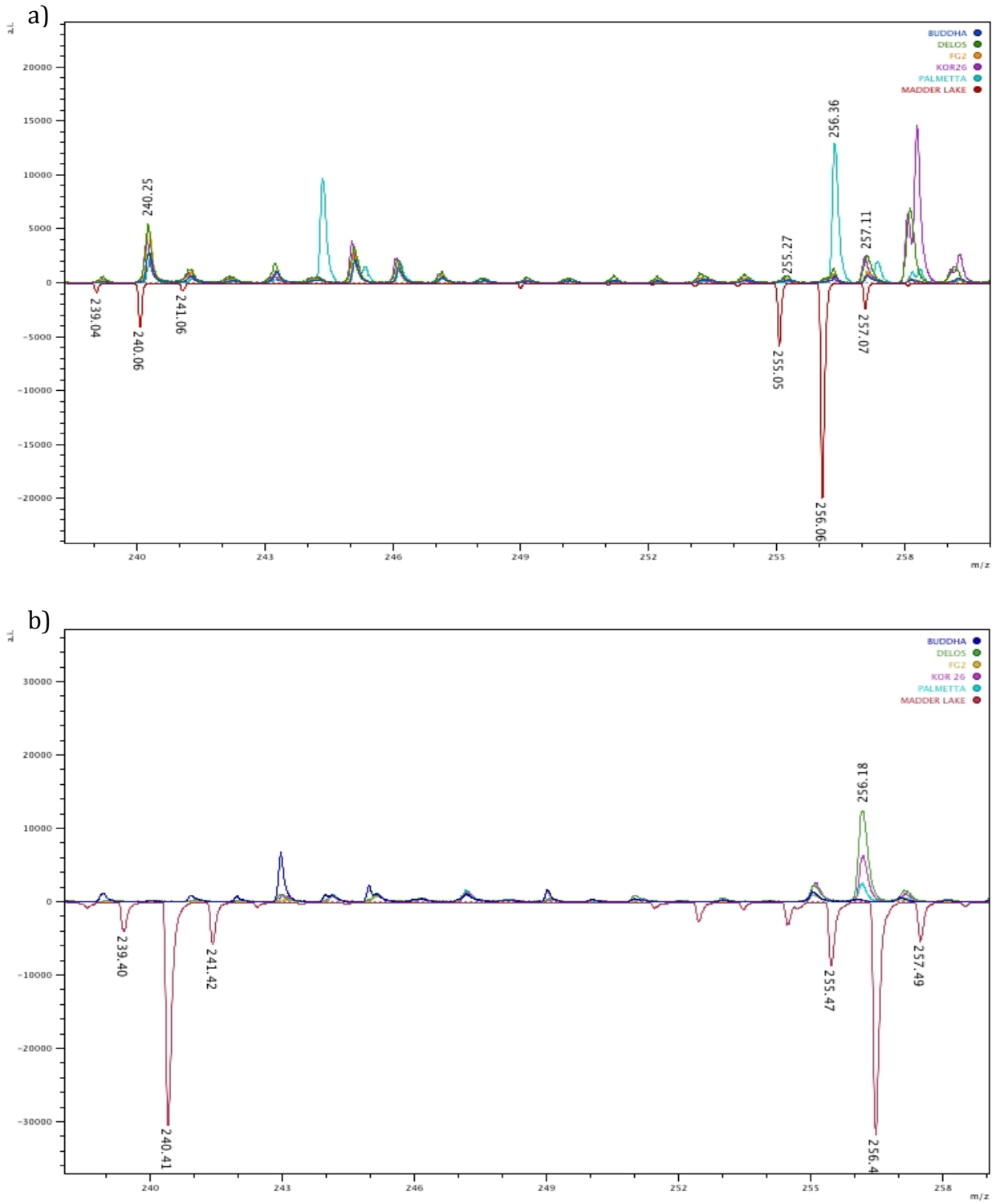


Figure 6.3: LDI-ToF mass spectra of samples of "Buddha", "Delos", "Kor 26", "FG 2" and "Palmetta" compared to madder lake and treated with HF method and acquired in **a)** positive mode and **b)** negative mode.

In order to confirm the identification of madder lake, fragmentation of m/z= 256 in samples of "Delos", "Kor 26" and "Palmetta" was performed and the tandem mass spectrum was compared to that of purpurin. The comparison of the tandem MS spectra is shown in Figure 6.4. Fragmentation of m/z= 240 was not performed because the intensity of the peak, especially in "Buddha" and "FG2", was nearly at blank level.

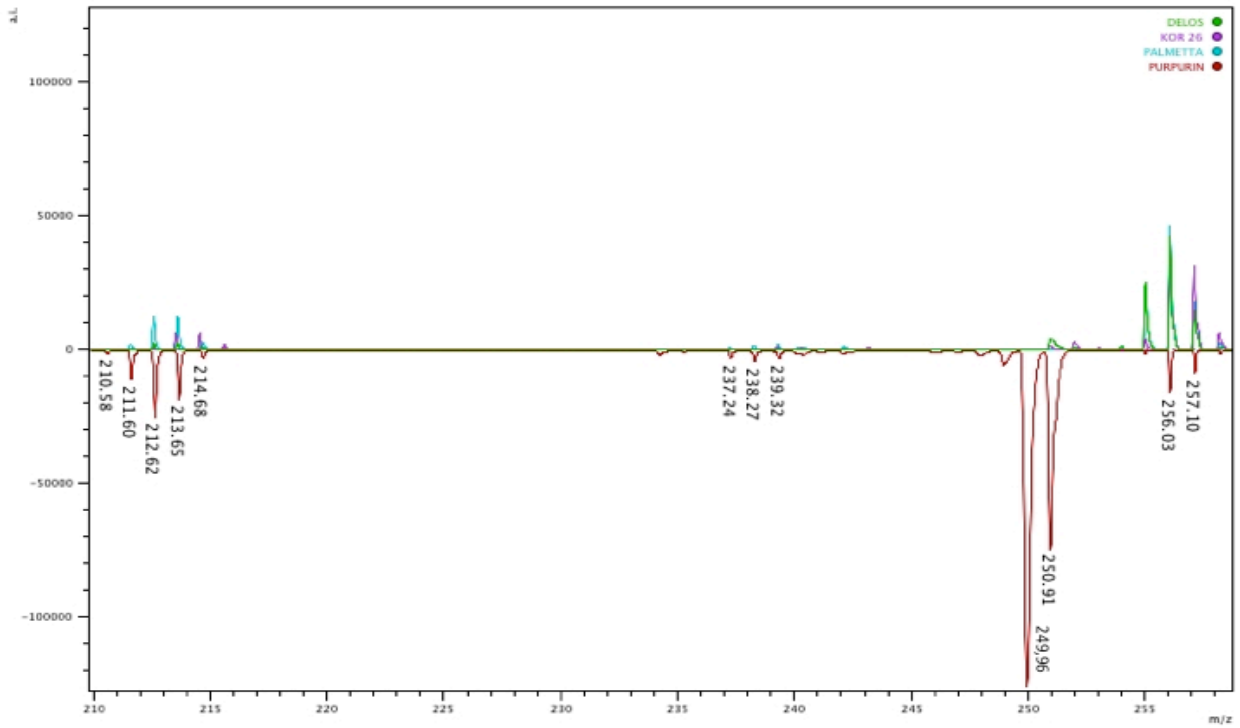


Figure 6.4: LDI-ToF tandem MS spectra of m/z= 256 of samples of "Delos", "Kor 26" and "Palmetta" compared to madder lake treated with HF method and analyzed in negative mode.

