Characterisation of Standard Tempera Painting Layers Containing Proteinaceous Binders by Pyrolysis (/Methylation)-Gas Chromatography-Mass Spectrometry

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Key Words

Pyrolysis-gas chromatography-mass spectrometry
Pyrolysis/methylation
Painting layer
Proteinaceous binder

Summary

Thirty standard painting layers were analysed by pyrolysis-gas chromatography/mass spectrometry (Py-GC-MS) and by Py-GC-MS in the presence of tetramethylammonium hydroxide (pyrolysis/methylation). Painting layers were prepared according to Renaissance recipes for tempera, employing proteinaceous binders (egg, glue and casein) and six different pigments. Thermal degradation products of proteins, carbohydrates and lipids were selected for semiquantitative analysis based on single/summed ion monitoring (SIM) mode. The relative distribution of these products was used to characterise binding media for the purpose of their identification in painting layers.

Introduction

Deterioration of artistic materials caused by environmental agents is one of the main problems concerning the preservation of cultural heritage. The optimum strategy for the examination/restoration of an artistic work could require a detailed knowledge of the composition of the materials employed by the artist. Among these, binders are particularly difficult to characterise being complex mixtures of organic substances, subjected to various biological and chemical degradation paths capable to modify the original composition [1].

Binders are important constituents of painting layers. In the Renaissance period painting layers were often based on tempera. Tempera is a fluid mixture of binder (or ligand, the organic medium), water and volatile additives. Pigments are dispersed into the tempera to form the tint, which gives rise to the paint after drying. The residual dried solid is composed of pigments and binders. Organic ligands used by Italian artists in the Renaissance period were primarily proteinaceous materials available from animal sources, such as eggs, animal glues and milk.

Characterisation of proteinaceous binders by means of chromatographic techniques has been recently reviewed [2]. The identification is commonly carried out by determining the amino acid composition of the binding media using gas chromatography (GC) [3, 4] or high-performance liquid chromatography (HPLC) [5,6] of pretreated samples. The principal disadvantages of these methods are related to the need of time-consuming chemical steps (hydrolysis, extraction, derivatization) which can cause loss of amino acids. Analytical pyrolysis does not require chemical treatment and minute amounts of sample are enough for analysis, thus this technique has been applied to the field of art and archaeology for the analysis of complex organic materials [7]. Recent studies have demonstrated the potentiality of pyrolysis in conjunction with gas chromatography and mass spectrometry (Py-GC-MS) for characterising proteinaceous binding media [8,9]. Actually, Py-GC-MS has been used to identify binders in painting layers of ancient Egypt [10].

The range of applicability of analytical pyrolysis has increased with the introduction of pyrolysis/methylation, that is pyrolysis in the presence of tetramethylammonium hydroxide (TMAH) [11]. Pyrolysis/methylation improves the detection of fatty acids with respect to conventional pyrolysis and for this reason has been applied to recognise binders with high lipid content, such as yolk and siccative oils [8, 12].

Analytical procedures for identifying binders have been often optimised employing pure organic materials, but pigments and grounds are known to affect the precision of the analyses [2]. Since reference materials are not commercially available, matrix effects can be taken into account using painting layers prepared in reputed laboratories. The Opificio delle Pietre Dure in Florence (It-

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Table I. Preparation of temperas and binder content (% dry weight).

Binder	Tempera	%
Animal glue	20 g rabbit skin glue "Lefranc" brand in 200 mL hot distilled water	9.1
Milk (casein)	Fresh pasteurised skimmed milk	8.1
Whole egg	1 vol. Glair (whipped, separated and naturally liquefied again after 24 hours); 1 vol. egg yolk 1 vol. white vinegar; 3 vol. Distilled water	11.9
Glue/milk	Mixture 1/1 v/v glue tempera and milk tempera	8.6
Glue/egg	Mixture 1/1 v/v glue tempera and egg tempera	10.5

aly) has prepared standard painting layers following Renaissance recipes which have been supplied to several research groups in Italy, co-ordinated in a research program aimed at evaluating different analytical procedures for the identification of binders This study reports on the application of both Py-GC-MS and pyrolysis/methylation-GC-MS in conjunction with the above mentioned standard painting layers to develop a semiquantitative method to characterise proteinaceous binding media occurring in Renaissance paints.

