ELSEVIER

Journal of Chromatography A, 846 (1999) 101–111

JOURNAL OF CHROMATOGRAPHY A

Two procedures for suppressing interference from inorganic pigments in the analysis by gas chromatography–mass spectrometry of proteinaceous binders in paintings

Maria Perla Colombini[a,*], Francesca Modugno[b], Ambrogio Giacomelli[b]

[a]Department of Environmental and Earth Science, University of Milan "Bicocca", Via Emanueli 15, 26126 Milan, Italy

[b]Department of Chemistry and Industrial Chemistry, University of Pisa, Via Risorgimento 35, 56126 Pisa, Italy

Abstract

Two methods for suppressing the interference of inorganic pigments in the determination of amino acids in hydrolysates of wall painting samples by gas chromatography–mass spectrometry are described. One is based on the extraction of proteinaceous matter from the sample by a 2.5 *M* ammonia solution prior to the hydrolysis step, and the other on the elimination of inorganic ions from the hydrolysate by means of a cation-exchange resin. The proteinaceous binders present in the paint layer were identified using principal component analysis on the relative amino acid percentage. Some samples from "Giudizio Universale" in Florence Cathedral and from the Monumental Cemetery in Pisa (Italy), were analysed using both procedures. The presence of milk binder as the main organic component of the tempera was highlighted. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Art analysis; Principal component analysis; Pigments; Amino acids

1. Introduction

The identification of proteinaceous binders (egg, animal glue, milk or casein) used in paintings [1,2] provides both art historians and restorers with very useful information in order to gain a better knowledge of the techniques used by the artists and to plan correctly any restoration. In particular, these binders play a fundamental role in old wall paintings since several pigments could not be used directly on fresco, as they react with calcium hydroxide, and therefore the tempera technique was extensively used on dried walls [1,2]. Their identification gives rise to several analytical problems, above all related to the very low amount of sample available (maximum 1 mg), to the very low content of organic binder (about 10%, w/w), and to the very high amount of pigments and calcium carbonate from ground. Thus analytical methods are needed which offer high sensitivity and are unaffected by pigment interferences. The characterisation of proteinaceous binders is generally based on acid hydrolysis, followed by the derivatization and quantitative determination of amino acids by gas chromatography [3–13] or liquid chromatography [3,9,14–19]. Unfortunately most of these procedures are subjected to serious interferences from inorganic pigments, e.g. S. Giovanni white (calcium carbonate), ochre and earth (iron oxides), malachite and azurite (copper acetate) [4,7–10,13,15–17]. According to the derivatization reaction employed and to the amount of pigment present, the degree of interfer-

*Corresponding author. Present address: Department of Chemistry and Industrial Chemistry, University of Pisa, Via Risorgimento 35 Pisa, Italy. Fax: +39-50-502589.

E-mail address: perla@dcci.unipi.it (M.P. Colombini)

0021-9673/99/$ – see front matter © 1999 Elsevier Science B.V. All rights reserved.
PII: S0021-9673(99)00192-2

ence may be so high that results may be unreliable. Pigments prevent the complete derivatization of amino acids since quite strong complexes between metallic cations and amino acids occur in the hydrolysate. To overcome this problem some analytical procedures [13,15,17] have been proposed. However, they only solve the problem partially and moreover they have never been applied to old wall painting samples where the ratio between protein and inorganic salt contents is about 1:90. In particular, one method [15] proposing a 1 h sample extraction with water is unable to solubilize casein and egg properly; while another [13], suggesting the use of NaOH (1 *M*, 80°C for 3 h), introduces such a high content of sodium so that it enables most of the derivatisation reactions.

This paper describes two methods for suppressing the interference of inorganic pigments on the quantitative determination of amino acids in hydrolysated wall painting samples by GC–MS. One is based on the extraction of proteinaceous matter from the sample by an ammonia solution prior to the hydrolysis step, and the other on the elimination of inorganic ions from the acid hydrolysate by selective elution on a small home-made column packed with a cation-exchange resin. Reference wall painting samples obtained from the Opificio delle Pietre Dure in Florence (Italian Ministry of the Cultural Heritage) [20] were employed to test and calibrate the overall analytical procedure. The percentage contents of fourteen amino acids, obtained by the proposed procedures, were submitted to principal component analysis (PCA) in order to achieve the identification of the proteinaceous binders present in the sample [10]. Finally, the results relevant to samples from wall paintings from both the Monumental Cemetery in Pisa and Florence Cathedral are discussed.

2. Experimental

2.1. Chemicals and reagents

All solvents were Baker HPLC grade (Baker J.T. Italia, Milan, Italy); HCl and NH_3 (Baker) were suprapur grade. Hexadecane (Fluka, Milan, Italy), triethylamine (TEA, Fluka, purity >99%) and *N*-*tert*.-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA, Pierce, USA, purity >98%) were used without purification. Bidistilled water was used throughout. Amino acid standard solutions of both collagen hydrolysate (2.5 μmol/ml in 0.1 *M* HCl of each amino acid except for proline and hydroxyproline whose concentration was 12.5 μmol/ml) and food hydrolysate (2.5 μmol/ml in 0.1 *M* HCl of each amino acid), norleucine (nor) used as an internal standard, dried chicken egg yolk, dried chicken egg white, collagen (type III from calf skin) and Dowex 50W crosslinkage 8% resin in hydrogen form, 100–200 mesh, were supplied by Sigma (Sigma-Aldrich, Milan, Italy). Casein (from bovine milk, purity 95%) was supplied by Fluka.