The comparison among the tandem MS spectra allowed us to confirm the presence of purpurin in "Palmetta", "Delos" and "Kor 26".

Even if madder lake is better detectable in positive mode, as deduced from the analyses of the reference materials and discussed in Section 5.3.3.2, purpurin gives a higher peak in negative mode. This may explain the better identification of madder lake in the archeological samples in negative mode.

The detection of madder lake in "Palmetta" sample is consistent with previous studies, which highlighted the presence of alizarin and purpurin by HPLC-DAD and GC-MS techniques [6].

The other four samples ("Buddha", "Delos", "Kor 26" and "FG 2") were also analyzed by the HPLC-DAD procedure summarized in Figure 3.14. Palmetta was not further analyzed due to the low amount of sample available. The chromatograms obtained for the extracts of "Delos" and "Kor 26" samples are reported in Figures 6.5 a and b, the range from 20.0 min to 30.0 min is zoomed in order to visualize the peaks of interest. The chromatograms of the other samples showed peaks at blank level and thus are not reported. Therefore, the results collected for "Buddha" and "FG 2"does not allowed to affirm the presence of madder lake

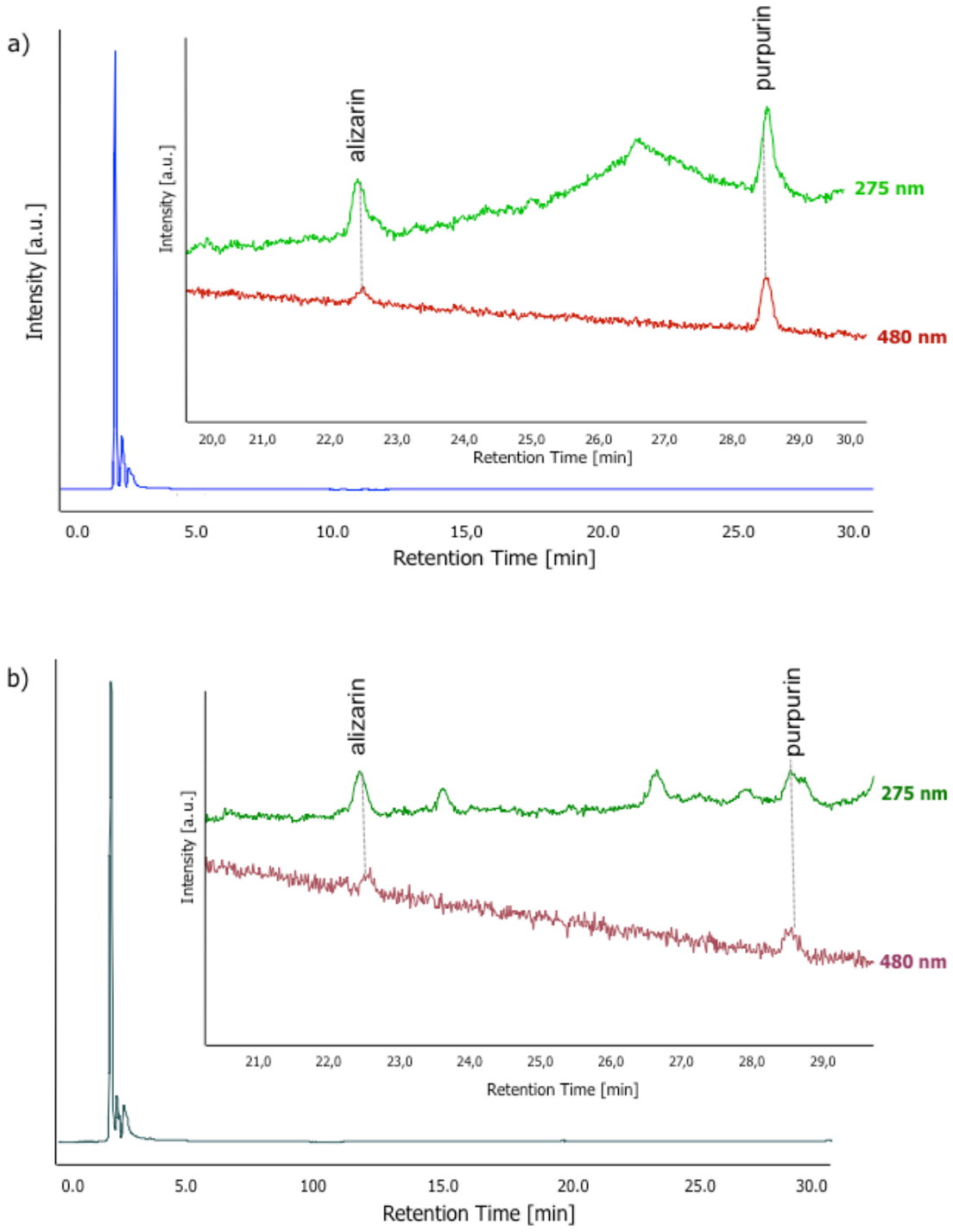


Figure 6.5: HPLC-DAD chromatogram acquired at 275 nm and 480 nm of **a)** Delos and **b)** Kor 26.

In both chromatograms two small peaks, but above the detention limit, are detected at c.a. 22.4 min and 28.5 min. Their absorptions at 275 nm and 480 nm and retention times allow the identification of alizarin and purpurin. In "Delos" sample the peak due to purpurin is higher than in "Kor 26" in agreement with LDI-ToF mass spectrum shown in Figure 6.3 b. The identification of madder lake can be confirmed in both cases.

6.4 Conclusion

The proteinaceous binders and the anthraquinoid dyes identified in “Buddha”, “Delos”, “Palmetta”, “FG 2” and “Kor 26” are summarized in Table 6.2, along with the analyses carried out in the course of the thesis. Previous reports on the same samples are also shown for comparison.

Table 6.2: Proteinaceous binders and anthraquinoid lakes identified in the historical samples analyzed (the techniques used for the detection are in brackets; *= analyses not performed in the course of the thesis).

	proteinaceous binder	**anthraquinoid dyes**
Buddha	egg (LC–MS/MS, GC/MS*)	-
Delos	-	alizarin + purpurin (LDI-ToF-MS, HPLC-DAD)
Palmetta	-	alizarin + purpurin (LDI-ToF-MS, HPLC-UV-Vis*, GC/MS*, DE-MS*)
FG 2	egg (GC/MS)	-
Kor 26	-	alizarin + purpurin (LDI-ToF-MS, HPLC-DAD)

With regard to the detection of the proteinaceous binding media, the MALDI-ToF-MS technique demonstrated not being successful when it comes to identify binders in historical samples. GC/MS resulted to be the best suited to identify proteinaceous materials used as paint binders and, when applied LC–MS/MS, showed its capabilities. The presence of non proteinaceous organic binders in the paint layers, or inorganic materials belonging to the support and not removed by the analytical procedure employed could account for the high background noise of MALDI-ToF-MS spectra. With regard to the detection of dyes, the simultaneous presence of alizarin and purpurin was revealed both by LDI-ToF-MS and HPLC-DAD, thus madder lake was the most probably used colorant material.

