Topics in Current Chemistry

Trends in HPLC for Cultural Heritage

--Manuscript Draft--

Manuscript Number:	TOCC-D-15-00005R1
Full Title:	Trends in HPLC for Cultural Heritage
Article Type:	T.C.: Analytical Chemistry for Cultural Heritage
Funding Information:	
Abstract:	The separation, detection and quantitation of specific species contained in a sample in the field of Cultural Heritage requires selective, sensitive and reliable methods. Procedures based on liquid chromatography fulfil these requirements and offer a wide range of applicability in terms of analyte types and concentration range. The main applications of High Performance Liquid Chromatography in this field are related to the separation and detection of dyestuffs in archaeological materials and paint samples by reversed-phase liquid chromatography with suitable detectors. The relevant literature will be revised, with particular attention to sample treatment strategies and future developments. Reversed phase chromatography has also recently gained increasing importance in the analysis of lipid binders and lipid materials in archaeological residues: the main advantages and disadvantages of the new approaches will be discussed. Finally, the main applications of ion chromatography and size exclusion chromatography in the field of Cultural Heritage will be revised in this chapter.
Corresponding Author:	Ilaria Degano, PhD Università di Pisa Pisa, ITALY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Università di Pisa
Corresponding Author's Secondary Institution:	
First Author:	Ilaria Degano, PhD
First Author Secondary Information:	
Order of Authors:	Ilaria Degano, PhD Jacopo La Nasa, PhD
Order of Authors Secondary Information:	
Author Comments:	Dear Prof. Mazzeo, Please find attached the revised version of the manuscript, "Trends in HPLC for Cultural Heritage", which I submitted to Topics in Current Chemistry. Accordingly to the reviewer's comments, in this new version the section on Ion Chromatography was improved. New references were added and the structure and selection of the topics of our paper were clarified, by adding the following paragraph: "In the field of Cultural Heritage related studies, IC has been mainly used as a robust routine technique to quantify inorganic anions and cations, and small organic acids in a plethora of samples. Few examples will be provided in this chapter of possible applications of this technique, focussing on a few classic case studies and presenting some new developments." We hope that the paper is now suitable for publication on ToC. Best regards Ilaria Degano
Response to Reviewers:	Reviewer #1: Manuscript focuses on revision of relevant literature on applications of HPLC for Cultural Heritage conservation. - First of all, Authors are suggested to review carefully the references section, as frequently there is not correspondence between the citations in the text - [number] -

	and the paper indicated in the reference section. For example in section 3 "Ion Chromatography" citations [49] and [50] are not correct. A - We thank the reviewer for his careful checking of the references. Indeed some errors in numbering occurred and we have now amended them in the revised version of the manuscript. - My major comments relate to section 3 on Ion Chromatography and are firstly linked to the fact that the use of this technique in the field of Cultural Heritage has larger application than the measurement of inorganic salts in damage layers and/or black crusts (for example damage due to rising damp on masonries). Additionally, the application of this technique for particulate matter characterisation cannot be discussed with just one sentence and without any reference (as done at page 7, line 10). It is fully understandable that in one paper all the aspects cannot be deeply developed: my suggestion to the Authors is to clearly define the framework of the topic that they want to discuss and present. A - We reckon that the use of IC in the Field of Cultural Heritage has a wide range of applications. Still, it is used as a routine technique in most cases: thus, we decided to present only representative case studies or some particular applications. The paper is in fact titled “Trends in HPLC” and not “HPLC methods”. In any case, more case studies dealing with the assessment of the causes of rising damp, and on the characterisation of particulate matter, were added and the paragraph was fully re-organized. The changes are marked in red in the manuscript. - It is not clear why Figure 6 (OC vs. oxalates) has been included: did the Authors want to show a relation between OC and oxalates? There is no connection with the sentence in the text, which concerns OC and EC. In addition nothing is discussed about the origin of these carbon fractions. I must also say that figures with new elaboration of data already presented in published papers are preferable than inclusion of figures already published. A - We remove the graphs in order to improve the readiness of the paper. - The section concludes with comments on air samples analyses: is the paper focusing on damage products analyses, environment characterisation or both? Being a "review manuscript" the selected points under discussion should be developed and the relating papers exhaustively cited. Summarizing, the paper will benefit in my opinion of a more focused structure and selection of the topics under discussion. A - We added some information on the characterisation of environment by citing proper literature. The paragraph was fully revised in order to fulfil the reviewer’s suggestions.
Suggested Reviewers:	Maarten van Bommel, dhr. prof. dr. ing. Professor of Conservation Science, Universiteit van Amsterdam M.R.vanBommel@uva.nl He's a renown expert in the analysis of dyes by High Performance Liquid Chromatography and was one of the first to apply UPLC to cultural heritage studies
	Klaas Jan van den Berg, PhD Researcher, Neatherland Institute for Cultural Heritage (ICN) klaas.jan.vd.berg@icn.nl Expert in the analysis of TAGs by HPLC-MS
	Alessandra Bonazza, PhD Researcher, Consiglio Nazionale delle Ricerche A.Bonazza@isac.cnr.it She is an expert in the analysis of inorganic deposit on historical buildings by HPLC

Trends in HPLC for Cultural Heritage

Ilaria Degano and Jacopo La Nasa

University of Pisa, Department of Chemistry and Industrial Chemistry
Via Moruzzi, 13, I-56124 Pisa (Italy)
ilaria.degano@unipi.it, jacopo.lanasa@for.unipi.it

Abstract

The separation, detection and quantitation of specific species contained in a sample in the field of Cultural Heritage requires selective, sensitive and reliable methods. Procedures based on liquid chromatography fulfil these requirements and offer a wide range of applicability in terms of analyte types and concentration range. The main applications of High Performance Liquid Chromatography in this field are related to the separation and detection of dyestuffs in archaeological materials and paint samples by reversed-phase liquid chromatography with suitable detectors. The relevant literature will be revised, with particular attention to sample treatment strategies and future developments. Reversed phase chromatography has also recently gained increasing importance in the analysis of lipid binders and lipid materials in archaeological residues: the main advantages and disadvantages of the new approaches will be discussed. Finally, the main applications of ion chromatography and size exclusion chromatography in the field of Cultural Heritage will be revised in this chapter.

Keywords
HPLC, Ion Chromatography, Size Exclusion Chromatography, RP-HPLC, black crusts, polymers in conservation, dyes, lipid binders

Acknowledgements

The authors graciously acknowledge the National project “PRIN 2010-2011”: Sustainability in Cultural Heritage: from diagnosis to the development of innovative systems for consolidation, cleaning and protection” granted by MIUR for financial support. They would also like to acknowledge their colleagues at University of Pisa professor Erika Ribechini and professor Francesca Modugno for the fruitful discussions.

Contents

1 Introduction

The separation, detection and quantitation of specific species contained in a sample in the field of Cultural Heritage requires selective, sensitive and reliable methods. The ability of a technique or protocol to provide information on single components of a sample, being them ions, small molecules or polymers, is of paramount importance when dealing with complex and unknown mixtures of organic and inorganic species, such as paint or archaeological samples. Moreover, analytical techniques generally need to be suitable for coupling with detectors, such as diode array spectrophotometers and mass spectrometers, which allow to run not only quantitative, but also qualitative analyses.

Liquid chromatography is a separation technique in which the mobile phase is a liquid and the elution is in the majority of cases carried out in a column [**1**]. The systems for liquid chromatography consist in a column containing a support and a stationary phase, and a pump for solvent delivery. For analytical purposes, an injection and a detection system are included. The only requisite for an analyte to be analysed by liquid chromatography is its solubility in a proper liquid solvent: thus, several materials present in Cultural Heritage samples can be analysed by this mean. In order to separate two or more analytes, they must exhibit a different retention, that depends on their interactions both with the stationary and the mobile phase. Small particles are used as stationary phase to enhance the differences in retention of the analytes, and thus to increase the efficiency of the columns. High performance liquid chromatography (HPLC) is the modern liquid-phase chromatography technique based on the use of small particles and high pressures. In the late 80s, 5 µm particles (diameter) were developed; the newest applications entail the use of 1.8 µm particles, which may be employed to perform Ultra-High Pressure Liquid Chromatography (UPLC, if pressures are higher than 400 bar). The main processes underneath separations in liquid chromatography are: adsorption, partition, ion exchange, size exclusion and affinity. In the field of Cultural Heritage studies, the great majority of applications of HPLC are based on partition (in particular, reversed-phase chromatography). RP-HPLC has been widely applied in the last 20 years to the characterisation of organic dyes: the identification of dyeing sources is usually achieved by comparing the chromatographic profiles of extracts of unknown samples to those obtained for known dyeing sources. Being most of the chromophores-containing molecules polar, water-soluble compounds, RP-HPLC is the method of choice for the analysis of natural dyes. The application of liquid chromatography, most often coupled to diode array detectors (HPLC-DAD) has lately become a routine strategy for the characterization of the colour palette in tapestry and textiles, and for the detection of organic lakes in paintings. The latest research trends today are mainly focused on sample preparation strategies, to the employment of MS based techniques for the detection and identification of unknown species, and on the application of Ultra HPLC.

Ion exchange chromatography has also been used in the field of Cultural Heritage, mainly as a step of a routine protocol aimed at identifying specific properties of particulates and deposits.

Size exclusion chromatography has also been used in some peculiar case studies, which will be reviewed in this Chapter.

Table 1 lists the main type of chromatographies described in the literature in the field, along with the type of samples analysed, and the most common detectors. Each type of chromatography will be discussed in detail in the following paragraphs.

2 Reversed-phase chromatography

Reversed-phase chromatography is the most used mode in HPLC analysis of organic molecules (RP-HPLC). The separation is prominently based on the relative hydrophobicity of the solutes, being the stationary phase made of low polarity packings such as octadecylsilane or octylsilane phases bonded to silica or neutral polymeric beads. The mobile phase is usually water and/or water miscible solvents, such as alcohols or acetonitrile [**1**]. Mobile phase modifiers include acid or alkaline buffers, depending on the nature of the analyte. Several detectors may be coupled with RP-HPLC, the spectrophotometric ones being the most common (UV-Vis, DAD, Fluorescence). Mass spectrometry can be coupled to RP-HPLC separations, mainly by ESI or APCI interfaces [**2**]. RP-HPLC is applied in the field of Cultural Heritage related studies in four main fields:

- analysis of natural and synthetic dyes;
- analysis of lipids in archaeological samples or used as paint binders;
- proteomics;
- analysis of molecular markers in archaeological residues (e.g. wine residues, etc.).

In this chapter, we will review the main applications in the fields of natural and synthetic dyes analyses and in lipidomics applied to Cultural Heritage. Being the great majority of applications related to the qualitative and quantitative analysis of dyes, a brief paragraph on their characteristics will be included.

2.1 Natural and synthetic dyes

Natural organic colorants were used to prepare inks and lakes and most of all to dye fabrics. They can be classified accordingly to the application method, i.e. as mordant, direct or vat dyes, or to their chemical classes, as reported in Table 2. Synthetic dyes and pigments are classified in the Colour Index, a reference record for all the commercial dyes and pigments, created in 1924 by the Society of Dyers and Colourists. The pigments are recorded by a generic name and a number, being their chemical classes too numerous to be listed [**3**].

The relatively small amount of samples available, the low concentration of molecular markers in the original material, the presence of possible degradation products, and the lack of information on the original recipes are the main analytical problems that challenge the analyst in the characterization of organic dyes in works of art by micro-destructive techniques [**4**]. Generally, the identification of the dye source is based on the identification of molecular markers, followed by a qualitative and semi-quantitative comparison of analytical results with the profiles of known reference materials. Reversed-phase liquid chromatography is the method of choice for the analysis of natural dyes, because most of the chromophores-containing molecules are polar, water-soluble compounds. The application of liquid chromatography to the analysis of natural dyestuffs has been reviewed in detail in [**5**], [**6**], and [**7**]. The 2014 review contains a complete overview of latest sample treatments and analysis methods [**8**].

The latest applications mainly focused on innovative sample preparation strategies, to the employment of novel detection techniques (in particular, MS based techniques), on the characterization of previously unreported dye sources or of minor components of known raw materials, which may help in their unambiguous identification in historical samples. With regard to synthetic dyes, organic pigments were in most cases analysed by pyrolysis coupled with GC/MS (Py-GC/MS) [**9**] [**10**] being insoluble in most solvents. Only in selected cases RP-HPLC, mainly coupled with ESI-MS^2, was used for their study [**11**] [**12**]. On the other hand, synthetic organic dyestuffs were analysed by RP-HPLC, either with DAD or MS detection [**13**] [**14**] [**15**].