2.2. Reference wall painting samples.

These samples were provided by the Opificio delle Pietre Dure [20], and the composition of the painted film is reported in Table 1.

2.3. Old wall painting samples

Table 2 summarises the main features of the three samples (Z3, V9 and V11) collected from the ''Giudizio Universale'' in Florence Cathedral and of one sample (AFFD and AFFN are part of the same homogenised pool) from the back of the frescoes of the Monumental Cemetery in Pisa. Historically, it was known that this last sample contained casein binder and calcium carbonate in a ratio of approximately 1:3.

2.4. Apparatus and chromatographic conditions

A microwave oven mod. MLS-1200 MEGA Milestone (FKV-Milestone, Sorisole-BG, Italy) was used for the acid hydrolysis of proteins using the following programme: 10 min at 160°C and 250 W, 30 min at 160°C and 500 W, and 15 min of N_2 venting. A 5890A gas chromatograph (Hewlett-Packard, USA) equipped with an on-column injection port and with a mass spectrometric detector model 5971A was used to separate and identify the silylated amino acids. Hewlett Packard Chemstation software (B.04.02) was used for the integration of peaks and for the mass spectra evaluation.

Chromatographic separation was performed on a

Table 1
Composition of reference wall painting samples

Sample	Binder	Pigment	Binder in the painted film (%, w/w)	Protein in the painted film (%, w/w)
P1	Animal glue	Yellow ochre	34	33
P2	Milk	Yellow ochre	28	10
P3	Egg	Yellow ochre	32	15
P16	Animal glue	S. Giovanni white	27	26
P17	Egg	S. Giovanni white	26	12
P18	Milk	S. Giovanni white	24	9
P26	Animal glue	Smalt	27	26
P27	Milk	Smalt	20	7
P28	Egg	Smalt	30	12
PCO	Animal glue	Absent	100	98
PLA	Milk	Absent	100	36
PUO	Egg	Absent	100	46

chemically bonded fused-silica capillary column HP-5 MS (Hewlett-Packard), stationary phase 5% phenyl–95% methylpolysiloxane, 30 m×0.25 mm I.D., 0.25 μm film thickness, connected to a 2 m×0.32 mm I.D. deactivated fused-silica capillary pre-column. The chromatographic conditions were: initial temperature 100°C isothermal for 2 min, then 6°C/min up to 280°C isothermal for 15 min; the carrier gas was helium at a constant flow of 1.2 ml/min (initial pressure 76 KPa). The GC–MS interface temperature was 280°C.

2.5. Analytical procedure

The analytical procedure (described elsewhere [4]) can be summarised as follows: (1) the sample, wetted with 6 *M* HCl, was subjected to vapour phase acid hydrolysis assisted by microwave; (2) the hydrolysates were diluted with bidistilled water up to 1–1.5 ml (pH 1.5), and stored at 4°C; (3) the amino acid derivatization with MTBSTFA in pyridine of a dried aliquot of hydrolysate, mixed with a known amount of internal standard norleucine, was performed by adding 15 μl of MTBSTFA, 2 μl of TEA, 40 μl of pyridine, at 60°C for 30 min. After cooling, 5 μl of hexadecane solution (80 ng/mg in isooctane) was added. The norleucine was used to control the recovery of the derivatisation reaction and to calculate amino acid levels. The hexadecane peak was used as an internal standard to check the reproducibility of injection conditions; (4) 2 μl were injected and analyzed by GC–MS in the selected ion monitoring mode.

This procedure allows the determination of the following amino acids: alanine (Ala), glycine (Gly), valine (Val), isoleucine (Ile), leucine (Leu), metionine (Met), serine (Ser), phenylalanine (Phe), proline (Pro), aspartic acid (Asp), glutamic acid (Glu), lysine (Lys), hydroxyproline (Hyp), tyrosine (Tyr) and norleucine (Nor). The detection limit was 0.01 ng/μl for Ala, Gly, Val, Ile and Leu, and 0.05 ng/μl for the other amino acids. The linearities of regression equations of amino acid standards were verified in the concentration range 0.1–3 ng/μl. The relative percentage contents were obtained by getting the ratio of the contents of each amino acid in the final solution, expressed in ng/μl of pyridine solution, to the total contents of all fourteen. The absolute amount of protein present in the sample was calculated considering that the fourteen amino acids determined represent about 85%, 95% and 90% of the total amount of proteinaceous matter in egg, milk

Table 2
Main features of old wall painting samples

Sample	Author	Pigment	Sample weight (mg)
Giudizio Universale – Florence Cathedral			
Z3	Zuccari	Yellow ochre S. Giovanni white	0.1
V9	Vasari	S. Giovanni white	1.5
V11	Vasari	Red ochre	0.2
Monumental Cemetery of Pisa			
AFFD	Unknown	$CaCO_3$	11.1
AFFN	Unknown	$CaCO_3$	10.6

and glue respectively. Depending on the limit of quantitation for each amino acid, it is possible to calculate that the minimum amount of proteinaceous matter detectable in a sample should be about 200 ng.

In order to ensure the production of data within known values of accuracy and precision, two amino acid standard solutions were run between samples each day. All the data were plotted in control charts to check in real time the on-going analytical performance.