The analysis of organic materials contained in historical and archaeological paints still presents more problems than the methods developed theoretically. The difficulties due to the small amount of sample available and the mixture of compounds present in unknown proportions have to be considered. Moreover, organic materials are subjected to ageing and degradation processes giving rise to complex matrices arduous to characterize [3]. Thus, the discrepancy between the efficiency of the different methods of analysis applied on reference materials or paint model systems and archeological samples is not unexplained. Therefore, additional researches and developments have to be carried out in order to further optimize the methods and improve their efficiency for the analyses of historical samples. Nevertheless the multyanalytical approach applied lead to the unambiguous identification of some of the painting materials, thanks to the cross-checking among the results obtained by different techniques.

Bibliography

(1) A. Lluveras, *A First Insight into the Asian Clay Sculptures Painting Technique and Materials: Western and Eastern Buddhas of the Bamiyan Valley (Afghanistan) and Sculptures from Shuilu'an (Shaanxi Province, China)*, Atti dell' Art 11, 10th International Conference, 2011.

(2) I. Bonaduce, M. Cito, M. P. Colombini, *The development of a Gas Chromatographic-Mass Spectrometric Analytical Procedure for the Determination of Lipids, Proteins and Resins in the Same Paint Micro-Sample Avoiding Interferences from Inorganic Media,* Journal of Chromatography A, no. 1216, pp. 5931-5939, 2009.

(3) A. Lluveras, I. Bonaduce, A. Andreotti, M. P. Colombini, *GC/MS Analytical Procedure for the Characterization of Glycerolipids, Natural Waxes, Terpenoid Resins, Proteinaceous and Polysaccharide Materials in the Same Paint Microsample Avoiding Interferences from Inorganic Media*, Analytical Chemistry, no. 82, pp. 376-386, 2010.

(4) A. Lluveras Tenorio, J. Mazurek, A. Restivo, M.P. Colombini, I. Bonaduce, *The development of a New Analytical Mode for the Identification of Saccharide Binders in Paint Sample*, Plos One, vol. 7, no. 11, pp. 1-17, 2012.

(5) I. Degano, M.P. Colombini, internal report, 2006.

(6) M.P. Colombini, I. Degano, E. Ribechini, *A Multi Analytical Approach to Determine Madder Lake in a Funerary Clay Vessel found in a chamber tomb in Taranto*, Dyes in History and Archaeology, accepted for publication.

Conclusions

The present study succeeded in developing a multi analytical approach for characterizing anthraquinoid lakes and proteinaceous binders in the same sample.
In detail:

- an HPLC-DAD and LC-MS/MS method was developed for the analyses of anthraquinoid lakes allowing us to separate dyes from the organic binders in a unique paint sample (Chapter 3). Ethylenediaminetetraacetic acid (EDTA) resulted the best extraction agent for our purposes, although the best working conditions varied according to the chemical proprieties of the different dyes analyzed. Alizarin and purpurin shown greater affinity to an organic solvent as dimethylformamide (DMF), and higher recoveries were achieved with H_2O : DMF with 0.1% EDTA as extraction solution and DMF : MeOH as solvent of injection. Carminic acid and laccaic acids, instead, due to their acidity, prefer the aqueous EDTA 0.1% extraction solution and the more polar 85% H_2O : 15% ACN solvent of injection. The final procedure is a compromise and consists in an extraction with 0.1% EDTA solution and a second one using ethylacetate (AcOEt), and allows to separate dyes from the binder getting rather high recoveries and low CVs for all of anthraquinones. The procedure was then successfully tested on anthraquinoid lakes;
- naturally aged and freshly prepared paint model systems were analyzed both by HPLC and by GC/MS in order to evaluate the cross-influence of binder-lake on the analysis of dyes and binders (Chapter 4). HPLC-DAD detected all the lakes in the presence of the different types of binder, and no peaks ascribable to the binders were present in the chromatograms. Nonetheless, analyses on naturally aged paint model systems revealed lower yields of extraction in comparison to those obtained for the freshly prepared ones, probably due to aged and possibly polymerized binding media. The evaluation of lipid, saccharide and amino acid contents of the anthraquinoid raw materials with an already published multistep GC/MS procedure was performed to investigate the contribution of the lake on the identification of the binder. The GC/MS chromatograms highlighted a composition mostly consisting in monocarboxylic acids. The different natural sources of the material analyzed influences the profiles of the raw materials: madder lake, prepared from roots, is mainly constituted by saccharides; Kermes and Armenian cochineal, extracted from the family of *Coccus* insects, are rich in lipids and showed an amount of proteinaceous material above the detection limit; lac dye contained only lipids. Theoretical profiles, calculated for 1:1 mixtures of reference anthraquinoid raw materials and Arabic gum, whole egg, milk and animal glue, were evaluated and the profiles obtained do not appear modified due to the presence of the lakes in the samples. Thus, the presence of the anthraquinoid lakes will not prevent the correct identification of the paint binding medium;
- MALDI and LDI-MS methods for the identification of anthraquinoid dyes and proteinaceous binders were developed and/or optimized at the Department of Biochemistry and Microbiology of ICT in Prague (Chapter 5). An already published MALDI-ToF-MS method for the analyses of proteins using trypsin cleavage has been optimized for paint model

systems introducing some steps for the removal of the lakes. All the proteinaceous binders were positively identified on the basis of their characteristic peptides or fragments in the mass spectra or in their tandem mass spectra, respectively. S/N ratio is highly dependent by the concentration of the proteinaceous binder and by the composition lake/ binder of the sample. Carmine was found to be the only lake with a proteinaceous content, consistently with the results of GC/MS obtained for kermes and Armenian cochineal. Two LDI-ToF-MS methods, one requiring any samples pre-treatment (direct method) and another employing hydrofluoric acid (HF method) were used for analyses of dyes in reference materials and paint model systems and were tested both in positive and in negative mode. The method of election for all dyes was the HF one in positive mode, for madder lake and Indian lac, and in negative mode for carmine. No peak ascribable to any binder was present in the mass spectra, therefore no influence of paint medium on dyes identification was revealed;

- the multi-analytical approach using the techniques applied and optimized in the course of the thesis (MALDI-ToF-MS, LC–MS/MS, LDI-ToF-MS, HPLC-DAD) allowed us to identify the presence of egg in a sample belong to a polychrome Buddha statue and madder lake in wall paintings in Delos and Corinth (Chapter 6).

Summarizing, the optimized analytical procedures allowed the successful analysis of anthraquinoid lakes by HPLC-DAD, LC-MS/MS and LDI-ToF-MS and of proteinaceous binding media by MALDI-ToF-MS. The cross-influence of lake-binder on the analyses of dyes and binders has been evaluated both with chromatographic techniques and desorption ones.

Further analyses have to be carried out in order to improve the database of reference materials and to increase the number of analyzed case studies. Procedures should be further optimized by the study of artificially aged model paint systems. Nonetheless, the results of this thesis are a good starting point for the further development of combined, multi-analytical techniques that allow for maximizing the information obtained while minimizing the amount of sample needed.