Experimental

Standard materials

Painting layers were prepared by the Opificio delle Pietre Dure upon solid supports made of rectangular briquettes (21 × 15 mm) formed with a double plaster layer. A mixture of gypsum, lime and sand (2:1:1) was used for the first layer forming the rigid support (15–20 mm thickness). The second layer (3–6 mm thickness) was made of lime and thin sand (1:1.5) in accordance with the traditional composition of the "intonachino" used for "fresco" painting. Briquettes were air-dried for 15 days at room temperature, then tints were applied onto the surfaces. Tints were spread five times to obtain a consistent paint material. Thirty different tints were prepared employing five binders and six pigments. The main components of the tints are listed in Table I (binders and aqueous tempera). The following pigments were employed: red ochre, yellow ochre, vine black, lime white ('S. Giovanni's white'), green earth and smalt. All pigments were furnished by Zecchi, Florence (Italy) with the exception of green earth (Kremer N. 4184).

Analyses

Pyrolysis experiments were carried out with a CDS 1000 Pyroprobe platinum heated filament pyrolyzer interfaced to a Varian 3400 gas chromatograph and a Varian Saturn III ion trap mass spectrometer. Samples (about 0.5 mg) were detached from the rigid support, put into a quartz tube and pyrolyzed (triplicate runs) at a set temperature of 700 °C for 10 s. Pyrolysis products were separated on a SPB-5 capillary column (Supelco, 30 m × 0.32 mm i.d., film thickness: 0.25 μm) using a linear temperature program from 50 °C (13 minutes) to 290 °C (10 minutes) at 5 °C min^{-1}. The Py-GC interface was set at 250 °C and the injector at 290 °C. Mass spectra (1 scan s^{-1}) were recorded under electron impact (70 eV) from 40 to 450 m/z.

Pyrolysis/methylation experiments were carried out by adding 5 μL of TMAH aqueous solution (25 %) into the quartz holder containing the sample and pyrolysing as previously described.

Pyrrole, diketodipyrrole, toluene, furanmethanol, maltol (2-methyl-3-hydroxy 4(H)-pyran-4-one), indole, cholesterol, mono and dicarboxylic fatty acid methyl esters were used as standard compounds for the identification of pyrolysis products and were purchased from Aldrich with the exception of diketodipyrrole which was synthesised according to a published procedure [13]. In the absence of standard compounds the identification of pyrolysis products was based on comparison of their mass spectra with those of literature data or on mass spectral interpretations.

Results

Py-GC-MS

Typical pyrograms of three different painting layers containing glue, casein and egg are shown in Figures 1a, 2a and 3a, respectively. Among the various pyrolysis products, a set of twelve compounds was selected for semiquantitation according to their importance as markers of binder constituents. These products are listed in Table II together with their retention times and the ions used for peak integration in the single/summed ion monitoring (SIM) mode. Examples of SIM mode pyrograms of some target ions are shown in Figures 1b, 2b and 3b. Relative peak area, calculated as percentages relative to the total area of selected markers, are presented in Table III together with estimated standard deviations. Standard deviations represent scatter of data resulting: 1) from repeated pyrolysis experiments on the same painting layer ($n = 3$) and 2) between painting lay-

