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Characterization of proteinaceous glues in old paintings by separation of the *o*-phtalaldehyde derivatives of their amino acids by liquid chromatography with fluorescence detection

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Abstract

A HPLC-fluorescence method for characterization of proteinaceous glues from binding media used in pictorial works of art prior to conservation or restoration treatment is proposed. Fluorescence derivatization of amino acids released by acid hydrolysis of standard proteins is studied. The derivatization reagent was *o*-phtalaldehyde with 2-mercaptoethanol as catalyst. Mobile phase was a programmed gradient among two eluents (water buffered at pH 5.8 wit 5% THF, and methanol) and is able to satisfactorily resolve the amino acid derivatives in 45 min. Peak area ratios among amino acid derivatives and the leucine derivative are useful to characterize the proteins. The method shows good sensitivity and adequate linearity between 2.0×10^{-3} and 3.3 mmol/l of each amino acid, with a limit of detection of 6.0×10^{-4} mmol/l. The proposed method has been successfully applied to artistic samples from items of the cultural heritage of Valencia (Spain).

Keywords: HPLC; Protein; Amino acids; Derivatization; Art analysis; Fluorescence

1. Introduction

Binding media are natural materials which can be found in pictoric works of art. They are organic substances employed to cover and provide cohesion to the pigments and protect the outer coat of the pictoric works of art. The most common organic materials used as binding media are drying oils, waxes, gums and proteinaceous glues [1,2]. These organic materials are difficult to analyze, due mainly to the small quantity of sample available, their complexity and low purity [1]. The difficulty is additionally increased by modification of the organic material through the time, due to the environmental conditions and the presence of pigments [3].

The proteinaceous glues have been frequently used as binding media in artistic paintings from immemorial time. The moisture can hydrolyze the peptide linkages, and it improves the growth of fungis and bacteria which also break the peptide linkage. Light also provokes the breakdown of the peptide bond and the modification of structure of the protein. However, the proteins and their constituents, the amino acids, are more stable to oxidation, temperature and environmental conditions than other organic materials [4]. Among the proteinaceous glues, the beef and porcine gelatines, albumin, casein and egg protein are the most employed as binding media in tempera pictoric works of art [2–4].

Spectrophotometric techniques, such as FTIR [5,6], Raman [6] have been studied to determine the presence proteins in pictorial samples. These techniques allow to found proteinaceous material by means of the study of the characteristic groups and the tertiary structure, but not the nature of the proteins. In order to characterize the proteins, separative techniques, such as ion exchange chromatography [5,6] or electrophoresis [5,6], have been used. However, in the case of works of art, the structure of the proteins can be modified, while the evolution of indi-

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vidual amino acids is less important [4]. The proteins can be analyzed by its relative amount of amino acids. The sample must be hydrolyzed in order to release the amino acids, and these obtained amino acids are analyzed by separative techniques as electrophoresis [7], gas chromatography [2,3,8] or liquid chromatography [9–17].

Liquid chromatography with electrochemical [9] or fluorescence [10–17] detection has been used to analyze amino acids. However, the fluorescence has more sensitivity and is usually chosen. As the amino acids have not fluorescence properties, a previous derivatization step is needed. The reagents 6-aminoquinolyl-*N*-hydroxysuccinimidylcarbamate [11], carbazole-*N*-(2-methyl)acetyl-chloride [14], 9-fluorenylmethyl chloroformate [15], 4-fluoro-7-nitro-2,1,3-benzoxadiazole [16] and phenylthiocabamate [17] have been used as derivative reagents for amino acids. In this paper, reagent *o*-phtalaldehyde with 2-mercaptoethanol (OPA/2-ME) as catalyst was employed as derivatizant [9,10]. The formed derivatives are separated by a gradient in an apolar C18 column [10]. The analysis of amino acids can be impaired by the presence of some pigments because the metallic cations of the pigments can form complexes with some amino acids, changing the relative amount of free amino acid. The use of a cation sequestering reactive as ethylendiamine tetraacetic acid (EDTA) can avoid the interference of the pigments [3]. Asparagine and glutamine are not analyzed, because of the hydrolysis turns them in aspartic and glutamic acid [2]. The glutamic acid is also partially converted into pyroglutamic acid during hydrolysis [2]. The hydrolysis destroys tryptophane, and it is not determined [2]. The proline and the cysteine do not appear in the study, because their *o*-phtalaldehyde derivatives are weak [10,12].