Appendix A

Anthraquinoid dyes and lakes

A.1. Natural anthraquinoid dyes of plant and animal origin

Since ancient times, anthraquinoid lakes have been prepared from natural dyes of plant and animal origin.

The vegetal world is full of plants and roots from which mankind, through centuries, has been able to extract dying substances. Antraquinoid dyes can be for instance extracted from roots of different species of *Rubiacee* family [1]. Different species of roots with their common and botanical names, geographical origins and specific composition in terms of their antraquinonoid chromophores containing molecules [2] are shown in Table A.1.

Table A.1: Common names, botanical names and geographical origins of vegetal antraquinoid dyes and specific composition in terms of their chromophores containing molecules (adapted from [2]).

common name	botanical name	origin	composition						
			ali	pu	xa.pu	mun	ps.pu	mor	rub
madder	*Rubia tinctorum L.*	Europe, Middle East, India	X	X	X	X	X	-	-
wild madder	*Rubia penegrina L.*	Mediterranean countries, Mesopotamia	-	X	-	-	X	-	X
munject	*Rubia cordifolia L.*	India, China, Japan, tropical Africa	X	X	X	X	X	-	-
Japanese madder	*Rubia akane Nakai*	Japan	-	X	-	-	X	-	-
chay root	*Oldenlandia umbrellata L.*	India, Burma, Abyssinia, Ceylon, Java	X	-	-	-	-	-	-
galium species	depending on species	Europe, Asia, North and South America, North Africa	X	X	X	-	X	-	-
relbunium	*Rebulnium hypocarpium L.*	South and Central America	-	X	X	X	X	-	-
morinda	*Morinda citrifolia*	South-East Asia	X\-	-	-	-	-	X	-

Antraquinoid dyes contained in vegetal species are only substituted on one aromatic ring [1].

The most famous and commonly used species was *Rubia tinctorum*, a rhizomatous herbaceous perennial plant native of Southern Europe. Alizarin is the principal chromophore containing molecule; purpurin, xanthopurpurin, munjistin, pseudopurpurin, morindone and rubiadin are secondary ones [3]. The detection of purpurin is useful to distinguish natural alizarin from the synthetic one. Wild *Rubia* plant and dried roots are shown in Figures A.1 a and b.

Figure A.1: a) Wild *Rubia* plant [4]; **b)** *Rubia* dried roots [5].

Antraquinoid dyes sources exist also in the animal world and insects from the *Coccidae* family, plants parasites, are the most important ones.
Different species of *Coccus* insects with their common and scientific names, geographical origins and percentages of antraquinonoid chromophores containing molecules [6] are shown in Table A.2.

Table A.2: Common names, zoological names and geographical origins of animal antraquinoid dyes with their % composition (adapted from [6]). (*dcII stands for *Dactylopius coccus* component II [7].)

common name	scientific name	origin	composition (%)
kermes	*Kermes vermilio Planchon*	South Europe, Near Est Turkey	kermesic acid (75-100), flavokermesic acid (0-25),
Polish cochineal	*Porphyrophora Polonica Linneaus*	Middle and East Europe, Germany, Poland, Ukraine	carminic acid (75-100), kermesic acid+ flavokermesic acid (12-38)
Armenian cochineal	*Porphyrophora haemli Brandt*	Armenia, Azerbaijan, Ararat valley	carminic acid (95-99), kermesic acid+ flavokermesic acid(1,0-4,2), dcII (0,1-1,2)
cochineal	*Dactylopius coccus Costa*	Mexico, South America	carminic acid (94-98), kermesic acid+ flavokermesic acid(0,4-2,2), dcII (1,4-3,8)
lac dye	*Kerria Lacca Kerr*	India, Thailand, Cambodia, Sumatra, Moluccas	laccaic acid A (71-96), laccaic acid B (0-20), flavokermesic acid(3,6-9,0)

Antraquinoid dyes contained in animal species are substituted on both aromatic rings [1].
Coccus is a genus of scale insects of *Coccidae* family. There are some important species of *Coccus* as *Kermes vermilio Planchon, Dactylopius coccus Costa* and *Kerria Lacca Kerr*.
Kermes vermilio Planchon female insect does not have any wings and it tends to live on *Quercus coccifera* branches, typical of South Europe. Kermesic acid is the principal anthraquionid

chromophore containing molecule, flavokermesic acid is the secondary one and it is responsible for kermes coloration [3].

Dactylopius coccus Costa or *Coccus cacti* female insect generally lives on *Cactus opunzia* branches typical of Central and South America [8]. Carminic acid is present in all kinds of cochineal although in different concentrations. Kermesic, flavokermesic and *Dactylopius coccus* component II (dcII) acids are secondary anthraquinoid chromophores containing molecules useful to identify *Coccus* species. Cochineal is the only insect dye that contains dcII [7].

Kerria Lacca Kerr produces a resinous substance called stick-lac which covers the eastern plants of *Ficus religiosa*, *Ficus indica and Butea frondosa*. Lac dye is characterized by a mixture of laccaic acids: A and B are the most important [9]. *Kermes vermilio Planchon, Dactylopius coccus Costa* and *Kerria Lacca Kerr* are shown in Figures A.2 a, b and c.

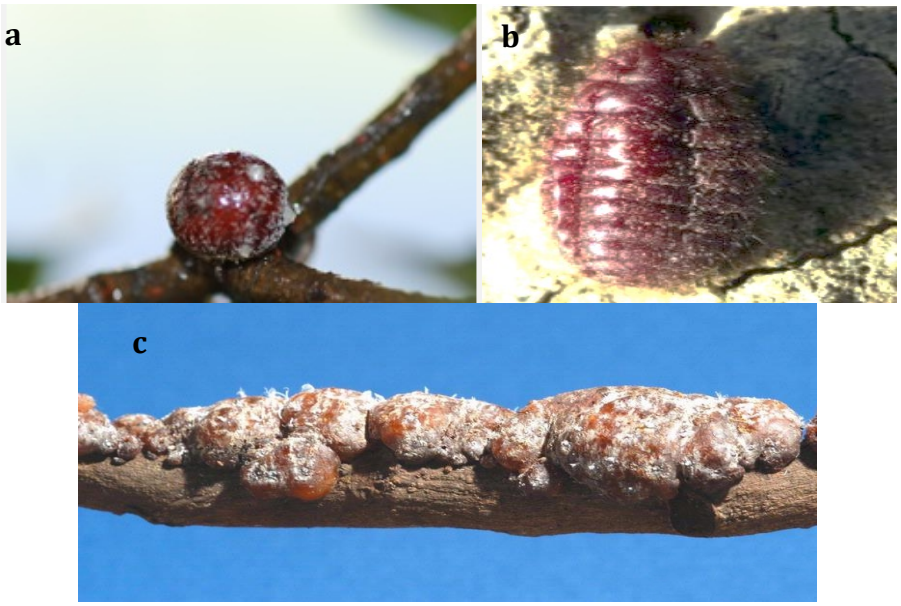


Figure A.2: a) *Kermes vermilio Planchon* insect [10]**; b)** *Dactylopius coccus Costa* insect [11]; **c)** *Kerria Lacca Kerr* colony of insect [12].

