Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1025 (2004) 277–285
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Suppression of pigment interference in the gas chromatographic analysis of proteinaceous binding media in paintings with EDTA☆

J. de la Cruz-Cañizares[a], M.T. Doménech-Carbó[a,*], J.V. Gimeno-Adelantado[b], R. Mateo-Castro[b], F. Bosch-Reig[b]

[a] *Department of Conservation and Restoration of Cultural Heritage, Polytechnic University of Valencia, Camino de Vera 14, 46022 Valencia, Spain*
[b] *Department of Analytical Chemistry, University of Valencia, Avda. Dr. Moliner s/n, 46100 Burjassot, Spain*

Received 7 March 2003; received in revised form 21 October 2003; accepted 30 October 2003

Abstract

A method to suppress the interference of pigments in the analysis of proteinaceous media used in paintings is presented in this paper. This method is based on the formation of metallic ion–ethylenediaminetetraacetic acid (EDTA) complexes previous to the derivatisation process, using ethyl chloroformate (ECF), to transform the amino acids in *N*(*O*,*S*)-ethoxycarbonyl (EOC) ethyl esters. Test specimens, containing different proteinaceous media such as albumin, porcine gelatine and casein mixed with lead white, chalk, verdigris and raw Sienna have been prepared for carrying out this study. Different pH conditions have been probed for the different pigments studied. Values of peak area ratio of amino acids relative to the alanine, obtained using the proposed method on a series of protein–pigment test specimens, have been compared to those from specimens of pure protein in which direct method of derivatisation was applied. Finally, the method has been successfully applied to the analysis of 18th century wall paintings in which animal glue was used as binding medium.

Keywords: Art analysis; Derivatisation, GC; Paints; Pigments; Amino acids; Ethyl chloroformate; Proteins

1. Introduction

Most of the traditional painting techniques, commonly employed in Fine Arts, are based on the use of a proteinaceous medium such as animal glue, egg or casein together with the pigments. Art treatises written since the antiquity to nowadays have included detailed descriptions of these materials, their preparation methodology and applications [1–6]. From the point of view of the chemical analysis, as a necessary step of the diagnostic work, the accurate determination of the chemical composition of pictorial samples permits not only the identification of the materials used but also the characterisation of the artistic technique. Many deterioration processes that are undergone by the art objects are due to the higher degree of reactivity of the organic binders by comparison to the inorganic pigments also found in works of art. Therefore, the identification of organic materials used as binding media is, additionally, of great interest.

Gas chromatography (GC) has been used, in the last decades, in the field of conservation and restoration of art works to obtain complete information on organic compounds and, in particular, on proteinaceous media due to its high sensitivity [7–17]. Characterisation of proteinaceous binding media of paintings using GC, usually, involves a procedure consisting in a first hydrolysis step and then, a derivatisation treatment of free amino acids using a specific reagent. Nevertheless, this analysis is often hindered by the pigments also present in the paint samples that complex the amino acids obtained as product of the hydrolysis of proteins, in particular, lead, copper, calcium, iron and manganese pigments [13,18,19]. In particular, calcium could be found in pictorial samples not only as pigment but also as major component of easel painting and wall grounds (gypsum or calcium carbonate). Pollution is other frequent source of calcium ions.

Different strategies have prompted their use to suppress the interference from inorganic pigments [19–21]. Thus, inclusion of a step, previous to the hydrolysis, in which

☆ Presented at the 2nd Meeting of the Spanish Society of Chromatography and Related Techniques, Barcelona, 26–29 November 2002.

* Corresponding author. Tel.: +34-96-3877310; fax: +34-96-3877319.
E-mail address: tdomenec@crbc.upv.es (M.T. Doménech-Carbó).

0021-9673/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2003.10.118

the proteins are extracted with ammonia. In this way, the binding medium is separated of the inorganic sample matrix and then, derivatised. Other method consisting in the addition of a cation exchange cleaning up step in which amino acids and cations are selectively retained by the resin has also been proposed [22]. Finally, use of Na_2EDTA as sequestering agent of metallic cations which interfere the analysis of amino acids has been suggested [21] but study of the specific conditions and methodology has not been established.

A method to suppress the interference of pigments is presented in this paper that is based on the formation of metallic ion–ethylenediaminetetraacetic acid (EDTA) complexes, previous to the derivatisation process using ethyl chloroformate (ECF) to transform the amino acids in *N*(*O*,*S*)-ethoxycarbonyl (EOC) ethyl esters of amino acids in presence of ethanol and pyridine [11]. The advantage of the use of ECF as derivatising agent has been already described in previous papers [11,17], and consists in the notable reduction of the necessary time to perform the analysis, minimal handling of the sample and, mainly, the use of an aqueous medium for the reaction that enables a high solubility of amino acids.

Test specimens containing different proteinaceous media such as albumin, porcine gelatine and casein mixed with lead white, chalk, verdigris and raw Sienna have been prepared for carrying out this study. Copper and lead pigments, in particular, verdigris and white lead have been frequently found in paint layers of wall, canvas and panel painting. Raw Sienna has been commonly used as pigment and, from the 16th to 19th centuries, was the main component of canvas painting grounds. Finally, chalk has been the main component of grounds of wall and panel paintings. Acid hydrolysis has been carried out in order to obtain the amino acids present in the samples. Then, a new step in which ethylenediaminetetraacetic acid is added, has been introduced in the method. After this, the free amino acid are transformed in ethyl esters by reaction with ethyl chloroformate followed by extraction in chloroform to complete the separation of the remaining EDTA–metal complexes. Different pH conditions have been probed for the different pigments studied. Values of peak area ratio of amino acids relative to the alanine, obtained using the proposed method on a series of protein–pigment test specimens, have been compared to those from specimens of pure protein in which direct method of derivatisation was applied. Finally, the method has been successfully applied to the analysis of 18th century wall paintings in which animal glue was used as binding medium.

2. Experimental

2.1. Solvents and reagents

The following reagents were used to treat the samples: ethyl chloroformate (purity >98%) and absolute pyridine (Fluka, Buchs, Switzerland). Hydrochloric acid at 37% for analysis (Scharlau, Barcelona, Spain). Chloroform at 98% for GC (Acros, Cambridge, USA). Absolute ethanol for analysis and sodium hydrogencarbonate for analysis (Panreac, Barcelona, Spain). Ethylenediaminetetraacetic acid disodium salt, ammonia and ammonium chloride (Probus, Badalona, Spain).

