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Garry Thomson, Scientific Adviser
Martin Wyld, Chief Restorer

Ashok Roy: Editor

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The Characterization of Proteinaceous Binders in Art Objects

Raymond White

Introduction and review

Perhaps one of the most taxing problems for a museum analyst to face is that of attempting to characterize adhesives and paint media based on proteinaceous material. In the case of European easel paintings, much useful information can be derived from an assay of the relative amounts of fatty acids present in the accompanying glycerides [1,2,3,4]. If we consider egg tempera paint, although there are no fatty acid components which are specific to that source, nevertheless a pattern of acids that is low in azelaic, but with a pronounced presence of palmitic and stearic acids, together with a modest proportion of oleic acid would suggest to the experienced analyst that an egg binder is the principal adhesive of the sample. Apart from cholesterol, which would appear to offer the possibility of a reliable indicator for egg tempera [5], there is no other specific lipid indicator that points unambiguously to an egg source. Furthermore, with a degree of experience, the analyst can deduce from the overall quantity of non-drying lipids within the sample, the presence of non-fatty binders such as animal glue, especially when combined with simple solubility tests. So a careful examination of the fatty constituents can be a useful diagnostic when taken in context and assessed with caution.

Nevertheless, it has been apparent for some time, that there is a very real need for an adequate analytical technique for the characterization of the nitrogen-containing components of which the bulk of a glue binder or tempera paint is composed. These are chiefly proteins, of course, which are constructed of amino acid units joined one-to-another via the peptide linkage (−CO−NH−) and represent the main binding components in glues and tempera, following gelling and dehydration.

A variety of analytical methods have been applied to this problem over the years and the literature is full of reports on analytical techniques which attempt to characterize these proteins, either by studying the whole protein or yet again by studying the individual amino acid residues which go to construct a given protein. Perhaps the earliest and simplest tests for proteinaceous materials were simple pyrolysis tests, in which alkaline fragments — mainly amines — are formed thermally [6]. This has been updated by combination with gas-liquid-chromatography whereby in a single unit, the pyrolytic products may be examined both qualitatively and quantitatively [7]. So-called 'wet tests' were developed at an early stage and include the ninhydrin test, which is based on the reaction of the material with indanetrione hydrate reagent to give a characteristic colouration [8,9]. In addition there is a more specific 'wet test', the Ehrlich test [10], which enables hydroxyproline to be assayed within the sample.

Paper chromatography [11,12] and thin-layer chromatography [13,14,15] have found wide application, but have the disadvantage of not being readily amenable to quantitative interpretation. Furthermore, electrophoretic methods are available to split-up the various composite proteins into discreet bands [16,17]. Amino acid analysis of protein hydrolysates has been undertaken using ion-exchange chromatography [18]. Gas-chromatography of amino acids formed by hydrolysis has been undertaken using trimethylsilyl derivatives [19,20].

The purpose of this article is to relate the experience gained by the National Gallery Scientific Department laboratory in the analysis of protein hydrolysates by gas-chromatographic methods. The method is based on that reported by Gherke *et al.* [21], with some modifications. Commonly, about twenty amino acid residues are found in the hydrolysis products of proteins found in living systems. These include: alanine, valine, glycine, isoleucine, leucine, proline, hydroxyproline, serine, threonine, glutamic acid (as hydrolysis product of glutamine), aspartic acid (as hydrolysis product of asparagine), phenylalanine, lysine, hydroxylysine; sulphur-containing amino acids such as cystine, methionine, arginine, tryptophan, histidine and tyrosine.

In addition, one may see artefacts such as ornithine and citrulline (derived from arginine), α- and γ-aminobutyric acids (degradation of glutamic acid). These are commonplace in geological samples. Furthermore, amino sugars may be of importance in natural materials such as chitin and the organic matrix of shells. Glucosamine and galactosamine are typical.

Although proteins appear in bacteria, silk, sponge, haemoglobin and various enzymes, it is mainly those materials of which proteins are structural components, from such sources as animal/fish skin, bones, egg albumen and milk whey and curds (casein) that are of interest to the museum analyst.

As far as the latter is concerned, the range of natural nitrogen-containing adhesives can be broken down into three main groups:

1. Animal collagen, gelatin or fish glue.
2. Egg albumen, whether it be from the whole egg, egg white (albumen), or egg yolk.
3. Milk or casein glue.

Unfortunately nearly all of the major, common amino acids (approximately thirteen) appear in each of the protein types listed above and apart from hydroxyproline, which only occurs in collagen, a simple qualitative, specific indicator is lacking. However the range of proportions of certain amino acids to others does offer a means of classification. Each protein group is outlined below.

1. Animal and fish collagen

This material has found wide application in museum objects as a strong glue adhesive for wood, as a pigment binder in paint and as a binder in the preparation of grounds. It is produced by the treatment of certain animal or fish tissues with hot water. On cooling the leached material, the solution sets to a jelly. The components which are responsible for this gelling are a result of the partial breakdown of tissues. Less drastic treatment, for example extraction at lower temperatures generally results in gelatins of light colour and giving a clear solution. Glues tend to be darker, thicker, more turbid and certainly contain more impurities than gelatin. Pliny relates that glue was cooked from the hides of bulls. It has been reported that fish glues have a reduced structural stability to that of animal collagens and this is believed to be linked with a reduction in the proportion of hydroxyproline and proline residues present.

2. Egg albumen and yolk

This has been used as a pigment binder and when used in this way, the paint is called tempera. The white of the egg can be used, in which case it is called glair. It has also found application as temporary varnish and as a sealant or priming over grounds. Whole egg can be beaten to form an emulsion and used as medium or just the yolk to give a somewhat richer medium. The use of the latter should lead to the detection of cholesterol in the sample and so would provide an alternative indicator [23,5].

3. Casein glue and whole milk tempera

Cow's milk contains about 5.5% fat, 4.9% lactose and between 3% and 5% protein. Unlike egg white, which contains but one non-glycoprotein (lysozyme), milk has been found to contain κ-, α-caseins and immunoglobulin G. Amongst the glycoproteins we may enumerate β-, γ-caseins, albumin, α-lactalbumin and β-lactoglobulin [24]. Clearly when whole milk has been used, in addition to the information afforded by amino acid analysis, examination of the fatty acid components should reveal augmented lauric and myristic acids with respect to palmitic and stearic acids.

Experimental

Ideally, the proteinaceous sample should be hydrolysed by hot, constant-boiling hydrochloric acid overnight in a sealed, evacuated tube. In the absence of a borosilicate vacuum-line and a glass-blower's torch, there is now available a teflon-sealed hydrolysis/reaction tube, fitted with a side-arm to which a rotary vacuum-pump may be attached and the system evacuated and sealed (Pierce Vacuum Hydrolysis Tube, 10mm × 100mm, 5ml volume). However, a perfectly satisfactory and much cheaper substitute can be effected by the use of a Quickfit B10-stoppered semi-micro tube (Quickfit MF24/0, 4ml volume).