2.6. Pretreatment and clean-up

2.6.1. Ammonia extraction

The sample was extracted twice with 1 ml of 2.5 *M* NH_3 for 3 h at 60°C in an ultrasonic bath. Depending on the insoluble component present in the sample, a variable amount of residue may remain after the solid–liquid separation step: actually, it was observed that grounded wall painting sample sizes lower than 0.5 mg gave a barely visible insoluble residue which was experimentally difficult to separate. Ammonia phases were combined and evaporated to dryness in a water bath at 40°C under a gentle stream of nitrogen. The residue was immediately wetted with 6 *M* HCl and processed for acid hydrolysis and the analysis of amino acids as previously described [4].

2.6.2. Cation-exchange clean up

The sample was hydrolysed according to the procedure described in Section 2.5, and the acid hydrolysate (1–1.5 ml) was passed through a laboratory-made glass column (5×0.5 cm) packed with a strong cation-exchange resin (250 mg of Dowex 50W in hydrogen form, 5 mequiv./g). Conditioning of this column was carried out with 5 ml of water–methanol (1:1), 10 ml of 2 *M* HCl, followed by washing with water until it became neutral. The column was loaded with the hydrolysate, and amino acids and cations were selectively retained by the resin. After washing with 3 ml of 0.02 *M* HCl, amino acids were eluted with 4 ml of 2.5 *M* NH_3. The eluate was evaporated to 1 ml at 40°C under a mild nitrogen stream, and aliquots were then analysed.

2.7. Statistics

Principal component analysis (PCA) is a multivariate statistical method used as the basis of a clustering method. When performed on predictor variables (in the present case on the fourteen amino acid relative percentage contents), PCA reduces the size of a data set to only two or three components by choosing those principal components which account for at least 70% of the variance. The principal components are linear combinations of predictor variables and their main characteristic is that they are orthogonal to each other. The result of each linear combination is a score value which represents the new co-ordinate of the object in the space of principal components. A plot with the first two principal components as co-ordinates permits the mapping of the objects into two-dimensional space (score plot), so that clusters can be observed visually. Objects are grouped according to similarities between original variables. On this basis the identification of a new object is graphically achieved by fitting it into a cluster. All the relative percentage contents of the amino acids were processed with this well-known method [21] using Scan – Software for Chemometric Analysis (Release 1.1) (Minitab, State College, PA, USA).

3. Results and discussion

3.1. Pigment interference

Unpigmented reference samples (PUO, PLA and PCO) and pigmented reference samples (P1, P2, P3, P16, P17, P18, P26, P27 and P28) were processed according to the selected analytical procedure without any clean-up step [4]. Fig. 1 reports the bar plots of the relative amino acid percentage contents of unpigmented samples and of those containing S. Giovanni white, and shows significant differences in the relative amino acid compositions. In particular, the relative amino acid composition of unpigmented samples is similar to that reported in literature for pure proteins [3,4,7,16], while in pigmented samples the relative contents of pro, hyp, glu and asp are lower than that of unpigmented samples. This is accompanied by a corresponding increase in the