A standard solution mixture was prepared at a concentration of 0.5 μg/μl of each amino acid. The amino acids used to prepare the standard mixture were alanine (A), glycine (G), valine (V), leucine (L), isoleucine (I), proline (P), threonine (T), serine (S), glutamic acid (E), aspartic acid (D), methionine (M), hydroxyproline (Hp) and phenylalanine (F). All the above standards were obtained from a Sigma LAA-21 kit (St. Louis, MO, USA).

Casein (from bovine milk, 95% purity), albumin from chicken egg (ovoalbumin grade II), gelatine type A (from porcine skin, 98% purity) were supplied by Sigma. White lead (Charbonnel 13, quai Montebello, 75005 Paris, France) was supplied by RCM Productos de Conservación, Barcelona, Spain. Raw Sienna, verdigris and chalk (Kremer, Farbmühle, D-88317 Aichstten/Allgäu, Germany) were supplied by AP Fitzpatrick, London, UK.

2.2. Test specimens

A series of test specimens were prepared in which aqueous solutions of gelatine (70 g/l) and albumin (70 g/l) and an ammonium caseinate solution obtained dissolving 67 g of casein in 1 l of 4.5 M NH_3 aqueous solution. Chalk, verdigris, white lead and raw Sienna pigments were dispersed in the above solutions in a proportion of 30% (w/w) and were spread as thin layers on glass slides. Then the test specimens were dried at room temperature (during 15 days) and kept in the fridge until analysis.

2.3. Art work samples

The Palace of the Marqués de Montortal was build at the end of the 18th century as a nobiliary mansion located in Carcaixent (Valencia, Spain). Eight rooms are decorated with wall paintings in academic, “trompe-l’oeil” classicist and local 18th century style. Examination of cross-section using light microscope has shown different paint strata in which pure pigments were applied on a gypsum white ground. White lead, vermilion, minium, Mars black, Ultramarine blue, chrome orange, chrome yellow, iron oxide red, raw Sienna, among other pigments, have been identified using scanning electron microscopy (SEM) and electron dispersive X-ray spectrometry (EDX) [23].

The samples were taken and the paint layers were mechanically separated using a scalpel. Then, the samples were ground and homogenised in a small agate mortar. Finally, 5–10 mg of the sample were weighed in order to apply the proposed experimental procedure of analysis.

2.4. Experimental procedure

2.4.1. Direct method of derivatisation (D.M.)

0.1 mg of the sample (standards or test specimens) were placed in 3 μl minivials (Supelco Bellefonte, PA, USA) and hydrolysed with 100 μl of 6 M HCl for 24 h at 110 °C in an Ar atmosphere. The resulting solution was evaporated to dryness and then, 50 μl of water and 50 μl of chloroform were added. After this, the solution was vigorously shaken to facilitate the extraction of amino acids in the aqueous phase. After separation of the two phases, 50 μl of ethanol–pyridine (4:1) solution were added to the aqueous phase. This solution was treated with 20 μl of ECF, and this reaction mixture was shaken for about 10 s and extracted with 50 μl of chloroform containing 1% ECF. Separation of the two phases was carried out and then, 50 μl of a saturated solution of $NaHCO_3$ were added to the organic phase and after this, the two layers (aqueous and chloroform) were vigorous but careful shaking. Finally,1 μl of the organic phase was injected into the gas chromatograph.

2.4.2. EDTA method for suppressing the pigment interference (EDTA.M.)

After the resulting hydrolysed solution was evaporated to dryness, 50 μl of 0.2 M Na_2EDTA, solved in a buffer ammonia/ammonium chloride (pH 9), were added instead of water. The rest of the experimental procedure was performed in the same way that in the direct method above described.

2.5. Instrumentation

GC–flame ionisation detector (FID) analysis was carried out on a Hewlett-Packard 6890 Series II gas chromatograph, controlled by HP 3365 ChemStation software (Hewlett-Packard, Avondale, PA, USA). The injector and detector temperatures were 250 and 300 °C, respectively. Separation was achieved on an HP-1701 (25 m × 0.25 mm i.d., 0.15 μm film thickness) fused-silica capillary column. Oven temperature was programmed from 100 to 275 °C (held 8 min) at 40 °C/min. Helium, at an inlet pressure of 115 kPa, was the carrier gas. Split injection, at a split ratio 1:20, was used. Volume injected was 1 μl. Pressure was maintained constant.

For confirmation of chemical composition of the compounds analysed, an Agilent 5973N mass spectrometer coupled to an Agilent 6890N gas chromatograph (Agilent Instruments, USA) was used. Agilent Chemstation software (MSD) was used for the integration of peaks and for the mass spectra evaluation. GC separation was achieved in a chemically bonded fused-silica capillary column HP-5-MS (Agilent, USA), (stationary phase 5% phenyl–95% methylpolysiloxane, 30 m × 0.25 mm i.d., 0.25 μm film thickness). Inlet temperature was 250 °C and the oven temperature was programmed from 120 to 150 °C with a first ramp of 10 °C/min and from 150 to 290 °C with a second ramp of 40 °C/min. Temperature of 290 °C was finally maintained for 5 min. Electron impact (EI) was used as ionisation technique with an electronic energy of 70 eV and a spectrometer scan rate of 0.52 s/scan with *m*/*z* range between 18 and 800. The carrier gas was He with inlet pressure of 75.2 kPa and 1:20 split ratio. Flow was maintained constant.

Purity of the pigments was determined using a Jeol JSM 6300 scanning electron microscope operating with a Link-Oxford-Isis X-ray microanalysis system. The analytical conditions were: 20 kV accelerating voltage, 2×10^{-9} A beam current and 15 mm as working distance. Samples were carbon coated to eliminate charging effects. Semiquantitative microanalysis was carried out using the ZAF method for correcting interelemental effects. The counting time was 100 s for major and minor elements. The standards used were natural minerals: albite (Na), MgO (Mg), Al_2O_3 (Al), quartz (Si), GaP (P), FeS_2 (S), MAD-10 (K), Fe (Fe), Mn (Mn), wollastonite (Ca), Cu (Cu), Pb (PbF_2).