Hydrolysis

Constant-boiling hydrochloric acid was prepared by diluting concentrated MAR hydrochloric acid to approximately 6 Molar, followed by distillation until the binary azeotrope distilled at 108/9°C. After cooling the reagent was stored in good quality borosilicate glass, previously boiled in 6 Molar hydrochloric acid. The paint or adhesive sample was placed in a ground-glass stoppered semi-micro test-tube and 1ml of constant-boiling HCl added. An anti-bumping granule was added and the tube heated over a bunsen flame until a vigorous boiling action had set in. The sides of the tube and the ground glass stopper were all bathed in the flame and after a minute or so of boiling to expel air, the tube was stoppered firmly, placed in a beaker and allowed to remain in an oven set at 100°C, overnight.

Clean-up and deionization

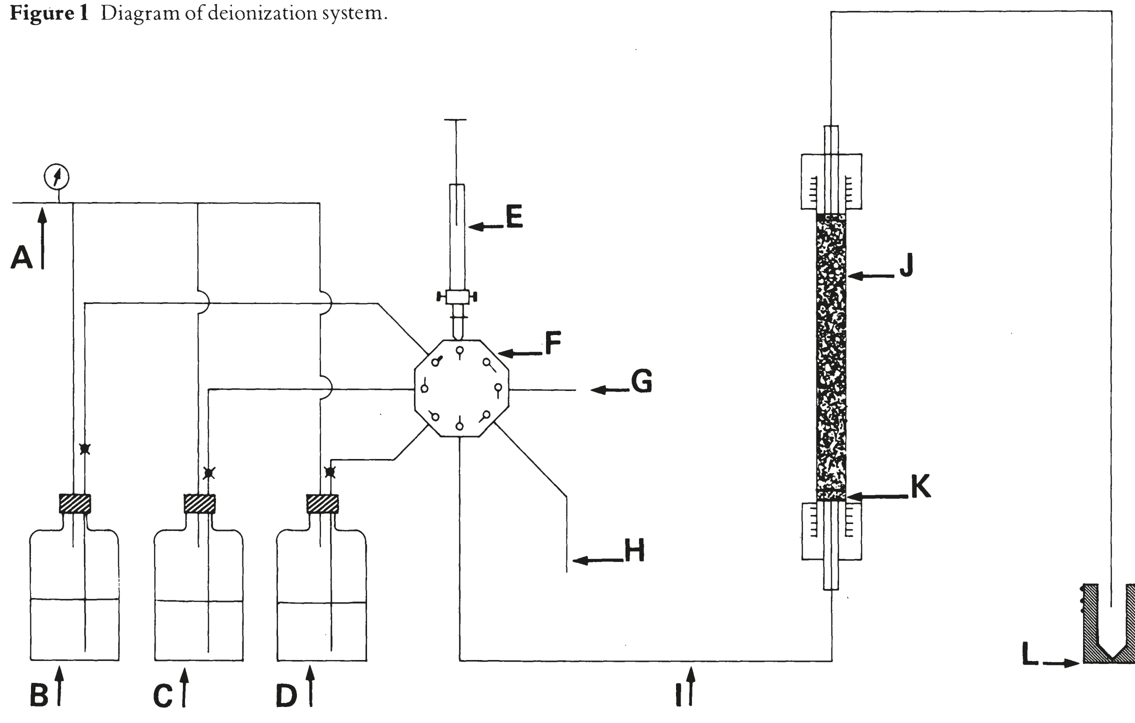
The hydrolysate was evaporated under a stream of dry nitrogen. Water (2.0ml) was added and the sample again taken down to dryness under nitrogen. Yet again, 2.0ml of water was added, warmed and filtered through a glass wool plug in a Pasteur pipette and set aside. A further 2.0ml of water was added to the test-tube, warmed and filtered through the same plug of glass wool. The filtrate was retained.

Deionization was carried out on a small column of Amberlite IR-120H ion-exchange resin (The Rohm and Haas Company, Philadelphia, USA). The system is depicted in Fig.1. The column consisted of a 10cm × 0.5cm glass column fitted with porous teflon frits and packed with the ion-exchange medium. The inlet to the chromatographic column was connected via narrow bore teflon tubing to an eight-port valve. The multiport selector was provided with supplies of 3 Molar double-distilled hydrochloric acid, for column regeneration, double-distilled water for washing and 7 Molar ammonium hydroxide (MAR grade) solution for elution. In addition one port was set aside for injection of the sample onto the column via a syringe and yet another port was used as a vent to enable purging of the system from air bubbles, when replenishing eluants. The solvent/eluant vessels were pressurized to approximately 1 bar above ambient.

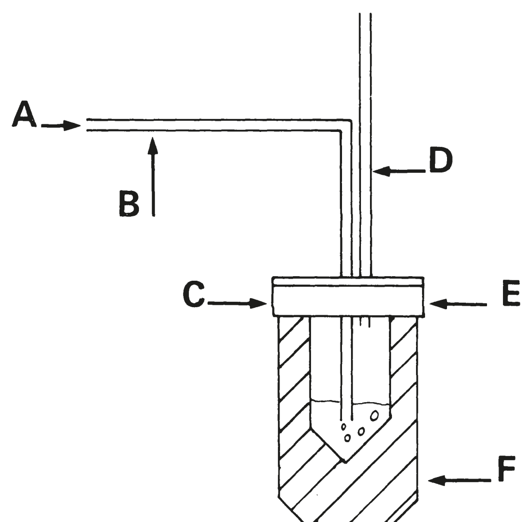
The column was regenerated, prior to application of samples of hydrolysate by the passage of 15ml of 7 Molar ammonium hydroxide at a rate of one drop every four seconds, followed by water at a rate of 1 or 2ml per minute until the effluent emerging from the column was at a pH of 6.5 to 7.0, as measured by narrow range pH paper. The column was then converted to its H^+-form by the passage of 12.0ml of 3 Molar hydrochloric acid at a rate of one drop every four seconds, followed by water at 1 or 2ml per minute until the effluent was between pH 6.5 and 7.0 once again.

The protein hydrolysate was taken up in a 1.0ml syringe with Luer fitting and injected slowly into the line, up-stream of the column, via the multiport valve. This procedure was repeated until all of the hydrolysate was injected and then the retained batch of water washings. The column was then flushed with water, initially at a rate of ten drops per minute (for 6.0ml of collected washings) and then at a flow of 2 to 3ml per minute until 40ml of washings had passed through the column. The washings were discarded unless it was necessary to check for sugars via the furfural test [22]. It was also found advisable to check for the absence of ninhydrin-positive materials at this stage.

Figure 1 Diagram of deionization system.