Chromatographic conditions entail the use of a reversed-phase C18 or C8 (or even C4) column; to separate the most polar coloured compounds, such as phenolic acids contained in tannins, polar embedded reversed-phase columns such as RP-amide were tested in a few cases [**16**]. The most commonly used eluents are water, acetonitrile, and methanol. Acetonitrile has important advantages over MeOH, including lower absorbance in the 200–275 nm range, lower UV cut-off, and lower back-pressure due to lower viscosity [**17**]. Elution takes place in gradient mode, with a constant percentage of an acid, used to reverse

the dissociation of analytes, most of which have acidic groups [**17**]. The nature and amount of mobile phase modifier is chosen not only on the basis of optimal peak shape and thus resolution, but also depending on the detection system adopted. Formic (FA), trifluoroacetic (TFA) and phosphoric acid are the most used modifiers in the analysis of natural dyes. In a few cases, methanesulfonic acid (MSA) was used to improve resolution of indigoid compounds [**18**]. Amongst these, only MSA and FA can be reasonably used when exploiting a mass spectrometric detection. FA is indeed the most used for LC-MS analyses.

Most separations are performed on 150 x 4.6, 5 µm particle size columns. The use of narrowbore columns can result in increased peak heights for analyzed compounds [**19**], [**18**], but their application never gained much importance in the field. UHPLC was successfully applied recently [**20**], [**21**], [**22**], showing the potentialities of this technique in this field. Owing to instrument improvements, UHPLC allows the use of sub-2 µm porous particles, while applying extreme pressures, faster flow rates and resulting, eventually, in shorter runtimes. As a consequence, lower solvent consumption and faster analyses can be achieved, while maintaining an high chromatographic efficiency. Further details on the three published applications of UHPLC to the analysis of natural dyes are reported in Table 3.

With regard to the sample treatments, most recent papers deal with the optimization of extraction conditions by comparison of different methods. The range of chemical classes exploited as coloring materials being surprisingly broad, the molecular markers of each dye differ in terms of solubility and reactivity in acidic and basic media [**7**]. In order to take into account these properties, distinct extraction procedures have been optimized for different classes of chromophores. Recent research deals with the quest for an optimal strategy to extract all type of dyestuffs from a textile, painting or ink sample. A comparative investigation of hydrolysis methods to analyze natural organic dyes by HPLC-PDA on 12 biological sources selected for the preparation of dyed fibers, pigments and paints is described in [**23**].The same type of study was performed on 6 dyestuffs in woolen textiles by Manhita and co-workers [**24**]and on 6 dyestuffs by Karapanagiotis and co-workers [**25**], while Sanyova proposed a new mild procedure for anthraquinone-based organic lakes [**26**]. For the analysis of inks on paper, specific sampling and extraction procedures were developed, such as the direct application of a brush imbibed with SDS [**27**].

Extraction methods can be divided into three main classes: hydrolysis in an acidic (for HCl) methanolic solution; application of complexation agents such as HF [**28**], [**26**], formic [**29**], oxalic [**30**], trifluoroacetic, acetic, EDTA (“mild extraction methods”); extraction with organic solvents such as pyridine, dimethylsulfoxide (DMSO), or dimethylformamide (DMF) [**5**]. Hydrolysis in acidic methanolic solution and the application of complexation agents is most often used in case of mordant dyes and organic lakes. The extraction of brominated indigoids, known molecular markers of purple, which are vat dyes, and of carthamin, the molecular marker of safflower, is mainly performed by an organic solvent such as DMF or DMSO. Moreover, being these latter analytes sensitive to acidic environments, the use of acids must be avoided if possible. The extractions take place at elevated temperatures, generally ranging between 40 and 120 °C, for a short time for labile dyes and up to one hour for the mordant dyes. In few cases the reaction is performed in an ultrasonic bath [**7**]. If HCl or HF is used, the extract is cooled and evaporated to dryness under vacuum or under nitrogen flow and redissolved in a small volume of the eluent, water-methanol mixture, DMF or DMSO (also in mixtures) depending on the nature of the analytes. The main difference between the use of the strong methods and the mild ones lays in the nature of the extracted compounds. In particular, the application of strong conditions lead to the breaking of glycosidic bonds [**30**], the decarboxylation or dehydration of molecular markers, the esterification of phenolcarboxylic compounds [**7**]. Moreover, the hydrolysis results in the complete disruption of the samples, leading in some cases to severe matrix effects, which may be overcome by introducing a liquid/liquid extraction step with a suitable solvent, such as ethylacetate [**31**], [**32**].No method of election has been identified yet, which is optimal for all the investigated materials. A comparison between different sample treatments applied to the extractions of madder components from reference lakes or textiles, reported in recent papers, is presented as an example in Figure 1.

2.2 Lipids

Vegetable oils consist of mixtures of triacylglycerols (TAGs), glycerol tri-esters of fatty acids. Their identification has proven valuable to establish the painting technique when oils are used as paint media, or to investigate ancient diet and pharmaceutical ointments when they are found as residues in potsherds. Reverse phase high pressure liquid chromatography is the preferred chromatographic approach for the separation of complex mixtures of triglycerides in oils, using several detectors including UV spectrophotometers, refractive index detectors, and mass spectrometers [**33**] [**34**] [**35**] [**36**]. Notwithstanding, only few applications of HPLC to the characterization of lipid materials in paints or archaeological materials have been published, mainly due to the difficulty to detect surviving TAGs after centuries of ageing, even in museum environments.

Mass spectrometry detection is fundamental for TAG profiling, since RP-HPLC is generally unable to separate the positional isomers of TAGs: mass spectrometry detects TAGs, and also identifies positional isomers [**37**] [**34**]. Both atmospheric pressure chemical ionization (APCI) mass spectrometry and electrospray ionization (ESI) tandem mass spectrometry have been successfully used for the differentiation of TAG positional isomers on the basis of their fragmentation patterns [**38**] [**39**] [**40**] [**41**] [**42**] [**35**] [**37**]. An example of APCI-MS spectrum obtained for 1,3-distearoyl-2-oleoyl glycerol (SOS) is presented in Figure 2.

In the field of archaeological materials characterisation, HPLC-APCI-MS was successfully applied for the study of the lipid fractions of residues extracted from late Roman cooking pots, along with the more traditional GC/MS approach [**39**]. The presence of beeswax in the residues was proven by identification of the constituting alkanes, mono and diesters. The main contribution of HPLC-APCI-MS was the detection of high amounts of saturated triacylglycerols (TAGs) that indicated that animal fat was processed in the pots. The lipid extracts were obtained by extraction with *n*-heptadecane and analysed using a reversed-phase column of the type Supelcosil LC18 (25 cm ×4.6 mm, Supelco) with a gradient of methanol and *iso*-propanol as mobile phase. The same approach was used in the study of residues in ancient oil lamps found at the archaeological site of Sagalassos (South-West Turkey) [**42**]. HPLC-APCI-MS allowed the detection of surviving TAGs whose nature and profile suggested that olive oil was used as illuminant for the archaeological oil lamps. The presence of relative high amounts of multiply unsaturated TAGs and traces of saturated TAGs suggested that also other oils and animal fat were added.

More recently, extracts of archaeological potsherds from the same archaeological site were analysed by three complementary chromatographic and mass spectrometric procedures. GC/MS, GC-IR-MS, HPLC-APCI-MS were used and led to the identification of animal fat. The samples for liquid chromatography were extracted with chloroform/methanol and subjected to silylation derivatization; the chromatographic set-up included a reversed phase column 15 cm Varian OmniSpher C18 with a diameter of 3 mm, which was held at 60 °C. A full 10 µL loop of the sample was injected. The same set-up was used to characterise burned greasy deposits found inside shells of the large Nile bivalve Chambardia rubens, excavated in a VIII-X century AD church of the Coptic monastery of Bawit (Egypt) and supposedly used as lamps. Low concentrations of unaltered TAGs were detected by HPLC-APCI-MS, which included TAGs with at least one dihydroxylated acyl chain. The preservation of this specie may be accounted by the dry climate of the excavation site. The type and distribution of the surviving TAGs suggested the illuminant was rapeseed (*Brassica napus* L.) or radish (*Raphanussativus* L.) oil.

HPLC-APCI-MS was also applied to the study of historical pharmaceutical ointments from the XVIII century [**43**]. The analytical protocol was set up using a comparative study based on the evaluation of TAGs compositions of raw natural lipid materials, in laboratory-reproduced ointments, and was then applied to residues contained in a series of apothecary jars. In order to characterize the triglyceride profile of the archeological samples, the chromatographic separation was performed using an Ascentis C18 column (150 x 4.6 mm,5 µm particle size, Supelco, U.S.A.) and *iso*-propanol and methanol as mobile phase. The samples were submitted to extraction with a mixture of chloroform/methanol at reflux and the extracted material was dried, suspended in hexane and washed three times in a separator funnel with a water/ethanol solution. The final residue in hexane was then dried and diluted in the elution mixture [**43**].

In the study of oil paints, HPLC-APCI-MS of triglycerides was performed to evaluate the effects of traditional processing methods of linseed oil on the composition of its triacylglycerols. The study aimed at evaluating how the triglyceride composition of linseed oil changed as a consequence of different oil

pretreatments such as washing with water, heating or treatment with lead-based dryers. The separation of the TAGs was performed using a NovaPack C18 column (150 x 4.6 mm, Waters, U.S.A.) and acetonitrile/ethanol as eluents. [**44**]. In order to characterize the lipid fraction of linseed oil after the processing methods the oil samples were dissolved in a acetonitrile/*iso*-propanol/hexane mixture and injected in the chromatographic system.

Recently developed non-porous core particles stationary phases [**45**] were also tested for the separation of TAGs in oils, which were used as binding media or for producing ointments, and proved to have a high efficiency at lower backpressures, compared to traditional porous particles [**36**] [**37**].

In particular, HPLC-ESI-MS equipped with a core shell stationary phase was used to determine the triglycerides profile of a set of fresh reference oils commonly used in modern and contemporary art. The separation of the triglycerides was performed using a Poroshell 120 EC-C18 column (50 mm x 3.0 mm, 2.7 µm particle size, Agilent, U.S.A.) with a high resolution ESI-Q-ToF tandem mass spectrometer as detection system using *iso*-propanol and methanol as eluents [**36**].HPLC TAGs analysis allows to characterize the different species in the sample (Figure 3). The triglycerides were extracted using *n*-hexane and the extracts were dried under nitrogen stream, diluted with the elution mixture before the injection. The optimized chromatographic method was applied for the characterization of the triglycerides profiles of drying oils used in modern paint tubes formulations and to characterize the lipids material in paint samples. The paint sample were submitted to a microwave extraction (600 W, 80°C) using a *n*-hexane/chloroform solution and the extracts were dried under nitrogen stream, diluted with the elution mixture and filtered before the injection [**46**].

The same analytical approach used for the characterization of fresh oils was applied to the characterization of the lipid composition of several alkyd resins. The characterization of the glycerides composition allowed to discriminate between different trademarks of commercial alkyd paints, to identify the raw materials used for the production of the resin and to define the synthesis process used for their preparation [**47**]. The same method based on liquid chromatography coupled with high resolution mass spectrometry and hexane extraction was applied to a set of industrial alkyd resins: the application of this method allowed not only the identification of the TAGs, but also the detection of pentaerythritol and phthalic acid esters deriving from the synthesis process of the resin [**48**] (tandem mass spectrum of PenLnLO and LGlyPhGlyLn sodiated adducts shown in Figure 4). The analytical approach used for the characterization of the fresh alkyd paint and resins was also applied for the characterization of the oxidized triglycerides in a set of alkyd paint layers exposed to acetic acid vapour. The application this HPLC procedure is suitable for the characterization of aged and fresh alkyd resins, for discriminating between different trademarks, and to evaluate the conservation state of an artwork [**49**].

A slightly modified method, still based on the use of a Poroshell 120 EC-C18 column with a high resolution electrospray ionization-quadrupole-time of flight tandem mass spectrometer as detection system, was optimized for the identification of triacylglycerols in complex archaeological residues [**37**]. Thanks to the unprecedented chromatographic separation and detection sensitivity of the set-up, it was possible for the first time to perform TAGs profiling in archaeological residues and reference materials with a dataset containing more than 500 molecular formula. In particular, it was possible to distinguish between plant oils and animal fats, and to identify pork suet even in complex mixtures with plant oils. The archaeological organic residues were homogenized in a mixture of dichloromethane/methanol using a ultrasonic bath. Then the mixture was filtered, dried under nitrogen stream, suspended in hexane and washed three times in a separator funnel with a water/ethanol solution. The final residue was then dried over $MgSO_4$, filtered and evaporated to dryness. The final material was diluted with the elution mixture and injected in the chromatographic system [**37**].