3. Results and discussion

3.1. Interferent effect of pigments in proteinaceous media analysis

Figs. 1 and 2, in which the gas chromatograms corresponding to the ECF derivatives of amino acids from two samples of albumin and albumin–verdigris have been represented, permit a qualitative approach to the problem of the interferent effect of the pigments in the analysis of proteinaceous media. A strong decreasing of the signal of the majority of the amino acids can be observed when the chromatogram represented in Fig. 2 is compared to the one in Fig. 1 in which pigment was not added. This fact is probably due to the significant decreasing in the amount of proteinaceous matter in the pigmented sample. Nevertheless, a more complete assessment of the influence of the pigments on the quantitation of amino acids can be made from the Fig. 3 in which the peak area ratios of glycine, valine, leucine, isoleucine, proline, and phenylalanine, relative to alanine for the four different test specimens prepared with chalk, raw Sienna, verdigris and white lead, bound with casein, are shown together with these from the pure casein. As can be seen, a remarkable decreasing in the peak area ratios of amino acids from the pigment–binder system is observed in the majority of cases and only a strong increasing in the peak area ratios of glycine, valine, leucine and isoleucine, corresponding to the verdigris–casein binary sample, is observed. Analogous behaviour is observed in the rest of the specimens prepared with gelatine and albumin. These results indicate that: (a) interference of the four studied pigments yields a reduction in the peak area ratios of the majority of amino acids used in the identification of proteinaceous binders; (b) the strong increasing observed in the peak area ratios of glycine, valine, leucine, isoleucine and phenylalanine from the three verdigris-binder systems suggests that this pigment

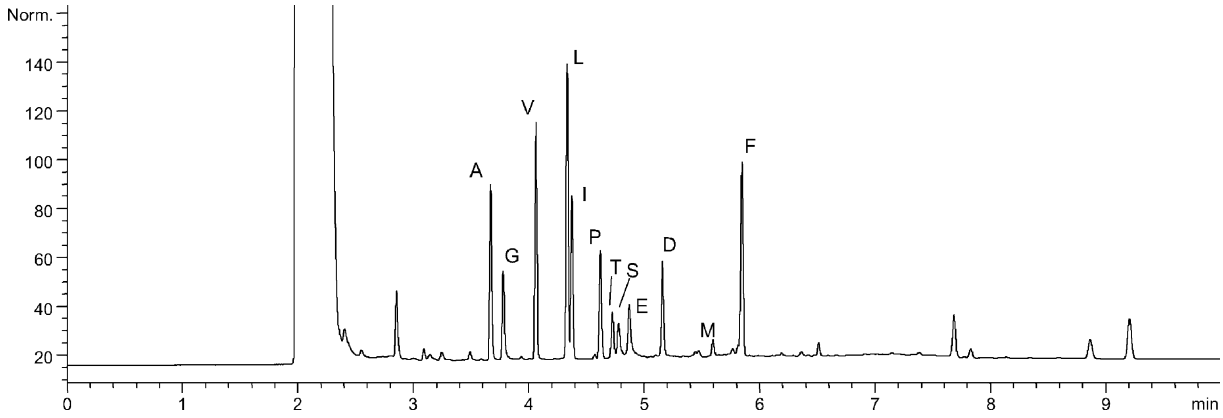

Fig. 1. Gas chromatogram of amino acids, present in albumin, as ECF derivatives after hydrolysis.

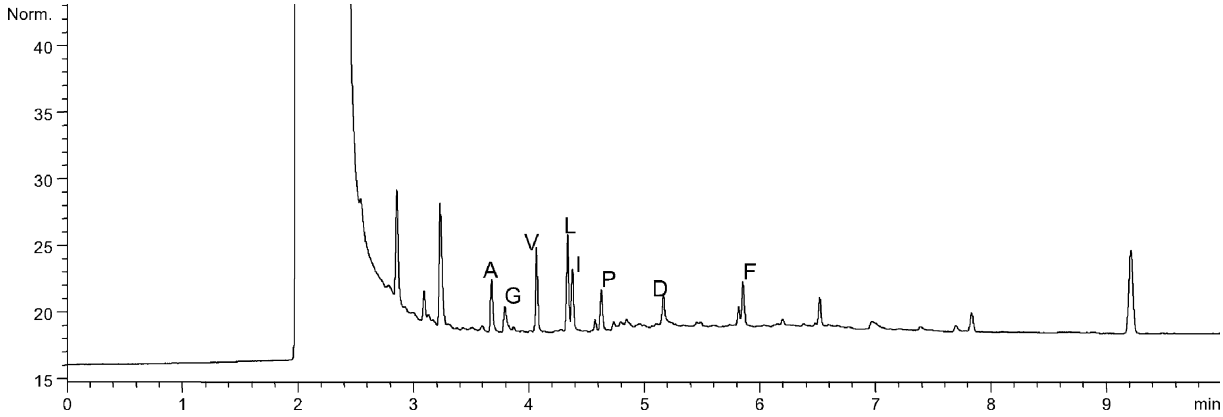

Fig. 2. Gas chromatogram of amino acids, present in the albumin–verdigris test specimen, as ECF derivatives after hydrolysis.

specially interferes the analysis of the proteinaceous media studied.

3.2. Use of Na_2EDTA for suppressing the pigment interference

A previous analysis attempting to determine the purity of the pigments was carried out using an X-ray microanalysis system coupled to a scanning electron microscope. Ca, Cu and Pb were the main metallic elements found in chalk, verdigris and white lead, respectively. Analysis of raw Sienna evidenced its "iron oxide pigment" composition with an 86.23% of Fe_2O_3 among other elements that are characteristic of natural earth pigments (see Fig. 4). The content of manganese (5.22% MnO) must be noted for the potential capacity of this metallic element to form highly stable complexes with many amino acids studied.

