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Study of binding media in works of art by gas chromatographic analysis of amino acids and fatty acids derivatized with ethyl chloroformate

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Abstract

The aim of this work is to identify proteinaceous and oil binding media used in paintings from art collections in the Region of Valencia (Spain). This information is extremely useful for conservation and restoration work. The proposed procedure involves protein and glyceride hydrolysis from sub-milligram samples by treatment with hydrochloric acid, followed by neutralization, partition with chloroform and derivatization with ethyl chloroformate (ECF) of both the aqueous and the organic phases. The ECF derivatives of amino acids and azelaic, myristic, palmitic and stearic acids are separated by capillary gas chromatography. Amino acids are mainly found in the aqueous phase and long chain fatty acids in the organic phase. The distribution of azelaic acid was approximately similar in the two phases. Amino acid and fatty acid ratios found proved extremely useful for identifying the binding media in the paintings analysed. © 1997 Elsevier Science B.V.

Keywords: Art analysis; Binding media; Derivatization, GC; Amino acids; Fatty acids; Pigments; Ethyl chloroformate

1. Introduction

Identification of the binding media in pigments is a difficult and complex matter in the field of restoration and conservation of works of art, especially wall paintings. Binding media are natural products of relatively complex nature that are used as film-forming substances to give cohesion to pigments and to protect them from deterioration. Natural binding media can be classified, according to their main components, into several categories. Proteinaceous binding media are animal glue (gelatine), caseine, milk, egg albumen (egg white) and egg yolk because they have a high proportion of proteins, although milk and egg yolk also contain a considerable level of fats. Fatty binding media are drying oils (linseed oil, walnut oil, poppyseed oil) and waxes. Other binding media have a high proportion of polysaccharides such as vegetable gums or mucilages (starch, guar, tamarind seeds, etc.) or are natural resins consisting mainly of terpenoids [1].

As binding media are only a part of the material to be studied and the amount of sample taken for analysis is usually very small (<1 mg) to avoid seriously damaging the paintings, very sensitive techniques such as capillary gas chromatography (GC) must be used. The use of amino acid and fatty acid ratios has been suggested for the identification of proteinaceous and fatty binding media [1]. Amino acid analysis requires protein hydrolysis. For separation by GC, the freed amino acids must be derivatized so they become volatile compounds.

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Ethyl chloroformate (ECF) reacts with amino acids in the presence of ethanol and pyridine to produce N(O,S)ethoxycarbonyl (EOC) ethyl esters of amino acids [2,3]. This procedure offers the advantage of speed as derivatives are yielded in a few seconds and the chromatographic separation in the capillary column can be accomplished in a few minutes, although arginine is not fully derivatized and is retained in the column. Other advantages are minimal handling of the sample, the use of an aqueous medium for the reaction and inexpensive reagents [2]. Fatty acids analysis in paint oils has been carried out by glyceride saponification followed by methylation to convert free fatty acids into methyl esters which are separated and determined by GC [4]. Direct treatment with BF_3 and methanol has also been used in this type of samples [1]. The reaction with alkylchloroformates leads to the rapid synthesis of fatty acid alkyl esters even in an aqueous medium [5–7]. In aged drying oils there are saturated fatty acids such as myristic, palmitic and stearic acids, as well as azelaic acid, which are formed as a result of the oxidation of fatty acids with a double bond at carbon 9 [1].

In this work the joint separation by GC of the ECF derivatives of amino acids and fatty acids was applied to the identification of proteinaceous binding media and drying oils in works of art from the artistic heritage in the Region of Valencia (Spain).