In conclusion, thanks to the improvements in columns efficiency and in resolution of the available mass spectrometers, HPLC-MS based techniques are gaining momentum in the analysis of triglycerides and their oxidation products in archaeological residues and in paintings. In the last three years several papers have described optimized procedure for their determination. On the one hand, the applicability of these protocols to actual case studies in the field of archaeology has been proven in several cases, in which the original lipid material was relatively well preserved, thanks to suitable conservation conditions (i.e. very dry climate and relatively anaerobic conditions, in the absence of direct light). On the other hand, the

application of TAGs profiling for the identification of the binding medium is complicated by the tendency to polymerization of siccative oils, to the exposure to direct light and conservation in unfortunate conditions. Thus, only few successful applications of this strategy in paint analyses have been presented so far [**46**]. The publication of new case studies in the future will prove the actual applicability of the technique to diagnostic analyses for paintings.

3 Ion Chromatography

Ion chromatography (IC) is an ion-exchange technique in which low concentrations of organic and inorganic anions and cations are determined using ion exchangers of low ion-exchange capacity with dilute buffers [**1**]. IC is mostly employed to separate organic and inorganic ions, amino acids, proteins or nucleic acids. Strong or weak acids or bases can be used as stationary phases to separate cations and anions respectively. The ion exchangers are supported on silica, polystyrene or carbohydrate-based polymers.

In the field of Cultural Heritage related studies, IC has been mainly used as a robust routine technique to quantify inorganic anions and cations, and small organic acids in a plethora of samples. Few examples will be provided in this chapter of possible applications of this technique, focussing on a few classic case studies and presenting some new developments.

Several papers describe the application of IC to the study of immovable cultural heritage, i.e. stone, masonries, etc., to characterise inorganic salts in damage layers or in black crusts. It has also been applied to characterise the VOCs and the particulate determined in environmental studies in musea, in semi-confined exhibition areas and in general in the urban environment where important monuments stand.

One of the first applications of ion chromatography to the characterization of anions in damage layers of outdoor building was published in 1995 [**50**]. An analytical procedure was set up for the dissolution of damage layers from stone monuments with the aim of performing ion chromatography analyses. The dissolution was achieved in water and purified from cations using cation resins and the ion chromatography analysis was performed with anionic micromembrane suppressor and two serial detectors (conductometer and spectrophotometer). The separation was undertaken on an Ionpac column (AS4A-SC). Halide anions (F^-, Cl^-, Br^-), nitrates and nitrites, phosphates, oxalates and carbonates were quantitated in samples from damaged layers of stone monuments, i.e. black crusts. The same approach and instrumental setup was adopted in [**51**], where the formate and acetate anions were also determined, and eventually applied to the complete characterisation of black crust in damage layers on historic buildings at different European urban sites [**52**]. The soluble anions, including formate, acetate and oxalate, were measured by ion chromatography analysis (IC) with a Dionex 4500i (IonPac AS14 column with a AMMS III suppressor in conjunction with a conductivity detector). Oxalates were the most abundant small (C1–C2) organic anion found at all the sites. The data reported show how OC and EC concentrations and OC/EC ratio are typical of each site and provide essential in-put for an exhaustive investigation of black crust formation.

Similar studies were presented in [**53**], where the degradation of archaeological porous stone in the medieval city of Rhodes was assessed, and in [**54**], which focuses on the effects of sulphation in the frame of a research study on the effects of atmospheric deposition on the cement mortar of the basement in a twentieth-century building. The results achieved by IC were complemented by analyses performed by visual observation, scanning electron microscopy, X-ray diffraction, differential and gravimetric thermal analysis and the quantitative determination of elemental carbon. Sulphation was found to be the main damage mechanism occurring on the cement mortar constituting the base section of a building since the concentration of sulphate increases from the inner to the outer layer at the expense of the carbonate. The same approach, system setup, and similar results were obtained for the case study of Michelozzo's Courtyard in Florence (Italy) [**55**]. The investigation adopted a holistic approach involving thermographic measurements on the wall paintings, microclimatic analysis, gaseous pollutant monitoring, atmospheric particles characterisation and dry deposition compositional analysis. Also in this case, the surface is undergoing a sulphation process. In particular, the presence of significant amounts of sulphite (SO_3^{2-}), with concentration values well correlated to the sulphate ones, indicates that sulphate is also produced by the oxidation of sulphite at the painting surface. The effects of rising damp on the durability of a plaster were studied by means of a similar, multi-analytical approach entailing Ion Chromatography in [**56**].

Summarizing, the application of a routine analytical technique such as IC, complemented by several other microscopy- or spectroscopy-based techniques, to the characterisation of black crusts and more generally, of superficial layers of stone and plaster, allowed the researchers to highlight specific behaviours of stone buildings depending on the environment.

A similar approach, in terms of use of a multi-analytical array of techniques (ICP-AES, SEM, ….) including ion chromatography, was adopted for the characterisation of particulate matter. This method is widely applied in environmental chemistry, so previously optimized methods are routinely applied in the field of Cultural Heritage. Notwithstanding, few interesting case studies will be discussed. To analyse the deposition of fine particulate matter (PM) on book surfaces in the National Library in Prague, Smolik and coworkers [**57**] used cellulose filters to sample the PM, and SEM and IC to characterise them. Ghedini and co-workers monitored the atmospheric aerosol in the area of the Florence Baptistery [**58**]. Total suspended particulate (TSP) was collected using circular filters with diameter of 47 mm. Atmospheric particles were sampled for the measurement of total weight, non-carbonate carbon (NCC) and major soluble ions by Ion Chromatography. Interesting results were obtained by comparing the data collected in different sampling points, both by a qualitative and quantitative point of view.

Besides the characterisation of damage on stone historical and modern buildings, IC was exploited to study metal corrosion in some archaeological and historical materials. A nice examples is given by the evaluation by IC of the atmospheric corrosion of historical organ pipes by volatile organic acids as described in [**59**]. The corrosivity of formic and acetic acid was investigated in laboratory exposing polished samples. Corrosion rate was measured gravimetrically and the corrosion products were analysed qualitatively and quantitatively by IC, equipped with an IONPAC AD9-SC Analytical Column. The results imply that acetic acid vapour is a very important corrosive agent for lead pipes in historical organs, while formic acid is slightly less corrosive than acetic acid. Also in this case, the application of a routine analytical protocol such as IC for the quantification of small organic acids to a peculiar case study allowed the researcher to highlight important characteristics of the objects under study.

Finally, ion chromatography has also been used to quantify the above mentioned volatile organic acids (namely, acetic and formic acid) in air samples in and outside the Cathedral of Cologne (Germany), after sampling the corresponding acids by passive Radiello® diffusion tubes [**60**]. Both acids were adsorbed by TEA and subsequently extracted with water. Four important gaseous pollutants were sampled simultaneously, i.e. NO_2 , SO_2 , acetic and formic acids, and their quantitation performed by IC by the same method described in [**61**]. The performances of two analytical columns (Allsep A-2 and IonPac) were compared in terms of detection limits, precision, and dynamic range. An example of separation is shown in Figure 5. With regard to confined environments, few analytical methods entailing IC have been published for the detection of acetic and formic acid vapours in musea [**62**]; one study even focuses on the detection not only of airborne acetate and formate, but also fluoride [**63**].

One last example presents the application of ion chromatography, along with several other techniques (such as mercury intrusion porosimetry; Fourier transform infrared spectroscopy; scanning electron microscope with energy dispersive spectrometry), to the evaluation of the best application technique for a novel hydroxyapatite-based consolidating treatment for limestone [**64**].

4 Size Exclusion Chromatography

Size exclusion chromatography (SEC), often referred to as gel permeation chromatography (GPC) in the case of the analysis of hydrophilic macromolecules, is a separation technique in which molecules are separated on the basis of their hydrodynamic molecular volume or size [**65**].

In the SEC chromatographic system a liquid mobile phase flows through the column at a fixed flow rate, setting up a pressure gradient across its length; the particles of the stationary phase are porous with controlled pore size. The smaller macromolecules are able to penetrate into these pores as they pass through the column, while the larger ones are too large to be accommodated and remain in the interstitial space. The smaller molecules are only temporarily retained and will flow throw the column until they encounter other particles' pores to enter meanwhile the larger molecules flow more rapidly throw the length of the column because they cannot reside inside the pores for any period of time [**66**].

SEC is widely used for separation of various natural and synthetic polymers. With proper column calibration and by the use of molecular-weight-sensitive detectors, such as light scattering, viscometry, or mass spectrometry, the molecular weight distribution and the statistical molecular weight averages can be obtained readily [**67**] [**66**] [**65**].

In Cultural Heritage studies, SEC has been used to characterize synthetic polymers used as paint binders or consolidants; to assess the degradation of cellulosic and wooden materials and Arabic gum used as binder; to study the binder-pigment interactions in protein-based paintings; to evaluate the polymerization or, in general, degradation processes undergone by terpenoid varnishes and lipid binders.

4.1 Synthetic polymers

A number of synthetic materials have been exploited by artists and restorers, and the wide variety of formulations of synthetics resins has allowed their extensive use as paint binders, plastic materials, consolidants, adhesives, coatings and varnishes and many other applications.

Size-exclusion chromatography interfaced with a refractive index detector (SEC-RID) was applied to evaluate the modification of the molecular weight distribution of the varnishes Laropal A81, Laropal A101, Laropal K80, Regalrez 1094 and Arkon P90 during artificial ageing and to evaluate the effects due to the addition of Tinuvin 292 as UV-stabilizer in the formulation of the resins. The resins were solubilized in tetrahydrofuran and directly injected in the LC system and the chromatographic separation was performed using two Polymer Laboratories (Netherlands) PL-gel 5µm mixed-D columns (300 mm × 7.5 mm) and tetrahydrofuran as eluent [**68**]. The results obtained for un-stabilized and stabilized Laropal K80 during accelerated light aging are reported in Figure 6 [**68**].

The modification of the molecular weight of the ketone resin Laropal K80 during ageing was also studied with the same chromatographic approach in the commercial formulation of BEVA 371, a heat-seal adhesive containing two EVA co-polymers, the ketone resin, a phthalate ester of a hydroabietyl alcohol and a paraffin wax [**69**].

SEC-RID was also applied to evaluate the photochemistry processes involved in the ageing poly(vinyl acetate) paints and to evaluate the ageing effects related to the presence of pigments and fillers in the paint formulation: the information on the molecular weight distribution obtained with SEC-RID were used to identify the main chemical reactions involved in the ageing of the paint and to predict its long-term life [**70**].

In all these cases, SEC allowed the researchers to evaluate the behavior of different polymeric resins upon ageing, and thus their long term stability and, as a consequence, suitability as conservation or restoration materials.

SEC coupled with Fourier transform infrared spectroscopy (SEC-FTIR) was in one case applied for the characterization of artists' acrylic emulsion paints [**71**]. The collection of the infrared data was performed using an off-line solvent-elimination interface. The use of the off-line interface is commonly preferred over on-line flow-cells system since the removal of the solvents used in the chromatographic separation allows to increase the signal to noise ratio and decreases the limits of detections [**72**]. The acrylic emulsions were solubilized in tetrahydrofuran and the chromatographic separation was performed using three Polymer

Laboratories (United Kingdom) PLgel 10 µm MIXED-B (300 X 7.5 mm) columns and tetrahydrofuran as eluent. Figure 7reports an example of a 3D plot obtained for the characterization of an acrylic emulsion paint [**71**].The use of infrared spectroscopy as a detector system allowed the simultaneous identification of the polymer matrix and the minor components, such as the surfactants and dyes used in the acrylic paint emulsions. This approach allowed a full characterisation of the material under study, but required a relatively high amount of sample. Thus, it can only be applied for studies on reference materials or for quality control of newly produced materials (e.g. during natural or artificial ageing experiments), and not for diagnostic purposes. Examples of the application of SEC to these type of problems follow.

4.2 Polysaccharides (wood, paper and gums)

The main chemical components in wood are cellulose, hemicellulose, lignin and extractives. Cellulose is a homo-polymer of β-1,4-glucose units with a highly regular H-bonded network between its layers, especially in the case of crystalline cellulose, while hemicelluloses is a carbohydrate hetero-polymer consisting of different monomers. Lignin is a tridimensional network macromolecular in which the more abundant monomers are substituted phenols bonded via ether bonds.

GPC was applied for the determine the chemical features and molecular weight distributions in wood and in the corresponding extracted lignin using different sample pretreatments [**73**] [**74**].The analyses were performed on both archeological and reference wood samples in order to evaluate the change in the molecular weight distribution occurring as an effect of ageing. The chromatographic separation of the wood components was performed using an Agilent (U.S.A.) PL 3 µm MIXED gel E (MW 220-400 K) and tetrahydrofuran as eluent. The detection of the analytes was performed using a diode array detector (DAD) set at 280 nm [**73**] [**74**].The same analytical approach was successfully applied for the characterization of the conservation state of archeological waterlogged wood artifacts [**75**].

