ELSEVIER

Journal of Chromatography A, 846 (1999) 113–124

JOURNAL OF CHROMATOGRAPHY A

Characterisation of proteinaceous binders and drying oils in wall painting samples by gas chromatography–mass spectrometry

Maria Perla Colombini[a,*], Francesca Modugno[b], Marina Giacomelli[b], Sandro Francesconi[b]

[a]*Department of Environmental and Earthy Science, University of Milan, Via Bicocca Emanueli 15, 20126 Milan, Italy*
[b]*Department of Chemistry and Industrial Chemistry, University of Pisa, Via Risorgimento 35, 56126 Pisa, Italy*

Abstract

A reliable analytical procedure has been developed for the characterisation of drying oils and proteinaceous binders in samples of painted artworks. The method is based on microwave assisted acid hydrolysis followed by the extraction of the lipid component with diethyl ether. Fatty acids were determined after saponification of the organic phase and derivatisation with *N-tert.*-Butyl methylsilyl-*N*-methyltrifluoroacetamide. Amino acids present in the acid aqueous phase were derivatised with the same silyl agent. The separation and quantitative determination of derivatives was performed by gas chromatography–mass spectrometry. Detection limits of fatty acids (lauric, myristic, palmitic, stearic, oleic, suberic, azelaic and sebacic acid) and amino acids were in the range 50–100 pg. The mean recovery of the procedure for lipid determination was about 70% and that for proteins 100% with a reproducibility better than 10% for 1 mg samples. The overall procedure was validated by analysing suitable reference wall painting samples. The identification of the proteinaceous binders was achieved by principal component analysis performed on the fourteen amino acid relative percentages while the identification of the drying oils was based on the values of palmitic to stearic ratio and azelaic to palmitic ratio. A general pattern recognition scheme based on these values and on the amounts of dicarboxylic acids and the presence of cholesterol was developed to distinguish between proteinaceous binder, ''tempera grassa'' and drying oils. The application of the proposed procedure to samples from ''*The Legend of True Cross*'' (1452) by Piero della Francesca allowed the identification of ''tempera grassa'' made of egg and linseed oil.

Keywords: Oils; Art analysis; Binding media; Amino acids; Fatty acids

1. Introduction

A diagnosis of the state of conservation of a work of art involves knowledge not only of art history but also of the chemical composition of the original materials employed. As far as wall paintings are concerned, the characterisation of organic binders, used for their pigment fixative and dispersing qualities, is crucial since it is a source of important information both for reconstructing the working techniques used in a particular painting and for defining a programme for the restoration and conservation of the work of art itself. Over the centuries artists have experimented with a variety of binding media for their pigments, ranging from natural gums and proteinaceous materials to oils, either alone or mixed together. Gums (such as arabic gum, cherry gum) and proteinaceous tempera (milk or casein, egg and animal glue) have been used since antiquity, whereas drying oils began to be used in European paintings some time before the thirteenth century [1–3]. Above all linseed oil, walnut oil and

*Corresponding author. Tel.: +39-2-6441-4304; fax: +39-2-6447-4300.
E-mail address: perla@dcci.unipi.it (M.P. Colombini)

0021-9673/99/$ – see front matter
PII: S0021-9673(99)00344-1

poppyseed oil were used [2,4], both as raw and ''*boiled*''[1]. Of these Italian painters preferred linseed oil. Moreover, to obtain particular chromatic effects, they often used ''tempera grassa'', an emulsion of oil and egg or, less commonly, oil and casein [1,2] and the choice of the drying oil was mainly influenced by the pigment to be dispersed.