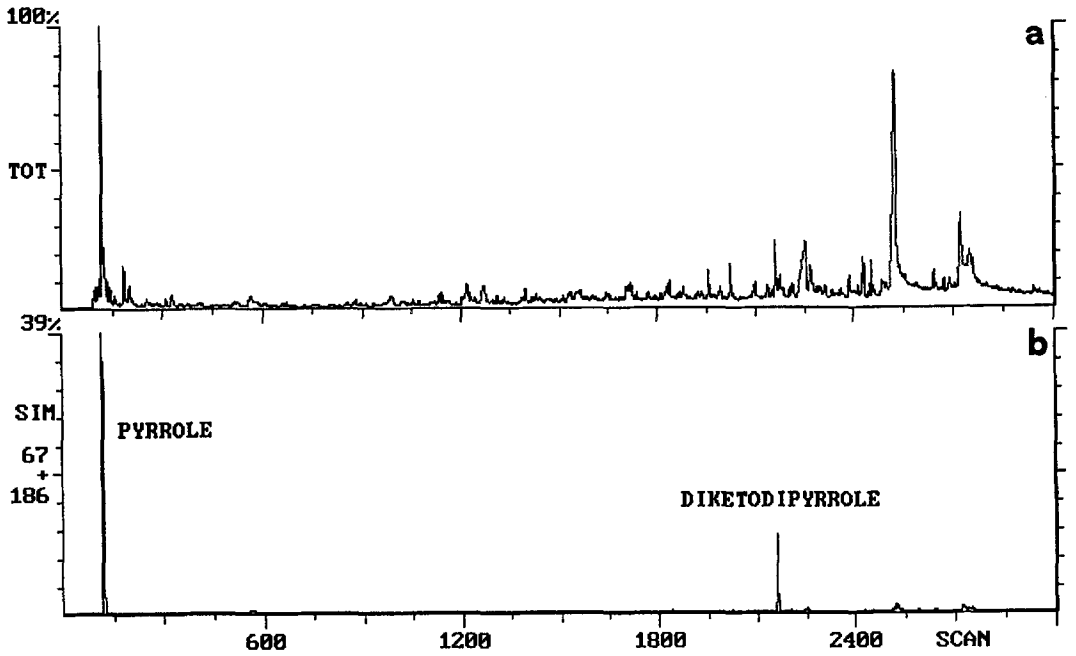

Figure 1
Py-GC-MS of glue-containing painting layer (pigment: yellow ochre): a) total ion chromatogram, b) the use of SIM to identify pyrrole (m/z 67) and diketodipyrrole (m/z 186).

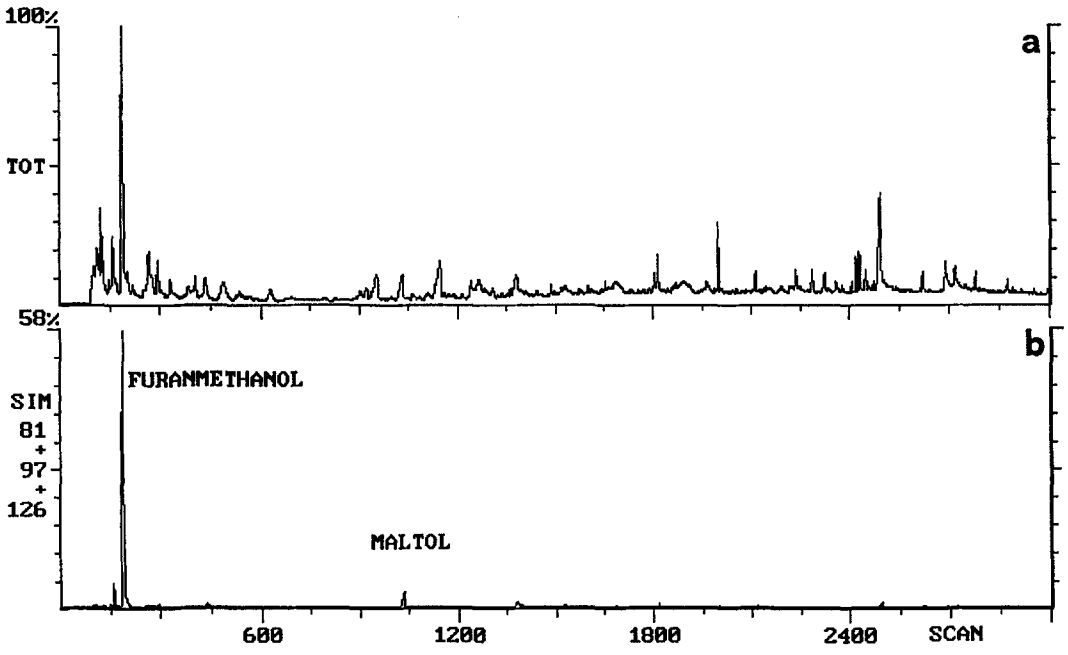

Figure 2
Py-GC-MS of casein-containing painting layers (pigment: vine black): a) total ion chromatogram, b) the use of SIM to identify furanmethanol (m/z 81 + 97) and maltol (m/z 126).

ers containing the same binder but different pigments ($n = 6$).