2. Experimental

2.1. Solvents and reagents

The amino acids used as standards were alanine (Ala), glycine (Gly), valine (Val), leucine (Leu), isoleucine (Ile), proline (Pro), threonine (Thr), glutamic acid (Glu), asparagine (Asn), serine (Ser), aspartic acid (Asp), methionine (Met), hydroxyproline (OH-Pro), phenylalanine (Phe), cysteine (Cys), glutamine (Gln), lysine (Lys), histidine (His), tyrosine (Tyr) and cystine (CC). Tryptophan and arginine were not used because the former is destroyed in acid hydrolysis and the latter is adsorbed on the chromatographic column. The amino acids were from the Sigma LAA-21 kit (St. Louis, MO, USA) as were the standards of hexadecanoic acid (palmitic acid, Pa), octadecanoic acid (stearic acid, St) and heptane-1,7-dicarboxylic acid (azelaic acid, Az). Tetradecanoic acid (myristic acid, My) was purchased from Aldrich (Steinheim, Germany). Chloroform, pyridine and ethyl chloroformate were from Fluka (Buchs, Switzerland). Hexane, ethanol, calcium carbonate, sodium hydrogencarbonate and hydrochloric acid were analytical grade reagents from Panreac (Barcelona, Spain)

2.2. Samples

Samples of binding media had been spread in thin layers on glass slides and left to stand at room temperature in the laboratory for 1 to 3 years to age them.

Work of art samples were taken from paintings from the collection held by the Archbishopric of Valencia and from wall paintings from an ancient church ("San Juan del Hospital", late Romanesque and Gothic, 13th–15th centuries) of Valencia (Spain).

2.3. Apparatus

GC–flame ionization detection (FID) analysis was carried out on a Hewlett-Packard 5890 Series II gas chromatograph, controlled by HP 3365 ChemStation software (Hewlett-Packard, Avondale, PA, USA). The injector and detector temperatures were 250 and 300°C, respectively. Separation was achieved on a HP-1701 (25 m×0.25 mm I.D., 0.15 μm film thickness) fused-silica capillary column. Oven temperature was programmed from 100 to 275°C (held 8 min) at 40°C/min. Helium at an inlet pressure of 115 kPa was the carrier gas. Split injection at a split ratio 1:20 was used. Volume injected was 2–3 μl.

For confirmation by GC–MS a Trio 1000 mass spectrometer coupled to a Fisons 8000 Series gas chromatograph (Fisons Instruments, Manchester, UK) was used. GC separation was achieved in the same HP-1701 capillary column. Inlet temperature was 250°C. Oven temperature was programmed from 100–125 to 275°C (10 min) at 30°C/min. The electron impact ionization (EI) technique was used. MS conditions were as follows: source temperature 200°C, electronic energy 70 eV, scan rate of the spectrometer 0.5 s/scan over the range m/z 15–750.

Helium at a head pressure of 47 kPa was the carrier gas. Split injection at a split ratio 1:20 was used.

2.4. Procedures

Binding media samples (0.5–1 mg) were introduced in 0.3-ml minivials (Supelco, Bellefonte, PA, USA) where they were hydrolyzed with 100 μl 6 *M* HCl for 24 h at 110°C in Ar atmosphere. The resulting solution was neutralized by careful addition of solid $CaCO_3$. 50 μl of chloroform was added to the minivial which was vigorously shaken in order to extract the least polar compounds. 50 μl of the aqueous layer was taken and mixed with 50 μl of an ethanol–pyridine (4:1) solution and with 8 μl of ECF. The reaction mixture was shaken for about 10 s and extracted with 50 μl of chloroform containing 1% of ECF. Then, 50 μl of a saturated solution of $NaHCO_3$ was added and after vigorous but careful shaking two layers were obtained. A 2–3-μl aliquot of the chloroform layer was injected for analysis. The organic layer obtained from the chloroform extraction of the solution neutralized with $CaCO_3$ was evaporated to dryness, redissolved in 50 μl of water–ethanol–pyridine (5:4:1) and derivatized in the same way as described for the aqueous layer, except that $CHCl_3$ with no added ECF was used.

The amino acid standards were dissolved separately and in mixture in water–ethanol–pyridine (7:4:1) at concentrations of 60–70 μg/ml (0.270–0.888 μmol/ml). Solubility of cystine and cysteine is low in this medium and increases with slight acidification. Myristic, palmitic and stearic acids were prepared separately and in mixture in hexane at a concentration of about 200 μg/ml. Azelaic acid was dissolved in absolute ethanol (200 μg/ml). Solutions were stored in refrigerator at 4–6°C. All standard solutions were derivatized before and after undergoing the same acid treatment and phase separation as the samples. Standard amino acids were derivatized by taking 50-μl aliquots of the solutions and treating them directly with ECF. Other aliquots were evaporated to dryness and the residues were treated with 6 *M* HCl in the same way as the samples. In the case of fatty acids aliquots (30–50 μl) of the solutions were evaporated to dryness. Then, the residues were dissolved in water–ethanol–pyridine (5:4:1), derivatized with ECF, and the derivatives extracted with $CHCl_3$. In other experiments, the residues were treated with HCl under the same conditions as the samples. After this treatment the neutralized liquid was subjected to chloroform extraction and the two phases were derivatized with ECF. For the extraction of ethyl esters $CHCl_3$ with no added ECF was used.