The chemical characterisation of such binders in historical paintings entails solving several analytical problems related to the complexity of these mixtures, the degradation of the materials as a result of ageing, and the small amount of sample available for analysis (generally, less than 1 mg with an organic content lower than 10%). Identifying mixed binders such as ''tempera grassa'' is, thus, extremely difficult. Generally, the characterisation of proteinaceous binders is based on the chromatographic determination of amino acids [1,5–10] and that of lipid binders on the determination of fatty acids [1,11–14]. The identification of these binders can be achieved through the statistical evaluation of amino acid distributions for proteinaceous binders [1,6,7,9,10] and of some selected ratios between fatty acids (e.g. azelaic/palmitic acid and palmitic/stearic acid) for drying oils [1,11,12]. Several analytical procedures have been reported in the literature for the analysis of such organic binders in works of art [1,5–16], but only two of these have been developed for the simultaneous characterisation of proteinaceous binders and drying oils [15,16]. In both cases, an acid hydrolysis is adopted to release amino acids from proteins and fatty acids from triglycerides, with a subsequent derivatisation reaction, which produces suitable derivatives for a single run gas chromatography analysis. Although these methods give valuable information on the constituents of the paint layer and with only one analysis, they are generally unable to determine several amino acids, some dicarboxylic acids (sebacic and suberic acid) and cholesterol (a marker of egg binder), which may cause errors in binder identification. Another major limitation of these procedures is that results are not reported in terms of the concentrations of the amino acids and fatty acids in the paint layer, and no recoveries are given. Results, if given in terms of chromatographic peak areas, can vary considerably depending on the procedure used, and thus comparisons with results obtained by different authors are not reliable.

This paper describes a quantitative analytical procedure for the characterisation of both drying oils and proteinaceous binders in the same sample. Two analytical methods, one previously developed for amino acid analysis [6] and a new one for fatty acid determination, have been combined for the characterisation of both protein and lipid binders. The procedure is based on the microwave assisted acid hydrolysis of the sample, followed by solvent extraction of the hydrolysate. Amino acids were determined in the aqueous phase, while fatty acids were determined in the organic phase after saponification. In both cases the analytes were derivatised with a silyl reagent and determined by gas chromatography–mass spectrometry (GC–MS). The silyl reagent, under the experimental conditions used, quantitatively gives both monocarboxylic and dicarboxylic acid derivatives. The overall analytical procedure was tested and calibrated by using reference wall painting samples which were prepared following old recipes at the Opificio delle Pietre Dure (Italian Ministry of Cultural Heritage). Proteinaceous binders were identified by principal components analysis (PCA) on the relative percentage content of fourteen selected amino acids, while the identification of drying oils was based on the presence of dicarboxylic acids, i.e. azelaic, sebacic and suberic acids and on the values of the ratio between the content of azelaic and palmitic acid, and palmitic and stearic acid. A general scheme for pattern recognition is suggested.

Finally, the proposed procedure was applied to some samples taken from the famous wall painting ''*The Legend of the True Cross*'' executed by Piero della Francesca in 1452 at S. Francesco Church (Arezzo, Italy). The identification of organic binders is discussed, with particular attention on ''tempera grassa'' which was suggested by some art historians [17].

[1]A ''*boiled*'' oil was obtained after exposing the raw oil to sunlight or by heating, often in presence of lead salts. In these conditions, carbon–carbon bonds are formed between the glyceride molecules in anaerobic conditions. The heating treatment improves drying properties, increases the refractive index, reduces light scattering at oil-pigment interface and thereby increases the saturation of the pigment colour [1,4].

2. Experimental

2.1. Chemicals and reagents

All solvents were Baker HPLC grade; HCl (Baker, Deventer, Holland) was suprapur grade, deionised water (Carlo Erba, Milan, Italy) was further purified by an Elgastat UHQ system (resistivity 18 MΩ at 25°C). *N-tert*.-Butylmethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA, purity >98%) (Pierce, Rockford, IL, USA) was used without any purification. Tridecanoic (i.s.2), dodecanoic (lauric, la), tetradecanoic (myristic, my), hexadecanoic (palmitic, pa), octadecanoic (stearic, st), (*Z*)-9-octadecenoic (oleic, ole), (*Z,Z*)-9,12-octadecadienoic (linoleic), (*Z,Z,Z*)-9,12,15-octadecatrienoic (linolenic), octadecanedioic (suberic, su), nonanedioic (azelaic, az), decanedioic (sebacic, se) acids, and cholesterol were supplied by Sigma (St. Louis, MO, USA) with a purity >99%, tripalmitin (purity >99%) was supplied by Aldrich (Steinheim, Germany). A standard solution containing la (5.6 ng/mg), my (6.0 ng/mg), pa (36.2 ng/mg), st (21.5 ng/mg), ole (9.4 ng/mg), su (16.7 ng/mg), se (12.2 ng/mg) and az (11.8 ng/mg) acids in acetone was used. Amino acid standard solutions of both collagen hydrolysate (2.5 μmol/ml in 0.1 *M* HCl of each amino acid except for proline and hydroxyproline whose concentration was 12.5 μmol/ml) and food hydrolysate (2.5 μmol/ml in 0.1 *M* HCl of each amino acid), norleucine (Nor), dried chicken egg yolk and Dowex-50W-crosslinkage 8% resin in hydrogen form, 100–200 mesh, were also purchased from Sigma. Hexadecane (Fluka, Buchs, Switzerland) was used as an internal standard. Linseed oil was kindly provided by the Opificio delle Pietre Dure (Florence, Italy).