Pyrrole, diketodipyrrole, toluene, indole and 3-methylindole (skatole) were selected as markers of proteins. Pyrrole and diketodipyrrole are thermal degradation products of hydroxyproline [14]. Table III shows that summed abundances of these two compounds represent more than 50 % of total area for glue-containing layers, and less than 10 % in the absence of glue. Toluene and indoles (indole and skatole) are pyrolysis products of phenylalanine and tryptophane residues of proteins, respectively [15] Toluene and indoles were present in the highest levels in the pyrograms of egg-based painting layers and in similar percentages in pyrograms of egg/glue and casein/glue mixtures.

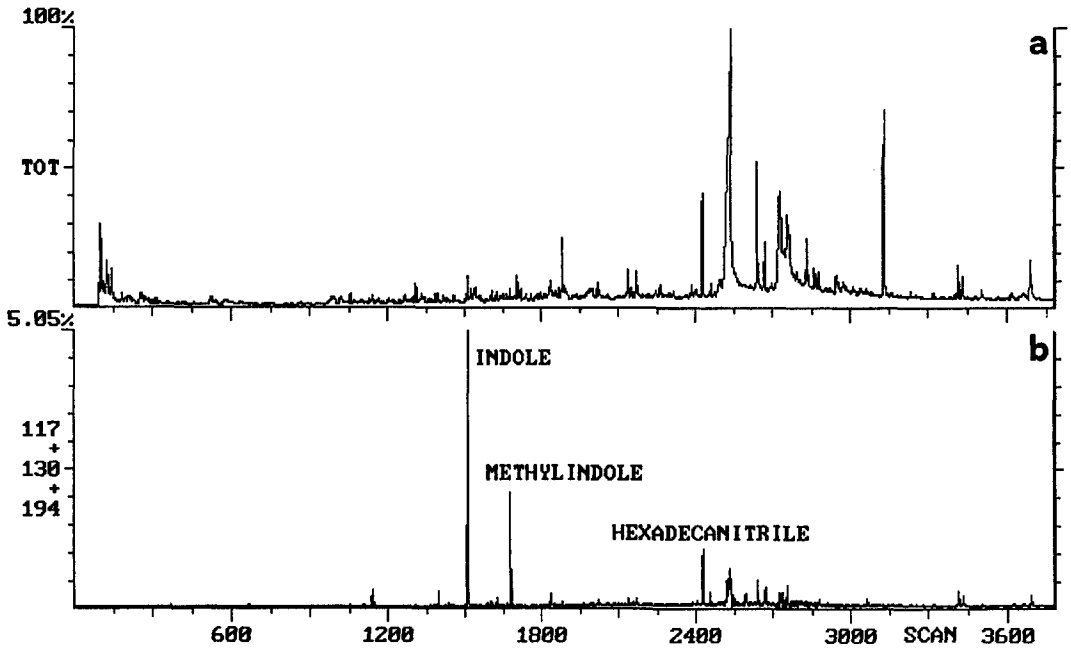

Figure 3
Py-GC-MS of egg-containing painting layers (pigment: vine black): a) total ion chromatogram, b) the use of SIM to identify indole (m/z 117), skatole (m/z 130) and hexadecanitrile (m/z 194).

Table II. Py-GC-MS of standard painting layers. Pyrolysis products selected for quantitation, retention times in secs (scan) and m/z of ions chosen for quantitation (QM).

Compound	Scan	QM	Source
Pyrrole	114	67	proteins
Toluene	126	91	proteins
Furanmethanol	196	81 + 97	carbohydrates
Maltol	1054	126	carbohydrates
Indole	1511	117	proteins
3-Methylindole	1680	130	proteins
Diketodipyrrole	2155	186	proteins
Hexadecanitrile	2428	180 + 194 + 208	lipids
Cholestatriene	3415	366	lipids
Cholestadiene	3433	368	lipids
Cholesterol	3684	386	lipids
Cholestadienone	3686	382	lipids

Furanmethanol and maltol were selected as markers indicative of carbohydrates since they were found in Py-GC-MS data of the disaccharide maltose (data not shown). Maltol is also formed upon pyrolysis of amylose and is considered a typical product in Maillard reactions [16]. The relative contents of furanmethanol and maltol were more abundant in painting layers containing casein than in those containing egg or glue. Looking at Table III it is apparent that the relative peak area of furanmethanol in the casein/glue mixtures was lower than that expected considering the separate contribute of each binder (that is 8.1 % instead of 35 %). This finding suggests that the pyrolitic behaviour of binders is affected by the composition of the organic medium.