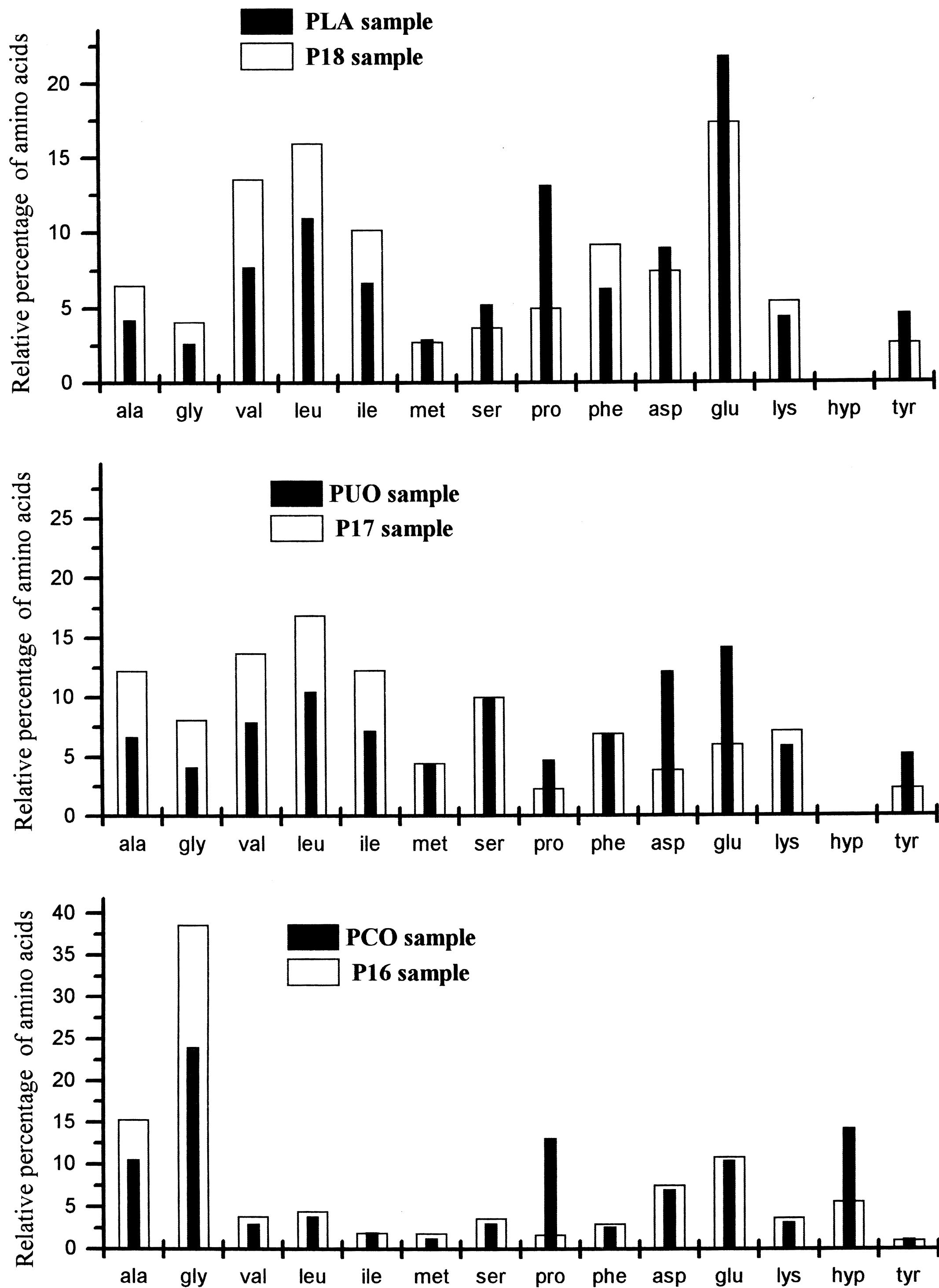

Fig. 1. Bar plot of average amino acid relative percentage contents of unpigmented reference samples PLA (milk), PUO (egg), PCO (glue), and of samples containing the same binders with S. Giovanni white: P18 (milk+$CaCO_3$), P17 (egg+$CaCO_3$), P16 (glue+$CaCO_3$).

relative percentages of ala, gly, val, leu and ile which is due only to the redistribution of percentage values. These findings highlight that the presence of S. Giovanni white interferes in the derivatisation reaction yield of several amino acids to different degrees; this is due to the formation of complexes between amino acids and calcium cation in the acid hydrolysate [8,9,15]. Varying recoveries of amino acids should be related to differences in the stability of complexes formed, according to the different functional groups and polarities of the amino acids. Moreover, the total protein content detected in the presence of calcium carbonate was lower than expected: 10% instead of 26% for animal glue, 5% instead of 12% for egg, and 5% instead of 9% for milk. Similar results were obtained for reference samples containing ochre, while no interference was exhibited by samples containing smalt (cobalt glass: SiO_2, K_2O, Al_2O_3, CoO). All the relative amino acid percentage data were subjected to PCA. Generally, the method is capable of spatially grouping the three proteinaceous materials considered into separate clusters [10], but the heavy interference of pigments containing iron and calcium drastically changes the amino acid composition, so that the binder identification is no longer reliable, as shown in Fig. 2 by the PCA plot of unpigmented reference samples and of those containing S. Giovanni white. Above all, the losses in pro, asp and glu content influence the PC2 values, with the result that the clusters of pigmented samples are located far from those of unpigmented ones, with an overlap between the cluster of unpigmented egg and pigmented milk samples.

3.2. Suppression of pigment interference

Two procedures have been developed. One is based on a preliminary ammonia extraction of the sample prior to the hydrolysis step other, and the on the cation-exchange clean-up of amino acids present in protein hydrolysates. In the following sections the main characteristics of both methods are reported.

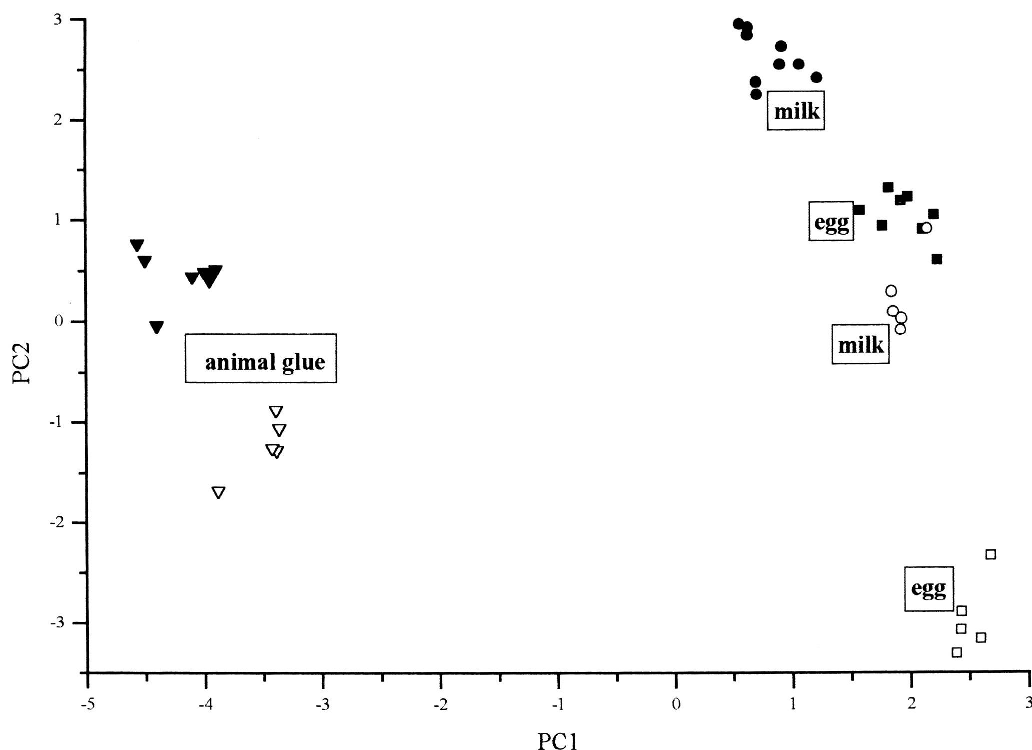

Fig. 2. PCA score plot of amino acid relative percentage contents of reference unpigmented samples PLA (●, milk), PUO (■, egg), PCO (▼, glue), and of samples containing S. Giovanni white: P18 (○, milk+$CaCO_3$), P17 (□, egg+$CaCO_3$), P16 (▽, glue+$CaCO_3$).