Use of Na_2EDTA for suppressing the pigment interference has been proposed in this work on the basis of its major ability for sequestering copper(II), iron(III), lead(II),

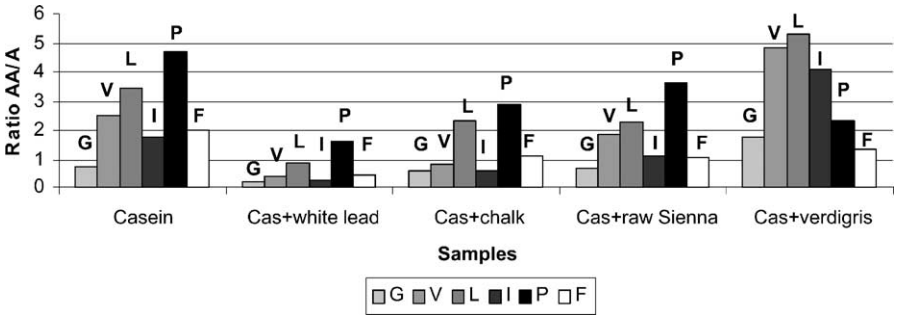

Fig. 3. Peak area ratios of amino acids relative to alanine for different test specimens which were prepared with chalk, raw Sienna, verdigris and white lead bound with casein. Amino acids from casein are obtained as ECF derivatives after hydrolysis (G: glycine; V: valine; L: leucine; I: isoleucine; P: proline; and F: phenylalanine).

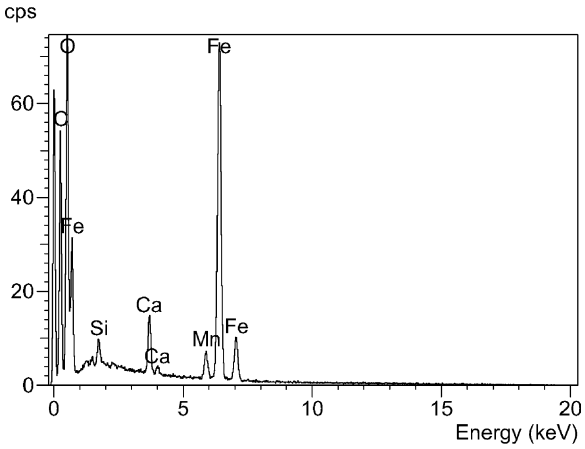

Fig. 4. Energy dispersive X-ray spectrum of raw Sienna pigment.

calcium(II) and manganese(II) than the amino acids present in proteinaceous media [24].

Formation of metallic ion–EDTA complexes is induced by the addition of 100 μl of 0.2 M Na_2EDTA solution to the extract obtained after leading to dryness the solution resulting of the hydrolysis of the samples. This solution consists in an ammonia/ammonium chloride buffer that provides a pH 9 in which the metallic ions studied quantitatively form the ethylenediaminetetraacetates [25]. The concentration of Na_2EDTA solution is adjusted on the basis of stoichiometric calculations from the content of pigment in the samples, in order to assure that the masking process is complete. Pigment content in the samples was evaluated on the basis of the concentration of the solutions prepared for the test specimens. After this, the sequence of steps leading to form the ECF derivatives of the amino acids is followed as described in prior works [11,17].

Attention should be drawn to the fact that the hexadentate metallic ion–EDTA complexes are unable to form the corresponding ECF derivatives due to the absence of free carboxylic and amine groups. It should be also noted that the 1% ECF solution in chloroform, which is added to the water–ethanol–pyridine phase, acts as an extraction system in which the more chloroform-soluble ECF derivatives of the amino acids and the $EDTA^{2-}$ excess are selectively separated of the metallic ion–EDTA complexes that are more soluble in the hydro-alcoholic phase.

A previous series of probes have been developed in order to check the possible interference of the ECF derivatives of the $EDTA^{2-}$ excess on the ECF derivatives of the free amino acids. Fig. 5 shows the gas chromatogram corresponding to a mixture of ECF derivatives of amino acids treated with $EDTA^{2-}$, following the proposed method. Experimental conditions in the gas chromatograph have been maintained as usual. The inspection of this chromatogram evidences that the ECF derivatives of amino acids, which exhibit retention time from 3.67 to 5.87 min are perfectly separated of the tetra and triethyl esters of EDTA that exhibit a higher retention time (7.28 and 8.32 min) and, therefore, interference does not occur at the experimental conditions used.

First column in Tables 1–3 summarises the average values for three independent replicates of peak area ratios of amino acids relative to the alanine corresponding to the three standard of proteinaceous media studied. Values of these quotients, obtained in the different binary specimens "proteinaceous medium–pigment", are also listed. The D.M. values have been obtained by applying the usual derivatisation procedure whereas the EDTA.M. values correspond to the derivatisation method including the addition of Na_2EDTA solution step. Values of peak area ratios for pure proteins according to the EDTA method were obtained in a second series of preliminary tests. These values have been in good agreement to those from direct method (D.M.) and none of them exceeded the standard deviation of pure protein according to the direct method.

It should be noted that values obtained using the proposed method for suppressing the pigment interference (EDTA.M.) are, in general, closer to these from the standard than the values obtained directly (D.M.) from the binary specimens proteinaceous medium–pigment. Results listed in Table 1, corresponding to albumin, show that binary specimens of

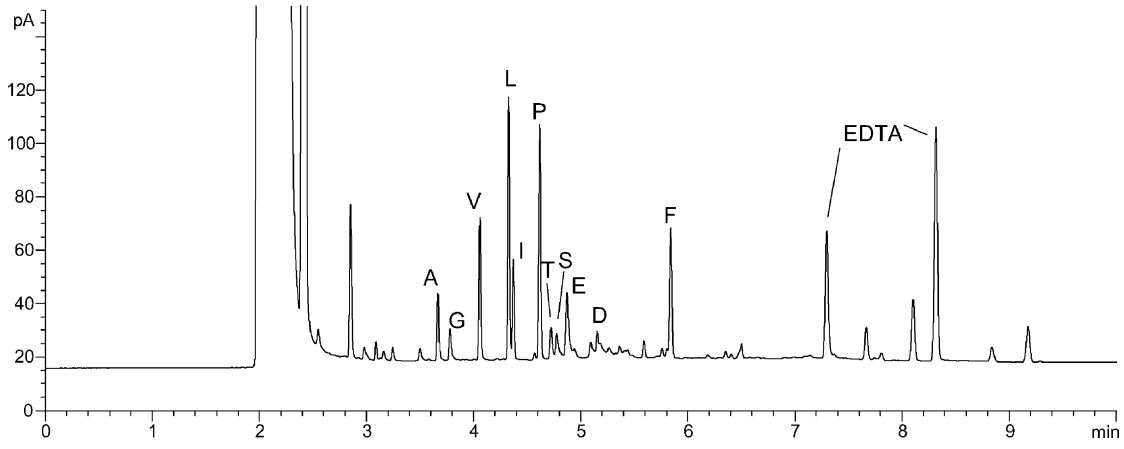

Fig. 5. Gas chromatogram of a mixture of ECF derivatives of amino acids treated with Na_2EDTA following the proposed method.