2.2. Reference wall painting samples

Table 1 reports the paint layer composition of samples obtained from the Opificio delle Pietre Dure of Florence (Ministry of Cultural Heritage). These reference samples belong to a wide collection (about nine hundred 10×15 cm tiles) stored at the Opificio's laboratory and developed under the project ''Safeguard of Cultural Heritage'' (Italian National Research Council). The support of the reference samples analysed was made by glass and/or plaster. Samples containing lead white were chosen since this pigment was the most commonly used in Italian Renaissance paintings. Sampling was performed on three areas of each paint layer of the tile after four months from sample preparation. Samples were stored in the dark at room temperature.

2.3. Old wall painting samples

Samples from the wall painting ''*The Legend of the True Cross*'' by Piero della Francesca in the Church of S. Francesco in Arezzo (Italy) were

Table 1
Composition of the paint layer of reference samples

Sample	Binder	Pigment	% (w/w) of lipids in the painted film	% (w/w) of proteins in the painted film
Ol	Linseed oil	Absent	100	–
On	Walnut oil	Absent	100	–
Op	Poppy oil	Absent	100	–
Ui	Egg[a]	Absent	42	46
Tg	Linseed oil+egg[b]	Absent	70	22
BpOl	Linseed oil	Lead white	26	–
BpOn	Walnut oil	Lead white	25	–
BpOp	Poppy oil	Lead white	29	–
BbUi	Egg[a]	Lead white	15	11
PbTg	Linseed oil+egg[b]	Lead white	25	7

[a] Egg yolk and egg white in ratio 1:1.
[b] Only egg yolk.