3. Results and discussion

3.1. Amino acid and fatty acid standards

Fig. 1 shows the separation obtained with the standard mixture of amino acids which agrees with the results reported by Hušek in columns with similar stationary phases [2,5]. In this case, the mixture was derivatized directly without HCl treatment or chloroform extraction and sodium hydrogencarbonate was not added after derivatisation. Cysteine and cystine peaks were smaller than expected possibly due to their low solubility in the medium and, in the case of cystine, to its minor molar concentration. The glutamic acid response is relatively low too, which, in accordance with a previous report [2] is attributable to the formation, by internal cyclization, of pyroglutamic acid whose peak follows that of serine and is assigned to this amino acid as it dominates the N(EOC) diethyl ester in the reaction conditions [2]. Likewise, the histidine response is relatively low, as reported by Nowik [8]. Separation can be considered to be generally acceptable. Analysis speed is high and could be further increased if the final temperature were raised to 300°C although this causes the stationary phase in the column to deteriorate more rapidly. Peaks of basic amino acids glutamine and asparagine decrease, especially the former, when the amino acid mixture is treated with 6 *M* HCl in the analysis conditions of the samples. This is attributable to their hydrolysis to glutamic and aspartic acids, respectively. The stability of these derivatives in $CHCl_3$ with 1% ECF at 4–6°C in a tightly closed vial was sufficient to maintain amino acid ratios with no significant differences for a week after derivatization. Amino acids were extracted mainly in the aqueous layer after the chloroform extraction following 6 *M* HCl treatment.