Table 1
Peak area ratios of amino acids relative to alanine corresponding to the albumin and the albumin–pigment binary specimens: (D.M.) direct method without application of suppressing pigment interference treatment and (EDTA.M.) application of the proposed suppressing pigment interference treatment

Amino acid	Albumin (D.M.)	Albumin + white lead		Albumin + Chalk		Albumin + Sienna raw		Albumin + verdigris	
		D.M.	EDTA.M.	D.M.	EDTA.M.	D.M.	EDTA.M.	D.M.	EDTA.M.
Glycine	0.45 ± 0.06	0.24 ± 0.06	0.26 ± 0.02	0.60 ± 0.03	0.56 ± 0.02	0.29 ± 0.09	0.15 ± 0.06	0.52 ± 0.03	0.64 ± 0.02
Valine	1.08 ± 0.07	1.4 ± 0.3	0.82 ± 0.05	1.26 ± 0.06	0.81 ± 0.04	1.2 ± 0.3	0.96 ± 0.09	1.41 ± 0.06	1.09 ± 0.05
Leucine	1.37 ± 0.05	1.9 ± 0.3	1.4 ± 0.1	1.72 ± 0.05	1.42 ± 0.08	1.6 ± 0.2	1.32 ± 0.08	1.71 ± 0.03	1.47 ± 0.07
Isoleucine	0.76 ± 0.09	1.1 ± 0.2	0.58 ± 0.03	0.90 ± 0.05	0.54 ± 0.03	0.8 ± 0.1	0.57 ± 0.08	1.07 ± 0.05	0.85 ± 0.08
Proline	0.63 ± 0.07	0.71 ± 0.09	0.55 ± 0.09	0.67 ± 0.06	0.55 ± 0.03	0.69 ± 0.09	0.75 ± 0.07	0.7 ± 0.1	0.49 ± 0.05
Threonine	0.26 ± 0.05	0.12 ± 0.05	0.15 ± 0.03	0.43 ± 0.03	0.21 ± 0.07	0.07 ± 0.02	[a]	0.19 ± 0.08	0.05 ± 0.01
Serine	0.20 ± 0.08	[a]	0.08 ± 0.05	0.47 ± 0.09	0.23 ± 0.04	0.04 ± 0.03	[a]	[a]	[a]
Glutamic acid	0.37 ± 0.06	[a]	[a]	0.40 ± 0.06	0.15 ± 0.06	[a]	[a]	[a]	[a]
Aspartic acid	0.5 ± 0.1	0.37 ± 0.09	0.17 ± 0.05	0.5 ± 0.1	0.24 ± 0.08	[a]	0.05 ± 0.02	0.13 ± 0.08	0.19 ± 0.09
Methionine	0.25 ± 0.04	[a]	0.20 ± 0.04	0.3 ± 0.1	0.21 ± 0.07	[a]	[a]	[a]	[a]
Hydroxyproline	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]
Phenylalanine	1.1 ± 0.2	1.08 ± 0.05	1.03 ± 0.08	1.43 ± 0.05	1.08 ± 0.08	0.9 ± 0.2	0.29 ± 0.03	1.07 ± 0.08	0.94 ± 0.07

[a] Peak was not detected or the ratio was <0.3.

Table 2
Peak area ratios of amino acids relative to alanine corresponding to the gelatine and the gelatine–pigment binary specimens: (D.M.) direct method without application of suppressing pigment interference treatment and (EDTA.M.) application of the proposed suppressing pigment interference treatment

Amino acids	Gelatin (D.M.)	Gelatin + white lead		Gelatin + chalk		Gelatin + Sienna raw		Gelatin + verdigris	
		D.M.	EDTA.M.	D.M.	EDTA.M.	D.M.	EDTA.M.	D.M.	EDTA.M.
Glycine	3.69 ± 0.04	2.9 ± 0.2	2.8 ± 0.1	2.7 ± 0.3	2.9 ± 0.1	3.1 ± 0.2	3.7 ± 0.2	2.5 ± 0.2	3.1 ± 0.2
Valine	0.31 ± 0.01	0.31 ± 0.01	0.21 ± 0.08	0.29 ± 0.07	0.19 ± 0.05	0.30 ± 0.02	0.28 ± 0.07	0.30 ± 0.03	0.29 ± 0.05
Leucine	0.36 ± 0.01	0.37 ± 0.02	0.37 ± 0.08	0.25 ± 0.08	0.34 ± 0.05	0.39 ± 0.06	0.39 ± 0.04	0.46 ± 0.03	0.41 ± 0.06
Isoleucine	0.13 ± 0.01	0.13 ± 0.01	0.10 ± 0.08	0.04 ± 0.03	0.08 ± 0.03	0.13 ± 0.05	0.13 ± 0.02	0.16 ± 0.02	0.14 ± 0.02
Proline	2.45 ± 0.08	1.96 ± 0.06	1.75 ± 0.08	1.3 ± 0.4	1.6 ± 0.1	1.9 ± 0.2	2.01 ± 0.06	1.1 ± 0.3	1.95 ± 0.08
Threonine	0.08 ± 0.02	0.06 ± 0.01	0.07 ± 0.02	[a]	0.06 ± 0.03	0.07 ± 0.01	0.12 ± 0.03	[a]	[a]
Serine	0.07 ± 0.03	0.05 ± 0.01	0.08 ± 0.02	0.06 ± 0.01	0.09 ± 0.02	0.06 ± 0.02	0.16 ± 0.05	[a]	[a]
Glutamic acid	0.37 ± 0.02	[a]	0.11 ± 0.06	0.15 ± 0.01	0.30 ± 0.03	0.05 ± 0.03	0.43 ± 0.07	0.20 ± 0.06	0.35 ± 0.07
Aspartic acid	0.30 ± 0.09	0.14 ± 0.05	0.05 ± 0.03	0.07 ± 0.06	0.15 ± 0.08	[a]	0.16 ± 0.05	0.15 ± 0.07	0.53 ± 0.07
Methionine	0.05 ± 0.04	0.05 ± 0.03	[a]	[a]	[a]	[a]	[a]	[a]	[a]
Hydroxyproline	0.85 ± 0.09	0.55 ± 0.06	0.86 ± 0.09	0.6 ± 0.1	0.83 ± 0.09	0.6 ± 0.2	1.2 ± 0.1	0.21 ± 0.01	0.65 ± 0.05
Phenylalanine	0.26 ± 0.05	0.24 ± 0.01	0.31 ± 0.04	0.12 ± 0.05	0.23 ± 0.06	0.27 ± 0.04	0.30 ± 0.04	0.25 ± 0.03	0.15 ± 0.05