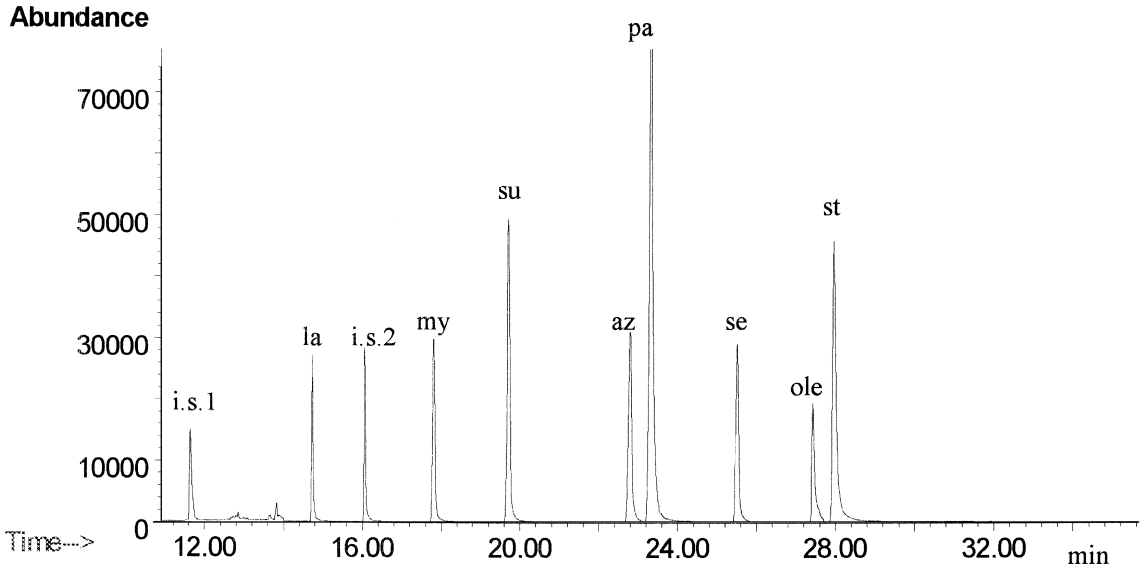

Fig. 1. Selected ion monitoring chromatogram of the derivatives of the fatty acid standard solution (injected amount: 1.5, 1.6, 4.5, 3.1, 9.7, 3.3, 2.5 and 5.7 ng. of la, my, su, az, pa, se, ole and st, respectively). Internal standards: i.s.1 hexadecane, i.s.2 derivative of tridecanoic acid.

identified as Nos. 4, 7, 8 and 9. Their masses were 1.4, 1.0, 1.1 and 0.6 mg, respectively, and they contained lead white as the main pigment [18].

2.4. Apparatus and chromatographic conditions

A microwave oven model MLS-1200 MEGA Milestone (FKV, Italy) was used for the acid hydrolysis of binders using the following programme: 10 min at 160°C and 250 W, 30 min at 160°C and 500 W, and 15 min of N_2 venting. A 5890 2 A gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with an on-column injection port and with a quadrupole mass spectrometric detector model 5971A (electron impact 70 eV, ion source temperature 180°C, interface temperature 280°C) was used to separate and identify the silylated amino acids and fatty acids. Chromatographic separation was performed on a chemically bonded fused-silica capillary column HP-5 MS (Hewlett-Packard), stationary phase 5% phenyl–95% methylpolysiloxane, 30 m× 0.25 mm I.D., 0.25 μm film thickness, connected to a 2 m×0.32 mm I.D. deactivated fused-silica capillary pre-column. The chromatographic conditions for the separation of silylated amino acids were: initial temperature 100°C, isothermal for 2 min, then 6°C/min up to 280°C, and isothermal for 15 min; the carrier gas was helium at a constant flow of 1.2 ml/min (initial pressure 76 KPa). For the separation of silylated fatty acids the chromatographic conditions were: initial temperature 80°C, isothermal for 2 min, 10°C/min up to 200°C, then 6°C/min up to 280°C and isothermal for 8 min; the carrier gas was helium at a constant flow of 1.3 ml/min (initial pressure 88 kPa). Hewlett-Packard Chemstation software B.04.02 was used for the integration of peaks and for the mass spectra evaluation.

2.5. Analytical procedure

The sample (0.1–2 mg) was hydrolysed with 6 *M* HCl in a microwave oven. The hydrolysate was extracted with 1 ml of diethyl ether three times. The determination of fourteen amino acids [alanine (Ala), glycine (Gly), valine (Val), isoleucine (Ile), leucine (Leu), metionine (Met), serine (Ser), phenylalanine (Phe), proline (Pro), aspartic acid (Asp), glutammic acid (Glu), lysine (Lys), hydroxyproline (Hyp), and tyrosine (Tyr)] was performed on the aqueous phase following the procedure described elsewhere [6–9]. The determination of fatty acids was made on the organic phase, as follows:

Table 2
Recovery of the overall analytical procedure

Sample	Lipid amount (μg)		Recovery (%)	Proteinaceous amount (μg)		Recovery (%)
	Expected[a]	Found[b]		Expected[a]	Found[b]	
Tripalmitin	952	670	70	–	–	–
Linseed oil	90	63	69	–	–	–
Dried egg yolk	222	160	72	311	304	98

[a] Expected lipid amounts are relevant to the content of palmitic acid in tripalmitin, palmitic and stearic acids in linseed and egg; expected proteinaceous amount is relevant to the content of fourteen amino acids in egg.
[b] Mean value on five replicate analyses.