The aim of this work was to perform an analytic study of the proteins by analysis of the relative amount of the amino acids released by acid hydrolysis, using HPLC as separative technique and fluorescence detector. The ratios between the peak area corresponding to each amino acid and the peak area of the leucine allow characterize the different proteins. Samples with different quantity of protein can be compared [2]. Leucine is a non-polar amino acid and it does not shows polar, basic or acid interactions. Then leucine derivative provides a chromatographic peak quite stable and with low variability [10]. The results would be applied to the study of proteinaceous glues used in artworks. The proposed method has allowed discriminating proteic binding media in samples from pictoric works of art of the Valencian Cultural Heritage in Spain.

2. Experimental

2.1. Reagents and solvents

The following reagents and solvents have been used for the treatment of the samples: *o*-phtalaldehyde (OPA), analysis grade (Carlo Erba, Val de Reuil, France), 2-mercaptoethanol (2-ME) analysis grade (Carlo Erba, Val de Reuil, France) (these reagents must be stocked at −4 °C protected from light). Methanol, analysis grade (Fischer Chemical, Loughborough, UK). Chlorhydric acid 37% analysis grade (Scharlau, Barcelona, Spain). Boric acid (Analysis grade, Panreac, Barcelona, Spain), potassium hydroxide (Analysis grade, Merck, Darmstadt, Germany). Ethylendiamine tetraacetic acid disodium salts, ammonia and ammonia chloride (Probus, Barcelona, Spain). Water (Nanopure II grade) was generated in laboratory by a Nanopure water production device (Sybron-Barnstead, Dubuque, IA, USA).

The following substances have been used for the chromatographic separation: Water (Nanopure II grade), sodium acetate (Analysis grade, Panreac, Barcelona, Spain), acetic acid (Analysis grade, Carlo Erba, Val de Reuil, France), tetrahydrofurane (THF) (HPLC grade, Carlo Erba, France) and methanol (HPLC grade, Val de Reuil, France).

The amino acids used as standards were: alanine (ala), arginine (arg), aspartic acid (asp), glutamic acid (glu), glycine (gly), histidine (his), isoleucine (ile), leucine (leu), lysine (lys), methionine (met), phenylalanine (phe), serine (ser), threonine (thr), tryptophane (trp), tyrosine (tyr) and valine (val). All were GC grade and purchased from Sigma (Saint Louis, Missouri, USA).

2.2. Solutions and chromatographic eluents

The derivatization solutions was made solving 100 mg of OPA in 25 ml of methanol, and adding 1.25 ml of 2-ME. This solution is maintained in the dark and kept at −4 °C. Under these conditions, the solution is stable during one week [10]. The borate buffer was made solving 6194 g of boric acid and 6524 g potassium hydroxide in 200 ml of nanopure water. The pH is adjusted to 10.4 adding chlorhydric acid.

The amino acid standard solutions were made solving the appropriate amount of amino acid in 0.1 M HCl aqueous solvent. No problems of solubility were detected.

Eluent A was made solving 6.804 g sodium acetate in 1 l of nanopure water. The pH was adjusted to 5.8 by acetic acid addition. After, 50 ml of THF were added. Eluent B was Methanol 100%.

2.3. Standards and samples

The proteins beef gelatine, porcine gelatine, albumin, casein were purchased from Sigma (Saint Louis). The egg protein was prepared mixing the white and the yolk of the egg, extending it in a slide, and irradiating it in a OSRAM L36/37 fluorescent lamp at 350–400 nm and 36 W, for 4 h at a distance of 12 cm.

The real samples were taken from the surface of pictorial works of art with a scalpel. The following pieces from artistic heritage of Valencia (Spain) were analyzed:

Sample 1: Altarpiece from San Miguel, by Vicente Maçip (1542).
Sample 2: Triptych of the “Magdalena” by “Maestro de Alzira” (XVI century).
Sample 3: Altarpiece from “Cinctorres” by Bernat Serra (XIV century).

Sample 4: Altarpiece from "Muro de Alcoi" by Jeronimo Rodríguez de Espinosa (XVII century).

2.4. Instrumentation

Chromatographic analysis was carried out using a 1100 Model Liquid Chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with quaternary pump, on-line vacuum degassing system, injector (20 μl loop volume), column oven and fluorescence detector. Signals were processed by the Agilent Chemstation for LC, Rev. A 09.03-847.