[a] Peak was not detected or the ratio was <0.3.

Table 3
Peak area ratios of amino acids relative to alanine corresponding to the casein and the casein-pigment binary specimens: (D.M.) direct method without application of suppressing pigment interference treatment and (EDTA.M.) application of the proposed suppressing pigment interference treatment

Amino acid	Casein (D.M.)	Casein + white lead		Casein + chalk		Casein + Sienna raw		Casein + verdigris	
		D.M.	EDTA.M.	D.M.	EDTA.M.	D.M.	EDTA.M.	D.M.	EDTA.M.
Glycine	0.69 ± 0.06	0.20 ± 0.08	0.63 ± 0.07	0.55 ± 0.06	0.55 ± 0.06	0.6 ± 0.1	0.63 ± 0.06	1.8 ± 0.5	1.02 ± 0.08
Valine	2.49 ± 0.07	0.38 ± 0.07	1.65 ± 0.07	0.8 ± 0.3	2.08 ± 0.08	1.9 ± 0.6	1.82 ± 0.07	4.8 ± 0.8	2.00 ± 0.08
Leucine	3.4 ± 0.1	0.9 ± 0.2	3.1 ± 0.1	2.3 ± 0.4	3.82 ± 0.04	2.3 ± 0.6	3.36 ± 0.09	5.3 ± 0.9	3.7 ± 0.2
Isoleucine	1.78 ± 0.05	0.23 ± 0.09	1.19 ± 0.04	0.6 ± 0.2	1.45 ± 0.09	1.1 ± 0.2	1.22 ± 0.05	4.1 ± 0.9	1.56 ± 0.05
Proline	4.7 ± 0.2	1.7 ± 0.2	3.9 ± 0.1	2.9 ± 0.4	3.89 ± 0.07	3.6 ± 0.3	4.15 ± 0.06	2.4 ± 0.8	4.2 ± 0.1
Threonine	0.63 ± 0.06	0.04 ± 0.03	0.56 ± 0.06	0.30 ± 0.09	0.55 ± 0.09	0.38 ± 0.05	0.43 ± 0.02	0.6 ± 0.1	0.36 ± 0.05
Serine	0.53 ± 0.05	[a]	0.47 ± 0.08	0.51 ± 0.06	0.48 ± 0.06	0.46 ± 0.06	0.43 ± 0.02	0.04 ± 0.01	0.20 ± 0.01
Glutamic acid	1.86 ± 0.06	0.59 ± 0.09	1.3 ± 0.1	0.70 ± 0.08	1.31 ± 0.09	1.1 ± 0.2	1.64 ± 0.07	0.10 ± 0.09	1.44 ± 0.08
Aspartic acid	0.96 ± 0.07	[a]	0.38 ± 0.03	1.09 ± 0.06	0.26 ± 0.02	0.3 ± 0.1	0.47 ± 0.06	0.59 ± 0.05	1.19 ± 0.09
Methionine	0.08 ± 0.02	0.12 ± 0.06	0.29 ± 0.05	[a]	0.5 ± 0.1	[a]	0.32 ± 0.08	[a]	[a]
Hydroxyproline	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]
Phenylalanine	2.0 ± 0.1	0.42 ± 0.03	1.84 ± 0.09	1.1 ± 0.3	0.49 ± 0.09	1.1 ± 0.7	1.97 ± 0.07	1.3 ± 0.1	2.18 ± 0.03

[a] Peak was not detected or the ratio was <0.3.

white lead, chalk and verdigris exhibit the best results and only aspartic acid exhibits a notably higher deviation in the peak area ratios obtained using Na_2EDTA than from those obtained without suppressing the interference of the pigment. More significant differences in values of glycine, isoleucine, and phenylalanine together with slight divergences in values of proline, threonine and serine are found in the raw Sienna specimen that offers the worse response.

Values summarised in Table 2, corresponding to the specimens prepared with gelatine, indicate that, in general, specimens prepared with chalk, raw Sienna and verdigris had the best response when Na_2EDTA was added. Amino acids such as valine, proline, aspartic acid, methionine and phenylalanine exhibited a higher deviation when Na_2EDTA was added to the specimens prepared with lead white.

The best results have been obtained in the specimens corresponding to the casein, as can be seen in Table 3. Only the values of methionine and threonine corresponding to the (EDTA.M.) showed, in general, significant higher deviations than those obtained applying the usual method (D.M.). Aspartic acid and phenylalanine, in particular, exhibited lower values of peak area ratios corresponding to EDTA.M than those from the direct method.

The values obtained for the standard deviation, ranging from 0.01 to 0.9, were similar in the three series of samples (standards, binary samples with and without addition of Na_2EDTA). This suggests that the inclusion of the steps for suppressing the pigment interference does not imply a decreasing of the repeatability of the method.

Fig. 6 shows the principal component analysis (PCA) score plot of sample of casein (C), test specimen prepared with casein bound with raw Sienna (CS), chalk (CC), verdigris (CV) and white lead (CP), and test specimens, in which complexation with Na_2EDTA was carried out, prepared with casein and raw Sienna (CSE), chalk (CCE), verdigris (CVE) and white lead (CPE). All peak area ratios of amino acids relative to alanine have been considered as variables for differentiating these binary specimens. Thus, two principal components that account for the 61% of the variance have been considered.