Hexadecanitrile and steroids are pyrolysis products associated to the lipid fraction of binding media. Several long chain alkylnitriles were detected in the pyrograms of painting layers, but only hexadecanitrile, the most abundant of them, was included in quantitation. Alkylnitriles are probably the products of secondary reactions between fatty acids and amines [17]. Amines are released through decarboxylation of aromatic amino acids under pyrolysis condition [15], while fatty acids were actually important components of painting layer pyrolysates. All the steroids listed in Table II were derived from flash vaporisation and thermal decomposition of free or bound cholesterol as confirmed by pyrolysing pure cholesterol. Data of Table III show that the highest levels of lipid markers were observed in egg-based painting layers.

Pyrolysis/methylation

Pyrolysis of painting layers in the presence of tetramethylammonium hydroxide (TMAH) produced a series of methylated carboxylic acids, hydroxybenzenes and aromatic amines. A typical pyrogram obtained from pyrolysis/methylation of painting layers is depicted in Figure 4. Products selected for semiquantitation are listed in Table IV. Relative peak area, determined in the SIM mode and summed up for compound classes, are reported in Table V. Comparing the values of standard deviations of Table V with those of Table III it is apparent that pyrolysis/methylation was less precise than conventional Py-GC-MS. This could be partly explained by additional sources of errors associated with the use of the methylating agent and by the fact that fatty acids can be

Table III. Py-GC-MS of standard painting layers. Average relative peak area and relative standard deviations of pyrolysis products listed in Table II. Means were calculated from 18 pyrograms corresponding to triplicate analyses of six different coloured painting layers containing the same binding media.

	Glue	Casein	Egg	Egg/Glue	Casein/Glue
Pyrrole	65 ± 6	8.2 ± 1	6.8 ± 1	41 ± 4	55 ± 4
Diketodipyrrole	16 ± 3	0.6 ± 0.4	0.4 ± 0.1	8.1 ± 3	8.6 ± 3
Toluene	9.1 ± 2	9.8 ± 4	26 ± 5	15 ± 4	15 ± 2
Furanmethanol	4.5 ± 2	67 ± 7	0.9 ± 0.5	I.2 ± 0.7	8.1 ± 3
Maltol	0.7 ± 0.3	3.5 ± 2	0.4 ± 0.2	0.3 ± 0.1	3.4 ± 1
Indoles	1.4 ± 0.3	9.1 ± 1.6	22 ± 1	11 ± 1	8.4 ± 1.6
Hexadecanitrile	1.0 ± 0.6	traces	9.1 ± 3	4.4 ± 1.3	0.5 ± 0.2
Steroids	2.9 ± 0.4	traces	34 ± 9	20 ± 4	1.9 ± 0.2