(a) The organic extract, containing free fatty acids and not hydrolysed triglycerides, was dried under a mild flow of nitrogen and the residue was admixed with 1 ml of 10% KOH and stirred for 4 h at 80°C. It was left to stir at room temperature for 12 h. The solution was acidified with HCl to pH 1 and free fatty acids were extracted three times with 1 ml of diethyl ether. The recovery of this extraction procedure was quantitative for monocarboxylic acids and 85% for dicarboxylic acids;

(b) An aliquot of the extract, admixed with a known amount of tridecanoic acid standard solution in order to control the recovery of the derivatisation reaction, was dried under a gentle flow of nitrogen. The dried sample was processed by modifying a procedure proposed for short-chain carboxylic acids [19]. In particular, it was admixed with 20 μl of MTBSTFA and stirred for 5 min at room temperature. Then, 200 μl of isooctane were then added and the mixture was warmed at 60°C for 30 min. After cooling, hexadecane was added as an internal standard and 2 μl were injected into the gas chromatograph–mass spectrometer.

MTBSTFA addition followed by solvent dilution was the most suitable combination for the derivatisation reaction of both mono- and dicarboxylic acids, thus solving problems due to differences in acid solubilities. Fig. 1 shows the selected ion monitoring (SIM) chromatogram of the fatty acid diluted standard solution after derivatisation, obtained acquiring the ion fragment M-57. This particular fragment (*m*/*z*: 271.1, 257.1, 285.1, 345.1, 359.1, 313.2, 373.1, 339.2, 341.2 for tridecanoic, la, my, su, az, pa, se, ole, and st acids, respectively) is originated by the loss of a *tert*.-butyl group, and it was chosen for SIM acquisition since it was the most abundant in the mass spectrum of both mono- and di-TBDMS esters. These derivatives, obtained under quite mild experimental conditions, are stable for two days when stored at room temperature, and show linear calibration curves in the range 0.2–20 ng/mg, with a peak area reproducibility better than 6% at 0.5 ng/mg level. Low detection limits ranging between 0.03 and 0.1 ng/mg for la, my, pa, st and ole, and between 0.08 and 0.2 ng/mg for the dicarboxylic acids, characterise this procedure.

Analytical quality control was checked by running daily a fatty acid standard solution between samples and plotting all the data in control charts. The percentage content of fatty acids was obtained by ratioing the content of each acid in the final solution, expressed in ppm (ng/mg), to the total content of all eight.

2.6. Recovery of the overall analytical procedure

The overall procedure was tested by performing five analyses each of 1 mg raw samples of tripalmitin, linseed oil and dried egg yolk, whose lipid and protein composition is well known from the literature [20]. The recovery for proteinaceous material was calculated determining the concentration of the 14 amino acids in the dried egg yolk samples, and considering that the expected protein content is 31% (w/w) [21]. The recovery for lipid material was estimated by quantitatively determining the concentration of palmitic acid in tripalmitin, and the sum of palmitic and stearic acid concentration in linseed oil and egg yolk. Expected lipid amounts are related to the contents of palmitic and stearic acids, and were calculated taking into account that: the contribution of glycerol is 5% (w/w) for tripalmitin and egg

lipids, the total lipid content of dried egg yolk is 59%, and the content of saturated monocarboxylic fatty acids is 10% in linseed oil [12] and is 37% in egg yolk lipids [21,22].

Table 2 reports the results obtained, and shows that the final recovery was nearly 100% for proteins and 70% for lipids, with a relative standard deviation of less than 10% on five replicate measurements.