A	From nitrogen pressure supply	**G**	To reserve deionization column
B	3 M hydrochloric acid	**H**	Purge to drain
C	Water	**I**	Teflon capillary connection tube
D	7 M ammonium hydroxide	**J**	Deionization column
E	1 ml syringe, Luer fitting with teflon slide-valve	**K**	Porous Teflon frits
F	Teflon 8-port valve	**L**	Sample vial



A From HCl cylinder and valve, via anti-suck-back Drechsel bottle
B 1 mm id glass capillary drawn to *c.* $\frac{1}{2}$ mm id
C Screw-cap
D 1 mm id glass capillary vent connected to moisture trap
E Teflon-faced silicone septum, pierced to accept two capillary tubes
F 300 µl heavy-wall reaction vial with conical bore

Figure 2 Reaction vial showing cap and septum for passing hydrogen chloride gas.

The amino acids were then eluted by the passage of approximately 20 ml of 7 Molar ammonium hydroxide at around ten drops per minute, followed by a water wash of 10 ml at 2 or 3 ml per minute. The effluent was collected and fed slowly into a 300 microlitre conical reaction vial placed in a heating block. Thereby, it was possible to concentrate the total eluant volume and final washings just to dryness under nitrogen. Aliquots of anhydrous benzene were added — up to a total volume of 4.0 ml — each time taken to dryness gently under the nitrogen stream. The residue was capped and set aside for derivatization.

Methylation

Approximately 0.2 ml of anhydrous redistilled methanol (MAR grade, stored under nitrogen and over molecular sieve 5A) was added to the amino acid residues above. The teflonized septum cap, fitted with side arms (Fig.2), was screwed onto the vial and a brisk stream of anhydrous hydrochloric acid gas was passed from a small cylinder (Matheson Gas Products, hydrogen chloride, technical, size LB). The contents of the vial tended to get hot and the methanol would reflux. At this stage the flow of gas was moderated to prevent excessive loss of the methanol and the HCl was passed for a further ten minutes, topping up with anhydrous methanol as necessary via a hypodermic syringe passed through the septum.

Finally the vial was capped with a teflon valve and

Table 1 Amino acid percentage peak areas

Amino Acid	Bologna (sample 2a)	Bellini ground	Bellini priming	Beccafumi ground	Laboratory aged sample of casein ground	glair/ chalk	egg yolk/ chalk	rabbit-skin glue/chalk
Alanine	2.3	10.9	7.5	14.4	3.9	10.2	5.1	10.0
Valine	19.0	1.5	4.8	1.3	4.4	4.6	6.9	2.4
Glycine	3.3	17.5	3.3	27.9	1.5	4.1	7.4	19.4
Isoleucine	4.0	0.6	2.6	0.4	2.3	3.1	5.2	1.1
Threonine	0.1	1.4	4.2	1.4	4.1	4.9	5.4	2.2
Leucine	10.6	3.9	13.0	4.7	16.4	15.5	11.8	4.0
Serine	8.1	2.6	7.9	3.6	5.8	9.8	7.0	2.9
Proline	14.4	20.6	6.3	8.3	21.0	7.7	5.4	19.6
Aspartic Acid	7.4	4.6	11.6	7.0	8.0	15.6	11.1	5.1
Hydroxyproline	0.2[1]	15.7	—	13.7	—	—	—	14.2
Methionine	—	0.0	0.2	0.2	0.4	0.1	1.1	0.3
Glutamic Acid	18.1	11.6	18.1	13.1	14.9	11.3	13.2	8.6
Phenylalanine	11.8	4.1	13.6	2.8	5.4	8.0	9.1	3.9
Lysine	0.4	4.2	6.1	0.7	11.2	4.4	10.7	5.6

1. This component may not be hydroxyproline.
(All ratios corrected for blank)

Table 2 Amino acid ratios for casein/chalk test sample (row entry/column entry)

	alan.	**val.**	**gly.**	**iso.**	**thre.**	**leu.**	**ser.**	**pro.**	**asp.**	**hy-pro.**	**met.**	**glu.**	**phe.**	**lys.**
Alanine	1.00	4.11	0.52	8.76	4.50	2.48	3.43	0.51	1.95	0.71	28.77	1.17	2.52	1.78
Valine	0.24	1.00	0.13	2.13	1.09	0.60	0.83	0.12	0.47	0.17	7.00	0.28	0.61	0.43
Glycine	1.93	7.92	1.00	16.88	8.67	4.78	6.60	0.99	3.75	1.36	55.46	2.26	4.86	3.43
Isoleucine	0.11	0.47	0.06	1.00	0.51	0.28	0.39	0.06	0.22	0.03	3.29	0.13	0.29	0.20
Threonine	0.22	0.91	0.12	1.95	1.00	0.55	0.76	0.11	0.43	0.16	6.40	0.26	0.56	0.40
Leucine	0.40	1.66	0.21	3.53	1.81	1.00	1.38	0.21	0.79	0.28	11.60	0.47	1.02	0.72
Serine	0.29	1.20	0.15	2.56	1.31	0.72	1.00	0.15	0.57	0.21	8.40	0.34	0.74	0.52
Proline	1.95	8.02	1.01	17.09	8.77	4.84	6.68	1.00	3.80	1.38	56.14	2.28	4.92	3.47
Aspartic acid	0.51	2.11	0.27	4.50	2.31	1.27	1.76	0.26	1.00	0.36	14.77	0.60	1.30	0.91
Hydroxyproline	1.42	5.32	0.74	0.74	12.41	3.51	4.85	0.73	2.76	1.00	40.77	1.66	3.58	2.52
Methionine	0.03	0.14	0.02	0.30	0.16	0.09	0.12	0.02	0.07	0.02	1.00	0.04	0.09	0.06
Glutamic acid	0.85	3.51	0.44	7.48	3.84	2.12	2.93	0.44	1.66	0.60	24.57	1.00	2.16	1.52
Phenylalanine	0.40	1.63	0.21	3.47	1.78	0.98	1.36	0.20	0.77	0.28	11.40	0.46	1.00	0.70
Lysine	0.56	2.31	0.29	4.92	2.53	1.39	1.93	0.29	1.09	0.40	16.17	0.66	1.42	1.00