Fig. 2 shows the separation of myristic, palmitic

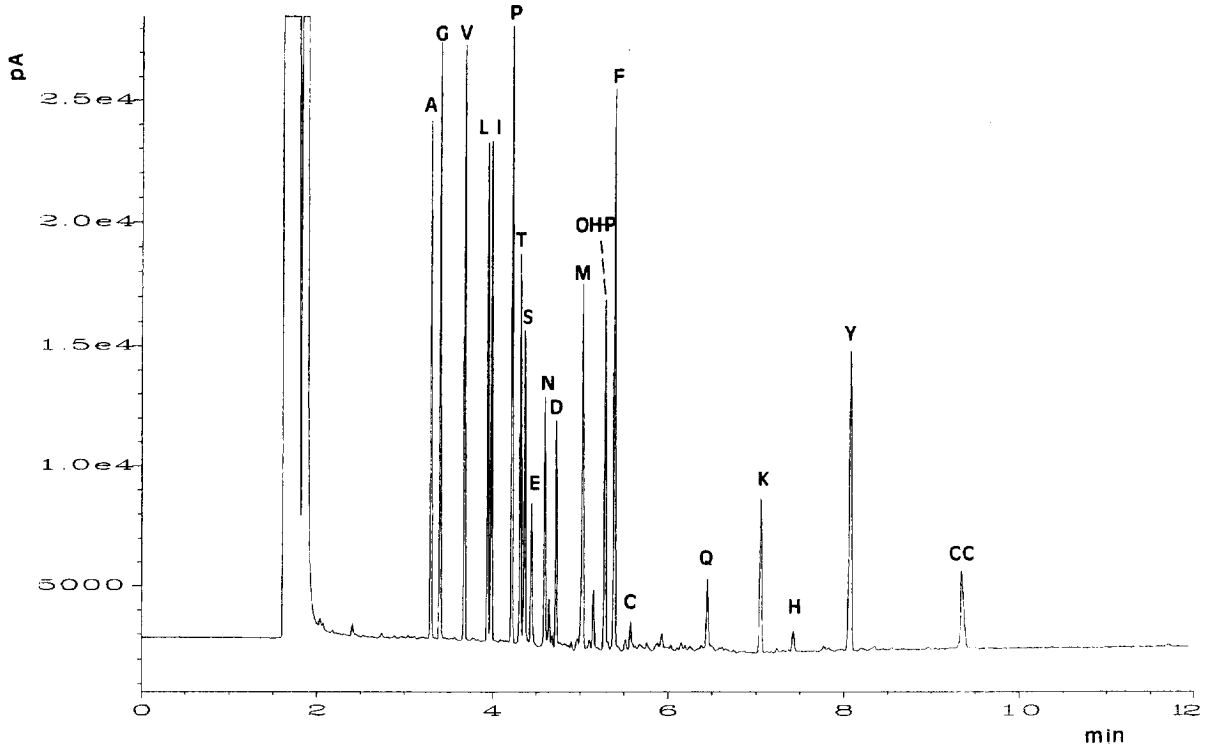

Fig. 1. Gas chromatogram of N(O,S)ethoxycarbonyl ethyl esters of amino acids separated on a HP-1701 fused-silica capillary column. A mixture of standards was derivatized directly with no acid treatment, chloroform extraction or sodium hydrogencarbonate addition. Temperature was increased from 100°C at 40°C/min to 275°C (held 8 min). Helium head pressure: 115 kPa. Split ratio: 1/20. Peaks: A, alanine; G, glycine; V, valine; L, leucine; I, isoleucine: T, threonine; S, serine; E, glutamic acid; P, proline; N, asparagine; D, aspartic acid; M, methionine; OH-P, hydroxyproline; F, phenylalanine; C, cysteine: Q, glutamine; K, lysine; H, histidine; Y, tyrosine; CC, cystine. Injected amounts: 120–140 ng of underivatized standards.

and stearic acid ethyl esters and azelaic acid diethyl ester. There are no resolution problems even though Az and My are separated by only 0.1 min. Unsaturated fatty acids are also present in fresh drying oils but oxidation and polymerization lead to their gradual disappearance with time so that finally only saturated fatty acids and azelaic acid are reported in aged paint oils [1]. My, Pa and St were extracted mainly in the organic layer after chloroform partition following HCl treatment. Az was partitioned into both layers at similar levels, so the Az:Pa peak area ratio is lower in the chromatograms from the organic layer.

The separation of ECF derivatives in a mixture of amino acids and fatty acids is possible although myristic and aspartic acids cannot be resolved as shown in Fig. 3. However, the corresponding peak can be used to evaluate the amount of either of the two compounds if a suitable identification technique is used (e.g., GC–MS). Where both My and Asp coexist, as fatty acids are recovered mainly in the organic phase, the peak from the chromatogram of the aqueous layer may be attributed to Asp with low error. The remaining compounds show sufficient separation from their neighbors. The differences in retention times of the pairs of peaks Asn–Az and Pa–Phe were about 0.03 min but high column efficiency allowed for adequate resolution.

Vigorous shaking in the different stages of the derivatization reaction is necessary, especially for extracting the derivatives in the chloroform layer and for the final mixing with $NaHCO_3$ to eliminate HCl dissolved in the chloroform and hydroalcohol layers. HCl in the organic solution may have adverse effects