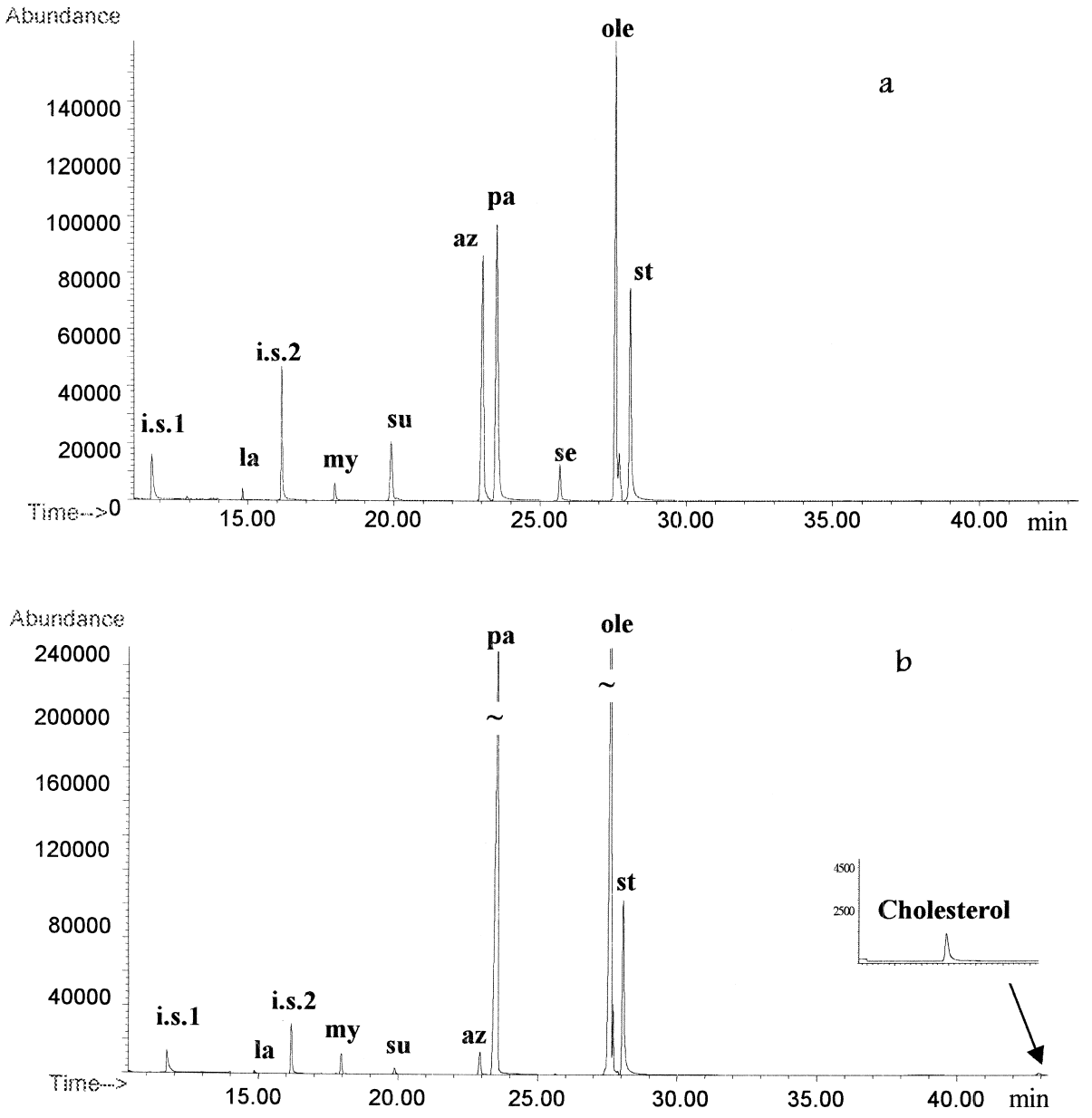

Fig. 2. Selected ion monitoring chromatogram of the derivatives of the fatty acid (a) from a sample containing linseed oil (1.0 mg), and (b) from a sample containing egg (1.4 mg).

3. Results and discussion

Using the analytical procedure described above it is possible to detect fourteen amino acids and eight fatty acids at the level of 0.1 ng. The saponification step which follows the acid hydrolysis improves the recoveries of fatty acids with respect to the procedures proposed in literature [15–17], which only use acid hydrolysis. The strong acid conditions, required in the hydrolysis step, imply that fatty acids are only partially released from lipids, because of the occurrence of equilibrium reactions [23] and of their low solubility. In fact, analysing an acid hydrolysate of tripalmitin it was found that the yield in palmitic acid was only about 25%, while a quantitative recovery was achieved following the analytical procedure described here (see Table 2).

The whole procedure was tested by analysing in triplicates the reference samples described in Table 1. The chromatograms in Fig. 2 show the fatty acid patterns obtained for a sample containing linseed oil (Ol, 1.0 mg) and another containing egg (Ui, 1.4 mg). Note that the drying oil is characterised by the presence of dicarboxylic acids az, su and se, which are practically absent in egg. These acids are formed during the drying process of the paint layer and come from cross-linking and oxidation reactions undergone by unsaturated fatty acids which are abundant in the fresh oil [1,11,13].