A.2 Different kinds of lakes

From natural anthraquinoid dyes it is possible to prepare lakes as explained in Section 1.2.1.2. The most important anthraquinoid lakes used as painting materials are: madder lake, cochineal lake, kermes lake and Indian lac. Features, history, preparation methods and selected remarks are reported below for each lake.

A.2.1 Madder lake

Madder lake has been considered the most beautiful and stable among all lakes, but its preparation is the most difficult. The coloring matter is derived from *Rubia tinctorum* roots. They are red and cylindrical (20-30 cm length, 1.2 cm thick) with a yellow internal layer [7] containing xanthin, a coloring matter soluble in cold water [8].

In Rubia roots dyes are present as glucosides, molecules in which a sugar (glucose) is linked to another functional group by a glycosidic bond. Several plants store glucosides in an inactive form

that are generally activated by enzymeatic hydrolysis. The enzymes are already present in the plant and induce the breaking of the glycosidic bonds: free sugars are therefore obtained in case of need. Rubierythric acid is the principal glycoside of madder and it is separated in glucose and alizarin by the enzyme erytozina [13].
The process of dyeing textiles with Rubia was reported in ancient Arabic, Indian, Greek, Roman and Germanic documents (since 2000 b.C). Some evidences of ancient madder lake applications are Tutankhamon's red belt [14] and Romans' military dresses [15]. Madder lake has been one of the most important textile natural dyes for centuries in the whole Europe. Only with alizarin synthesis, that took place in 1871, the natural red dye was replaced worldwide [3]. In recent years, research has turned its interest to natural madder lake to use it for valuable art crafts and as food colorant. However it has been discovered to be not completely harmless for men because of it contains lucidin, a mutagen compound. Notwithstanding, the aqueous extract is not dangerous [16].
Roots are collected in the third month of the year when the dye percentage is about the 2%, but the concentration and relative amount of the colorant also depend on the origin place and growth conditions [7]. Dye compounds are extracted from roots boiled for at least one day with the occasional addiction of barn (in order to help enzymatic hydrolysis and the release of aglycones) and of basic compounds as sodium carbonate to help the solubilization of the dyestuff. Afterwards, the recipes entail the precipitation on alum, the centrifugation and drying of the lake [9]. Great attention has to be paid to the presence of iron, which is a common impurity in natural alum, because alizarin has a higher tendency to complex it instead of aluminium, giving a darker color in the final lake [3]. In Table A.3, different alizarin lake nuances are reported.

Table A.3: Alizarin lake nuances in relation to the kind of mordant used.

mordant	color
alum	rust red
tin salts	orange
chrome salts	chestnut brown
copper salts	brownish
iron salts	dark brown

A.2.2 Kermes lake

Kermes lake and cochineal lake are often called with the same unspecific name of carmine. Even if they are extracted from different species of *Coccus* insects they have similar chemical proprieties and colors in relation to pH and mordant used. Nevertheless, their composition is different: the presence of kermesic acid in kermes lake instead of carminic acid makes it soluble in ether, ethanol, glacial acetic acid and in cold water, insoluble in benzene and chloroform [17].
Kermes coloring matter is derived from *Kermes vermilio Planchon* eggs. The colorant is extracted when the egg are still inside the insect, which has a two months life span [13].
Kermes lake has been used since prehistory (evidences of dyed textiles have been found in a Neolithic French cave [18]); however kermes dying was mostly used in Middle Age. From the beginning of XVI century, American cochineal became competitive with kermes because of its brighter nuances and higher tinting power, even if it was less durable [3].
The procedure for preparing the lake consisted in: picking up insects during reproductive period, killing the insects by immersion in acetic acid, drying and powdering the corpses, extracting the

colorant thanks to water and alcohol. Afterwards alum is added to precipitate the lake, which is then filtered and left to dry [3].

A.2.3 Cochineal lake

Cochineal coloring matter is achieved from *Dactylopius coccus Costa* or *Coccus cacti* female insects. This dyestuff was already used by pre-Columbian cultures and during colonial period some *Cactus opunzia* plants and their parasites were brought to Europe. *Coccus cacti* was rather economically important and it became the third highest income for New Spain after gold and silver exportations. In the Middle Age Cochineal dying was widely used and substituted the more expensive purple throughout Europe. Mexico was the first producer of cochineal during the colonial period, but, today, the greatest quantity of cochineal is obtained from regions of Peru, Canary Islands, and Chile where cochineal has almost no natural enemies [19]. In the middle of the XIX century synthetic colorants took the place of natural ones because of their lower cost: a large number of insects was required for making lakes with sufficient color strength. Nowadays cochineal is used as colorant in food industry with the name of E120 [20].
The procedure for extracting the colorant and preparing the lake consisted in: scarping off the insects from the bark of the tree during their reproductive period, killing the insects with hot water, drying and powdering them, boiling the powder with potassium carbonate or potassium hydroxide, filtrating, boiling again for several hours, adding alum, precipiting and drying. All this purification and drying steps may be repeated several times [9].

A.2.4 Indian lac

Lac dye is obtained from stick-lac that is a resinous substance produced by *Kerria Lacca Kerr.* The hue of Lac is similar to that of lakes obtained from cochineal and kermes. The color of the dye/lake can be tuned from violet to red and brown by choosing the right mordant [9].
The use of Indian lac was spread in the East and it can be traced back to 250 A.D thanks to Claudius Aelianus evidences [21]. This dye remained widely used until the late 19th century. Beyond textiles dying, indian lac was used as skin cosmetic and nowadays is exploited as food colorant (in juices, carbonated drinks, wines, jams, sauces and candies) and in medicine (in hepatoprotective and anti-obesity drugs) [22].
Kerria Lacca Kerr secrection takes some months to stiffen and, after that, it contains about 10% of dying colorant material [3]. For extracting this one it is necessary to put the stick-lac in boiling water or in acqueous basic solution (Na_2CO_3). The precipitation of indian lac is finally obtained thanks to the addition of alum. The residue of the extraction is called shellac.

A.3. Hypothesis on the structure of the alum based lakes

Anthraquinoid lakes structures, especially the alizarin one, have been studied thanks to X-Ray Diffraction (XRD), Fourier Transform Infrared Spectroscopy (FT-IR), Secondary Ion Mass Spectrometry (SIMS). Structures hypothesized on the basis of the data collected by these techniques are listed below [16].

Alizarin is able to chelate aluminium cation with its phenolic groups, in suitable pH conditions. The phenolate groups chelate the metal ion by forming a highly stable structure. Some questions are still open about the number of alizarin functional groups involved in complexation, which causes differences in proprieties and coloration of the lake. Several different structures have been hypotesized for alizarin lake during last century and a brief history about them is herein reported [16].
In 1893 Liebermann suggested the presence of bonds between aluminium and two deprotonated phenolic groups of alizarin [23]. This structure is shown in Figure A.3.

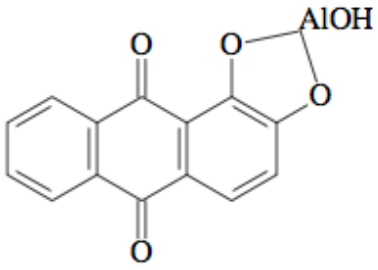


Figure A.3: Alizarin structure proposed by Liebermann [23].