Separation of fatty acids was achieved in a Zorbax XDB-C18 C_{18} main column (15 cm × 4.6 cm i.d., particle size 5 μm) thermostated at 30 °C. A pre-column filled with the same stationary phase was used. The selected excitation and emission wavelengths were 340 and 450 nm, respectively [10].

2.5. Hydrolysis of proteins

Proteins standards (0.5–1 mg) were placed in a 0.3-ml microvessel (Supelco, Bellefonte, USA) and treated with 100 μl of 12 mol/l HCl for 24 h at 110 °C [2]. The HCl is evaporated and after cooling, 250 μl of 0.1 M HCl aqueous was added and the mixture was shaken in order to favour extraction. Three extractions were made. Finally, the three extractions were mixed in a Pyrex test tube (Bibbi Sterilin, Staff, UK), and dried at 70 °C. The residue was dissolved in 0.2 ml of 0.1 M HCl aqueous. After cooling the mixture was ready to be derivatized [2].

For real sample analysis, solid parts scalpered from the pictorial work of art (0.5–1 mg) were derivatized in the same way as proteins standards.

2.6. Derivatization procedure

A volume of 200 μl of 0.1 M HCl aqueous solution containing the amino acid was introduced in a Pyrex test tube, and 0.1 ml of borate buffer was added. A volume of 0.4 ml of the OPA/2-ME solution was added [10]. After shaking during 4 min, the mixture is filtered though a 0.45 μm i.d. nylon filter and immediately 20 μl were introduced in the chromatographic system.

2.7. Derivatization of the samples to avoid the effect of the pigments

A volume of 0.2 ml of Na_2EDTA at pH 9 with ammonia/ammonumchloride is added instead of aqueous 0.1 M HCl. The rest of the experimental procedure is carried out as in Section 2.6.

2.8. Chromatographic elution

The chromatographic separation was carried out by a complex gradient program, eluent A is a water buffered at 5.8 with acetate buffer and 5% of THF as modifier. The selected program gradient is shown in Table 1.

Table 1
Gradient elution and column clean up protocol (flow rate 1 ml/min)

Time (min)	Eluent A%	Eluent B%
0	90	10
12	72	28
20	70	20
22	65	35
31	63	37
35	53	47
38	45	55
50	35	65
Time (min)	**Cleaning protocol**	
52	0	100
58	0	100
60	90	10

3. Results and discussion

3.1. Derivatization conditions

The OPA/2-ME is a well-known derivatization reagent in fluorescence analysis of amino acids. The conditions used in this work are similar that presented by Soufleros et al. [10], however the use of 100 ml/25 ml MeOH instead of 25 mg/25 ml MeOH, allows to analyze sample with more quantity of amino acids with great robustness.

In order to optimize the reaction time, some analyses were made at different times. For the analysis, alanine, an apolar amino acid, serine, a polar amino acid, histidine, a basic amino acid and glycine, the amino acid without lateral chain were chosen. A solution 1.25 mM of each one is derivatized and injected into the chromatographic system, several reaction times were tested. The results can be seen in Fig. 1 and show that after 3 min the yield remains roughly constant. A time of 4 min is taken for the derivatization reaction.

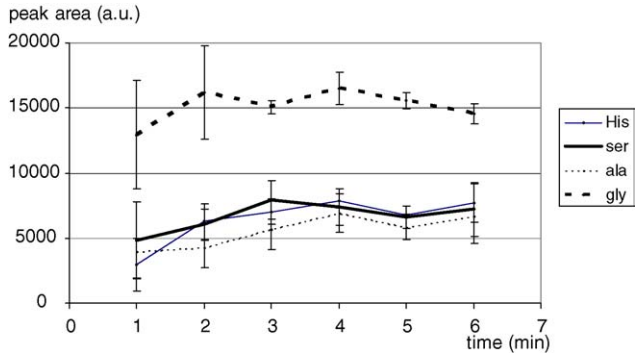

Fig. 1. Values of peak area corresponding to the OPA derivatives of alanine, histidine, serine and glycine at different times. Standard deviation were calculated for $n = 5$.

3.2. Chromatographic separation

The chromatographic conditions and the detection parameters applied to this work are as Soufleros et al. [10]. However, in order to reduce the pressure over the column, the flow is reduced to 1 ml/min, adjusting the time to maintain the gradients. The temperature is fixed to 30 °C in order to improve the stability of the retention time. As the last amino acid appears at nearly 44 min, at 50 min a cleanage procedure is applied. At 52 min the eluent was methanol 100%, running for 6 min, then the eluent rate is adjusted to the initial conditions for the following analysis.

