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ANALYSIS OF DANSYL DERIVED AMINO ACIDS OF PROTEINACEOUS GLUES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract. The nature of proteinaceous binding materials used in works of art can be estimated before restoration by their amino acid composition. Standard binders and samples from paintings were hydrolyzed into their constituent amino acids and the amino acids were labelled by derivation with dansyl chloride. High performance liquid chromatography was used for identification of individual amino acids. The relative content of amino acids in skin glue, gelatine, sturgeon glue, casein, albumin, egg yolk, and egg white were determined and compared with the amino acid composition of samples from two icons from the 19th century. The proteinaceous glues were successfully identified by the method described.

Key words: amino acids, proteinaceous glues, restoration, conservation.

INTRODUCTION

Artists use a wide range of materials as pigment binders or adhesives. These include natural proteinaceous materials such as egg (tempera), milk casein, and collagen glues prepared from animal skin or fish skeletons. The general category of glues can be determined by qualitative methods of analysis: spot tests and paper and thin layer chromatography. Several studies can be found in this field [1, 2]. Proteinaceous glues can be identified by determining the relative content of amino acids in the materials.

Glues such as gelatine and skin glue can be distinguished from other glues on the basis of the content of proline and hydroxyproline. Amino acids can be convincibly analysed and quantified by HPLC after pre-column derivation with

dansyl chloride, *o*-phthalaldehyde, phenylisothiocyanate, or 9-fluorenylmethyl chloroformate [3, 4]. The last reagent has also been successfully applied in the analysis of proteinaceous artists' materials with casein, egg yolk, egg albumen, whole egg, and animal glue used as standards [4]. Halpine described an analysis method for the same purpose using phenylisothiocyanate for derivation [5].

We preferred dansyl chloride, which offers sufficient sensitivity of analysis, permits analysis of secondary amino acids, and requires a simple derivation procedure. This paper describes the application of the method for the analysis of proteinaceous glues.

EXPERIMENTAL

Amino acid standards

Pure amino acids were derived with dansyl chloride. 100 μL amino acid solutions (0.72 mM) were derived with 200 μL dansyl chloride (3.6 mM in acetonitrile) for 40 min at 25 °C [6].

Pure proteinaceous glues

Gelatine (Bio-Rad), casein (Nutritional Biochemicals Corporations), egg white, egg yolk, skin glue (technical), sturgeon glue (technical), albumin, which are used in the base of fresco and paints, were used as standard samples of proteinaceous binding materials. Before hydrolysis proteinaceous glues were treated with performic acid to convert methionine to methionine sulfone and cystein to cysteic acid. Performic acid was prepared by mixing one volume of 30% hydrogen peroxide with nine volumes of 99% formic acid containing phenol at a concentration of 0.5% and the mixture was allowed to stand at room temperature for 2.5 h. The reagent and the hydrolysis tube were cooled to 0 °C in an ice bath and the cold reagent was added to the sample. After a reaction time of 2 h at 0 °C the reaction mixture was diluted with 1 mL ice-cold water, lyophilized and hydrolyzed with 6 N hydrochloric acid for 24 h at 105 °C [7, 8].

Model systems and samples

The model systems were prepared from chalk, gypsum, and ageing proteinaceous glues, such as gelatine, egg yolk, and skin glue [9]. Samples of paint and adhesive were removed from the surface of icons (19th century) with a scalpel.

For removal of pigments from proteinaceous materials a one-hour water-extraction method has been developed [5]. The extraction procedure separates the sample into water-soluble and water-insoluble components. The model systems and samples (50 mg) were thoroughly mixed with water (7 mL) and allowed to stand at room temperature for one hour. To ensure that the larger particles were broken up the samples were sonicated in an ultrasonic water bath at 25 °C for 15 min before centrifugation at 3000 rpm for another 15 min. After

centrifugation the sample solutions (5 mL) were placed in a hydrolysis tube (5 mm i.d., 20 cm long), evaporated to dryness and hydrolyzed. Hydrolyses of model systems and samples were performed under similar conditions. The hydrolysate was cooled, diluted with water, evaporated to dryness, and the residue was dissolved in isopropyl alcohol. Then the pH of the solution was adjusted to 6–7 (universal indicator paper) using sodium carbonate solution (analytical grade). Salts were separated by gel chromatography or ion exchange chromatography.