In all these cases, GPC provided important data on the depolimerization of lignin in different conservation conditions, which can be correlated with the conservation state of wood in archaeological context.

GPC was also used for the ageing studies of paper related to the presence of metals. The presence of copper ions that migrate from the pigments can catalyze the degradation of cellulose and discoloration of the paper. In order to evaluate the effects of this metal on paper, a GPC-Fluorescence-multiangle laser light scattering detector (MALS) apparatus was used. The use of this system allowed to determine not only the absolute molar mass moments by MALS, but also the contents of carbonyl groups measured by a fluorescence detector after a carbonyl specific chemical labeling [**76**].Gel permeation chromatography was also applied for the characterization of cellulose nitrate, a semi-synthetic material deriving from the industrial processing of cellulose, which was one of the first semi-synthetic plastics to be commercially exploited. Many museums contain a large number of artifacts containing this plastic. In order to evaluate the main degradation pattern of this material GPC-RID was applied for the characterization of several samples from selected artifacts in various stages of preservation and on a set of artificially aged reference cellulose nitrate films [**77**] [**78**].

In both cases, GPC was mainly adopted to highlight the depolymerization reactions occurring in paper or nitrocellulose as results of hydrolysis, possibly catalyzed by metal ions.

Gum Arabic, or acacia gum, is the oldest and best known of all the tree gum exudates and has been used as binder and traded for more than 5000 years. Structural studies of the proteic and polysaccharide components of gum Arabic were performed using GPC coupled with a UV-RID-MALS [**79**]. The effects of the ultraviolet irradiation on the physicochemical and functional properties of gum Arabic were also evaluated using GPC-RID-UV: the modification of the molecular weight distribution of the gum polymer were used to identify the cross-linking and depolymerization processes occurring during the exposure to UV light [**80**].

4.3 Protein paint binders

Proteinaceous materials, such as egg, casein and animal glue, have commonly been used as paint binders historically.

Size exclusion chromatography was applied on paint replicas of casein and ovalbumin with cinnabar (HgS) in order to evaluate the ageing pathways of these proteins in presence of such pigment. The separation of the proteins was performed using a SEC system coupled to UV and cold vapor generation atomic fluorescence spectrometry detector (SEC-UV-CVGAFS). CVGAFS technique is based on the pre-column derivatization of protein thiol groups with a mercurial probe in order to selectively speciate and detect mercury-protein complexes by CVGAFS. The separation of the proteins was performed using a Biosep SEC S2000 column (Phenomenex, U.S.A.) and an isocratic elution in 50 mM Phosphate Buffered Saline pH 7.4, 0.15 M NaCl at 1.0 mL/min [**81**].The interaction of casein and ovalbumin with azurite ($Cu_3(CO_3)_2(OH)_2$), calcium carbonate ($CaCO_3$), hematite (Fe_2O_3) and red lead (Pb_3O_4) were also evaluated using the chromatographic conditions described above, with a DAD detector (Figure 8) [**82**].The results highlighted that pigments may act directly on the stability of the protein structure because of their interaction with amino acid functional groups or indirectly, promoting oxidation.

4.4 Triterpenoid resins

Triterpenoid resins are the main components of traditional varnishes used by artists and often identified in archeological amorphous materials. Polymerization and degradation of triterpenoid resins entails radical polymerization, cross-linking and condensation, oxidative modifications and shortening of side-chains.

In order to evaluate the effects on the molecular weight distribution of these chemical processes, dammar and mastic resins were studied using a high performance size exclusion chromatography system (HP-SEC) coupled with RID and UV/Vis detectors [**83**]. This technique was not applied to other cases of terpenoid resins due to their poor solubility in the majority of the samples. Nonetheless, the results highlighted in [**83**]proved that the ageing processes are related to the thickness of the varnish layer, as an example, condensation reactions that leads to cross-linking and polymerization were identified only in the first portion of the resin layer and are related to the UV wavelengths exposure.

4.5 Lipids

Drying oils are one of the most ancient binders used in paints for both decorative and protective purposes. The oils used as paint binder are usually pre-processed by heating, chemical bleaching and treatment with metallic salts in order to enhance their siccative proprieties (ability to form films).

The effects of these processes on linseed oil were studied using an HP-SEC-RID-UV/Vis-DAD system. The oil samples were solubilized using tetrahydrofuran and the chromatographic separation was performed using a PLGEL 5 µm 1000 Å column (300 x 7.5 mm, Polymer Laboratories, Netherlands) and tetrahydrofuran as eluent [**84**].The application of SEC analysis allowed the identification of the main chemical modification involved during the preparation of linseed oil. Two major chemical processes were observed for the oils: oxidation and oligomerisation. This is accompanied by a relative decrease of the percentage of the triple unsaturated fatty acid, the most reactive component of the TAGs.

Alkyd paints, introduced in the ‘40s, represent an evolution of modern drying oils. They are oil-modified polyesters, characterized by higher drying rates than classic drying oils. Size exclusion chromatography coupled with a MALS and a RID detector was applied to evaluate the structure of an alkyd resin produced using soybean oil at different polymerization reaction time in [**85**].The application of SEC coupled with a MALS detector provides information about the branching and on the molecular weight distribution of the resin.

5 Summary and conclusions

The review of the several applications of liquid chromatography to the study of materials in the field of Cultural Heritage highlighted that several protocols were optimized for the detection and, in several cases, quantitation, of a great number of analytes. Almost all types of HPLC have been exploited with the aim of characterizing different properties of different materials.

Until now, most of the research has focused on the application of reversed-phase HPLC to the analysis of organic dyes and lakes, which has almost become a routine in diagnostic campaigns. Besides the future perspective for proteomics in this field, reviewed in Chapter xx, the analysis of dyes and lipid residues or binders represents the most promising application up to now. The main improvement that is gaining momentum at the time is related to the development and validation of UHPLC methods for dyes analyses, which allow faster separations without losses in chromatographic resolution. Moreover, the key issue in both these applications (namely, dyes and lipid analysis) is the optimization of sample treatment, in order not only to avoid matrix effects, which have not been evaluated in any of the published papers, but also to lower detection and quantitation limits.

Less common applications of HPLC include ion chromatography and size exclusion chromatography, which have both been successfully applied to selected cases.

Ion chromatography has been mainly used as routine analytical technique to quantify cations and anions in damage layers and patinas, mostly on stone building. Robust protocols entailing sample treatment and complementation of IC results with those of other techniques have been published, which set the standard for future applications.

Size exclusion chromatography has been applied in a few cases to the study of polymeric materials upon ageing (also thanks to accelerated ageing tests). Both natural (proteins, polysaccharides, lignin) and synthetic (alkyd binders and low molecular weight resins) polymers have been studied, with particular attention to their behavior in the presence of inorganic salts, which may influence depolymerization or polymerization reactions. This technique has an high potential for the characterization of the ageing of materials, given the possibility to detect and characterize the products. On the one hand, we may expect future developments to go in the direction of coupling this technique with mass spectrometric detection that has already proven effective in the study of protein behavior in biological studies, and in polymer chemistry since more than 10 years. On the other hand, the lack of proper standards in the field of Cultural Heritage related studies might represent a drawback for this kind of applications.

References

1 *The chromatography and sample preparation terminology guide*. 2013.

2 Colombini, Maria Perla and Modugno, Francesca. *Organic Mass Spectrometry in Art and Archeology*. John Wiley & Sons, Chichester, U.K., 2009.

3 Lomax S.Q., Learner T.. A Review of the Classes, Structures, and Methods of Analysis of Synthetic Organic Pigments. *Journal of the American Institute for Conservation*, 45, 2 (2006), 107-125.

4 Nowik, W., Desrosiers, S., Surowiec, I., and Trojanowicz, M. The analysis of dyestuffs from first- to second-century textile artefacts found in the Martresde-Veyre (France) excavations. *Archaeometry*, 47 (2005), 835–848.

5 Surowiec, I. Application of high-performance separation techniques in archaeometry. *Microchimica Acta*, 162 (2008), 289–302.

6 Rosenberg, E. Characterisation of historical organic dyestuffs by liquid chromatography–mass spectrometry. *Analytical and Bioanalytical Chemistry*, 391 (2008), 33-57.

7 Degano, I., Ribechini, E., Modugno, F., and Colombini, M.P. Analytical methods for the characterization of organic dyes in artworks and in historical textiles. *Applied Spectroscopy Reviews*, 44 (2009), 363–410.

8 Pauk, V. and Bartak, P., Lemr, K. Characterization of natural organic colorants in historical and art objects by high-performance liquid chromatography. *Journal of Separation Sciences*, 37 (2014), 3393–3410.

9 Russell, J., Singer, B.W., Perry, J.J., and Bacon, A. The identification of synthetic organic pigments in modern paints and modern paintings using pyrolysis-gas chromatography–mass spectrometry. *Analytical and Bioanalytical Chemistry*, 400 (2011), 1473-1491.

10 Ghelardi, E., Degano, I., Colombini, M.P., Mazurek, J., Schilling, M., and Learner, T. Py-GC/MS applied to the analysis of synthetic organic pigments: characterization and identification in paint samples. *Analytical and Bioanalytical Chemistry*, 407 (2015), 1415-1431.

11 Carlesi, S. and co-workers. Discovering "The Italian Flag" by Fernando Melani (1907-1985). *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* (2015).

12 Lech, K., Wilicka, E., Witowska-Jarosz, J., and Jarosz, M.. Early synthetic dyes – a challenge for tandem mass spectrometry. *Journal of Mass Spectrometry*, 48 (2013), 141-147.

13 van Bommel, M.R., Vanden Berghe, I., and Wallert, A.M. High-performance liquid chromatography and non-destructive three-dimensional fluorescence analysis of early synthetic dyes. *Journal of Chromatography A*, 1157 (2007), 260-272.

14 Confortin, D., Neevel, H., Brustolon, M., Franco, L., Kettelarij, A.J., Williams, R.M., and van Bommel, M.R. Crystal violet: study of the photo-fading of an early synthetic dye in aqueous solution and on paper with HPLC-PDA, LCMS and FORS. *Journal of Physics: Conference Series*, 231 (2010), 1-9.

15 de Keijzer, M., van Bommell, M.R., Hofmann-de Keijzer, R., Knaller, R., and Oberhumer, E. Indigo carmine: Understanding a problematic blue dye. In *Contributions to the Vienna Congress 2012* (Vienna 2012), S87-S95.

16 Restivo, A., Degano, I., Ribechini, E., and Colombini, M.P. Development and optimisation of an HPLC-DAD-ESI-Q-TOF method for the determination of phenolic acids and derivatives. *PlosOne*, 9 (2014), e88762.

17 Halpine, S.M. An improved dye and lake pigment analysis method for high performance liquid chromatography and diode-array detection. *Studies in Conservation*, 41 (1995), 76–94.

18 Nowik, W., Marcinowska, R., Kusyk, K., Cardonc, D., and Trojanowicz, M. High performance liquid chromatography of slightly soluble brominated indigoids from Tyrian purple. *Journal of*

Chromatography A, 1218 (2011), 1244-1252.

19 Surowiec, I., Quye, A., and Trojanowicz, M. Liquid chromatography determination of natural dyes in extracts from historical Scottish textiles excavated from peat bogs. *Journal of Chromatography A*, 1112 (2006), 209-217.

20 Taujenis, L. and Olšauskaitė, V. Identification of main constituents of historical textile dyes by ultra performance liquid chromatography with photodiode array detection. *Chemija*, 23 (2012), 210-215.

21 Serrano, A., van Bommel, M., and Hallett, J. Evaluation between ultrahigh pressure liquid chromatography andhigh-performance liquid chromatography analytical methods for characterizing natural dyestuffs. *Journal of Chromatography A*, 1318 (2013), 102-111.

22 Troalen, L.G., Phillips, A.S., Peggie, D.A., Barran, P.E., and Hulme, A.N. Historical textile dyeing with Genista tinctoria L.: a comprehensive study by UPLC-MS/MS analysis. *Analytical Methods*, 6 (2014), 8915-8923.

23 Wouters, J., Grzywacz, C.M., and Claro, A. A comparative investigation of hydrolysis methods to analyze natural organic dyes by HPLC-PDA. *Studies in Conservation*, 56 (2011), 231-249.

24 Manhita, A., Ferreira, T., Candeias, A., and Barrocas Dias, C. Extracting natural dyes from wool—an evaluation of extraction methods. *Anlytical and Bioanalytical Chemistry*, 400 (2011), 1501–1514.