As can be seen in this PCA score plot, specimens in which complexation method was not applied exhibit a notably deviation on the value of the pure casein. On the contrary, values of the specimens treated with the EDTA appear, in general, closer to that from pure casein than the specimens in which EDTA treatment was not applied. Only the chalk–casein specimen (CCE) shows a value slightly better than the same chalk–casein specimen (CC) in which EDTA treatment was not applied. It suggests that the proposed complexation method enables for a satisfactory procedure for suppressing the interference of pigments.

3.3. Effect of the pH on the stability of the metallic ion–EDTA complexes

Effect of the pH on the stability of the metallic ion–EDTA complexes has been studied. Based on the data from the literature [26], decreasing in the value of pH 8 results, in general, in a strong decreasing of the stability of Ca–EDTA. This same behaviour is observed for Mn–EDTA at pH 6, Cu–EDTA and Pb–EDTA at pH values lower than 3.5 whereas Fe–EDTA, Mn–EDTA complexes remain stable until pH values near to 1. Stability of amino acids, as species in solution, is also depending on the pH. In addition, optimum conditions for carrying out the further steps in the sequence of analysis require to work at a slightly basic pH.

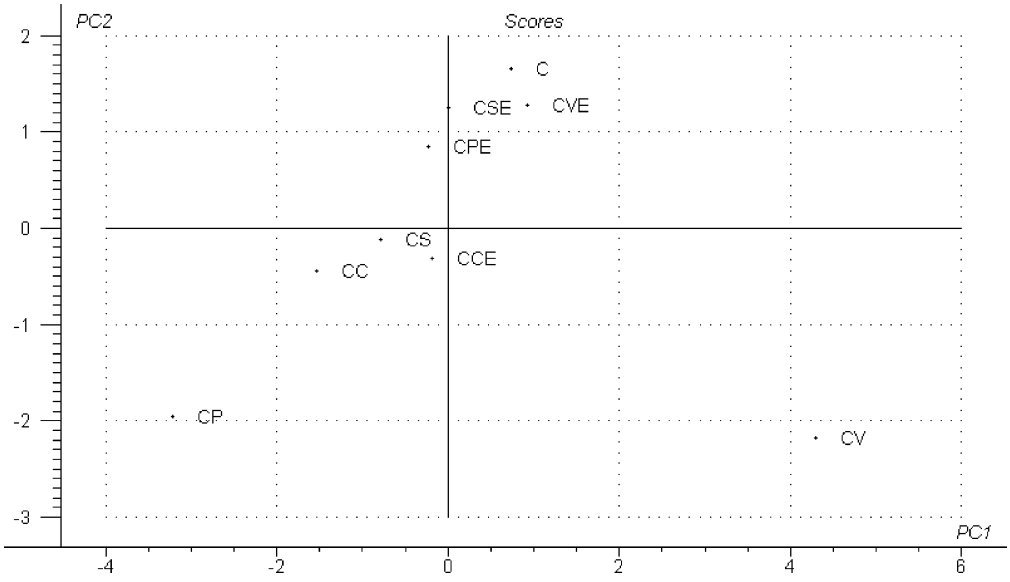

Fig. 6. PCA score plot of sample of casein (C) and test specimens prepared with casein bound with Sienna raw (CS), chalk (CC), verdigris (CV), white lead (CP) using the direct method, and specimens in which complexation with Na_2EDTA was carried out prepared with casein and Sienna raw (CSE), chalk (CCE), verdigris (CVE), white lead (CPE).

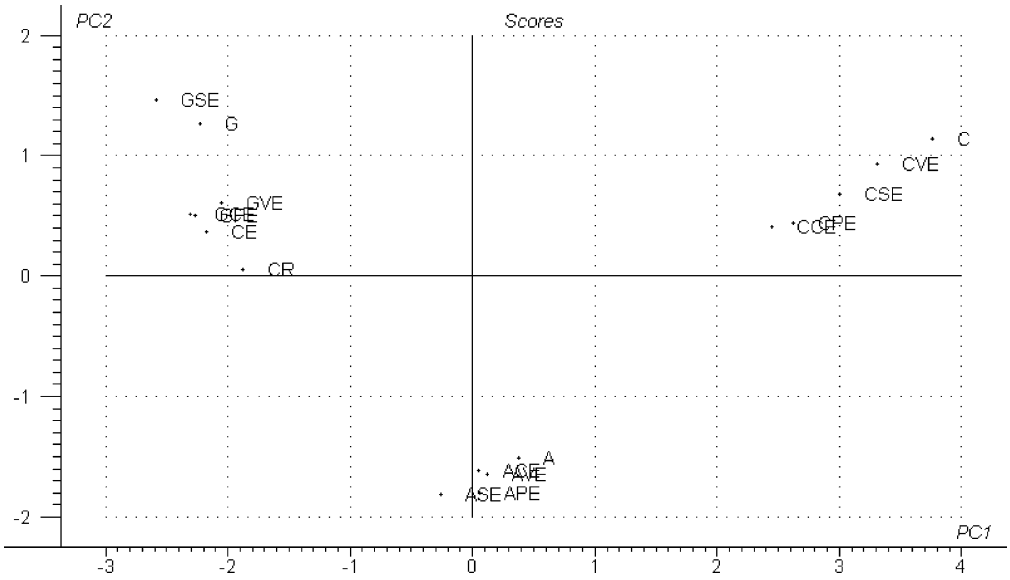

Fig. 7. PCA score plot of samples of casein (C), albumin (A), gelatine (G) using the D.M. test specimen in which complexation with Na_2EDTA was carried out prepared with raw Sienna (GSE), (CSE), (ASE), chalk (GCE), (CCE), (ACE), verdigris (GVE), (CVE), (AVE) and white lead (GPE), (CPE), (APE), and both samples from the "Fumoir" room in the Marqués de Montortal Mansion (18th century, Carcaixent, Spain) prepared with and without applying the complexation method (CE and CR).