Table 3 Amino acid ratios for rabbit-skin glue/chalk test sample (row entry/column entry)

	alan.	val.	gly.	iso.	thre.	leu.	ser.	pro.	asp.	hy-pro.	met.	glu.	phe.	lys.
Alanine	1.00	0.89	2.50	1.67	0.94	0.24	0.67	0.19	0.49	—	8.52	0.26	0.72	0.35
Valine	1.13	1.00	2.82	1.88	1.88	0.27	0.76	0.21	0.55	—	9.61	0.29	0.81	0.39
Glycine	0.40	0.36	1.00	0.67	0.38	0.10	0.27	0.07	0.20	—	3.41	0.10	0.29	0.14
Isoleucine	0.60	0.53	1.50	1.00	0.56	0.14	0.40	0.11	0.29	—	5.11	0.16	0.43	0.21
Threonine	1.07	0.95	2.66	1.78	1.00	0.25	0.72	0.20	0.52	—	0.09	0.28	0.77	0.37
Leucine	4.19	3.71	10.45	6.98	3.93	1.00	2.81	0.78	2.05	—	35.67	1.09	3.01	1.46
Serine	1.49	1.32	3.72	2.49	1.40	0.36	1.00	0.28	0.73	—	12.70	0.39	1.07	0.52
Proline	5.38	4.77	13.43	8.97	5.05	1.29	3.61	1.00	2.63	—	45.85	1.41	3.86	1.87
Aspartic acid	2.05	1.81	5.11	3.41	1.92	0.49	1.37	0.38	1.00	—	17.43	0.54	1.47	0.71
Hydroxyproline	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	—	0.00	0.00	0.00	0.00
Methionine	0.12	0.10	0.29	0.20	0.11	0.03	0.08	0.02	0.06	—	1.00	0.03	0.08	0.04
Glutamic acid	3.82	3.39	9.55	6.38	3.59	0.91	2.57	0.71	1.87	—	32.59	1.00	2.75	1.33
Phenylalanine	1.39	1.24	3.48	2.32	1.31	0.33	0.93	0.26	0.68	—	11.87	0.36	1.00	0.48
Lysine	2.87	2.55	7.15	4.80	2.70	0.69	1.93	0.53	1.41	—	24.50	0.75	2.06	1.00

Table 4 Amino acid ratios for glair/chalk test sample (row entry/column entry)

	alan.	val.	gly.	iso.	thre.	leu.	ser.	pro.	asp.	hy-pro.	met.	glu.	phe.	lys.
Alanine	1.00	2.22	2.46	3.32	2.06	0.66	1.04	1.32	0.66	—	79.15	0.91	1.28	2.29
Valine	0.45	1.00	1.11	1.50	0.93	0.30	0.47	0.60	0.30	—	35.69	0.41	0.58	1.03
Glycine	0.41	0.90	1.00	1.35	0.84	0.27	0.42	0.54	0.27	—	32.23	0.37	0.52	0.93
Isoleucine	0.30	0.67	0.74	1.00	0.62	0.20	0.31	0.40	0.20	—	23.85	0.27	0.39	0.69
Threonine	0.48	1.08	1.19	1.61	1.00	0.32	0.51	0.64	0.32	—	38.38	0.44	0.62	1.11
Leucine	1.52	3.36	3.72	5.03	3.12	1.00	1.58	2.01	1.00	—	119.92	1.38	1.94	3.47
Serine	0.96	2.12	2.35	3.18	1.98	0.63	1.00	1.27	0.63	—	75.85	0.87	1.23	2.20
Proline	0.76	1.67	1.85	2.51	1.56	0.50	0.79	1.00	0.50	—	59.77	0.69	0.97	1.73
Aspartic acid	1.52	3.36	3.73	5.04	3.13	1.00	1.58	2.01	1.00	—	120.08	1.38	1.95	3.48
Hydroxyproline	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	—	0.00	0.00	0.00	0.00
Methionine	0.01	0.03	0.03	0.04	0.03	0.01	0.01	0.02	0.01	—	1.00	0.01	0.02	0.03
Glutamic acid	1.10	2.44	2.70	3.65	2.27	0.73	1.15	1.46	0.73	—	87.08	1.00	1.41	2.52
Phenylalanine	0.78	1.73	1.91	2.59	1.61	0.51	0.81	1.03	0.51	—	61.69	0.71	1.00	1.79
Lysine	0.44	0.97	1.07	1.45	0.90	0.29	0.46	0.58	0.29	—	34.54	0.40	0.56	1.00

Table 5 Amino acid ratios for egg yolk/chalk test sample (row entry/column entry)

	alan.	val.	gly.	iso.	thre.	leu.	ser.	pro.	asp.	hy-pro.	met.	glu.	phe.	lys.
Alanine	1.00	0.74	0.69	0.99	0.95	0.44	0.74	0.95	0.46	—	4.54	0.39	0.56	0.48
Valine	1.35	1.00	0.93	1.34	1.28	0.59	0.99	1.28	0.63	—	6.11	0.53	0.76	0.65
Glycine	1.45	1.07	1.00	1.44	1.38	0.63	1.07	1.38	0.67	—	6.57	0.57	0.82	0.70
Isoleucine	1.01	0.75	0.70	1.00	0.96	0.44	0.74	0.96	0.47	—	4.57	0.39	0.57	0.49
Threonine	1.05	0.78	0.72	1.04	1.00	0.46	0.77	1.00	0.49	—	4.76	0.41	0.59	0.51
Leucine	2.30	1.70	1.59	2.28	2.19	1.00	1.69	2.19	1.07	—	10.42	0.90	1.30	1.11
Serine	1.36	1.01	0.94	1.35	1.29	0.59	1.00	1.29	0.63	—	6.15	0.53	0.77	0.65
Proline	1.05	0.78	0.72	1.04	1.00	0.46	0.77	1.00	0.49	—	4.76	0.41	0.59	0.51
Aspartic acid	2.15	1.60	1.49	2.14	2.05	0.94	1.59	2.05	1.00	—	9.76	0.84	1.22	1.04
Hydroxyproline	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	—	0.00	0.00	0.00	0.00
Methionine	0.22	0.16	0.15	0.22	0.21	0.10	0.16	0.21	0.10	—	1.00	0.09	0.12	0.11
Glutamic acid	2.56	1.90	1.77	2.54	2.44	1.11	1.89	2.44	1.19	—	11.61	1.00	1.44	1.23
Phenylalanine	1.77	1.31	1.22	1.76	1.69	0.77	1.31	1.69	0.82	—	8.04	0.69	1.00	0.85
Lysine	2.08	1.54	1.43	2.06	1.98	0.90	1.53	1.98	0.96	—	9.42	0.81	1.17	1.00

back-up septum ('Mininert Valve', Applied Science Laboratories Inc.) and the sealed mixture was warmed for thirty minutes. The cap containing inlet and outlet tubes was refitted, the vial was warmed and dry nitrogen passed until the sample volume was reduced to about 50µl. This was then transferred by syringe, with washings, to a conical 100µl microvial ('Minivial' 0.1 ml capacity, Applied Science Laboratories Inc.) and taken to dryness, followed by the addition of 50µl of anhydrous dichloromethane. Having evaporated just to dryness under a nitrogen stream, the vessel was capped and sealed.

It was found advisable, at this stage to check that all ninhydrin-positive material had been removed from the ion-exchange column, prior to regeneration of the resin.