3.2.1. Ammonia extraction procedure

The efficiency of the extraction procedure was tested using casein, collagen, and dried chicken egg yolk and white. In the experimental conditions adopted, collagen, casein and egg white were completely soluble in ammonia and gave quantitative recoveries; egg yolk recovery, with respect to the expected protein content, was 89%. Eleven percent of the egg yolk protein was not soluble in ammonia, as found by analysing the sample residue after extraction. The relative standard deviation for the whole procedure using a 1 mg sample was 15% on five replicate experiments. With the exception of copper salts, the inorganic salts used in wall paintings are insoluble at the high pH used for extraction.

3.2.2. Cation-exchange clean-up procedure

Replicate measurements on a standard amino acid solution (20 ng/μl) containing 0.15 M Ca^{2+} at pH 1.5 passed through the column, showed that the average recovery was 74–100% of the initial amino acid content, as reported in Table 3; and that calcium concentration was less than 10^{-4} M, as verified by spectrophotometric measurements with methyl thymol blue at 630 nm [22]. The selectivity coefficients of Dowex 50W resin are quite high with respect to hydrogen ion (i.e. 3.9, 2.5, 12 and 2.9 for Pb^{2+}, Fe^{2+}, Fe^{3+}, and Cu^{2+}, respectively), so the resin capability of cleaning-up amino acid hydrolysates would to be suitable even for samples with a pigment content as high as 99%, w/w.

3.2.3. Analysis of unpigmented and pigmented reference samples

The two procedures were tested by analysing unpigmented and pigmented reference samples. The relative amino acid percentage contents achieved with both methods are reported in Table 4: there is no significant difference between the relative amino acid percentages of unpigmented and pigmented reference samples. Moreover, the recovery for each binder is very close to the expected value. Fig. 3 shows the results of PCA analysis: the first two components (PC1 and PC2) take into account 81% of the total variance of data. It highlights that cleaned-up samples belong to the right cluster, regardless of the experimental procedure used and the kind of sample. Both methods are thus able to eliminate pigment interference as well. Cation-exchange resin clean-up seems to be of a more general use since it is able to eliminate the majority of inorganic cations from a hydrolysate [23], with a sufficient recovery (>68%) and reproducibility (R.S.D.<18%) of amino acid determination even for a sample size of 0.1 mg. Ammonia extraction is a valuable alternative when copper is absent as a pigment, giving quantitative recoveries for casein and animal glue binder, and about a 90% recovery for egg.

3.3. Analysis of old wall painting samples

The amino acid percentage contents of the old wall painting samples are reported in Table 5, and the relative results of PCA analysis in Fig. 4. The proteinaceous content (as the sum of amino acids quantified) of Z3, V9 and V11 samples was very low (0.2, 8.2 and 0.7 μg, respectively), while the AFF sample contained 0.6 mg. No reliable binder identification was obtained for these samples when analysed without clean-up procedures. On the other hand, using the proposed methods, the PCA values for all the old samples (75% of the total data variance) are very close to the milk binder cluster (Fig. 4); this may suggest the presence of casein. Samples AFFD and AFFN, which were known to contain casein,

Table 3
Average percentage recovery and relative standard deviation (R.S.D.) of amino acids from the cation-exchange resin column. Recovery and R.S.D. were calculated on five replicate analyses of a standard solution of amino acids containing 0.15 M Ca^{2+}

Amino acid	Ala	Gly	Val	Leu	Ile	Met	Ser	Pro	Phe	Asp	Glu	Lys	Hyp	Tyr
Recovery (%)	100	90	99	96	89	80	82	79	68	77	77	78	74	74
R.S.D. (%)	6	12	6	5	9	15	15	16	18	12	12	10	10	15

Table 4
Relative amino acid percentage contents of reference unpigmented and pigmented wall painting samples following the cation-exchange resin clean up (D) and the ammonia extraction (N) procedures