25 Valianou, L., Karapanagiotis, I., and Chryssoulakis, Y. Comparison of extraction methods for the analysis of natural dyes in historical textiles by high-performance liquid chromatography. *Analytical and Bioanalytical Chemistry*, 395 (2009), 2175-2189.

26 Sanyova, J. Mild extraction of dyes by hydrofluoric acid in routine analysis of historical paint micro-samples. *Microchimica Acta*, 162 (2008), 361–370.

27 Blanc, R., Espejo, T., Lopez-Montes, A., Torres, D., Crovetto, G., Navalon, A., and Vilchez, J.L. Sampling and identification of natural dyes in historical maps and drawings by liquid chromatography with diode-array detection. *Journal of Chromatography A*, 1122 (2006), 105-113.

28 Sanyova, J. and Reisse, J. 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*, 7 (2006), 229-235.

29 Lech, K. and Jarosz, M. Novel methodology for the extraction and identification of natural dyestuffs in historical textiles by HPLC–UV–Vis–ESI-MS. Case study: chasubles from the Wawel Cathedral collection. *Analytical and Bioanalytical Chemistry*, 399 (2011), 3241-3251.

30 Mouri, C., Laursen, R. Identification of anthraquinone markers for distinguishing Rubia species in madder-dyed textiles by HPLC. *Microchimica Acta*, 179 (2012), 105-113.

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

32 Vanden Berghe, I., Gleba, M., and Mannering, U. Towards the identification of dyestuffs in Early Iron Age Scandinavian peat bog textiles. *Journal of Archaeological Science*, 36 (2009), 1910-1921.

33 Colombini, M.P., Carmignani, A., Modugno, F. et al. Integrated analytical techniques for the study of ancient Greek polychromy. *Talanta*, 63 (2004), 839-848.

34 A. Stolyhwo, H. Colin, G. Guiochon. Analysis of Triglycerides in Oils and Fats by Liquid Chromatography with the Laser Light Scattering Detector. *Analytical Chemistry*, 57 (1985), 1342-1354.

35 L.C. Herrera, M. Potvin, J. Melanson. Quantitative analysis of positional isomers of triacylglycerols via electrospray ionization tandem mass spectrometry of sodiated adducts. *Rapid Communication in Mass Spectrometry* , 24 (2010), 2745–2752.

36 W.C. Byrdwell, W.E. Neff. Dual parallel electrospray ionization and atmospheric pressure chemical ionization mass spectrometry (MS), MS/MS and MS/MS/MS for the analysis of triacylglycerols and triacylglycerol oxidation products. *Rapid Communication in Mass Spectrometry*, 16 (2002), 300-319.

37 J. La Nasa, E. Ghelardi, I. Degano, F. Modugno, M.P. Colombini. Core shell stationary phases for a novel separation of triglycerides in plant oils by high performance liquid chromatography with electrospray-quadrupole-time of flight mass spectrometer. *Journal of Chromatography A*, 1308 (2013), 114-124.

38 F. Saliu, I. Degano, M.P. Colombini. Identification of triacylglycerols in archaelogical organic residues by core–shell reversed phase liquid chromatography coupled to electrospray ionization-quadrupole-time of flight mass spectrometry. *Journal of Chromatography A*, 1346 (2014), 78-87.

39 Mottram, H.R. and Evershed, R.P. Structure analysis of triacylglycerol positional isomers using atmospheric pressure chemical ionisation mass spectrometry. *Tetrahedron Letters*, 37 (1996), 8593-8596.

40 Kimpe, K., Jacobs, P.A., and Waelkens, M. Mass spectrometric methods prove the use of beeswax and ruminant fat in late Roman cooking pots. *Journal of Chromatography A*, 968 (2002), 151-160.

41 Kimpe, K., Jacobs, P.A., and Waelkens, M. Analysis of oil used in late Roman oil lamps with different mass spectrometric techniques revealed the presence of predominantly olive oil together with traces of animal fat. *Journal of Chromatography A*, 937 (2001), 87-95.

42 Romanus, K., Van Neer, W., Marinova, E. et al. Brassicaceae seed oil identified as illuminant in Nilotic shells from a first millennium AD Coptic church in Bawit, Egypt. *Analytical and Bioanalytical Chemistry*, 390 (2008), 783-793.

43 Romanus, K., Poblome, J., Verbeke, K., Luypaerts, A., Jacobs, P., De Vos, D, and Walekens, M. An evaluation of analytical and interpretative methodologies for the extraction and identification of lipids associated with pottery sherds from the site of Sagalassos, Turkey. *Archaeometry*, 49 (2007), 729-747.

44 F. Saliu, F. Modugno, M. Orlandi, M.P. Colombini. HPLC APCI-MS analysis of triacylglycerols (TAGs) in historical pharmaceutical ointments from the eighteenth century. *Analytical and Bioanalytical Chemistry*, 401 (2011), 1785-1800.

45 J.D.J.v.d. Berg, N.D. Vermist, L. Carlyle, M. Holcapek, J.J. Boon. Effetcs of traditional processing methods of linseed oil on the composition of its triacylglcyerols. *Journal of Separation Science*, 27 (2004), 181-199.

46 Chester, T.L. Recent Developments in High-Performance Liquid Chromatography Stationary Phases. *Analytical Chemistry*, 85 (2013), 579−589.

47 Jacopo La Nasa, Marco Zanaboni, Daniele Uldanck, Ilaria Degano, Francesca Modugno, Hartmut Kutzke, Eva Storevik Tveit, Biljana Topalova-Casadiego, Maria Perla Colombini. Novel application of liquid chromatography/mass spectrometry for the characterization of drying oils in art: Elucidation on the composition of original paint materials used by Edvard Munch (1863–1944). *Analytica Chimica Acta*, 896 (2015), 177-189.

48 J.L. Nasa, I. Degano, F. Modugno, M.P. Colombini. Alkyd paints in art: Characterization using integrated massspectrometry. *Analytica Chimica Acta*, 797 (2013), 4- 80.

49 J.L. Nasa, I. Degano, F. Modugno, M.P. Colombini. Industrial alkyd resins: characterization of pentaerythritol and phthalic acid esters using integrated mass spectrometry. *Rapid Communication in Mass Spectrometry*, 29 (2015), 225-237.

50 J.L. Nasa, I. Degano, F. Modugno, M.P. Colombini. Effects of acetic acid vapour on the ageing of alkyd paint layers: Multianalytical approach for the evaluation of the degradation processes. *Polymer Degradation and Stability*, 105 (2014), 257-264.

51 Gobbi, G., Zappia, G., and Sabbioni, C. Anion determination in damage layers of stone

monuments. *Atmospheric Environment*, 29 (1995), 703-707.

52 Sabbioni, C., Ghedini, N., and Bonazza, A. Organic anions in damage layers on monuments and buildings. *Atmospheric Environment*, 37 (2003), 1261–1269.

53 Bonazza, A., Sabbioni, C., and Ghedini, N. Quantitative data on carbon fractions in interpretation of black crusts and soiling on European built heritage. *Atmospheric Environment*, 39 (2005), 2607–2618.

54 Moropoulou, A., Koui, M., Kourteli, Ch., Theoulakis, P., and N.P., Avdelidis. Integrated methodology for measuring and monitoring salt decay in the medieval city of Rhodes porous stone. *Mediterranean Archaeology and Archaeometry*, 1 (2001), 57-68.

55 Tittarelli, F., Moriconi, G., and Bonazza, A. Atmospheric deterioration of cement plaster in a building exposed to a urban environment. *Journal of Cultural Heritage*, 9 (2008), 203-206.

56 Nava, S., Becherini, F., Bernardi, A. et al. An integrated approach to assess air pollution threats to cultural heritage in a semi-confined environment: The case study of Michelozzo's Courtyard in Florence (Italy). *Science of the Total Environment*, 408 (2010), 1403–1413.

57 Fassina, V., Favaro, M., Naccari, A., Pigo, M. Evaluation of compatibility and durability of a hydraulic lime-based plaster applied on brick wall masonry of historical buildings affected by rising damp phenomena. *Journal of Cultural Heritage*, 3 (2002), 45–51.

58 Smolik, J., Maskova, L., Zikova, N., Ondrackova, L., Ondracek. Deposition of suspended fine particulate matter in a library. *Heritage Science*, 1, 7 (2013).

59 Ghedini, N., Ozga, I., Bonazza, A., Dilillo, M., Cachier, H., Sabbioni, C. Atmospheric aerosol monitoring as a strategy for the preventive conservation of urban monumental heritage: The Florence Baptistery. *Atmospheric Environment*, 45 (2011), 5979-5987.

60 Niklasson, A., Johansson, L-G., and Svensson, J-E. Atmospheric corrosion of historical organ pipes: influence of acetic and formic acid vapour and water leaching on lead. In *Proceedings of Metal 2004 National Museum of Australia Canberra ACT 4–8 October 2004* (Canberra 2004), National Museum of Australia, 273-280.

61 Kontozova-Deutsch, V., Krata, A., Deutsch, F., Bencs, L., and Van Grieken, R. Efficient separation of acetate and formate by ion chromatography: Application to air samples in a cultural heritage environment. *Talanta*, 75 (2008), 418-23.

62 Ghedini, N., Sabbioni, C., Bonazza, A., and Gobbi, G. Chemical−Thermal Quantitative Methodology for Carbon Speciation in Damage Layers on Building Surfaces. *Environmental Science and Technology*, 40 (2006), 939–944.

63 Hodgkins, R.E., Grzywacz, C.M., Garrell, R.L. An improved ion chromatography method for analysis of acetic and formic acid vapours. *e-preservation science*, 8 (2011), 74-80.

64 Kontozova-Deutsch, V., Deutsch, F., Bencs, L., Krata, A., Van Grieken, R., De Wael, K. Optimization of the ion chromatographic quantification of airborne fluoride, acetate and formate in the Metropolitan Museum of Art, New York. *Talanta*, 86 (2011), 372-376.

65 Franzoni, E., Sassoni, E., Graziani, G. Brushing, poultice or immersion? The role of the application technique on the performance of a novel hydroxyapatite-based consolidating treatment for limestone. *Journal of Cultural Heritage* , 16 (2015), 173–184.

66 H.G. Barth, B.E. Boyes, C. Jackson. Size Exclusion Chromatography and Related Separation Techniques,. *Analytical Chemistry*, 70 (1998), 251R-278R.

67 Wu, C.S. *Handbook Of Size Exclusion Chromatography And Related Techniques: Revised And Expanded*. Taylor & Francis, 2003.

68 Berek, D.A. Size exclusion chromatography – A blessing and a curse of science and technology of synthetic polymers. *Journal of Separation Science*, 33 (2010), 315–335.

69 C.A. Maines, E.R.Da la Rie. Size-exclusion chromatography and differential scanning calorimetry of low molecular weight resins used as varnishes for paintings,. *Progress in Organic*

Coatings, 52 (2005), 39-45.

70 R. Ploeger, E.R. De la Rie, C.W. McGlinchey, M. Palmer, C.A. Maines, O. Chiantore. The long-term stability of a popular heat-seal adhesive for the conservation of painted cultural objects. *Polymer Degradation and Stability*, 107 (2014), 307-313.

71 J.L. Ferreira, M.J. Melo, A.M. Ramos. Poly(vinyl acetate) paints in works of art: A photochemical approach. Part 1. *Polymer Degradation and Stability*, 95 (2010), 453-461.

72 D. Scalarone, O. Chiantore. Separation techniques for the analysis of artists' acrylic emulsion paints. *Journal of Separation Science*, 27 (2004), 263–274.

73 G.W. Somsen, C. Gooijer, N.H. Velthors, U.A.T. Brinkman. Coupling of column liquid chromatography and Fourier transform infrared spectrometry. *Journal of Chromatography A*, 811 (1998), 1-34.

74 M.P. Colombini, J.J. Lucejko, F. Modugno, M. Orlandi, E.-L. Tolppa, L. Zoia. A multi-analytical study of degradation of lignin in archaeological waterlogged wood. *Talanta* , 80 (2009), 61-70.

75 A. Salanti, L. Zoia, E.L. Tolppa, G. Giachi, M. Orlandi. Characterization of waterlogged wood by NMR and GPC techniques. *Microchemical Journal*, 95 (2010), 345-352.

76 L. Zoia, A. Salanti, M. Orlandi. Chemical characterization of archaeological wood: Softwood Vasa and hardwood Riksapplet case studies. *Journal of Cultural Heritage* (2014).

77 K. Ahn, A. Hartl, C. Hofmann, U. Henniges, A. Potthast. Investigation of the stabilization of verdigris-containing rag paper by wet chemical treatments. *Heritage Science*, 2 (2014).