Acylation

Redistilled trifluoracetic anhydride (30µl) was added to the reaction vial, through the septum, by means of a 100µl hypodermic syringe fitted with isolation valve. 10µl of anhydrous dichloromethane was added in a similar way. The mixture was capped with a fresh septum lined with a thick coating of teflon and warmed at about 70°C for four hours.

The mixture was concentrated with great care, by a gentle stream of nitrogen at no more than room temperature and with swilling of the sides of the vessel to bring materials deposited on the sides back into the bulk of the solution. The N-acetyl, methyl esters of the amino acids are very volatile and the mixture must not be taken to dryness under any circumstance otherwise marked loss of some components will be inevitable.

Gas-chromatography

One microlitre aliquots of the trifluoracyl, methyl esters were distributed on a 9 ft $\times \frac{1}{4}$ inch column packed with 1% XE60 cyanosilicone gum on 100/120 mesh Diatomite CQ support, coated from acetone. The chromatographic conditions were as follows:

Injection temperature: 200°C
Detector temperature: 250°C
Carrier flow-rate: 45 ml per minute
Carrier gas: argon
Column temperature: 80°C × 3°C/min. to 210°C

The column was treated with 5 × 1µl aliquots of pure trifluoracetic anhydride prior to sample injection and cycled to 215°C, briefly.

Results and discussion

It has to be admitted that the method presented above has many pitfalls and is far from ideal. Notwithstanding this, it does enable the museum analyst to undertake, in favourable cases, a more thorough characterization of proteinaceous binders from art objects.

In general a sample size of the order of two or three times that required for fatty acid analysis must be taken, although this can vary according to the leanness of the medium present. The larger sample size is necessary to offset losses at each stage of the clean-up and derivatization procedure.

Major losses can occur during the acid hydrolysis stage should moderate amounts of sugars and carbohydrates be present, in addition to the protein and minerals, by the elimination of amino acids as humins. The latter side-products cause darkening of the hydrolysate and insolubles may be formed. It is claimed that humin formation may be averted by undertaking hydrolysis in 80% aqueous ethanol in the presence of Amberlite IR-112(H) ion-exchange resin for six to ten hours at 95°C in a sealed tube under nitrogen. Presumably the amino acids are removed from solution and immobilized on the resin via the nitrogen function, thereby preventing the formation of Schiff's base condensation products between amino groups and keto/aldehyde groups of the sugars. The amino acids were subsequently eluted from the Amberlite resin by 10% ammonium hydroxide [26].

Incomplete derivatization can be occasioned by the presence of traces of metal ions, particularly iron salts and calcium salts (chloride). Great care should be taken with the deionization stage. Calcium chloride probably affects the efficiency of derivatization by occluding traces of moisture.

Whenever derivatization with trifluoracetic anhydride is taking place and in the final work-up of the mixture prior to injection on the gas-chromatograph, transfers and sample manipulation should be kept to a minimum, preferably in a sealed glove-box.

Table 1 illustrates the relative peak areas of the fourteen most common amino acids found in living systems for test samples and a selection of museum objects. The figures represent the percentage of the summed peak area for the amino acids listed, from alanine to lysine, assayed as their trifluoracetyl, methyl esters (TFA, methyl esters).

The right-hand section of the table is devoted to samples removed from test films of casein, glair, egg yolk and rabbit-skin glue, which had been mixed with chalk and aged under ambient laboratory conditions for ten years. The left-hand section of the table is concerned with samples of paint and primings taken from easel paintings and polychrome architecture.

Some general observations on the test sample amino acid profiles can be made from a preliminary examination of Table 1.

1. Animal collagen (rabbit-skin glue) was the only source with significant amounts of the amino acid hydroxyproline (*c.*14%). In addition approximately 20% proline and 20% glycine were found. However, leucine (*c.*4%) appeared at a level of about one-fifth of that for proline (Table 3).
2. Casein contained no measurable hydroxyproline. Approximately 20% of proline is present, whilst leucine was about 4 to 5-fold of that amount and glutamic acid was at a level of some 3 to 5 times the peak area of proline (Table 2).
3. Egg white (glair) had an entry which was dominated by leucine and aspartic acid, both amounting to twice as much as proline (7.77%). It might seem possible that there could be confusion with casein but, clearly, the latter had a markedly larger proline content (Table 4).
4. In general, egg yolk seemed to have a more even distribution with respect to its leucine/aspartic acid levels. The leucine peak had approximately twice the area of the chromatographic peak due to proline. It

appeared that there was slightly less leucine and aspartic acid present as a percentage of the summed areas in egg yolk than in glair (Table 5).

Procedure for classifying protein type

Hydroxyproline should be searched for and if present in the range of ten to seventeen per cent one can conclude that the sample contained either animal or fish glue. One should also check that there are similar amounts of proline and glycine present. Nevertheless if hydroxyproline was evident, but at a level nearer the lower limit of that given above, the investigator should monitor the proline level carefully. Should it be in the region of six to ten per cent — that is, some overall deficiency in imino acids, it may well be that the sample contained fish collagen, as was discussed in the introductory section above.

Only one sample of isinglass has been examined in this laboratory, but it was found to have a slight deficiency in imino acids (26.49% total proline + hydroxyproline, of which the latter came to only 11.4%). This can be compared with about 34% (proline + hydroxyproline) of which hydroxyproline accounted for 14.27%.

Should hydroxyproline be absent, the analyst may be confident that the binder is limited to casein, egg white, egg yolk or whole egg.

Having made the broad classification outlined above there are several useful indicator ratios of amino acids which may point to an egg or casein source. These include: leucine/alanine; proline/alanine; proline/serine; proline/leucine; glumatic/aspartic acid.

Once the use of an egg tempera has been established, some idea as to which part of the egg has been used can be gained from such ratios as: proline/isoleucine; alanine/isoleucine; alanine/glycine.

Tables 2 to 5 show the amino acid ratios for the four main test samples (see Figs.3–6), whilst Tables 6 and 7 show useful criteria for assessing an egg or casein binder and, in the case of egg, yolk or glair.

From the tables it becomes clear that for casein, the glutamic acid/aspartic acid ratio is of the order 2:1, as opposed to a somewhat lower ratio for the two types of egg medium (*c*.1:1). In addition, the proline/serine ratio is greater than unity for casein, but below unity for tempera, whilst that for proline/leucine is greater than unity for the former and of the order 0.5 for the latter. A substantial difference can be noted in the proline/aspartic peak area ratio, which can vary from 2 or above for casein, but only 0.5 for egg.

Moreover, three other ratios have a powerful diagnostic function: leucine/alanine of the order of 4 to 5 for casein, but from 1.5 (the case of glair) to 2.5 (egg yolk); the proline/alanine ratio is about 1.0 for egg, but is five or six times larger for casein.

If the conclusion drawn is that some form of egg has been used, it may be possible to be more specific. The general lipid levels will give some indication of yolk or white of egg, but an examination of alanine/valine, alanine/glycine, alanine/isoleucine ratios may well make the precise origin clear.