Sample	Ala	Gly	Val	Leu	Ile	Met	Ser	Pro	Phe	Asp	Glu	Lys	Hyp	Tyr
PUOD1	7.1	4.8	8.9	11.5	7.9	3.7	9.0	5.9	6.7	11.5	12.5	4.9	0	6.6
PUOD2	7.5	3.8	8.2	11.7	7.3	3.2	8.1	6.2	7.6	11.4	13.1	6.0	0	6.1
PUON1	6.4	3.9	7.4	10.7	7.3	3.1	8.5	6.0	7.5	13.5	14.3	6.8	0	4.6
PUON2	7.9	5.1	7.7	10.1	6.9	4.2	7.0	5.1	8.5	11.7	13.0	6.6	0	6.5
PUON3	7.5	4.8	7.3	10.5	7.1	4.5	8.0	5.5	7.8	11.0	14.0	6.5	0	5.5
PLAD1	3.8	1.9	9.5	14.0	6.7	3.1	7.5	12.1	6.0	8.5	21.8	2.8	0	2.2
PLAD2	3.5	2.2	10.4	13.5	7.5	3.4	5.9	11.5	6.1	7.7	19.4	4.4	0	3.5
PLAN1	3.9	2.3	8.7	11.8	7.0	3.7	4.7	15.1	6.9	8.1	17.5	5.1	0	5.1
PLAN2	4.3	3.1	6.7	11.8	6.8	3.0	5.7	12.4	8.1	10.8	19.3	3.2	0	4.9
PLAN3	4.7	2.5	9.4	13.2	6.7	3.7	6.2	11.8	6.6	8.4	20.1	3.2	0	4.5
PCOD1	15.9	24.2	5.1	5.9	2.7	1.3	4.9	11.5	3.0	6.1	8.8	4.3	6.0	1.0
PCOD2	14.3	24.1	5.0	6.0	2.4	1.3	5.1	12.6	3.1	5.3	7.8	3.7	8.2	1.2
PCON1	15.9	24.2	5.1	5.9	2.7	1.3	3.9	11.5	3.0	6.1	8.8	4.3	7.2	1.2
PCON2	15.5	25.9	4.4	5.3	2.4	1.0	2.8	13.1	2.9	6.1	8.4	3.7	7.1	1.4
PCON3	13.5	25.0	3.4	4.5	2.3	1.6	4.4	9.4	4.0	6.8	9.4	4.0	8.9	1.5
P1D	11.7	24.2	3.2	3.5	2.1	1.3	7.2	10.2	2.7	6.9	12.2	5.0	8.5	1.2
P1N	12.4	26.9	3.4	4.4	2.1	2.3	5.6	10.6	3.0	6.5	10.1	3.6	7.3	1.7
P2D	4.9	3.6	7.7	11.8	7.0	4.1	4.9	14.5	6.0	6.1	17.2	7.1	0	6.0
P2N	4.3	3.1	6.4	11.4	6.2	4.7	3.6	14.1	6.9	7.9	16.9	7.6	0	6.6
P3D	6.3	4.9	8.2	10.7	7.4	4.5	8.2	6.4	7.9	11.2	12.8	7.2	0	5.3
P3N	6.5	4.8	7.0	10.6	7.4	4.6	6.1	8.4	9.0	10.6	12.4	6.1	0	6.5
P16D	9.5	21.0	3.2	4.1	2.4	1.7	3.9	11.8	3.9	6.4	11.4	5.1	13.2	2.5
P16N	8.4	19.0	3.1	4.8	3.3	2.4	4.0	12.4	3.6	7.8	10.7	3.6	14.7	3.2
P17D	7.0	4.2	7.4	11.5	7.2	3.7	6.5	7.9	8.4	9.9	12.0	7.9	0	6.6
P17N1	6.0	3.7	6.2	9.6	6.2	4.8	6.8	6.0	8.2	11.2	11.8	10.9	0	8.7
P17N2	6.9	4.0	7.3	10.9	7.1	4.7	5.1	6.2	7.9	10.2	10.7	10.6	0	8.4
P18D	3.0	3.0	7.6	7.0	5.1	3.3	4.4	16.4	7.9	10.5	18.8	7.1	0	5.8
P18N1	3.8	2.6	6.1	10.3	6.1	4.0	3.0	15.2	6.5	7.9	17.2	8.7	0	7.0
P18N2	4.5	2.9	7.3	10.2	6.9	3.8	3.1	14.9	6.6	7.8	17.9	7.7	0	6.5

were located sufficiently well, confirming that the suppression of pigment interference allows the proteinaceous binder to be correctly identified. The presence of a low amount of Hyp in samples AFF and V11 suggests that animal glue was added to milk to achieve better adhesive properties, and partially explains the value shift from the centre of the cluster. The contents of some amino acids (Lys, Met, Ser, Tyr) in old wall paintings are reduced or zeroed, probably as a result of oxidation reactions during ageing [24], therefore the amino acid percentage contents of old samples differ slightly from the unaged reference samples, thus explaining the shift in PCA values for V9, while Z3 seems to be unaffected by ageing.

4. Conclusions

The two procedures developed suppress the pigment interference on amino acid determination and provide satisfactory results for binder characterisation in old wall painting samples. The main advantage of using the cation-exchange column method is to eliminate all the inorganic salts used as pigments in wall paintings, while ammonia extraction prior to the hydrolysis step achieves a better recovery of proteins. Statistical treatment by principal component analysis of the relative percentage amino acid contents obtained by the proposed methods, groups into clusters samples containing the same binder. The protein can thus be easily identified. Wall samples