In 1911 Pfeiffer tried to obtain a reaction between tin tetrachloride ($SnCl_4$) and alizarin in a benzene solution and the resulting elimination of hydrochloric acid implied a hydroxyl deprotonation. Anyway the hydroxyl group in C-1 position is less acid than the one in C-2 because of the hydrogen bond stabilization with the carbonyl group of the other ring. The addition of a basic compound permits to salify the hydroxyl group in C-1, thus $SnCl_4$ is able to break the hydrogen bond and to create a complex with alizarin [24]. The structure, which has been hypothesized, is shown in Figure A.4.

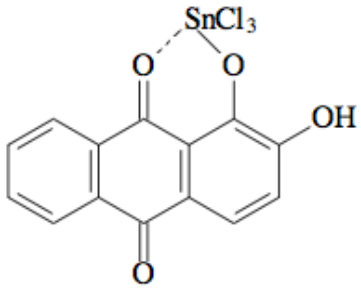


Figure A.4: Alizarin structure proposed by Pfeiffer [24].

In 1937 Hoffmann proposed a new structure based on studies performed by the precipitation of the lake in pyridine. A complex of $(Al(aliz)_2)^+$ is bonded to three calcium ions with O-Ca-O- and Al--OH—Ca--OH—Al bonds [25]. Two pyridine and seven water molecules completed the formula. In 1940 Fierz- David and Rutishauer suggested a similar structure with only one Al-O-Ca bond per each aluminium atom [26]. The two structures are shown in Figures A.5 a and b.

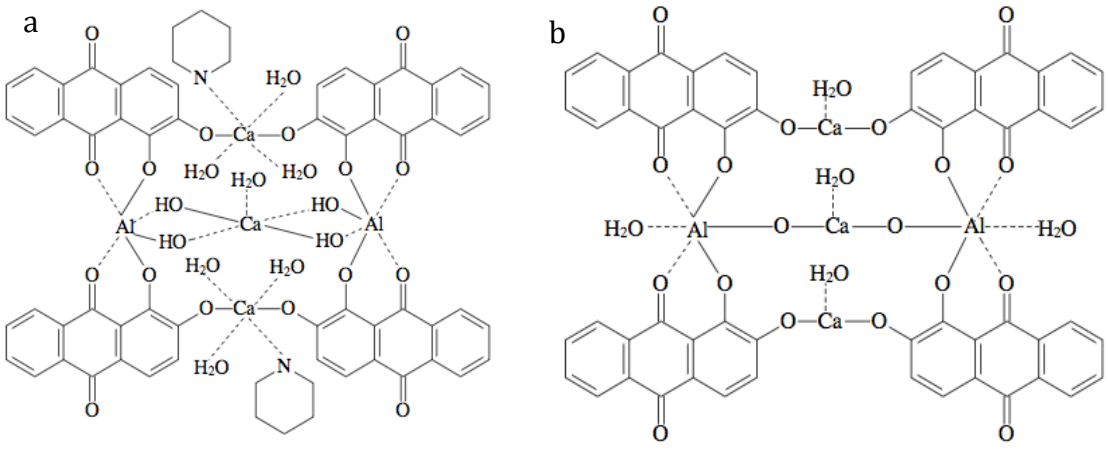


Figure A.5: a) Alizarin structure proposed by Hoffmann [25]; **b)** Alizarin structure proposed by Fierz- David and Rutishauer [26].

These hypothesis did not convince Kiel and Heertjees who in 1963 suggested $CaAl(OH)(aliz)_2.xH_2O$ formula whose stoichiometry is Al:2aliz:Ca. Covalent coordination bonds fix the aluminium atom to a hydroxyl ion and to two deprotonated alizarin molecules through carbonyl groups and oxygen in C1 position. Aluminium interacts also with water molecules and there is an ionic bond between calcium and the deprotonated oxygen in C2 position [27]. A variable number of coordination water molecules is present. The structure is displayed in Figure A.6.

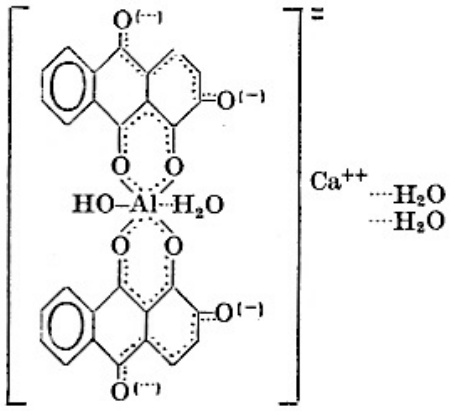


Figure A.6: Alizarin structure proposed by Kiel and Heertjees [27].

This model has been considered valid for many years and only in the last decade of the 20th century Soubayrol [28] and Wunderlich [29] proposed two other possibilities on the basis of X-ray analyses. These two structures are shown in Figures A.7 a and b.

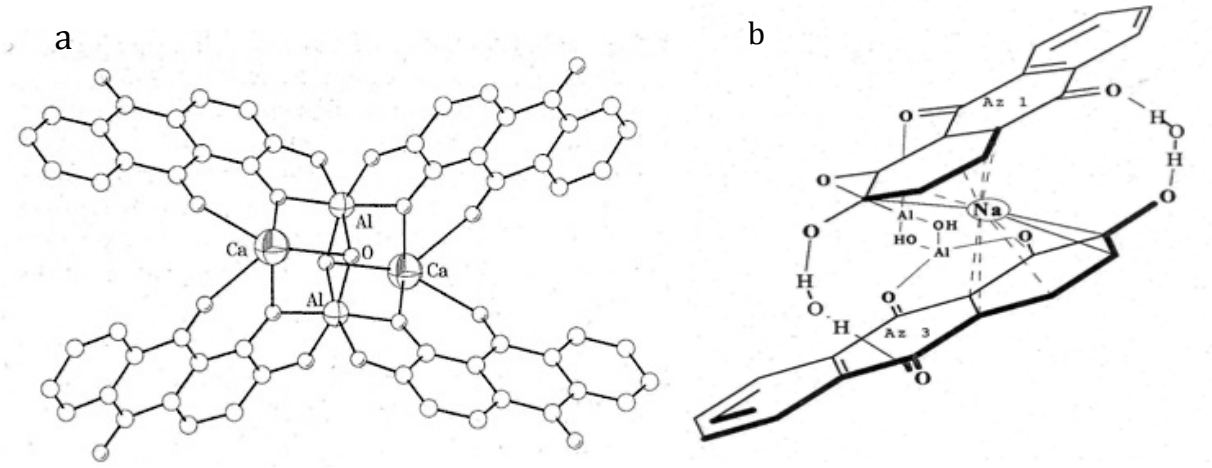


Figure A.7: a) Alizarin structure proposed by Soubayrol [28]; **b)** Alizarin structure proposed by Fierz- David and Wunderlich [29].

Bibliography

(1) I. Degano, *A Multiple-Analytical Approach for the Characterisation of Organic Dyes in Textiles*- ph. D thesis in Chemical Science, Università degli studi di Pisa, Facoltà di Scienze Matematiche, Fisiche e Naturali, Dipartimento di Chimica e Chimica Industriale, 2009.