The four examples of paint/ground samples chosen and listed below were selected to illustrate that despite modest variations between their amino acid ratios and those of the test samples, the analyst is still able to positively assign them to a particular protein category.

Table 6 Useful criteria for egg and casein binders

Amino Acids	Casein	Egg White	Egg Yolk
glu/asp	1.87	0.73	1.19
pro/ser	3.61	0.79	0.77
pro/leu	1.29	0.50	0.46
pro/asp	2.63	0.50	0.49
leu/alan	4.19	1.52	2.30
leu/asp	2.05	1.00	1.07
ser/iso	2.49	3.18	1.35
pro/alan	5.38	0.76	1.05

Table 7 Useful criteria for egg white/egg yolk

Amino Acids	Egg White	Egg Yolk
alan/val	2.22	0.74
alan/gly	2.46	0.69
alan/iso	3.32	0.99
pro/iso	2.51	1.04

(A) Sample of gesso from Giovanni Bellini, *The Madonna of the Meadow* (No.599).

The chromatogram is shown in Fig.7. This sample contains a substantial amount of hydroxyproline (15.76%) and clearly comes under the heading of collagen. This interpretation is supported by a major glycine (17.54%) and proline (20.69%) content. There can be little hesitation in assigning the binder used in this ground layer to the animal or fish category, the former being the more likely since the percentage of the hydrolysate assayed as imino acid is quite normal for an animal source.

(B) Ground layer from Domenico Beccafumi, *Tanaquil* (No.6368).

The chromatogram of the amino acids as their trifluoracetyl, methyl esters is depicted in Fig.8. As in example (A), above, hydroxyproline is evident (13.70%) together with a major peak for glycine (27.97%) and a pronounced proline component (8.36%). Nevertheless, the total imino acid percentage sums to about 22%, which is decidedly lower than the normal 34% fairly typical of most animal glues so far examined. It is possible that this sample may represent the use of a fish glue as binder rather than animal glue, but there appear to be no references to its use for such purposes amongst old manuscripts other than that of De Mayerne [25].

(C) Upper red layer from top of column (sample 2a), Facade of San Petronio, Bologna.

Fig.10 shows the chromatogram obtained for this sample. Here we see a somewhat different pattern to that of the two samples (A) and (B). Only a trace of hydroxyproline was found and possibly represented an insignificant

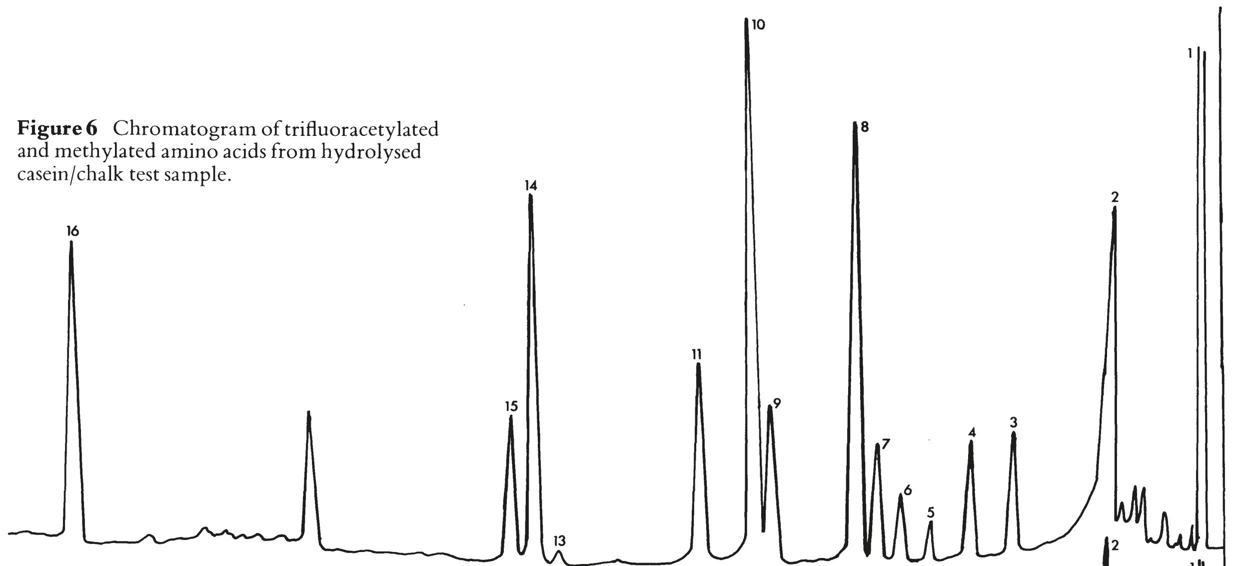


Figure 6 Chromatogram of trifluoroacetylated and methylated amino acids from hydrolysed casein/chalk test sample.

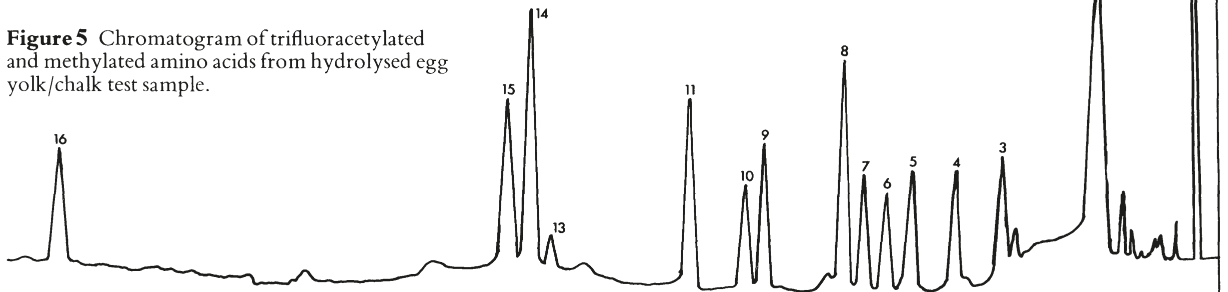


Figure 5 Chromatogram of trifluoroacetylated and methylated amino acids from hydrolysed egg yolk/chalk test sample.