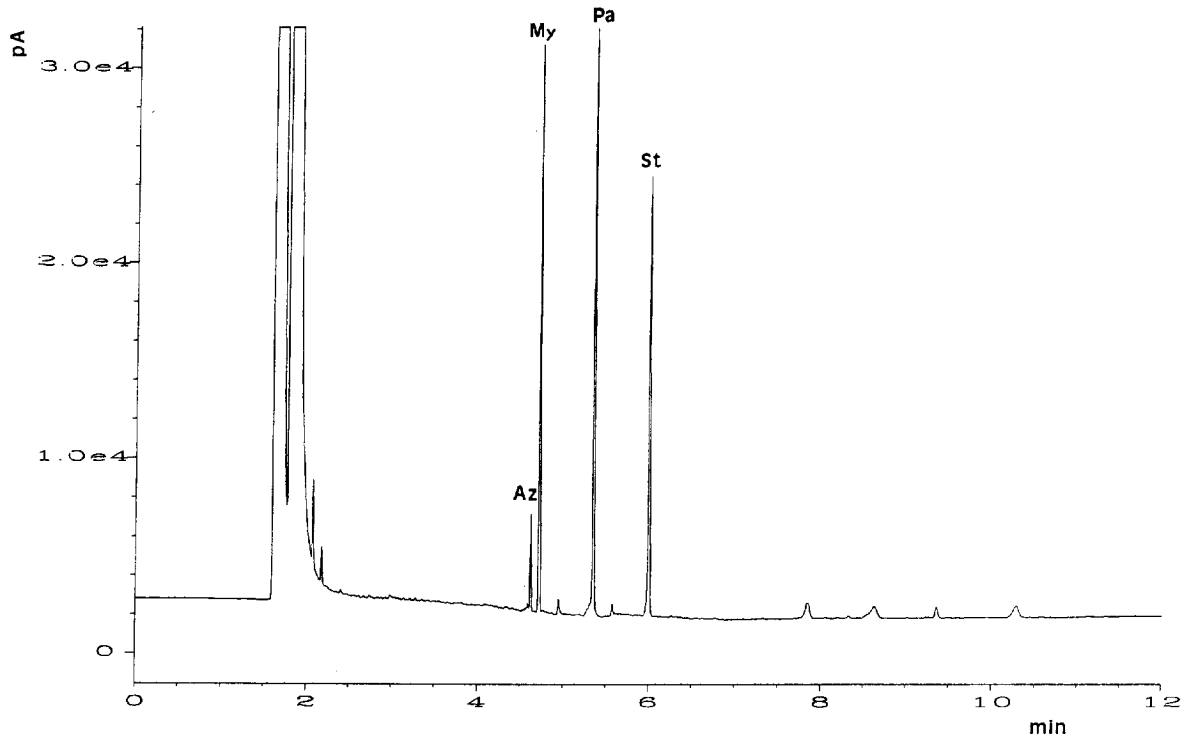

Fig. 2. Gas chromatogram of azelaic acid and fatty acids as ECF derivatives. Standards were derivatized directly with no acid treatment or chloroform extraction. Chromatographic conditions as in Fig. 1. Peaks and injected amounts of underivatized standards are: Az (azelaic acid, 35.2 ng); My (myristic acid, 164 ng); Pa (palmitic acid, 188 ng); St (stearic acid, 164 ng).

on the syringe and the column. An irregular chromatographic base line after the solvent peak and/or low recovery of analytes was observed when this final shaking did not take place.

3.2. Binding media

The mean values found for the ratios between the peak area of each amino acid and the peak area of alanine (AA:Ala) in the different proteinaceous binding media studied are listed in Table 1. Normalization against Ala has been used for comparative purposes according to other studies [8] but different ratios can be used [1]. Columns A and O list the ratios from the aqueous and organic layers, respectively. Data from Asp in egg yolk and whole egg might be affected by the possible presence of My, given the differences of the Asp:Ala peak area ratios in aqueous and organic layers. The AA:Ala ratios in columns A and O are generally alike but the absolute values of peak areas are greater in the chromatograms from the aqueous layer where amino acids are dissolved preferentially after chloroform partition, as indicated above. The observed differences may be due to differences in the partition constants. The Pro:Ala ratio is always slightly higher in the organic layer. Comparison of the AA:Ala peak area ratios with the pertinent ratios calculated from tabulated data of amino acid composition of the products studied is not suitable because correction factors have not been established as they were not essential to our purposes.