Gel chromatography: 200 mL of water was added to 5 g of dry Sephadex G-50 (Pharmacia) and the gel was allowed to swell for 12 h, then the swollen gel was carried into the column. The dimensions of the column were 1.6 cm × 40 cm, the thickness of layer was 29 cm. The empty volume of the column was determined with Dextran Blue 2 000 000. The sample was eluted with water as described in [10].

Ion exchange chromatography: salts were separated on the columns with Amberlite IRA-400 (Bio-Rad) (H^+ form, pH = 5.0) and with Dowex 50W × 10 (Bio-Rad) (OH^- form, pH = 7.0) [11]. The dimensions of the columns were 1.4 cm × 10 cm and 1.8 cm × 10 cm, the layers were respectively 20 and 23 cm thick.

The presence of protein in the samples was detected with 0.1% ninhydrin solution in ethanol after treatment with 6 N hydrochloric acid for 24 h at 105 °C. The mixture of about 0.1 mL of the sample and 0.5 mL of ninhydrin solution was boiled. The test was regarded as positive if the colour changed to violet.

After column chromatography the samples were concentrated by rotary evaporation. The residue was dissolved in isopropyl alcohol followed by derivation with dansyl chloride [6].

20 μL of the mixture was injected into a Supelcosil LC-18 column (150 mm × 4.6 mm, 3 μm). The HPLC system consisted of Gilson model 302 pumps with a dynamic mixer (model 811B) and manometric module (model 802C), UV and fluorescence detectors (models 116 and 121), Supelcosil LC-18 (Supelco) column, and a computer for data processing.

The elution was done in the gradient mode applying 0.1 M Na phosphate buffer and acetonitrile [MeCN] (Fluka). The gradient flow was programmed from 20% MeCN to 100% MeCN over 50 min (flow rate 1 mL/min). The temperature of the Supelcosil LC-18 column was 36 °C. The chromatogram was monitored with a UV-detector at 254 nm.

RESULTS AND DISCUSSION

Table 1 shows the retention times of the dansyl derivatives of standard amino acids on the Supelcosil LC-18 column under the described conditions. Table 2 shows the results obtained for seven main proteinaceous binding materials.

Table 1. Retention times of dansyl derivatives

No.	Peak name	Retention time	No.	Peak name	Retention time
1	Aspartate	5.55±0.64	9	Proline	17.13±0.86
2	Glutamate	5.78±0.88	10	Methionine	20.11±0.11
3	Hydroxyproline	8.89±0.73	11	Isoleucine	22.25±0.71
4	Serine	11.53±0.43	12	Leucine	22.75±0.87
5	Glycine	11.84±0.69	13	Phenylalanine	25.55±0.63
6	Arginine	12.62±0.74	14	Lysine	34.85±0.75
7	Threonine	11.71±0.27	15	Histidine	37.46±0.57
8	Valine	15.11±0.86	16	Tyrosine	38.81±0.18

Table 2. Average amino acid composition (%) of standards of proteinaceuos binding materials

Amino acid	Skin glue	Gelatine	Sturgeon glue	Casein	Egg yolk	Albumin	Egg white
Aspartate	6.35	8.50	2.68	8.32	11.90	7.71	7.14
Glutamate	11.20	14.48	4.20	18.90	16.58	13.90	13.98
Hydroxyproline	12.05	11.99	3.47	–	–	–	–
Serine	4.27	4.56	2.96	5.35	9.21	8.47	9.72
Glycine	26.50	23.82	9.63	4.10	4.56	3.98	3.38
Arginine	8.26	8.13	16.96	3.34	7.73	4.70	5.53
Threonine	2.45	1.70	–	–	4.46	3.56	4.51
Valine	2.83	2.32	1.37	7.36	7.25	6.63	6.98
Proline	13.90	11.83	12.04	12.05	4.15	4.10	4.06
Methionine	–	–	1.93	4.98	2.83	6.01	5.87
Isoleucine	1.76	1.81	1.02	8.03	7.97	6.34	6.91
Leucine	3.49	3.84	1.17	5.32	8.40	8.78	9.91
Phenylalanine	2.15	2.13	0.58	5.85	2.98	7.12	8.05
Lysine	4.12	3.97	4.31	7.26	5.38	5.59	6.80
Histidine	0.69	–	1.22	4.47	2.93	3.42	3.50
Tyrosine	–	0.92	–	4.67	3.67	3.98	3.66

– Not present or below detection limit.