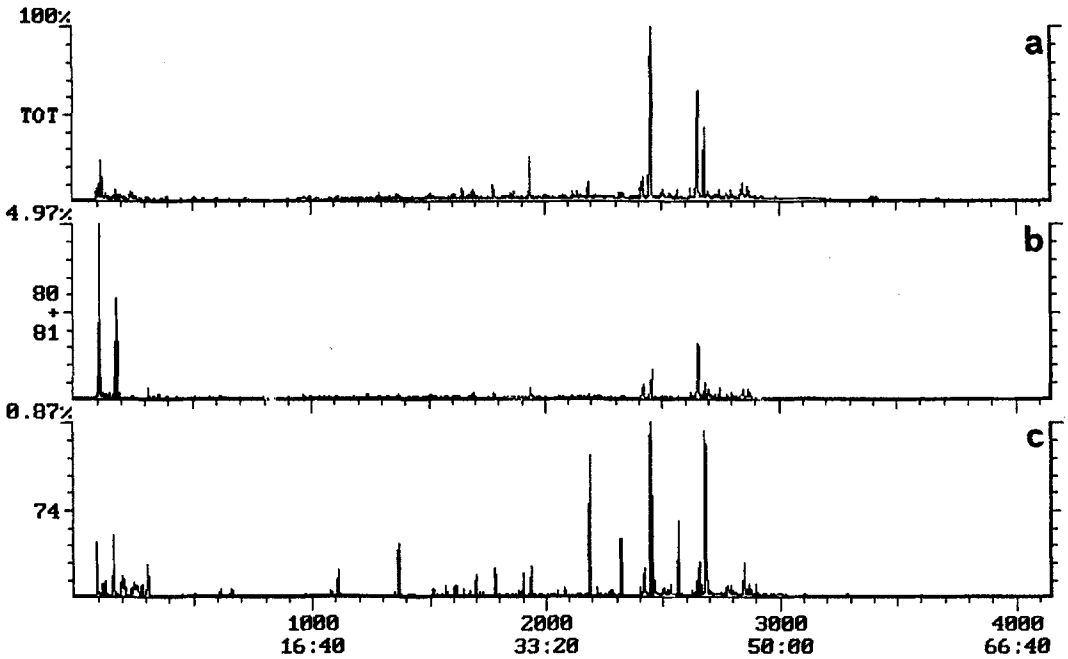

Figure 4
Pyrolysis/methylation of painting layer containing a mixture of egg and glue (pigment: yellow ochre): a) total ion chromatogram, b) the use of SIM to identify methyl pyrroles (m/z 80 + 81) and c) fatty acid methyl esters (m/z 74).

adsorbed onto the Py-GC interface causing memory effects [7].

Fatty acid methyl esters were the principal compounds observed in the pyrograms of all the binding media. Dicarboxylic acids (suberic and azelaic) methyl esters were also identified. They are degradation products of unsaturated fatty acids formed during the drying stage of tempera preparation.

Markers of proteins are difficult to quantify because of the presence of various derivatives associated to the same parent compound. For instance, pyrrole occurred together with at least three methylated isomers, and indole together with methyl and dimethylindoles.

Mono, di and trimethoxybenzenes were important pyrolysis products released by casein-containing painting layers and are probably derived from the TMAH thermochemolysis of carbohydrates. Actually, trimethoxybenzene has been identified in the pyrolysis/methylation of carbohydrates [18].

Discussion

The use of diagnostic markers in analytical pyrolysis to recognise binders in painting layers is quite straightforward for binders belonging to different chemical groups. However, discrimination between similar binding media (e.g. those made with proteinaceous materials) or in the case of mixtures, markers are less specific and a semiquantitative approach should be more appropriate. In the present study, a semiquantitative Py-GC-MS method is proposed for characterising proteinaceous binders using selected pyrolysis products representative of different chemical groups, namely proteins, carbohydrates and lipids.

Table IV. Pyrolysis/methylation of standard painting layers. Pyrolysis products selected for quantitation, retention times in s (scan) and m/z of ions chosen for quantitation (QM).

Compound	Scan	QM	Source
N-methyl pyrrole	105	80 + 81	proteins
Pyrrole	114	67	proteins
2-Methyl pyrrole	180	80 + 81	proteins
3-Methyl pyrrole	190	80 + 81	proteins
Methylmethoxybenzene	597	77 + 122	carbohydrates
Dimethoxybenzene	1161	95 + 123 + 138	carbohydrates
Dimethoxybenzene	1193	95 + 123 + 138	carbohydrates
Dimethoxyphenol	1389	125 + 139 + 154	carbohydrates
N-methyl indole	1426	130 + 131	proteins
Indole	1511	1117	proteins
Dimethyl indole	1618	144 + 145	proteins
Trimethoxybenzene	1641	110 + 1 53 + 168	carbohydrates
2-Methyl indole	1658	130 + 131	proteins
Methyltrimethoxybenzene	1746	139 + 167 + 182	carbohydrates
Suberic acid, dimethyl ester	1772	138 + 171 + 203	lipids
Dodecanoic acid, methyl ester	1896	74 + 87 + 214	lipids
Azelaic acid, methyl ester	1933	185 + 152 + 217	lipids
Tetradecanoic, methyl ester	2183	74 + 87 + 242	lipids
Pentadecanoic, methyl ester	2313	74 + 87 + 143	lipids
Palmitoleic acid, methyl ester	2414	236 + 268	lipids
Palmitic acid, methyl ester	2439	74 + 87 + 270	lipids
Heptadecanoic acid, methyl ester	2561	74 + 87 + 143	lipids
Oleic acid, methyl ester	2644	264 + 296	lipids
Stearic acid, methyl ester	2678	74 + 298	lipids