The most characteristic parameters of gelatine are the presence of hydroxyproline (our mean value for the OH-Pro:Ala ratio was 0.55) which does not appear in the other binding media [1,8] and a Gly:Ala ratio>2.5. The Pro:Ala ratio was the next highest with a mean value of 1.58. The proportion Gly:Phe>10 for gelatine and lower than unity for the

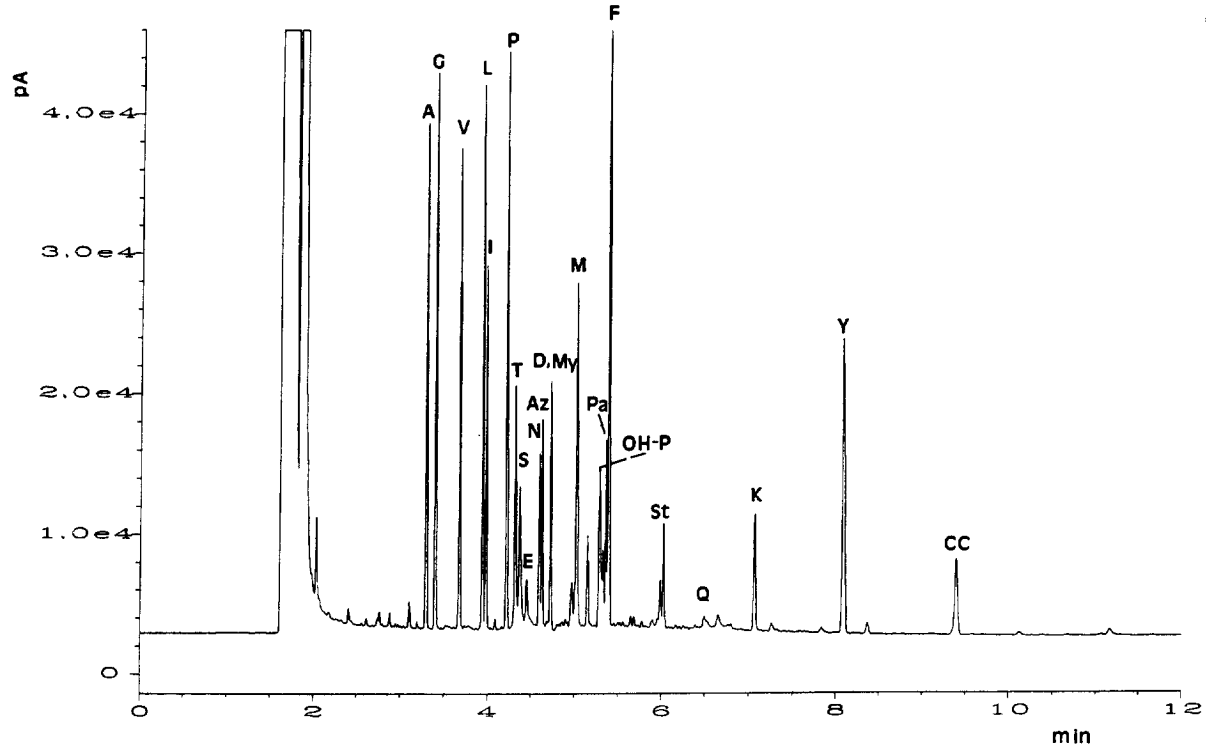

Fig. 3. Gas chromatogram of a mixture of amino acids and fatty acids as ECF derivatives after treatment with 6 *M* HCl; with no chloroform extraction but with sodium hydrogencarbonate addition. Chromatographic conditions and peaks as in Figs. 1 and 2. Injected amounts of underivatized standards: Az (105.6 ng); My (98.4 ng); Pa (112.8 ng); St (98.4 ng); amino acids (36–42 ng).

other binding media is another interesting ratio which agrees with previous reports [1].

Caseine showed a Pro:Ala index of 2.3, which is even higher than that for animal glue and egg. Other amino acids that help to identify this medium were high proportions of tyrosine, threonine and glutamic acid.

Samples of aged egg albumen, egg yolk and whole egg were analysed. The higher AA:Ala ratios correspond to Leu, Phe and Val but they cannot be considered as characteristic. The Ser:Ala ratios in our samples are not as high as those reported by Pancella and Bart [1] but they agree quite well with Nowik's results, although this author was not able to resolve Ser and Glu [8]. At amino acid level the differentiation between egg (white and yolk) and gelatine is the lack of OH-Pro and a Gly:Ala ratio< 1. A Pro:Ala and Glu:Ala ratio lower than 1 and 0.6, respectively, may help to distinguish egg from caseine.