3.3. Assay with amino acids

A mixture of alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophane, tyrosine and valine 0.8 mM was analyzed by the proposed method. As it can be seen in Fig. 2, the amino acids were well-separated and can be quantified, even if histidine and glutamic acid have near retention time. Glycine and lysine show double peaks [10]. To quantify these amino acids the sum of their two peaks were taken as analytical parameter. Peaks seem approximately Gaussian but areas are different for each fatty acid, which may be due to different derivatization yield or different fluorescence yield of each derivative. Leucine provides adequate sensitivity and stability and it is chosen as reference to calculate the relative amount of amino acids. Ratios among the peak area of the amino acids and the leucine remains roughly constant.

The calibration parameters for the amino acids analysis were determined using 0.5 mM tryptophane (trp) as internal standard. The method was tested for alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tyrosine and valine and found analytical parameters are shown in Table 2, where the regression line equation were obtained with the linear range and the limit of detection, which was calculated as three times the standard derivation of the blank divided by sensitivity of each amino acid [18].

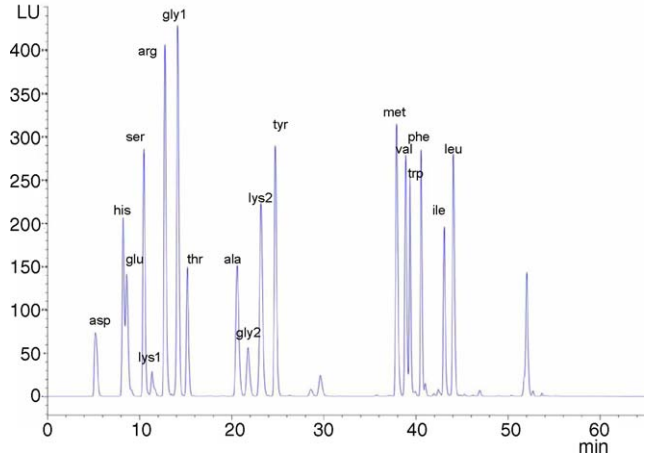

Fig. 2. Liquid chromatogram obtained by optimized derivatization with OPA/2-ME of 0.8 mM each amino acid alanine (ala), arginine (arg), aspartic acid (asp), glutamic acid (glu), glycine (gly), histidine (his), isoleucine (ile), leucine (leu), lysine (lys), methionine (met), phenylalanine (phe), serine (ser), threonine (thr), tyrosine (tyr), tryptophane (trp) and valine (val). Conditions: mobile phase: gradient as explained in Section 2.7 at 30 °C. Flow-rate: 1 ml/min. Excitation wavelength 350 nm, emission wavelength 450 mn, injected volume: 20 μl.

3.4. Assay with proteins

Proteins have been analyzed by HPLC using the proposed method, and characteristically chromatograms were found for each kind of protein. The chromatogram corresponding to egg protein analysis can be seen in Fig. 3. There are no new peaks which interfere with the amino acid derivatives peak. For each protein, the ratio between the peak area of each amino acid derivative and the area of the leucine derivative was calculated. The results for five different replicates of each protein are shown in Table 3.

Table 2
Analytical parameters of the suggested analytical method for the selected amino acids

Amino acid (aa)	$A(\text{aa})/A(\text{trp}) = (a \pm s_a)[\text{aa}] + (b \pm s_b)$		R^2	Linear range (mM)	Limit of detection ($\times 10^{-4}$ mM)
	Slope	Ordinate in origin			
asp	1.16 ± 0.02	−0.04 ± 0.02	0.998	0.002–5.0	6
his	1.75 ± 0.01	−0.02 ± 0.01	0.9995	0.002–3.3	4
glu	1.42 ± 0.01	0.03 ± 0.01	0.9991	0.002–3.3	5
ser	1.71 ± 0.01	0.02 ± 0.01	0.9992	0.002–3.3	4
arg	2.08 ± 0.05	0.12 ± 0.05	0.993	0.002–3.3	3
thr	1.31 ± 0.01	−0.02 ± 0.01	0.9990	0.002–5.0	5
ala	1.63 ± 0.01	−0.02 ± 0.02	0.9991	0.002–5.0	4
tyr	1.52 ± 0.08	0.04 ± 0.04	0.994	0.002–3.3	4
met	2.42 ± 0.03	0.03 ± 0.02	0.998	0.002–1.6	3
val	2.42 ± 0.03	0.00 ± 0.02	0.998	0.002–1.6	3
phe	1.62 ± 0.02	−0.01 ± 0.01	0.9990	0.002–3.3	4
ile	1.16 ± 0.01	0.05 ± 0.02	0.9990	0.002–5.0	6
leu	1.34 ± 0.03	0.05 ± 0.03	0.996	0.002–3.3	5
lys	3.37 ± 0.06	0.11 ± 0.07	0.996	0.002–3.3	2
gly	3.15 ± 0.05	0.00 ± 0.06	0.998	0.002–3.3	2