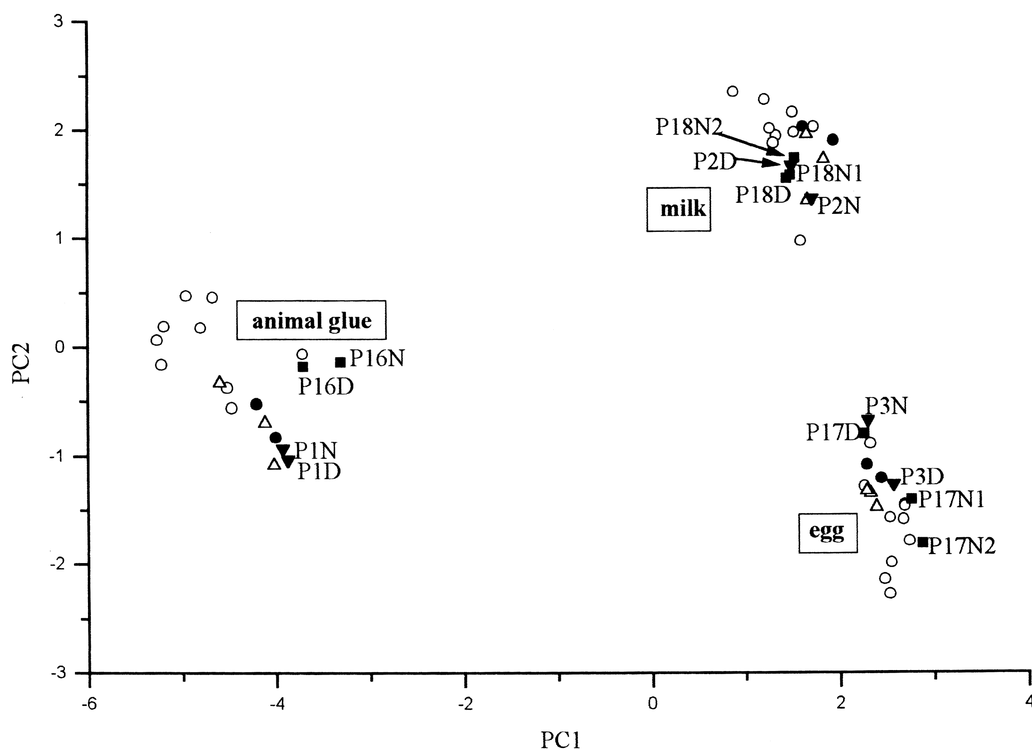

Fig. 3. PCA score plot of amino acid relative percentage contents of unpigmented reference samples and of pigmented samples P1, P2, P3, P16, P17, P18 submitted to ammonia extraction (N) and to cation-exchange resin clean up (D). Sample labels are as follows: ○ unpigmented reference samples analysed without any purification treatment; ● unpigmented reference samples cleaned up with cation-exchange resin; △ unpigmented reference samples extracted with ammonia; ▼ samples containing yellow ochre submitted to both procedures; ■ samples containing S. Giovanni white submitted to both procedures.

from "Giudizio Universale" in Florence Cathedral and from the Monumental Cemetery in Pisa, for which in the absence of a clean up procedure a reliable binder characterisation was not achieved, were processed according to the proposed methods. Milk binder was found to be the main component of

Table 5
Relative amino acid percentage contents of old wall painting samples following the cation-exchange resin clean up (D) and the ammonia extraction (N) procedures

Sample	Ala	Gly	Val	Leu	Ile	Met	Ser	Pro	Phe	Asp	Glu	Lys	Hyp	Tyr
Z3D	4.1	6.9	6.6	7.0	5.5	6.9	7.0	11.0	12.0	8.8	22.0	0.0	0.0	1.0
V9N	8.0	4.2	7.2	9.3	6.2	1.5	5.0	8.2	9.2	17.1	20.4	0.0	0.0	3.9
V9D	9.0	3.5	8.0	9.0	5.3	1.9	4.7	11.2	6.7	15.6	20.6	0.0	0.0	4.7
V11D	5.1	6.6	6.7	7.0	5.2	3.9	8.3	12.9	4.2	9.2	26.8	0.0	3.0	1.0
AFFD	6.4	9.5	7.8	9.5	6.0	3.6	6.1	12.2	4.9	5.5	17.2	5.4	2.7	3.4
AFFN	7.2	9.0	8.1	9.2	6.5	3.0	6.5	11.1	5.6	6.3	16.5	4.1	3.0	3.4