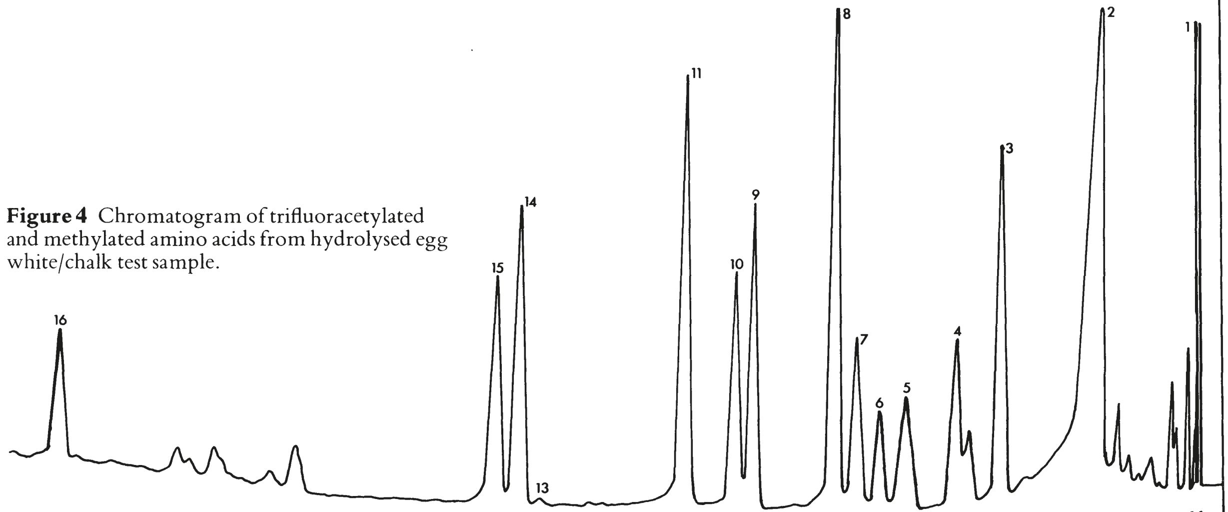


Figure 4 Chromatogram of trifluoroacetylated and methylated amino acids from hydrolysed egg white/chalk test sample.

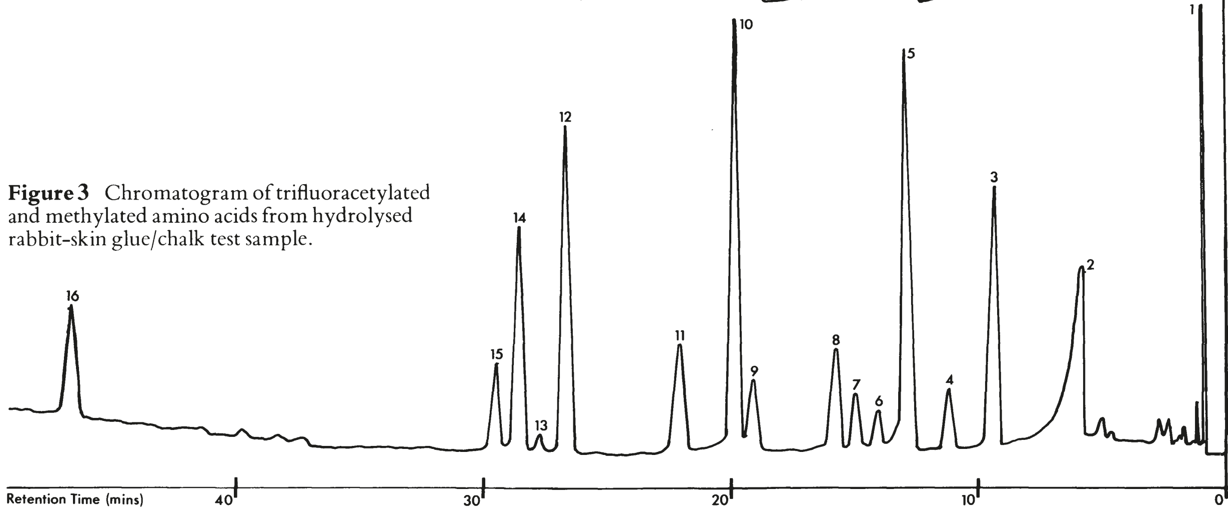


Figure 3 Chromatogram of trifluoroacetylated and methylated amino acids from hydrolysed rabbit-skin glue/chalk test sample.

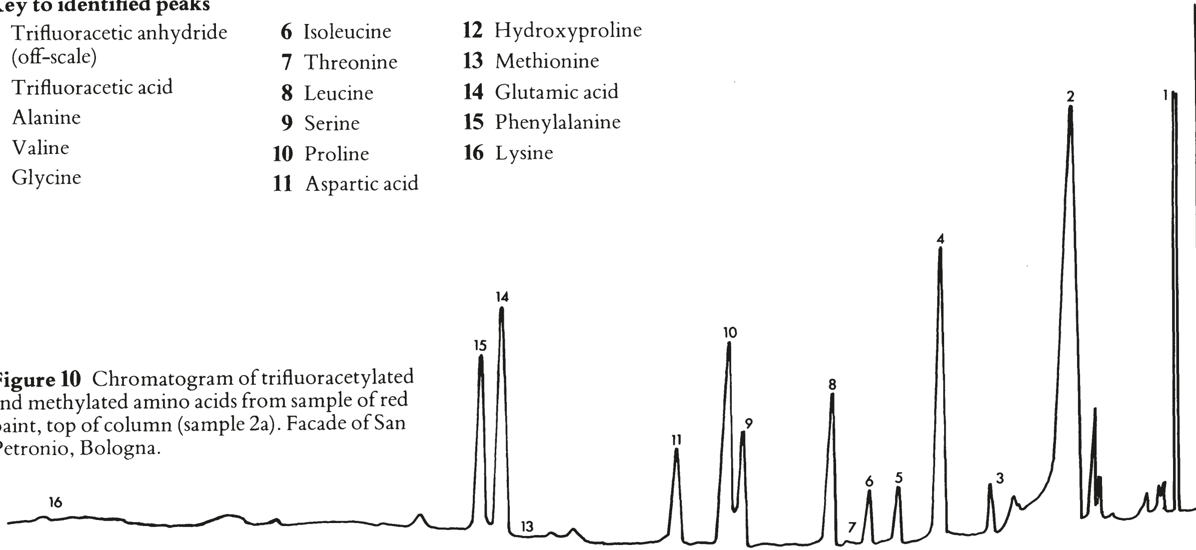


Figure 10 Chromatogram of trifluoracetylated and methylated amino acids from sample of red paint, top of column (sample 2a). Facade of San Petronio, Bologna.