The calibration lines were made with 13 points.

Table 3
Calculated ratios among the peak area of the amino acid derivatives and the peak area of the leucine derivative for the five standards

Amino acid	Values of the *A*(aa)/*A*(leu) ratio				
	Albumin	Casein	Beef gelatine	Porcine gelatine	Egg protein
asp	0.37 ± 0.07	0.3 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.013 ± 0.006
his	0.30 ± 0.09	0.4 ± 0.1	0.06 ± 0.05	0.3 ± 0.2	0.13 ± 0.03
glu	0.9 ± 0.3	1.1 ± 0.6	0.8 ± 0.9	0.8 ± 1.0	0.007 ± 0.005
ser	0.6 ± 0.2	0.4 ± 0.2	0.03 ± 0.04	0.01 ± 0.02	0.4 ± 0.2
arg	1.6 ± 0.3	0.77 ± 0.08	4.2 ± 0.6	5.4 ± 0.7	1.0 ± 0.2
thr	0.5 ± 0.1	0.41 ± 0.06	0.2 ± 0.01	0.3 ± 0.1	0.24 ± 0.03
ala	0.8 ± 0.1	0.7 ± 0.3	4.7 ± 0.7	5.2 ± 0.8	2.0 ± 0.1
tyr	0.48 ± 0.09	0.46 ± 0.07	0.06 ± 0.02	0.11 ± 0.04	0.43 ± 0.08
met	0.64 ± 0.03	0.40 ± 0.06	0.29 ± 0.05	0.36 ± 0.06	0.35 ± 0.05
val	1.0 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	1.2 ± 0.1
phe	0.62 ± 0.6	0.44 ± 0.05	0.50 ± 0.05	0.53 ± 0.08	0.40 ± 0.03
ile	0.7 ± 0.1	0.59 ± 0.07	0.7 ± 0.1	1.0 ± 0.2	0.9 ± 0.1
lys	3 ± 1	3.6 ± 0.7	7 ± 1	6.7 ± 0.7	0.05 ± 0.3
gly	6 ± 2	2.1 ± 0.6	50 ± 20	80 ± 20	0.84 ± 0.2

Values are average ± S.D. for $n = 3$.

The amino acids glycine and lysine present high variability, because of they present double peaks. The calculate areas are the sum of the areas of the two peaks, so the deviation standard is added. The asparaguine is changed into aspartic acid in the hydrolysis, so the obtained values for the aspartic acid represent in fact the amount of aspartic acid and asparaguine in the protein. Glutamine is changed into glutamic acid as the same way. In this case, the glutamic acid is partially converted to pyroglutamic acid [2]. These phenomena difficult the accurate measure of their amount. However, the amino acids with apolar groups generally show less variability than those with polar amino acids, because of the less reactivity of apolar groups. The cysteine, proline [10] and tryptophane [2] cannot be found using this method.

The ratios for some amino acids have high deviation standard, this is due to the natural origin of the standard, and the biosynthesis of the proteins is sensible to the environmental conditions and may present high variability for some amino acids [4].