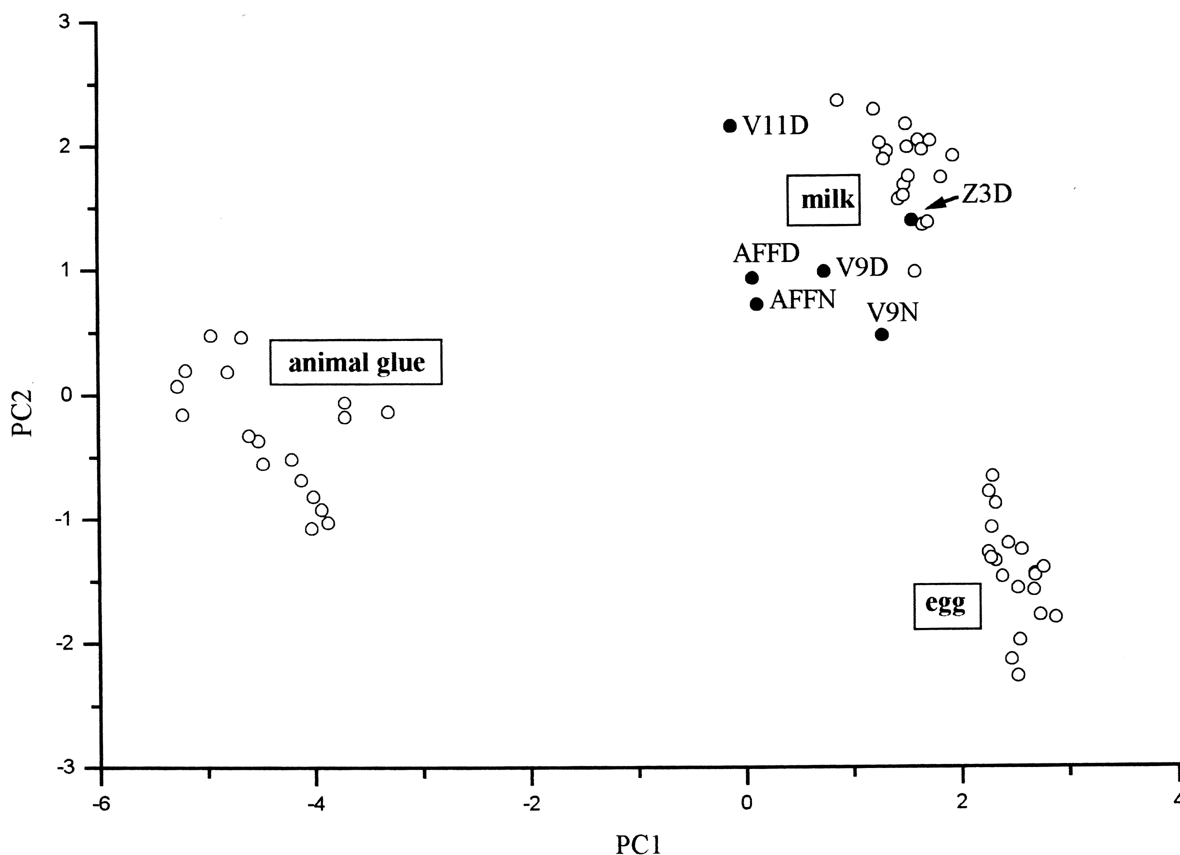

Fig. 4. PCA score plot of amino acid relative percentage contents of reference samples (○) and of old wall painting samples (●) V11, V9, Z3 from *Giudizio Universale* and AFF from Pisa Monumental Cemetery, obtained by suppressing pigment interference following the ammonia extraction method (N) or the cation-exchange resin method (D).

the tempera made by two 15th century Italian painters Zuccari and Vasari, in agreement with the execution techniques generally used in this historical period. Casein was confirmed to be the adhesive used for the frescoes of the Monumental Cemetery in Pisa

Acknowledgements

The authors gratefully acknowledge the National Research Council (Committee for "Science and Technology for the Cultural Heritage") and the University of Pisa (ex 60%) for financial support.

References

[1] C. Cennini, Il Libro dell'Arte (1437), Neri-Pozza, Vicenza, 1971.
[2] P. Bensi, in: Le Pitture Murali, Centro Di, Firenze, 1990, p. 73.
[3] J.S. Mills, R. White, The Organic Chemistry of Museum Objects, Butterworths, London, 1987.
[4] M.P. Colombini, R. Fuoco, A. Giacomelli, B. Muscatello, Stud. Cons. 42 (1998) 3.
[5] J.S. Mills, R. White, Nat. Gallery Tech. Bull. 4 (1980) 65.
[6] R. White, Nat. Gallery Tech. Bull. 8 (1984) 5.
[7] M.R. Shilling, H.P. Khanjian, L.A.C. Souza, J. Am. Inst. Conserv. 35 (1996) 45.
[8] M.R. Shilling, H.P. Khanjian, J. Am. Inst. Conserv. 35 (1996) 123.
[9] S.L. Vallance, Analyst 122 (1997) 75R.

[10] M.P. Colombini, R. Fuoco, A. Giacomelli, B. Muscatello, N. Fanelli, Science and Technology for Cultural Heritage 7 (1) (1998).
[11] A. Casoli, P.C. Musini, G. Palla, Fresenius J. Anal. Chem. 352 (1995) 372.
[12] A. Casoli, P.C. Musini, G. Palla, J. Chromatogr. A 731 (1996) 237.
[13] F. Ronca, Stud. Cons. 39 (1994) 135.
[14] M.P. Colombini, R. Fuoco, A. Giacomelli, B. Muscatello, C. Baracchini, G. Caponi, in: Scienza e Beni Culturali: La pulitura delle superfici dell'architettura, Vol. XI, Libreria Progetto Editore, Pisa, Italy, 1995, p. 227.
[15] S.M. Halpine, in: K.B. Anderson, J.C. Crelling (Eds.), Amber, Resinite, and Fossil Resins, ACS Symposium Series, 617, American Chemical Society, Washington, DC, 1995, p. 234.
[16] S.M. Halpine, Stud. Conserv. 37 (1992) 22.
[17] S.L. Vallance, LC·GC Int. 10 (1997) 48.
[18] C.M. Grzywacz, J. Chromatogr. A 676 (1994) 177.
[19] G. Kenndler, K. Schmidt-Beiwl, F. Maringer, M. Pohm, Fresenius J. Anal. Chem. 342 (1992) 141.
[20] M. Matteini, Science and Technology for Cultural Heritage 7 (1) (1998) 1.
[21] The Data Analysis Handbook, Elsevier, 1994.
[22] E. Bishop, Indicators, Pergamon, Oxford, 1972.
[23] C.W. Gehrke, L.W. Larry, J.S. Absher, F.E. Kaiser, R.W. Zumwalt, in: R.W. Zumwalt, K.C. Kuo, C.W. Gehrke (Eds.), Amino Acid Analysis by Gas Chromatography, Vol. I, CRC Press, Boca Raton, FL, 1987, p. 1.
[24] A. Karpowicz, Stud. Cons. 26 (1981) 153.