In order to assess the possible influence of the pH on the efficiency of the formation of complexes, a series of experiences in which the four studied pigments were bound with casein and gelatine have been performed at different pH values. Results obtained suggest that, in agreement with the theory, pH 9 is the optimal value for carrying out the complexation process. To assure the fixed pH to be constant, an ammonia/ammonium chloride buffer solution is added to the extract obtained after leading to dryness the solution obtained from the hydrolysis of the specimens. Then, 100 μl of 0.2 M Na_2EDTA solution are added.

3.4. Analysis of old samples

Fig. 7 shows the PCA score plot of samples of casein (C), albumin (A), gelatine (G) in which direct method of derivatisation was applied and test specimens prepared with raw Sienna (GSE), (CSE), (ASE), chalk (GCE), (CCE), (ACE), verdigris (GVE), (CVE), (AVE) and white lead (GPE), (CPE), (APE) in which complexation with Na_2EDTA was carried out.

Samples providing from the wall paintings of the "Fumoir" room in the Marqués de Montortal Mansion (18th Century, Carcaixent, Spain) have been analysed with and without applying the complexation method (CE and CR) and they are also shown in Fig. 7. White lead and raw Sienna have been identified in the paint layer of this area of the painting using SEM/EDX. A ground of gypsum has also been identified.

Two principal components that account for the 74% of the variance have been obtained. All quotients of amino acids have been considered for differentiating these proteinaceous media according to the chemical composition of them.

The depicted PCA score plot shows a satisfactory discrimination of the three different proteinaceous media not only for the samples of pure protein but also for the pigment–binder specimens in which complexation method was applied. Value of old sample treated with the EDTA complexation method (CE) satisfactorily fits with those from pigment–gelatine specimens. The old sample treated with the EDTA is closer to these from gelatine than that derivatised without application of the EDTA-complexation method (CR). These results suggest that the proposed complexation method for suppressing the interference of the pigment improves the usual method of discrimination of proteinaceous media and permits a more precise identification of this type of artist's materials.

4. Conclusions

1. A method to suppress the interference of pigments in the analysis of proteinaceous media based on the formation of metallic ion–EDTA complexes as a previous step to the derivatisation process using ethyl chloroformate has been proposed in order to tentatively improve the usual method of identification of proteinaceous media based on the calculation of the peak area ratios of amino acids to alanine.
2. The proposed method does not take into account the pigment interference on the alanine to whose value of peak area are referred the other amino acids. Nevertheless, the peak area ratios of amino acids exhibited for

proteinaceous binder–pigment specimens, in which the proposed method for suppressing the interference of pigments was applied, are closer to that from the pure protein than those in which direct method of derivatisation was applied.

3. The method has been successfully applied to the analysis of 18th Century wall paintings in which animal glue was used as binding medium.

Acknowledgements

Financial support is gratefully acknowledged from the Valencian Regional Government "I + D Generalitat Valenciana" GV01-161 and National Spanish "I + D + I CICYT" project BQU2001-2776-C03-01.

References

[1] Plinio Naturalis Historia Libro XXXV, 7, 50.
[2] M.P. Vitruvio, De Architectura Libri Decem, Dover Publications, New York, 1960.
[3] C. Cennini, Il Libro dell'arte, Akal, Vicenza, 1982.
[4] Leonardo da Vinci, Treatise of Painting, third ed., Akal, Madrid, 1995.
[5] A. Palomino, El Museo Pictórico y Escala Óptica, Aguilar, Madrid, 1947.
[6] R. Mayer, The Artist's Handbook of Materials and Techniques, fifth ed., Blume, Madrid, 1993.
[7] L. Masschelein-Kleiner, Pact 13 (1986) 185, and references cited therein.
[8] R. Pancella, R. Bart, Zeitschrift Kunsttechnol. Konservierung 3 (1989) 101.
[9] R. White, Nat. Gallery Tech. Bull. 8 (1984) 5.
[10] W. Nowik, Stud. Conserv. 2 (1995) 120.
[11] R. Mateo-Castro, M.T. Doménech-Carbó, V. Peris-Martínez, J.V. Gimeno-Adelantado, F. Bosch-Reig, J. Chromatogr. A 778 (1997) 373.
[12] A. Casoli, P.C. Musini, G. Palla, J. Chromatogr. A 731 (1996) 237.
[13] M.P. Colombini, R. Fuoco, A. Giacomelli, B. Muscatello, Stud. Conserv. 43 (1998) 33.
[14] A. Casoli, G. Palla, J. Tavlaridis, Stud. Conserv. 43 (1998) 150.
[15] R. Aruga, P. Mirti, A. Casoli, G. Palla, Fresenius J. Anal. Chem. 365 (1999) 559.
[16] M.P. Colombini, F. Modugno, M. Giacomelli, S. Francesconi, J. Chromatogr. A 846 (1999) 113.
[17] M.T. Doménech-Carbó, M.J. Casas-Catalán, A. Doménech-Carbó, R. Mateo-Castro, J.V. Gimeno-Adelantado, F. Bosch-Reig, Fresenius J. Anal. Chem. 369 (2001) 571.
[18] S.L. Vallance, Analyst 122 (1997) 75R.
[19] F. Ronca, Stud. Conserv. 39 (1994) 135.
[20] S.M. Halpine, in: K.B. Anderson, J.C. Crelling (Eds.), Amber, Resinite and Fossil Resins (ACS Symposium Series No. 617), American Chemical Society, Washington, DC, 1995, p. 234.
[21] S.L. Vallance, B.W. Singer, S.M. Hitchen, LC–GC Int. January (1997) 48.
[22] M.P. Colombini, F. Modugno, A. Giacomelli, J. Chromatogr. A 846 (1999) 101.
[23] C. Perez-Garcia, M.T. Doménech-Carbó, V. Guerola-Blay, P. Roig-Picazo, in: Proceedings of the 9th Congress on Conservation and Restoration of Cultural Heritage, Sevilla, 1992, p. 244.
[24] A.E. Martell, Stability Constants of Metal–Ion Complexes, Section 2: Organic Ligands, Chemical Society, London, 1964.
[25] C.N. Reilley, R.W. Schmid, Anal. Chem. 30 (1958) 947.
[26] D.A. Skoog, J.J. Leary, Análisis Instrumental, fourth ed., McGraw-Hill, Mexico, 1994.