78 A. Quye, D. Littlejohn, R.A. Pethrick, R.A. Stewart. Investigation of inherent degradation in cellulose nitrate museum artefacts. *Polymer Degradation and Stability*, 96 (2011), 1369-1376.

79 A. Quye, D. Littlejohn, R.A. Pethrick, R.A. Stewart. Accelerated ageing to study the degradation of cellulose nitrate museum artefacts,. *Polymer Degradation and Stability*, 96 (2011), 1934-1939.

80 A.M.Islam, G.O.Phillipsl, A.Sljivo, M.J.Snowden, P.A.Williams. A review of recent developments on the regulatory, structural and functional aspects of gum arabic. *Food Hydrocolloids*, 4 (1997), 493-505.

81 Y.-H. Kuan, R. Bhat, C. Senan, P.A. Williams, A.A. Karim. Effects of Ultraviolet Irradiation on the Physicochemical and Functional Properties of Gum Arabic. *Journal of Agricultural and Food Chemistry*, 57 (2009), 9154–9159.

82 C. Duce, L. Ghezzi, M. Onor, I. Bonaduce, M.P. Colombini, M.R. Tinè, M. Bramanti. Physico-chemical characterization of protein–pigmentinteractions in tempera paint reconstructions: casein/cinnabar and albumin/cinnabar. *Analytical and Bioanalytical Chemistry*, 402 (2012), 2183–2193.

83 C. Duce, E. Bramanti, L. Ghezzi, L. Bernazzani, I. Bonaduce, M.P. Colombini, A. Spepi, S. Biagi, M.R. Tine. Interactions between inorganic pigments and proteinaceous binders in reference paint reconstructions. *Dalton Transactions*, 42 (2013), 5945–6236.

84 C. Theodorakopoulos, J.J. Boon. A high performance size exclusion chromatographic study on the depth-dependent gradient in the molecular weight of aged triterpenoid varnish films. *Progress in Organic Coatings*, 72 (2011), 778-783.

85 J.D.J.Van der Berg, N.D. Vermist, L. Carlyle, M. Holcˇapek, J.J. Boon. Effects of traditional processing methods of linseed oil on the composition of its triacylglycerols. *Journal of Separation Science*, 27 (2004), 181–199.

86 D. Vareckova, S. Podzimek, J. Lebduska. Characterization of alkyd resins by size exclusion chromatography coupled with a multi-angle light scattering photometer. *Analytica Chimica Acta* , 557 (2006), 31-36.

Figure Captions

Figure 1: Examples of chromatograms obtained after the application of different sample treatments to reference lakes or textiles prepared with madder dyestuffs .

Figure 2: The APCI mass spectrum of 1,3-distearoyl-2-oleoyl glycerol (SOS) (reprinted with permission from **[**38**]**).

Figure 3: Extract ion chromatograms of linseed oil for 15 identified TAGs species (reprinted with permission from **[**36**]**).

Figure 4: Tandem mass spectrum and fragmentation pattern of a) PenLnLO and b) LGlyPhGlyLnsodiate adducts (reprinted with permission from **[**49**]**).

Figure 5: Separation of fluoride, acetate and formate on an IonPac AS14 in a real sample taken at the Cathedral of Cologne (reprinted with permission from **[**60**]**).

Figure 6: Changes in SEC of un-stabilized and stabilized Laropal K 80 during accelerated light aging (reprinted with permission from **[**68**]**).

Figure 7: 3D plot obtained by SEC-FTIR of an acrylic emulsion paint. Peak A has been identified as a poly(nBA-co-MMA) medium, peak B as an alkyl aryl polyethoxylate surfactant and peak C as Pigment Red 5 (reprinted with permission from **[**71**]**).

Figure 8: SEC-UV chromatograms of the soluble fraction of un-aged (continuous line, black) and aged (dotted line, red) ovalbumin/pigment (a) and casein/pigment (b) paint replica (reprinted with permission from **[**82**]).**

Table 1: Main applications of liquid chromatography in Cultural Heritage.

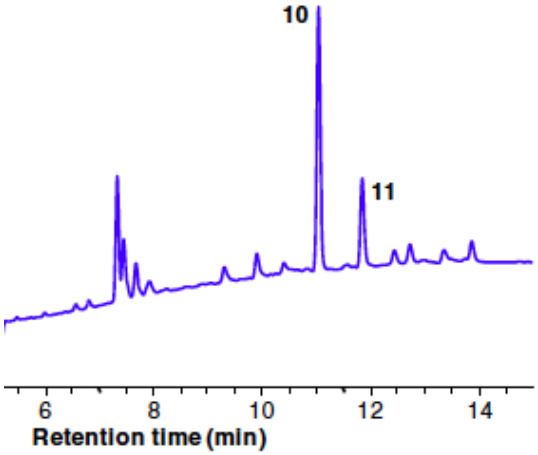
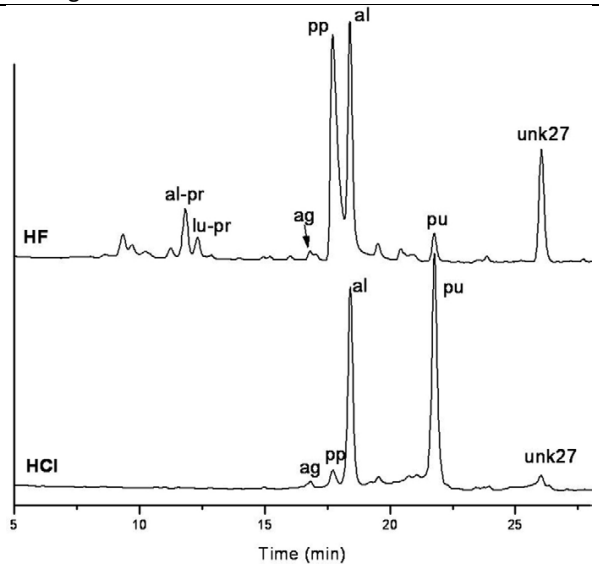
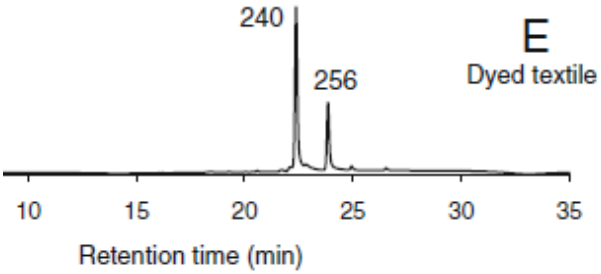
Type of LC	Type of samples	Detectors	References
Partition (Reversed-phase)	Dyes	UV/Vis detectors (UV/Vis) Diode array detector (DAD) Fluorescence detector (Fluo) API-Mass spectrometry (ESI-MS, APCI-MS)	[**3-33**]
	Lipids: drying oils; alkyd resins; archeological samples (amorphous residues)	Atmospheric pressure chemical ionization (APCI) mass spectrometry Electrospray ionization (ESI) tandem mass spectrometry	[**34-50**]
Ion chromatography	Inorganic salts (mainly anions) and small organic acids (formic, oxalic and acetic acids) in black crusts and generally damaged layers on stone buildings	Conductometer (with ion suppressor) and UV-Vis spectrophotometers	[**51-53**]
	Damaged layer due to atmospheric corrosion of historical organ pipes	Conductometer (with ion suppressor)	[**54-57**]
	Indoor pollutants	Conductometer (with ion suppressor)	[**58-59**]
	NO_2 , SO_2 , acetic and formic acids in their anionic forms in air in historical buildings	Conductometer (with ion suppressor)	[**60-65**]
SEC/GPC	Polysaccharides (wood, paper and gums)	Refractive index detector (RID) Fourier transform infrared spectroscopy (FTIR)	[**69-73**]
	Cellulose based materials	Diode array detector (DAD) Refractive index detector (RID) Fluorescence-multiangle laser light scattering detector (MALS)	[**74-81**]
	Protein paint binders	UV detector (UV) Diode array detector (DAD) Cold Vapour Generation Atomic Fluorescence Spectrometry (CVGAFS)	[**82-83**]
	Triterpenoid resins	UV/Vis detector (UV/Vis) Refractive index detector (RID)	[**84**]
	Lipids	UV/Vis detector (UV/Vis) Diode array detector (DAD) Refractive index detector (RID) Fluorescence-multiangle laser Light scattering detector (MALS)	[**85-86**]

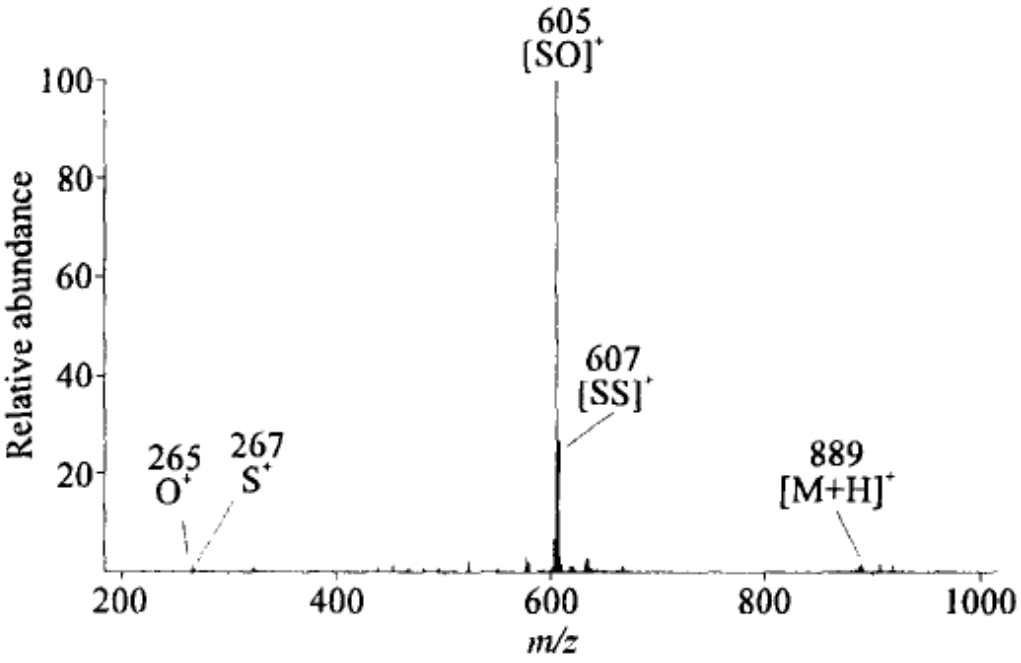
Table 2: Most common natural dyestuffs classified on their chemical class [**7**].