(2) J. Hofenk de Graaf*, The Colorful Past: Origins, Chemistry and Identification of Natural Dyestuffs*, Abegg-Stiftung an Archetype Publications Ltd., 2004.

(3) E. Martuscelli, *I Coloranti Naturali nella Tintura della Lana Arte, Storia, Tecnologia e 'Archeo-Materials Chemistry'*, Programma Nazionale di Ricerca Beni Culturali (MIUR) La Conservazione dei Tessuti Antichi, vol. I.

(4) *Monte Linas*, http://www.montelinas.it/florasarda/283-robbia-selvatica.

(5) *Etsy*, http://www.etsy.com/madder roots natural dye.

(6) E. Rosemberg, *Characterization of Historical Organic Dyestuffs by Liquid Chromatography-Mass Spectrometry, Analytical and Bionalytical Chemistry*, vol. 391, pp. 33-57, 2008.

(7) J. Wouters, *L'analyse de Colorants Organiques Naturels par Chromatographie Liquide haute performance et Traitement des Donnèes par Ordinateur*, Pigments and Colorants, pp. 331-338, 1990.

(8) J. Napier, A Manual of the Art of Dyeing, ed. Griffin & Co, Glasgow, 1853.

(9) M. Rosato, *Caratterizzazione delle lacche rosse in manufatti pittorici antichi*, tesi di laurea, Facoltà di Scienze matematiche, fisiche e naturali, corso di laurea in Chimica, Università degli Studi di Pisa, 1999-2000.

(10) J. Kirby, M.Van Bommel, A. Verhecken, *Natural Colorants for Dyeing and Lake Pigments, Practical Recipes and their Historical Sources*, Archetype Publications, 2014.

(11) *Gidateroru*, http:// gidateroru.tr.gg.

(12) *Forestry Images*, http://www.forestryimages.org.

(13) M. Brito-Arias, *Synthesis and Characterization of Glycosides*, Spinger 2007.

(14) R. M. El- Shishtawy, G.M. Shokry, N.S.E. Ahmed, M.M. Kamel, *Dyeing of Modified Acrylic Fibers with Curcumin and Madder Natural Dyes*, Fibers and Polymers, vol. 10, no. 10, pp. 617-624, 2009.

(15) C.A. Giner, *Purpureae Vestes I. Textiles y Tintes del Mediterráneo en Epoca Romana*, University of Valencia, 2004.

(16) J. Sanyova, *Contribution a' L'étude de la Structure et des Propriétés des Laques de Garance,* Université libre de Bruxelles, Faculté des Sciences Appliquées, Service de Chimie Organique, pp. 200, 2001.

(17) H. Schweppe, H Roosen-Runge, *Carmine- Cochineal Carmine and Kermes Carmine,* Artists' Pigment-A handbook of their Hystory and Characteristic, Cambridge University press, vol. 1, 1986.

(18) M.L. Vázquez de Ágredos Pascual, M.T. Doménech Carbó, D.J. Yusá-Marco, S.V. Palomino, L. Fuster López, *Kermes and Cochineal; Woad and Indigo. Repercussions of the Discovery of the New World in the Workshops of European Painters and Dyers in the Modern Age,* Archè Publicacion del Instituto Universitario de Restauration del Patrimonio de la UPV, 2, 2007.

(19) L. Portillo, A. L. Vigueras, *Natural Enemies of Cochineal (Dactylopius coccus Costa): Importance in Mexico*, Departamento de Botánica y Zoología, Universidad de Guadalajara, Mexico, 1998.

(20) N. Sugimoto, Y. Kawasaki, K. Sato, H. Aoki, T. Ichi, T. Koda, T. Yamazaki, T. Maitani, *Structure of Acid-Stable Carmine*, Journal Food Hygiene Society Japan, vol. 43, no. 1, pp. 18-23, 2002.

(21) C. Aelianus, *De Natura Animalium*.

(22) A. Flinn, *Shellac & Food Glaze*, Gentle World, 2011.

(23) C. Liebrmann, *Ueber Eine Neue Synthese der Allozimmtsäur*e, Berichte der deutschen chemischen Gesellschaft, vol. 26, no. 2, pp. 1571-1574, 1893.

(24) P. Pfeiffer, Zur Kenntnis des Farblacke I, *Berichte der Deutschen Chemischen Gesellschaft* , vol. 44, no. 3, pp. 2653-2670, 1911.

(25) E. Hoffmann, *Ueber die Konstitution des Turkischrotlackes*, thesis, Dresden, 1937.

(26) M. Rutishauser, *Zur Kennrnis des Alizarinlacke*, thesis, Zurich, 1940.

(27) E. G. Kiel, P.M. Heertjes, *Metal Complexes of Alizarin I- The Structure of the Calcium Lake of Alizarin*, Journal of the Society of Dyers and Colorist, vol. 79, pp. 21-27, 1963.

(28) P. Soubayrol, *Préparation et Eude Structurale des Complexes Formés Entre l'Aluminium et l'Alizarine*, thèse de doctorat à l'université Pierre et Marie Curie, Paris, 1996.

(29) C.H. Wunderlich, G. Bergerhoff, *Konstitution und Farbe von Alizarin- und Purpurin- Farblacken, Chemische Berichte*, vol. 127, 7, pp. 1185-1190, 1994.

Appendix B

Acid-stable carmine (dcIII) synthesis and characterization

A reaction with ammonia was suggested in order to explain some unexpected peaks present in the HPLC chromatograms and shapes of UV-Vis spectra of anthraquinoid dyestuffs treated with ammonia as extraction solution. Therefore acid stable carmine (dcIII) was synthesized to verify if nitrogen containing groups were added to carminic acid, as suggested in some papers. In literature, a procedure for dcIII synthesis is reported and entails the following steps[6]:

- 0.30 g of carminic acid and 0.36 g of citric acid are mixed in 1 mL of 25% NH_3 solution;
- the solution is heated for 70 min at 115-120 °C;
- the solvent is evaporated until dryness.

In Figure B.1 the scheme of reaction and 4-aminoanthraquinone, the most plausible product of the treatment explained above, are shown.

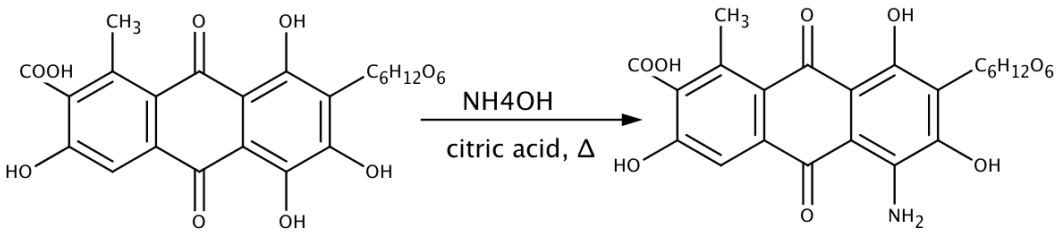


Figure B.1: Scheme of reaction (carminic acid on the left and 4-aminoanthraquinone on the right) [1].