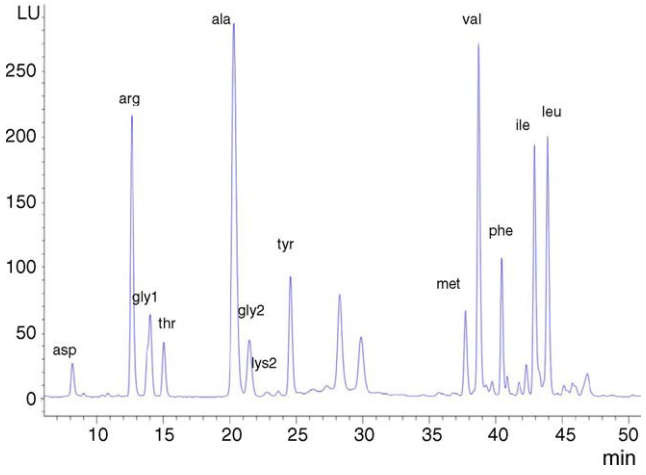

Fig. 3. Liquid chromatogram obtained by optimized derivatization with OPA/2-ME of the amino acids mixtured obtained by hydrolysis of egg protein. Conditions: mobile phase: gradient as explained in Section 2.7 at 30 °C. Flow-rate: 1 ml/min. Excitation wavelength 350 nm, emission wavelength 450 mn, injected volume: 20 μl.

The gelatines are differentiated from the other proteins due especially to their high amount of glycine. These gelatines also provide more lysine, arginine and alanine than the other studied proteins. The gelatines are obtained from the skin of the animal where they assure the cohesion, and then the amino acid composition is similar for different species. However, the porcine gelatine shows more amounts of glycine, isoleucine, methionine, tyrosine and histidine.

The egg protein shows low amount of lysine, glycine and glutamic acid. The relative amount of arginine and alanine are different from the other proteins and characteristic from the egg.

The group albumin/casein is differentiable from the other proteins by the amount of glycine, lysine, arginine, threonine, alanine and methionine. However, casein has less glycine, lysine, arginine and threonine than albumin.

Isoleucine, phenylalanine, valine were found approximatively in the same amount for the five studied proteins.

The proteins are characterized by their relative amount of amino acids [2]. However, for a sample the comparison of 44 amino acids with those of five proteins can be difficult and confusing. In order to clearly illustrate the differences among the proteins and better organize the information, a chemometric analysis by PCA was made. The glutamic acid is not included in the system, due to its partial alteration to pyroglutamic acid during hydrolysis [2]. As can be seen in Fig. 4, the five kind of proteins are clearly resolved, although the gelatines and the albumin have more dispersion then the other proteins.

3.5. Analysis of proteinaceous glues from painted works of art

3.5.1. Analysis without considering the pigments

Samples of real paintings were taken in order to identify which protein was used by artists using the developed method. Samples were analyzed in the same way as the standards (see

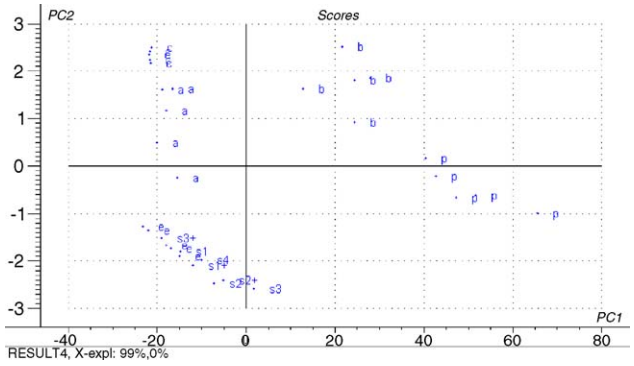

Fig. 4. Scoreplot for PC1 and PC2 obtained by means of a principal components analysis of the results obtained in the chromatographic analysis by the proposed method of (b) beef gelatina, (p) porcine gelatine, (a) albumin, (c) casein, (e) egg protein, samples 1, 2, 3 and 4 (s1, s2, s3, s4), and samples 1, 2 and 3 with adding EDTA to avoid the effect of the pigments (s1+, s2+, s3+).

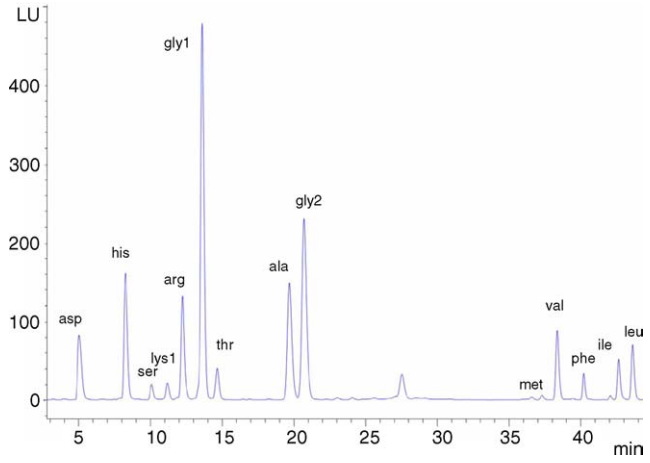

Fig. 5. Liquid chromatogram obtained by optimized derivatization with OPA/2-ME of the amino acid mixture obtained by hydrolysis of a sample taken with an scalpel from the altarpiece of San Miguel, by Vicente Maçip (sample 1). Conditions: mobile phase: gradient as explained in Section 2.7 from at 30 °C. Flow-rate: 1 ml/min. Excitation wavelength 350 nm, emission wavelength 450 mn, injected volume: 20 μl.