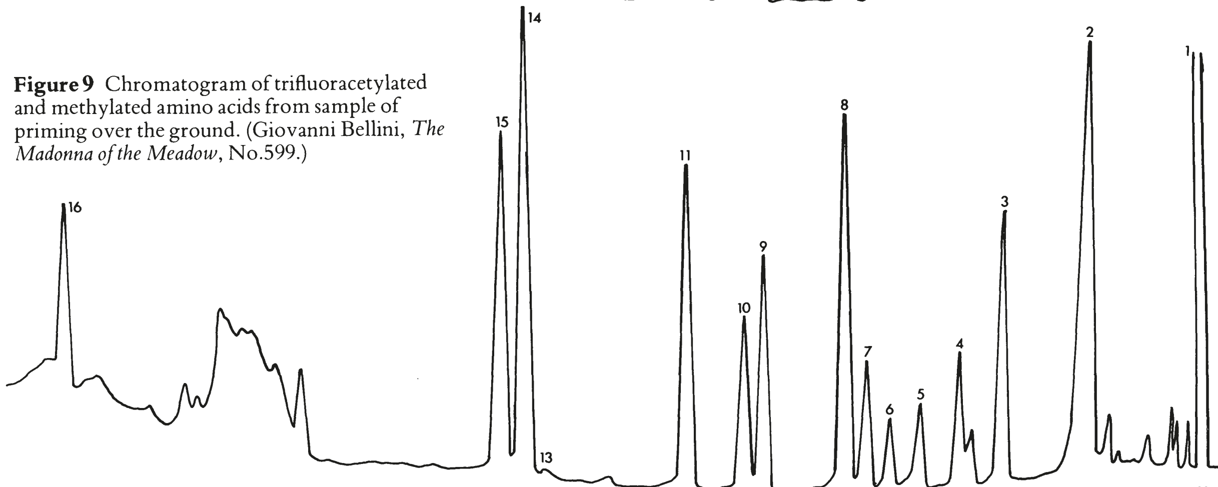


Figure 9 Chromatogram of trifluoracetylated and methylated amino acids from sample of priming over the ground. (Giovanni Bellini, *The Madonna of the Meadow*, No.599.)

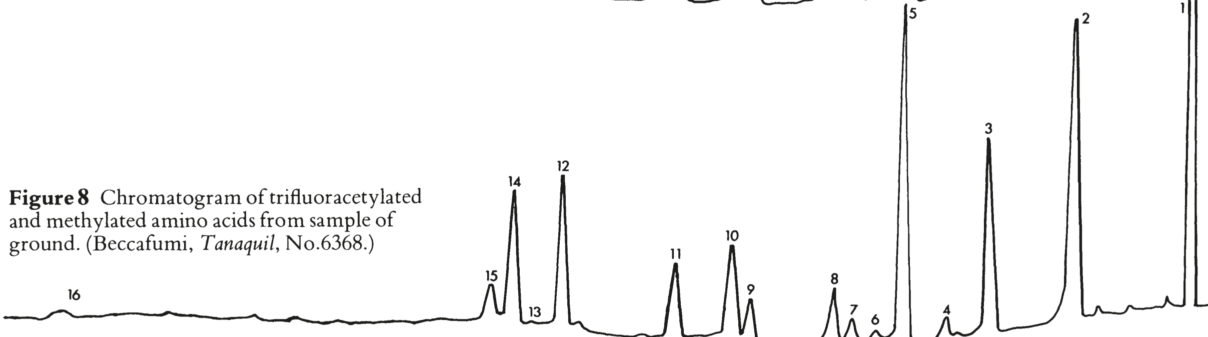


Figure 8 Chromatogram of trifluoracetylated and methylated amino acids from sample of ground. (Beccafumi, *Tanaquil*, No.6368.)

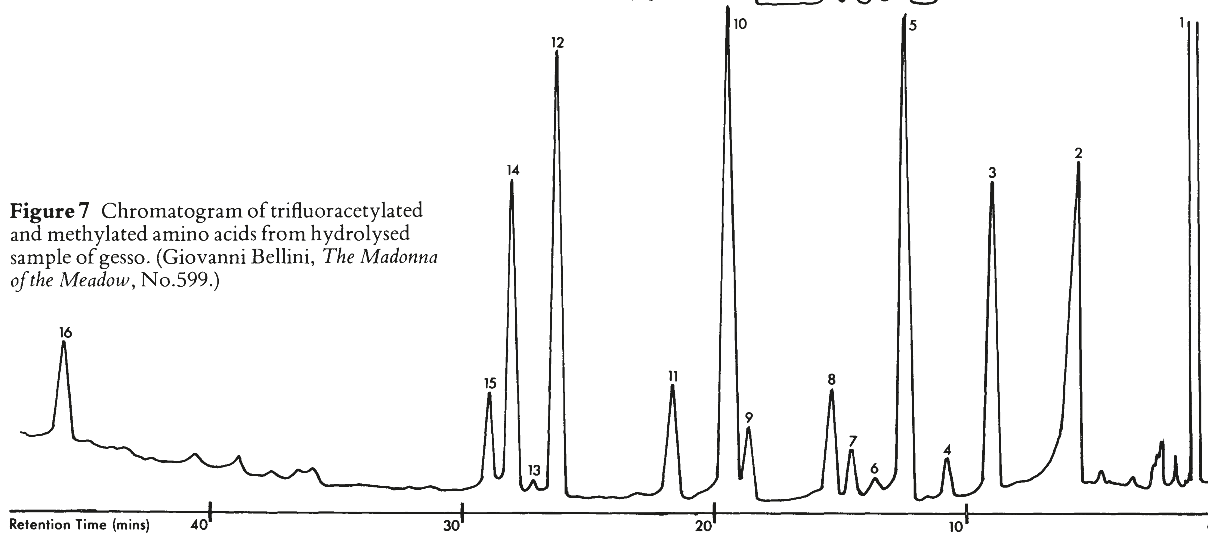


Figure 7 Chromatogram of trifluoracetylated and methylated amino acids from hydrolysed sample of gesso. (Giovanni Bellini, *The Madonna of the Meadow*, No.599.)

background contaminant with a retention time similar to that for hydroxyproline. However this may be, one must conclude that the proteinaceous component is not from an animal or fish collagen. Certainly the high leucine/alanine ratio of 4.59 is much too high for typical egg values (generally 1.5 to 2.5) and so would point to casein. This is supported by a proline/aspartic acid value of 1.95 (2–2.5 for casein, but *c*.0.5 for egg) and a proline/alanine ratio of 6.24 compared with 0.5 to 1.5 for egg.

(D) Unpigmented priming over ground from Giovanni Bellini, *Madonna of the Meadow* (No.599).

The hydrolysate from this sample gave no indication of animal/fish glue, since hydroxyproline was not detected in any significant quantity. Inspection of Table 1 or of the chromatogram (Fig.9) reveals that leucine, aspartic and glumatic acids account for a major part of the liberated amino acid residues. Clearly the results represent casein or some form of egg vehicle.

Whereas in the case of casein, one would anticipate a proline/aspartic ratio of about 2 to 3, a proline/alanine ratio of about 5 to 6 and leucine/alanine ratio of about 4 to 5, in fact ratios of 0.54, 0.84 and 1.73 respectively were actually found. Thus from Table 6 there can be little doubt that the layer is just an egg medium. Furthermore, with ratios of 1.55 for alanine/valine, 2.30 for alanine/glycine, 2.85 for alanine/isoleucine and 2.39 for proline/isoleucine, comparison with Table 7 would suggest that the material was essentially glair, that is egg white, rather than whole egg or egg yolk.

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