Table 2 shows the ratios between the peak areas of azelaic and saturated fatty acids for aged whole egg, egg yolk and linseed oil in both the aqueous and organic layers after partition of the neutralized hydrolysate. Peak area ratios have been normalized with respect to Pa. Egg white, caseine and animal glue do not show fatty acids in their composition so they are excluded from Table 2. The Az:Pa ratio is much lower in solutions from organic extracts than in solutions from aqueous extracts because of the higher polarity (and water solubility) of Az. The Az:Pa ratio was very small in egg yolk and whole egg, as found by other authors [1,8] and it was 0.34 ± 0.1 in linseed oil.

Although the My:Pa ratio is affected by a certain degree of error in aged egg due to coelution of My and Asp, there is no practical problem in analysis of O extracts where fatty acids predominate over amino acids. This ratio ranged from 0.02 to 0.04 in egg yolk, whole egg and linseed oil, and is of no interest

Table 1
Peak area ratios of amino acids relative to alanine for different proteinaceous binding media derivatized with ethyl chloroformate after acid hydrolysis

Amino acids	Gelatine		Caseine		Egg white		Egg yolk		Whole egg	
	A	O	A	O	A	O	A	O	A	O
Alanine	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Glycine	2.94	2.3	1.06	1.02	0.56	0.51	0.62	0.63	0.70	0.69
Valine	0.31	–	1.53	1.46	1.16	1.14	1.26	1.22	1.20	1.22
Leucine	0.41	–	1.82	1.70	1.58	1.44	2.13	1.59	1.89	1.64
Isoleucine	0.18	–	1.17	1.08	0.85	0.79	0.94	0.89	0.99	0.92
Proline	1.58	1.76	2.30	2.41	0.57	0.62	0.74	0.80	0.68	0.77
Threonine	0.08	–	1.03	0.86	0.34	0.26	0.42	0.34	0.53	0.46
Serine	0.1	–	0.79	0.58	0.47	0.21	0.44	0.37	0.71	0.55
Glutamic acid	0.12	–	1.13	0.98	0.33	–	0.53	0.12	0.46	0.38
Aspartic acid[a]	0.13	–	0.78	0.70	0.34	0.26	0.42	1.4	0.49	1.1
Metionine	0.04	–	0.78	0.54	0.46	0.37	0.44	0.17	0.53	0.53
OH-Proline	0.55	–	–	–	–	–	–	–	–	–
Phenylalanine	0.28	–	1.78	1.59	1.20	1.04	1.44	–[b]	1.35	–[b]
Lysine	0.04	–	0.72	0.65	0.31	0.15	–	0.13	0.20	0.69
Histidine	–	–	0.09	–	–	–	–	–	–	0.03
Tyrosine	–	–	1.29	1.15	0.31	0.22	0.17	0.14	0.22	0.21
Cystine	0.14	–	–	–	–	0.32	0.15	0.27	0.18	0.53

A:Derivatization of the aqueous extract after acid hydrolysis and neutralization.
O: Derivatization of the organic (chloroform) extract after acid hydrolysis and neutralization.
–: Peak was not detected or the ratio was <0.03.
[a]: Peaks from aspartic and myristic acids have the same retention time.
[b]: Interference from palmitic acid.

for their differentiation. The St:Pa ratio was about 1 in all samples but values from O extracts are more precise as the peak areas of both fatty acids were greater than values from A extracts. Pancella and Bart report St:Pa values of 0.5–1 for linseed oil and 0.31–0.42 for egg yolk [1], which agree with our results for the first binding medium. The similar values found make this ratio useless for differentiating between egg and linseed oil or an emulsion of both and the presence of amino acids is the key to discriminate between these two binding media. A peak eluting just before the stearic acid peak was noticed in both media as found in a previous report [8]. This peak was attributed by GC–MS to the ethyl ester of octadec-9-enoic acid (oleic acid) and it also appeared in 3-year-aged samples. The superficial layer of polymerised oil may act as a barrier protecting inner unsaturated fatty acids from rapid oxidation by air. Another peak found in egg yolk and whole egg chromatograms at retention time 11.1 min was identified by GC–MS as the ethyl ester of colest-5-en-ol. In some chromatograms a small peak