Sections 2.5 and 2.6). The resolution of the amino acids is not impaired by new peaks, and their peak area ratios can be calculated without difficulty, as can be seen for the analysis of the sample 1 in Fig. 5. As the case of the standard proteins, the ratios among peak area of amino acids and the peak area of the leucine and their standard deviation were calculated for three replicates (three different samples taken from the pictorial work of art). The results obtained for these ratios are shown in Table 4.

The standard deviations for samples were found higher than in the case of proteins. The considerable heterogeneity and complexity of the pictorial works of art may be the cause of this behaviour [4]. The ratios obtained for the painting samples sometimes differ from those found for the proteins. Standard ratios were calculated by means of fresh proteins. The real samples have undergone natural ageing which can partially hydrolyzed the protein. Real ageing process occurs by addition of some causes, such as slow attack by environmental substances (dioxygen, moisture, microorganisms, pollution agents) and irradiation from light, and exact effect undergone by the proteins of pictorical work of art are usually unknown [4,19]. The artist can also employ a mixture of glues and the sum of the amino acids of all the present proteins can appear.

In order to characterize the analyzed real samples, the obtained data for the ratios among the area peak of the amino acid derivatives and the area peak of the leucine derivative are introduced in the PCA model [18]. As can be seen in Fig. 4, the obtained scores for samples 1, 2 and 4 point to the use of egg protein in the artistic works of art. The samples scores deviated a little from the egg protein standard, maybe a little porcine gelatine has been added in the binding media. The obtained scores of the sample 3 seems egg protein, but its position in the score plots is quite far from the standards.

Table 4
Calculated ratios among the peak area of the amino acid derivatives and the peak area of the leucine derivative for samples from four pictorical works of art

Amino acid	Value of the A(aa)/A(leu) ratio			
	Sample 1	Sample 2	Sample 3	Sample 4
asp	1.2 ± 0.5	2.0 ± 0.3	2 ± 1	2.6 ± 0.4
his	2.0 ± 0.8	2.2 ± 0.2	0.13 ± 0.01	0.11 ± 0.03
glu	0.00 ± 0.00	0.4 ± 0.1	2.1 ± 0.7	2.4 ± 0.7
ser	0.2 ± 0.2	0.4 ± 0.2	0.5 ± 0.1	0.38 ± 0.05
arg	1.6 ± 0.6	1.3 ± 0.4	3.0 ± 0.4	1.6 ± 0.2
thr	0.5 ± 0.2	0.52 ± 0.05	0.6 ± 0.3	0.53 ± 0.02
ala	2.5 ± 0.9	2.3 ± 0.5	2.6 ± 0.9	1.9 ± 0.3
tyr	0.0416 ± 0.0001	0.46 ± 0.07	0.12 ± 0.02	0.14 ± 0.04
met	0.08 ± 0.01	0.40 ± 0.06	0.2 ± 0.1	0.4 ± 0.1
val	1.2 ± 0.1	0.7 ± 0.1	1.0 ± 0.1	1.2 ± 0.2
phe	0.38 ± 0.03	0.44 ± 0.05	0.45 ± 0.04	1.2 ± 0.2
ile	0.70 ± 0.05	0.59 ± 0.07	0.7 ± 0.2	1.7 ± 0.2
lys	0.39 ± 0.01	3.6 ± 0.7	1.0 ± 0.3	0.9 ± 0.3
gly	9 ± 5	2.1 ± 0.6	25 ± 5	14.0 ± 0.7
Suggested proteinaceous glue	Egg protein	Egg protein	Probably egg protein	Egg protein

Values are average ± S.D. for $n = 3$ (different samples taken from the pictorial works of art).