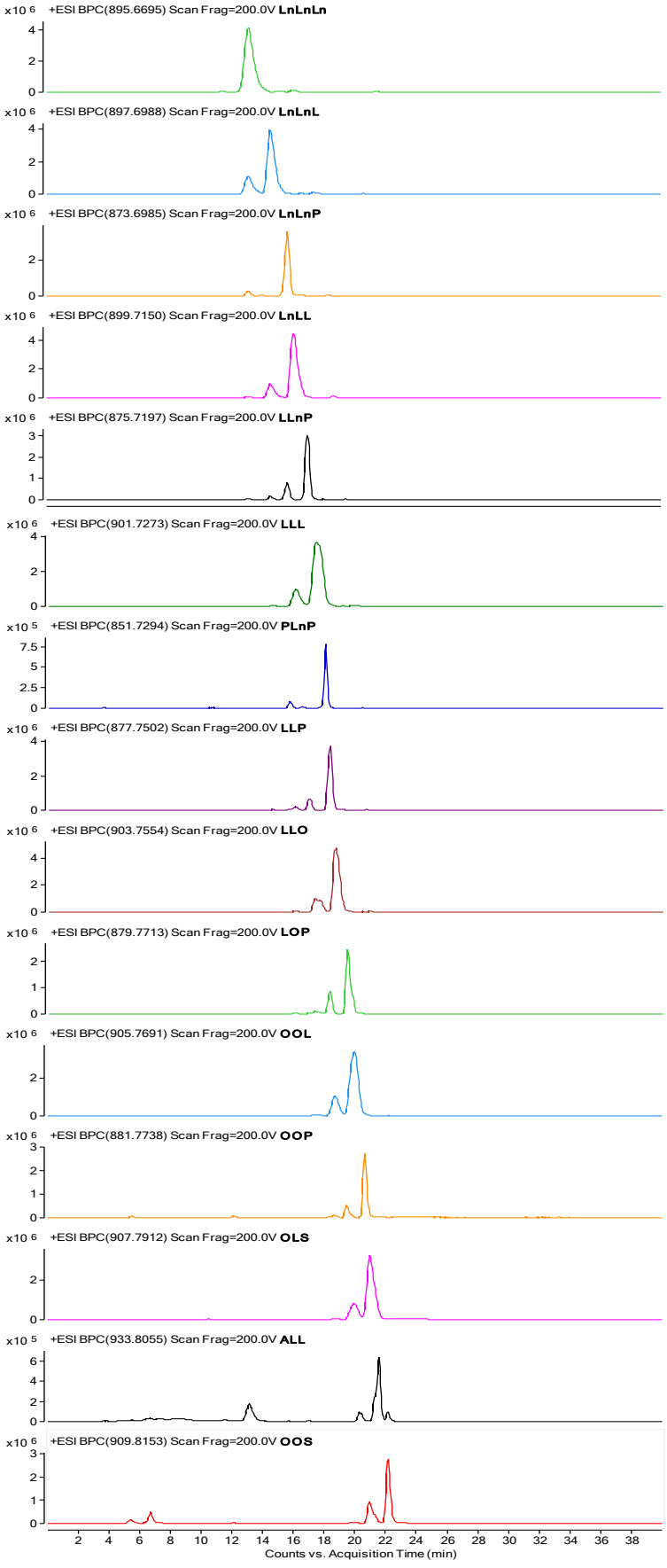
Dye source		Botanical name	Main molecular markers
Anthraquinoid dyes	Madder-type	*Rubia tinctorum* L., *Rubia peregrina*, *Rubia cordifolia*, *Rubia* akane, *Galium* species, *Relbunium*, *Morinda citrifolia* L.	alizarin, purpurin, xanthopurpurin, munjistin, pseudopurpurin
	Armenian, Polish and American cochineal	*Dactylopius coccus* Costa, *Porphyrophora polonica*, *Porphyrophora haemli* Brandt	carminic acid (main component), kermesic acid, flavokermesic acid, dcii, dciv, dcvii
	Kermes	*Kermes vermilio* Planchon	kermesic acid (75-100%), flavokermesic acid (0-25%)
	Lac dye	*Kerria Lacca* Kerr	laccaic acid a (71-96%), laccaic acid b, c and d (0-20%), flavokermesic acid (3.6-9.0%)
Flavonoid dyestuffs	Weld	*Reseda luteola* L.	luteolin, apigenin, chrysoeriol
	Dyer's broom	*Genista tinctoria* L.	luteolin, (apigenin), genistein
	Sawwort	*Serratula tinctoria*L.	luteolin, diosmetin, chlorogenic acid
	Persian berries	*Rhamnus* genus berries	quercetin, rhamnetin, kaempferol
	Young fustic	*Cotinus coggygria* Scop.	fisetin, fustin, sulfuretin
	Fustic	*Clorophora tinctoria* L.	morin, maclurin, kaempferol
	Quercitron	*Quercus velutina* Lam.	quercetin, quercitrin
	Safflower	*Carthamus tinctorius*	carthamin, cts, safflower yellow a, safflower yellow b, and precarthamin
	Brazilwood and sappanwood	*Caesalpinia* species	brazilin, brazilein, bw compounds
Indigoid dyestuffs	Logwood	*Haematoxylum campechianum*	hematein, hematoxylin
	Indigo	*Indigofera tinctoria*	indigotin, indirubin
	Woad	*Isatis tinctoria*	indigotin
	Purple	*Bolinus brandaris*L., *Hexaplex trunculus* L., *Stramonita haemastoma*, *Plicopurpura patula*, *Nucella lapillus*	6,6'-dibromoindigotin, 6-bromoindigotin, indigotin, 6,6'-dibromoindirubin, 6-bromoindirubin, indirubin
Lichen	Orchil	*Rocella tinctoria*	orcein
Tannins	Galls	gallnuts from *Cynips*, or *Quercu sinfectoria* Oliv.	gallic acid, ellagic acid
	Alder bark	*Alnus glutinosa*	gallic acid , quercetin, emodin
	Sumac	*Rhus* genus	gallic acid , elladico acid, quercitrin, kaempferol
	Black walnut	*Juglans nigra, Juglans regia* L.*, Juglans cinerea* L.	juglone
Naphto-quinones	Alkanna	*Alkanna tinctoria* Tausch.	alkannin
	Henna	*Lawsonia inermis*L.	lawsone
Polymethin dyes	Turmeric	*Curcuma longa* L.	curcumin, demethoxycurcumin, bisdemethoxycurcumin
	Saffron	*Crocus sativus*L.	crocin, crocetin
	Annatto	*Bixa orellana* L.	bixin

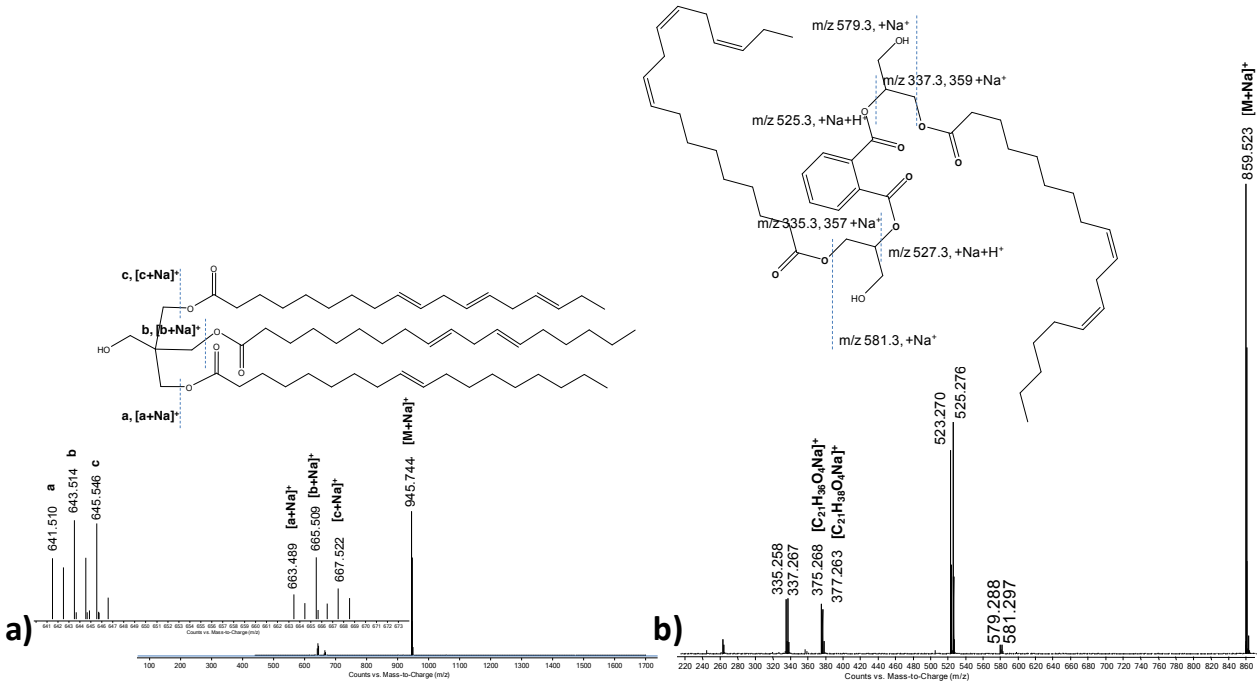
Table 3: Comparison of the UHPLC methods described in the literature for the characterization of natural organic dyes.

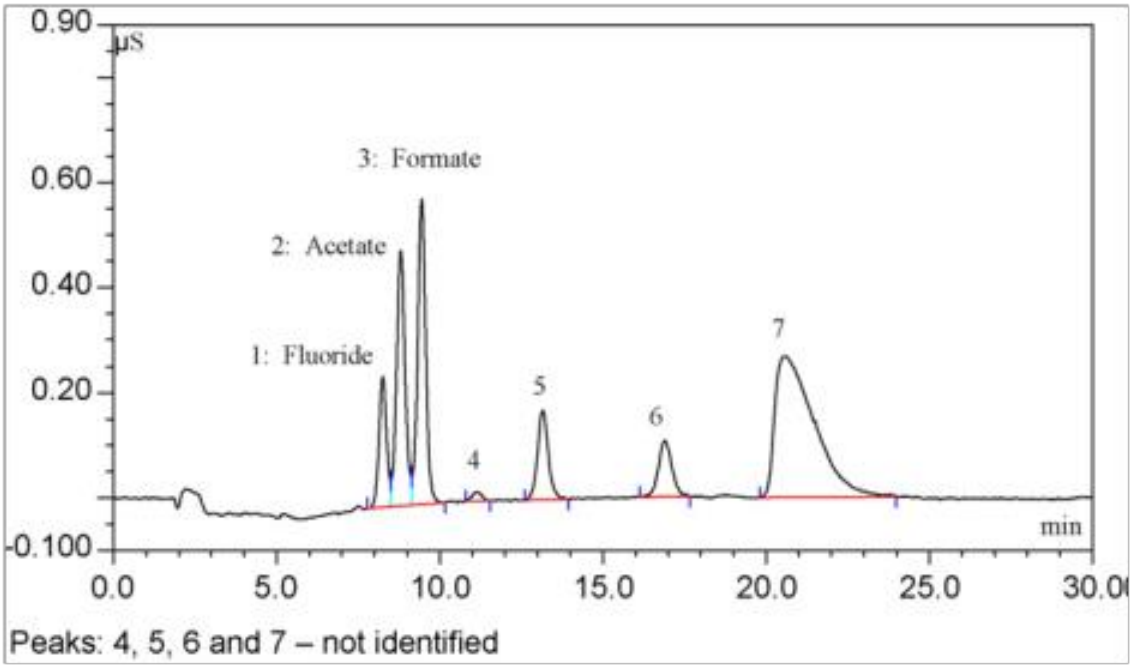
Standards and reference materials	Column(s) and detector(s)	Acquisition parameters	Advantages/disadvantages	Ref.
gallic acid, carminic acid, rutin, luteolin, quercetin, apigenin, alizarin, purpurin	Acquity UPLC BEH C18 100 mm x 2.1 mm ID, 1.7 µm particle size, Waters (best of three UHPLC columns tested) Detector: PDA	T= 30 °C flow rate: 0.25 mL/min eluents: H_2O and ACN with 0.1% formic acid V= 5 µL	short runtime (6 minutes, gradient) LODs not reported	[**16**]
standards for quantitative analysis: alizarin, apigenin, genistein, luteolin, purpurin; reference materials: cochineal, turmeric, indigo, Kermes; spectral database: 59 natural reference materials (85 compounds)	Acquity UPLC BEH C18 150 mm x 2.1 mm ID, 1.7 µm particle size, Waters (best of five UHPLC columns tested) Detector: PDA	T=40 °C flow rate: 0.2 mL/min eluents: H_2O and MeOH with 0.1% formic acid V= 0.1-4µL	40 minutes runtime(gradient) LODs (evaluated at the λ_{max} in the visible range for each analyte): <0.1 ng (injected) for flavonoids; 0.2-0.3 ng for anthraquinones; 0.3 ng for indigotin and 8.1 ng for curcumin	[**2**]
standards for quantitative analysis: luteolin, apigenin, genistein, chrysoeriol, diosmetin reference material: *Genistatinctoria* L.	PST BEH C18 150 mm x 2.1 mm ID, 1.7 µm particle size, Waters Detectors: PDA and ESI-MS/MS	T=55 °C flow rate: 0.25 mL/min eluents: H_2O with 0.02% formic acid and MeOH V= 5 µL	37 minutes runtime (gradient) LODs (evaluated at 254 nm): 0.5-1.9 ng injected	[**17**]