Table 2
Peak area ratios of azelaic acid and saturated fatty acids relative to palmitic acid in some binding media containing fats

Peak area ratio	Egg yolk		Whole egg		Linseed oil	
	A	O	A	O	A	O
Azelaic acid:palmitic acid	–	–	–	–	37	0.34
Myristic acid:palmitic acid[a]	0.45	0.02	3.25	0.04	–	0.04
Stearic acid:palmitic acid	1.13	0.97	1.47	0.89	1.1	0.85

A: Derivatization of the aqueous extract after acid hydrolysis and neutralization.
O: Derivatization of the organic (chloroform) extract after acid hydrolysis and neutralization.
[a]: Peaks from aspartic and myristic acids have the same retention times.

appearing at about 5.6 min was identified by both GC–FID and GC–MS as free palmitic acid.

3.3. Artistic paintings

Although other binding media could be present, we have tried to apply the method described to the identification of the binding media studied here using real samples from the artistic heritage of Valencia. Here are some results:

(a) Altarpiece of the "Magdalena", painted by the Master of Alcira (Archbishopric of Valencia). In the organic extract, the Az:Pa, My:Pa and St:Pa ratios were 0.17, 0.065 and 0.52, respectively. In the aqueous extract, the Az:Pa ratio was 3.4 whereas the St:Pa ratio was 0.51. There is a peak of oleic acid (its area was approximately half the area of the stearic acid peak). This suggests the presence of drying oil, probably linseed oil. In the aqueous extract there were also several peaks identified as amino acids. The high Gly:Ala (4.16) and Pro:Ala (1.97) ratios and, especially, the identification of OH-Pro point to the use of animal glue in this altarpiece.

(b) Panel of the "Virgen de los Desamparados" by Vicente López (Archbishopric of Valencia). Azelaic, palmitic and stearic acids were identified in the aqueous extract (Az:Pa ratio=6, St:Pa ratio= 0.78) but no amino acids. In the organic extract the same acids were identified but the ratios were different (Az:Pa=0.6, My:Pa=0.03 and St:Pa= 0.61). The oleic acid peak is still detectable and its area accounts for 16% of the stearic acid peak. These results suggest the presence of linseed oil and not proteinaceous binding media, as previously supposed.

(c) Church of "San Juan del Hospital" (Valencia). The samples analysed were taken from paintings recently discovered in the vault of a chapel in the oldest church in Valencia; their origin is unknown but they are thought to be Romanesque. The samples showed components soluble in 6 *M* HCl but on neutralization a precipitate appeared. In the aqueous extract, some minor peaks corresponding to amino acids were detectable. The presence of gelatine is suggested by the following ratios: Gly:Ala=2.9, Pro:Ala=1.13 and Val:Ala=0.5. A small peak coincident with that of hydroxyproline (OH-Pro:Ala= 0.49) supports this hypothesis. Azelaic acid was also detected. The organic extract revealed that azelaic, palmitic and stearic acid were present in the samples in ratios which suggest the presence of linseed oil in these paintings. Oleic acid was hardly detectable as its level was very low in comparison with palmitic and stearic acids. Although an in-depth study is needed, the results obtained so far suggest that, contrary to what was first thought, they are not frescos.

4. Conclusions

The proposed method allows for the rapid derivatization with ECF of mixtures of amino acids and fatty acids, which usually result from protein and glyceride hydrolysis in aged binding media. A single medium, water–ethanol–pyridine, can be used for both types of compounds. With the exception of myristic acid, which overlaps with aspartic acid, separation is good or acceptable and rapid. However, chloroform partition of the neutralized hydrolysate helps to clean up amino acids, mainly dissolved in the aqueous layer, from fatty acids, which are mainly dissolved in the organic layer. The distribution of azelaic acid was approximately similar in the two layers. So, GC analysis of ECF derivatives from the aqueous and organic phases is preferred for amino acids and fatty acids, respectively. The method provides ratios for amino acid and fatty (or azelaic) acids that can be used to characterize the binding media studied. When applied to several pictorial works of art the method proved useful for identifying the proteinaceous and oil binding media used, thus providing information of great interest for conservation or restoration treatments.

Acknowledgments

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