3.5.2. Analysis considering the effect the pigments

In order to avoid the effect of the pigments in the analysis, samples taken from the works of art 1, 2 and 3 were analyzed as Sections 2.5–2.7. The resolution of the amino acids was not prejudiced by new peaks, and their peak area ratios can be calculated. The ratios among peak area of amino acids and the peak area of the leucine and their standard deviation were calculated for three replicates (three different samples taken from the pictorial work of art). The obtained results were included in the PCA model [18]. As can be seen in Fig. 4, the dots for the samples 1 and 2 with EDTA are near the dots for samples 1 and 2 without EDTA, so the pigments have no influence in the analysis. However, the sample 3 with EDTA was found nearer from the egg protein standard than the sample 3 without EDTA, thus for the sample 3, pigments had interfered in the analysis.

4. Conclusions

A satisfactorily characterization of proteinaceous glues in pictorial artworks of art by HPLC-Fluorescence detection is possible. The amino acids were obtained from the proteins by means of acid hydrolysis. A derivatization with *o*-phtalaldehyde and 2-mercatpoethanol provide fluorescence properties with enough sensitivity to the amino acids acids. The mixture of the derivatives of these amino acids was clearly resolved, despite their similar polarity. The chromatograms for different standard proteins (beef gelatine, porcine gelatine, albumin, casein and egg) provide sufficient data to differentiate among them by means of amino acids peak area ratios and a chemiometric analysis by PCA of the obtained values. The suggested HPLC method permits the easy characterization of proteins used in pictorial works of art in the same way of the standards, which is essential to conservation or restoration treatments. Even if the ageing of proteins can affect the amino acid amount by unknown processes in pictorial works of art, the amino acids ratios allows to provide an hypothesis of the nature of the proteinaceous binders.

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References

[1] C. Marinach, M.C. Papillon, C. Pepe, J. Cult. Heritage 5 (2004) 231.
[2] J.V. Gimeno-Adelantado, R. Mateo-Castro, M.T. Doménech-Carbó, F. Bosch-Reig, A. Doménech-Carbó, J. De la Cruz-Cañizares, M.J. Casas-Catalan, Talanta 56 (2002) 71.
[3] J. De la Cruz-Cañizares, M.T. Doménech-Carbó, J.V. Gimeno-Adelantado, R. Mateo-Castro, F. Bosch-Reig, J. Chromatogr. A 1025 (2004) 277.
[4] J.S. Mills, R. White, The Organic Chemistry of Museum Objects, second ed., Butterworth, Oxford, 1994.
[5] S. Gorinstein, M. Zemser, F. Vargas-Albores, J.L. Ochoa, O. Paredes-López, Ch. Scheler, J. Salnikow, O. Martín-Belloso, S. Trakhtenberg, Food Chem. 67 (1999) 71.
[6] S.H. Chiou, W. Chen, Biochem. Int. 28 (1992) 401.
[7] M. Ummadi, B.C. Weimer, J. Chromatogr. A 964 (2002) 243.
[8] M.P. Colombini, F. Modugno, E. Menicagli, R. Fuoco, A. Giacomelli, Microchem. J. 67 (2000) 291.
[9] Y.V. Tcherkas, L.A. Kartsova, I.N. Krasnova, J. Chromatogr. A 913 (2001) 303.
[10] E.H. Soufleros, E. Bouloumpasi, C. Tsarchopoulos, C.G. Biliaderis, Food Chem. 80 (2003) 261.
[11] G.P. Palace, C.H. Phoebe, Anal. Biochem. 244 (1997) 393.
[12] K.S. Lee, D.G. Drescher, J. Biol. Chem. 254 (1979) 6248.
[13] T.P. Piepponen, A. Skujins, J. Chromatogr. B 757 (2001) 277.
[14] J. You, W. Lao, Q. Ou, X. Sun, J. Chromatogr. A 848 (1999) 117.
[15] R.A. Bank, E.J. Jansen, B. Beekman, J.M. Koppele, Anal. Biochem. 240 (1996) 167.
[16] K. Hamase, T. Inoue, A. Morikawa, R. Konno, K. Zaitsu, Anal. Biochem. 298 (2001) 253.
[17] A. Vasanits, I. Mólnar-Perl, J. Chromatogr. A 832 (1999) 109.
[18] J.N. Miller, J.C. Miller, Statistics and Chemiometrics for Analytical Chemistry, fourth ed., Prentice-Hall, Harlow, England, 2000.
[19] M.P. Colombini, F. Modugno, R. Fuoco, A. Tognazzi, Microchem. J. 73 (2002) 213.