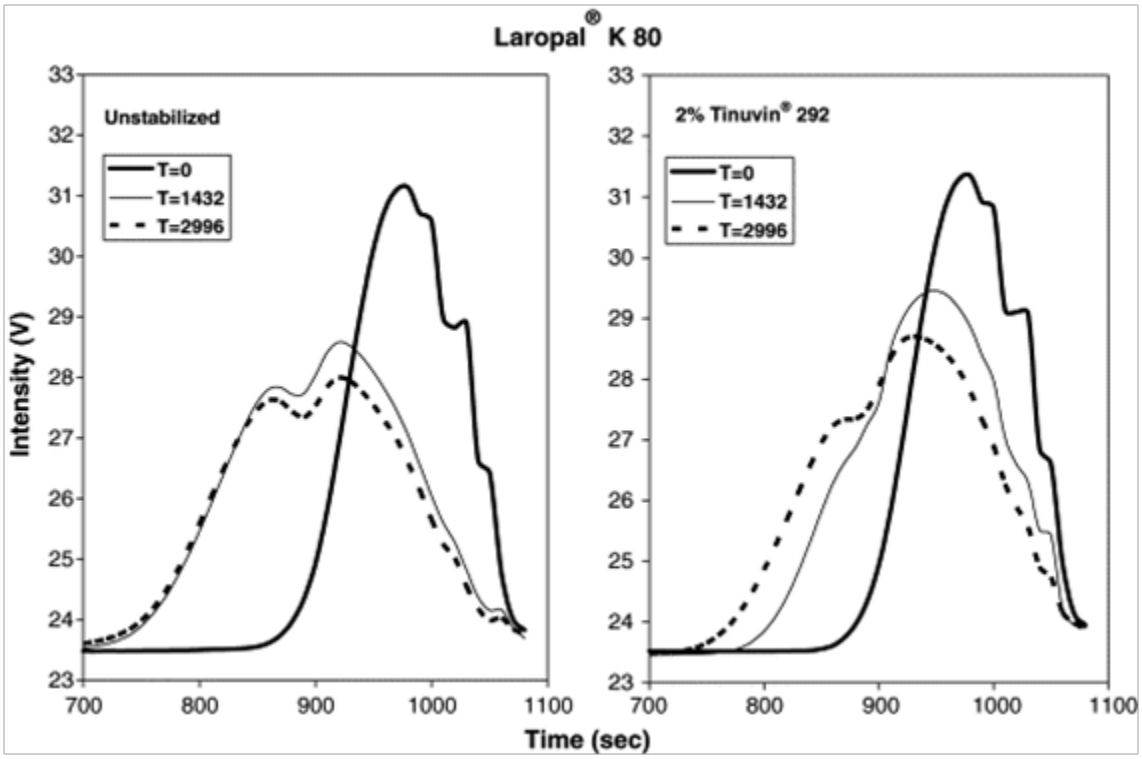
Chromatogram	Sample treatment	Ref.
 reference madder-dyed wool (alum-mordanted), chromatogram shown at 290 nm	2 mg of dyed wool was placed in vials, and 1.0 mL of 0.1% Na_2EDTA in H_2O/DMF (1:1, v/v) solution was added. The vials were capped and kept at 100 °C for 30 min. Vials were cooled to room temperature, and the solvent was evaporated under vacuum. 10 = alizarin; 11 = purpurin.	[24]
 Chromatograms (at 255 nm) of extracts of home-made madder lake RT2	Upper chromatogram: the samples (c. 0.2 mg in glass test tubes) were dispersed at room temperature in 250 µL of a 2/1/1/1 (v/v) mixture of HF aqueous solution (HF 4 M, DTPA 4 mM, LiF 120 mM), MeOH, DMF and AcEt. The supernatants were filtered on polyethylene frits (0.5 µm) before injection. Lower chromatogram: the samples (c. 0.2 mg in glass test tubes) were dispersed in 250 µL of a 2/1/1 mixture of concentrated HCl, methanol and water, and heated for 10 min at 110 °C. After evaporation to dryness under vacuum, the residues were taken up in 50 µL of MeOH/H2O (1:1, v/v), filtered on polyethylene frits (0.5 µm), and injected into the HPLC system.	[28]
 Chromatogram (at 450 nm) of extract of a textile dyed with *Rubia tinctorum* (240 = alizarin and 256 = purpurin)	heating approximately 0.1–1 mg of fibers in 200 µL of a solution of pyridine/water/1.0 M oxalic acid in water (95:95:10) at 100 °C for 15 min.	[30]

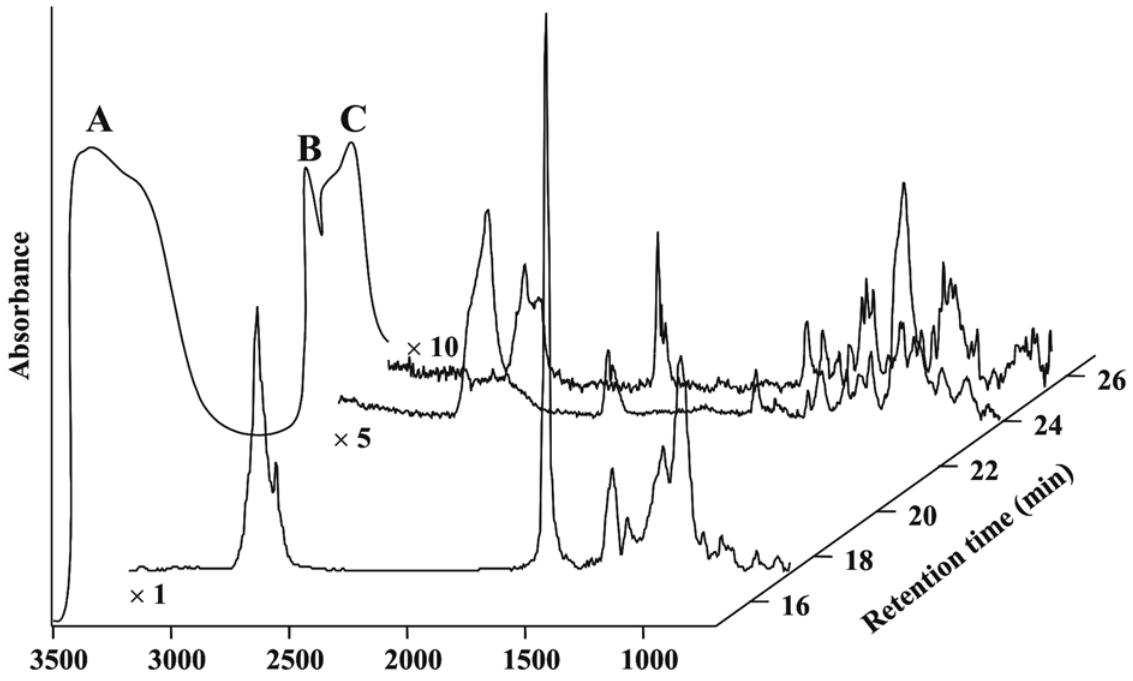

605
[SO]+
607
[SS]+
265
O+
267
S+
889
[M+H]+
Relative abundance
100
80
60
40
20
200
400
600
800
1000
m/z

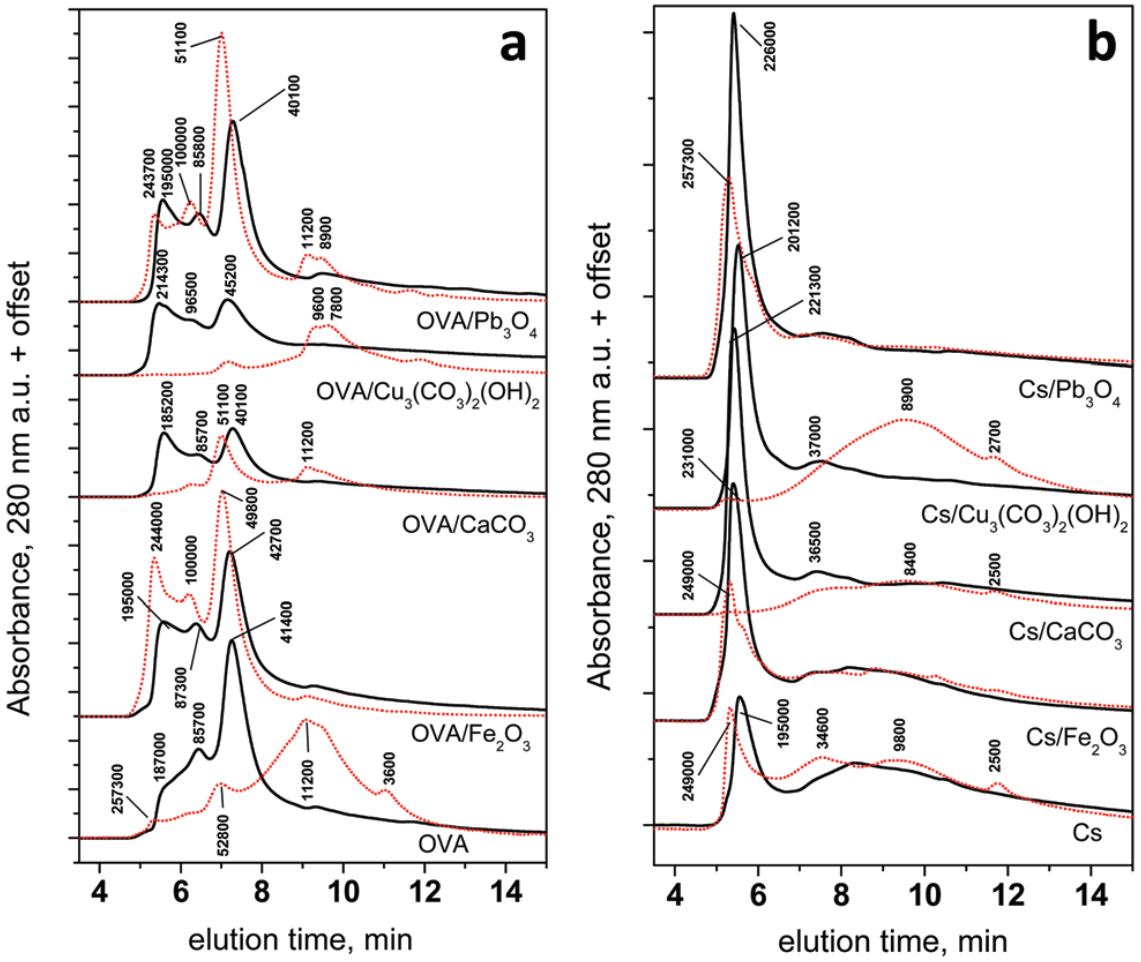

x10 6 +ESI BPC(895.6695) Scan Frag=200.0V LnLnLn
x10 6 +ESI BPC(897.6988) Scan Frag=200.0V LnLnL
x10 6 +ESI BPC(873.6985) Scan Frag=200.0V LnLnP
x10 6 +ESI BPC(899.7150) Scan Frag=200.0V LnLL
x10 6 +ESI BPC(875.7197) Scan Frag=200.0V LLnP
x10 6 +ESI BPC(901.7273) Scan Frag=200.0V LLL
x10 5 +ESI BPC(851.7294) Scan Frag=200.0V PLnP
x10 6 +ESI BPC(877.7502) Scan Frag=200.0V LLP
x10 6 +ESI BPC(903.7554) Scan Frag=200.0V LLO
x10 6 +ESI BPC(879.7713) Scan Frag=200.0V LOP
x10 6 +ESI BPC(905.7691) Scan Frag=200.0V OOL
x10 6 +ESI BPC(881.7738) Scan Frag=200.0V OOP
x10 6 +ESI BPC(907.7912) Scan Frag=200.0V OLS
x10 5 +ESI BPC(933.8055) Scan Frag=200.0V ALL
x10 6 +ESI BPC(909.8153) Scan Frag=200.0V OOS
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38
Counts vs. Acquisition Time (min)


a)
c, [c+Na]+
b, [b+Na]+
a, [a+Na]+
HO
641.510
643.514
645.546
663.489
665.509
667.522
945.744
[a+Na]+
[b+Na]+
[c+Na]+
[M+Na]+
Counts vs. Mass-to-Charge (m/z)
b)
m/z 579.3, +Na+
m/z 337.3, 359 +Na+
m/z 525.3, +Na+H+
m/z 335.3, 357 +Na+
m/z 527.3, +Na+H+
m/z 581.3, +Na+
OH
HO
335.258
337.267
375.268
377.263
523.270
525.276
579.288
581.297
859.523
[C21H36O4Na]+
[C21H38O4Na]+
[M+Na]+
Counts vs. Mass-to-Charge (m/z)


0.90
µS
0.60
0.40
0.20
-0.100
3: Formate
2: Acetate
1: Fluoride
4
5
6
7
min
0.0
5.0
10.0
15.0
20.0
25.0
30.0
Peaks: 4, 5, 6 and 7 – not identified


Laropal® K 80
Unstabilized
T=0
T=1432
T=2996
2% Tinuvin® 292
T=0
T=1432
T=2996
Intensity (V)
Time (sec)
33
32
31
30
29
28
27
26
25
24
23
700
800
900
1000
1100


A
B
C
× 10
× 5
× 1
Absorbance
3500
3000
2500
2000
1500
1000
16
18
20
22
24
26
Retention time (min)


a
b
Absorbance, 280 nm a.u. + offset
elution time, min
OVA/Pb3O4
OVA/Cu3(CO3)2(OH)2
OVA/CaCO3
OVA/Fe2O3
OVA
Cs/Pb3O4
Cs/Cu3(CO3)2(OH)2
Cs/CaCO3
Cs/Fe2O3
Cs
51100
40100
243700
195000
100000
85800
11200
8900
214300
96500
45200
9600
7800
185200
85700
244000
49800
42700
100000
41400
87300
187000
257300
52800
3600
226000
201200
221300
231000
37000
8900
2700
36500
8400
2500
249000
34600
9800