Gelatine and animal glue are both composed of collagen. Collagen contains hydroxyproline, therefore the content of hydroxyproline in samples indicates the presence of animal glues. Hydrolyzed skin glue contains a large amount of glycine (around 26%) and small amounts of such amino acids as valine and leucine, the ratio serine:glycine is approximately 1:6 and the ratio hydroxyproline:proline is 1:1. Gelatine is characterized by high levels of glycine (≈23.8%), proline (≈11.9%), glutamic acid (≈14.48%), and arginine (≈8.1%).

Hydrolyzed egg white shows well-separated peaks of leucine (≈9.9%), proline (≈4.0%), and glycine (≈3.3%). Aspartic acid is well represented. Egg yolk has higher levels of such amino acids as threonine (≈4.5%) and serine (≈9.7%).

Hydrolyzed casein is characterized by high levels of glutamic acid (≈18.9%), proline (≈12.0%), and aspartic acid (≈8.3%) and relatively low levels of such amino acids as leucine and methionine. Casein can be distinguished from egg proteins by higher levels of proline.

Hydrolyzed sturgeon glue contains large amounts of arginine (≈16.96%), glycine (≈9.6%), and proline (≈12.04%) and has low levels of serine (≈2.9%), valine (≈1.37%), and aspartic acid (≈2.6%). Sturgeon glue contains also hydroxyproline (≈3.47%).

The results obtained for the model systems show the nature of the glues used. The model systems (MS 1, MS 2) and samples (S 1, S 2) exhibit the same trends in amino acid composition as standards of proteinaceous binding materials (Table 3).

Table 3. Average amino acid composition (%) of known model systems and unknown samples. Model systems: MS 1, chalk, gypsum/skin glue; MS 2, umbra/egg yolk. Samples: S 1, *Iberian Icon of the Mother of God* (red area); S 2, *Seven Ephesian Adolescents* (brown area)

Amino acid	Model system 1 (MS 1)	Model system 2 (MS 2)	Sample 1 (S 1)	Sample 2 (S 2)
Aspartate	6.89	11.60	7.68	9.24
Glutamate	10.57	16.88	7.93	12.03
Hydroxyproline	11.90	–	12.34	4.47
Serine	5.09	9.43	7.37	9.31
Glycine	27.10	5.01	18.78	16.09
Arginine	7.34	7.63	4.97	5.00
Threonine	2.48	4.76	3.56	4.55
Valine	2.45	7.10	7.49	8.37
Proline	14.05	4.82	7.80	6.96
Methionine	–	3.04	3.00	2.32
Isoleucine	1.32	7.15	2.75	3.39
Leucine	4.53	8.64	5.60	6.45
Phenylalanine	2.10	3.16	2.20	2.01
Lysine	4.25	5.88	3.88	4.78
Histidine	0.53	2.32	1.68	1.98
Tyrosine	–	2.58	2.87	3.05

The two samples analysed were taken from *Iberian Icon of the Mother of God* and *Seven Ephesian Adolescents*. Both are 19th century panel paintings from the Museum of the University of Tartu.

Sample 1 was taken from the Madonna's robe (red area). Analytical results indicated that two kinds of proteinaceous paint binders had been used. The red area in particular was rendered in egg yolk whereas animal glue was used as a general binder. Animal glue was distinguished on the basis of the presence of hydroxyproline. Serine was the characteristic constituent as in egg yolk (Table 3). Sample 2 was taken from the back (brown area) of the painting. Amino

acid analysis indicated egg yolk and animal glue binders (Table 3). The results are similar to Sample 1.

CONCLUSIONS

The amino acid analysis of proteinaceous artists' glues by using high performance liquid chromatography and derivation with dansyl chloride offers useful information to conservators and art historians. This method is suitable for the analysis of samples taken from works of art and can be applied routinely in the region of 75 ng of sample.

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VALGULISTE LIIMAINETE DANSÜÜLITUD AMINOHAPETE ANALÜÜS KÕRGRÕHU VEDELIKUKROMATOGRAAFIA ABIL

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