Table V. Pyrolysis/methylation of standard painting layers. Average relative peak area and relative standard deviations of pyrolysis products listed in Table IV. Means were calculated from 18 pyrograms corresponding to triplicate analyses of six different coloured paint layers containing the same binding media.

Markers	Glue	Casein	Egg	Egg/Glue	Casein/Glue
Pyrroles	24 ± 9	21 ± 6	2.0 ± 0.7	11 ± 5	32 ± 9
Phenolic compounds	1.5 ± 1	28 ± 11	1.3 ± 1	1.7 ± 1	7.6 ± 1
Indoles	0.5 ± 0.3	5.9 ± 3	1.3 ± 0.6	1.4 ± 0.7	2.6 ± 1
Dicarboxylic FAMEs	traces	traces	3.2 ± 2	2.4 ± 1.7	1.3 ± 0.8
Monocarboxylic FAMEs	69 ± 14	43 ± 11	89 ± 19	83 ± 16	57 ± 10

The relative distribution of thermal degradation products of proteins observed in conventional Py-GC-MS can be used to distinguish glue from egg and casein. In fact, pyrograms of glue-based painting layers were characterised by the highest levels of hydroxyproline markers (pyrrole, diketodipyrrole) in accordance with the fact that hydroxyproline is largely more abundant in collagene, the principal protein of glue, than in the proteins of egg and milk [1]. Indoles are indicative of the presence of tryptophane, which is a difficult amino acid to determine by conventional GC or HPLC methods because it is decomposed during acid hydrolysis [2]. Low relative abundances of indoles (1–2 %) suggest glue as the principal ligand, in agreement with the lack of tryptophane in collagene [1], while higher contents of indoles indicate the presence of egg or casein.

Differentiation between egg and casein can be accomplished using thermal degradation products of carbohydrates and lipids in both conventional Py-GC-MS and pyrolysis/methylation. Pyrograms obtained from egg-containing painting layers yielded the highest proportion of lipid markers, while those obtained from casein-containing painting layers were characterised by the highest levels of carbohydrate markers. Since the method of binder preparation based on ancient recipes (see Table I) did not produce pure casein, which is a mixture of phosphoproteins [1], some milk carbohydrates were retained in the tempera. These carbohydrates gave furanmethanol and maltol upon conventional pyrolysis and methylated phenols when pyrolysis was performed in the presence of TMAH.

The relative peak area of fatty acid methyl esters could be used to distinguish between egg (89 %) and casein (47 %), but in the case of mixtures differentiation is not so obvious because of the low reproducibility. Steroids identified in conventional Py-GC-MS appear to be more specific markers to recognise egg, as their relative content was significantly larger in the pyrograms of painting layers containing egg than in those containing casein, even in the case of mixtures. It is worth pointing

out the sensitivity of Py-GC-MS considering that dried whole eggs contain some 2.5 % cholesterol [1] and samples of painting layers generally contain less than 10 % of binder.

The obtained results indicate that Py-GC-MS is an adequate method for characterising proteinaceous binders in painting layers, in particular, conventional pyrolysis is more simple, specific and precise than pyrolysis/methylation. Quantitative analysis of amino acids by means of conventional GC and HPLC may give a more detailed description than Py-GC-MS, nevertheless, the latter technique provides, in a single analysis, structural information on a wide range of organic media, comprising not only proteinaceous binders, but also waxes, gums, oils, natural resins and synthetic polymers [7]. In conclusion, Py-GC-MS finds its application as a simple and rapid tool for the identification of the principal organic materials whose individual constituents can be further analysed by specific chemical treatments followed by traditional chromatographic techniques.

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