NMR analyses reported in literature suggest the identification of the product of reaction (dcIII) as 4-aminoanthraquinone thanks to the values of the chemical shifts. In this compound one hydroxyl group of carminic acid is replaced by an amino group. The structure reported is the most plausible although the unambiguous determination of the specific product is not possible. The position of the replacement of OH with NH is not unequivocally defined because of the several functional groups of the carminic acid.

The compound synthesized was dissolved in 500 µL of 85% H_2O: 15%ACN and 20 µL and 2 µL of the solution were injected in HPLC-DAD and LC-MS/MS, respectively.

(1) [6] N. Sugimoto, Y. Kawasaki, K. Sato, H. Aoki, T. Ichi, T. Koda, T. Yamazaki, T. Maitani, *Structure of Acid-Stable Carmine*, Journal Food Hygiene Society Japan, vol. 43, no. 1, pp. 18-23, 2002.

The HPLC-DAD chromatogram acquired at 275 nm of the product obtained with UV-Vis spectra of peaks at 10.1 min and 13.7 min and the LC/MS-MS chromatogram are shown in Figures B.2 a and b respectively.

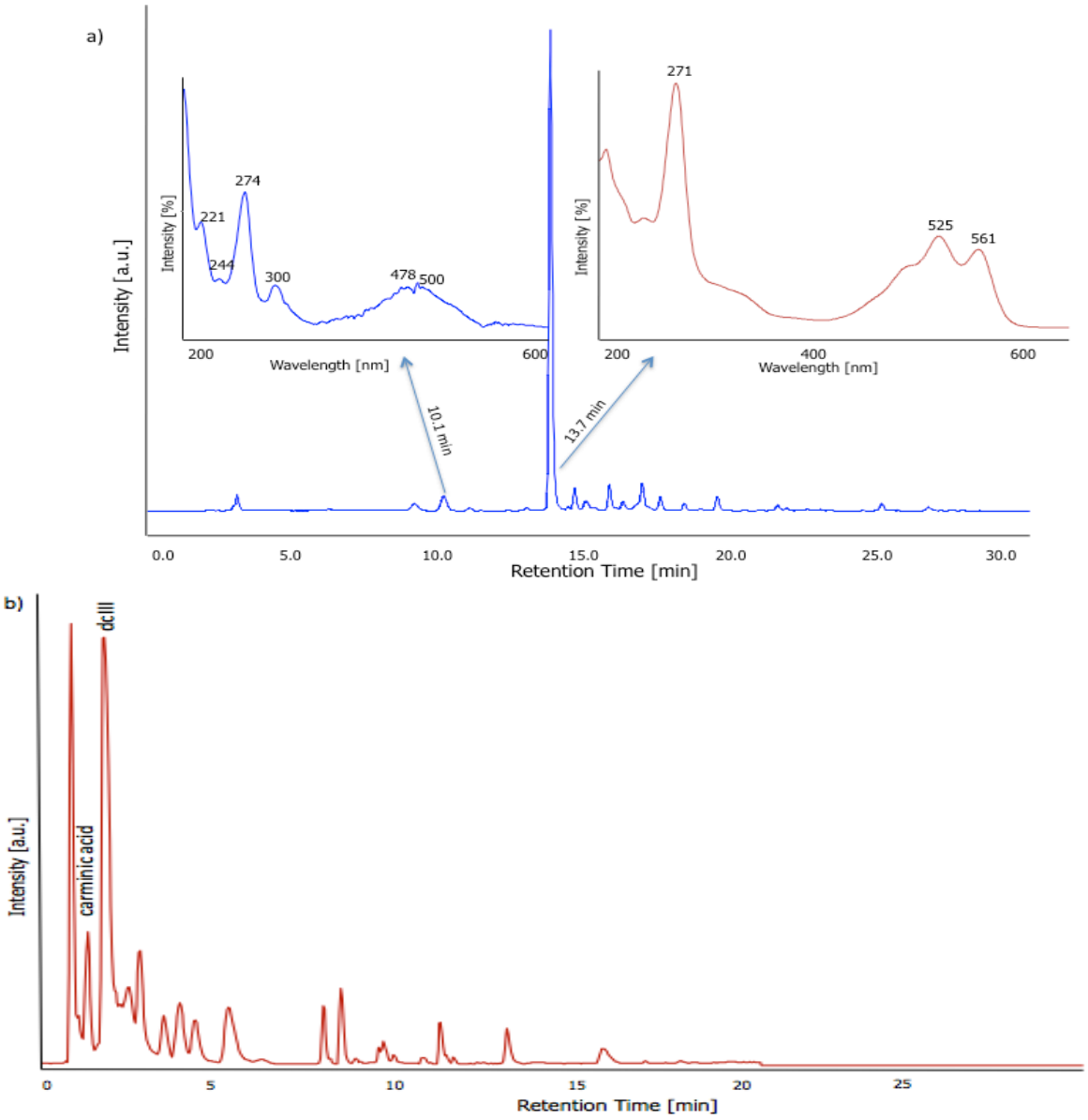


Figure B.1: a) HPLC-DAD chromatogram acquired at 275 nm of the product synthesized with UV-Vis spectra of peaks at 10.1 min and 13.7 min and **b)** LC/MS-MS chromatogram**.**

In the HPLC-DAD chromatogram a peak is present in correspondence to the retention time of carminic acid (10.1 min) but the highest peak is at 13.7 min. Comparison of the UV-Vis spectra with the reference ones allows the identification of acid carminic and dcIII respectively in the chromatogram. Some other small peaks are present in the range from c.a. 15.0 min to c.a. 19.8 min and their UV-Vis spectra would suggest other aminated forms. This hypotheses are confirmed by the LC/MS-MS chromatogram characterized by a peak at 1.317 min with m/z=491, pseudomolecular ion of carminic acid, and a very high peak at 1.79 min with m/z=490, pseudomolecolar ion of dcIII. From 1.791 min to 2.786 min other peaks with m/z=490 are present and the hypothesis of further aminated forms could be confirmed.

Appendix C

Synthesis of alizarin lake

Alizarin lake was synthesized and analyzed in order to investigate the low recoveries obtained for madder lake. In literature a procedure for alizarin lake synthesis is reported and entails the following steps[7]:

- 120 mg of alizarin are dissolved in 250 mL of bidistilled water (pH=4.4);
- the solution is submitted to ultrasonic bath for 10 min;
- 25 mL of an aqueous solution containing 2.37 g of $KAl(SO_4)_2$ $12H_2O$ (pH=2.8) is added to alizarin solution (Al/Alizarin=10);
- the solution is mixed at room temperature for 10 min;
- the solution is neutralized with an aqueous solution of K_2CO_3 0.1 M and left overnight to precipitate the resulting orange-red solid;
- the solution is filtrated;
- the precipitate is washed with acetone and bidistilled water and dried in the dessicator in the dark for three days.

Two steps of the alizarin lake preparation are shown in Figure C.1 a and b.

Figure C.1: a) Precipitation of the resulting orange-red solid and **b)** filtration.

[7] J. Sanyova, *Contribution a' L'étude de la Structure et des Propriétés des Laques de Garance,* Université libre de Bruxelles, Faculté des Sciences Appliquées, Service de Chimie Organique, pp. 200, 2001.