Egg binder, on the other hand, mainly contains pa, st and ole acids, and cholesterol is also detected by acquiring $m/z=443.3$ (M-57) in the time range 40–43 min, and used as a marker of the presence of egg yolk. Unfortunately, cholesterol cannot be quantitatively determined, because the yield of TBDMS-ether is incomplete and irreproducible, thus confirming data from the literature [23,24].

Table 4
Mean percentage contents[a] of fatty acids in reference wall painting samples

Sample	La	My	Pa	St	Ole	Su	Az	Se
Ol	0.1	0.7	20.4	14.9	40.4	3.4	20.1	2.3
BpOl	0.1	0.1	21.7	13.7	42.1	3.3	17.1	2.0
On	0.1	0.5	22.5	9.6	45.0	2.8	18.2	1.2
BpOn	0.1	0.3	21.0	9.9	45.2	3.0	19.6	1.1
Op	0.2	0.5	26.4	7.1	35.0	3.7	26.1	1.2
BpOp	0.1	0.4	26.2	6.8	32.4	4.7	27.8	1.9
Ui	0.1	0.6	34.0	11.2	53.1	0.2	0.9	0.0
BpUi	0.2	0.8	33.3	12.5	51.3	0.3	1.5	0.1
Tg	0.1	0.6	27.6	12.3	47.8	1.8	8.8	1.1
BpTg	0.1	0.6	27.4	13.7	46.7	1.3	9.7	0.7

[a] Data are average of three replicates, R.S.D.=2–9%.

Average amino acid and fatty acid relative percentage distributions for all the reference samples are reported in Tables 3 and 4, respectively. The most significant results are discussed in detail in the next sections.

3.1. Characterisation of proteinaceous binders

The data shown in Table 3 are in good agreement with those reported in the literature for amino acid egg distribution [1,10]. In order to verify the classification of the proteinaceous binder present in the sample, we applied PCA analysis to amino acid percentages, comparing the data obtained by this procedure with data obtained previously [6–9] and relative to egg, animal glue and milk (Fig. 3). The result shows that the samples containing egg binder and ''tempera grassa'', analysed by this method, are correctly classified in the egg cluster, which is well separated from milk and animal glue clusters. These

Table 3
Mean percentage contents[a] of amino acids in reference wall painting samples

Sample	Ala	Gly	Val	Leu	Ile	Met	Ser	Pro	Phe	Asp	Glu	Lys	Tyr
Ui	6.9	5.0	6.8	9.1	5.4	3.1	10.9	4.5	6.4	13.2	16.9	6.6	5.2
BpUi	6.3	5.1	5.2	10.9	7.1	2.3	9.2	5.0	7.9	13.9	15.9	6.7	4.2
Tg	5.3	3.9	5.5	9.0	5.9	1.8	12.2	5.7	5.3	14.3	19.7	6.5	5.6
BpTg	6.5	4.1	5.3	9.2	6.4	2.3	11.9	5.3	6.3	14.2	17.6	4.5	5.2

[a] Data average of three replicates, R.S.D.=5–13%.

results once again highlight the efficiency of this pattern recognition method.

3.2. Characterisation of lipid binders

The data shown in Table 4 highlight that:

1. Fatty acid concentrations are very similar in the absence and in the presence of lead white pigment. A graphical representation of all the data, once subjected to principal component analysis, is shown in Fig. 4: five clusters are spatially grouped and well characterise each binder, showing also that the procedure does not undergo pigment interference. Preliminary data obtained on samples containing smalt and cinnabar support this finding;
2. In the drying oils, the content of pa acid increases and that of st decreases in the following order: linseed, walnut and poppyseed oil; az represents about 20% of the total content and ole about 40%. Although these amounts seem quite high, they reveal that the drying process of the paint layer had not yet been completed. In fact, the total ion chromatogram showed the presence of unsaturated fatty acids that had not yet been oxidised. Therefore, the values herein reported for dicarboxylic and oleic acids are representative of the state of conservation of the reference samples analysed. The increase in dicarboxylic acids content (D) and the decrease in oleic acid content are to be expected during the ageing of these samples. Saturated fatty acids, being less reactive, undergo small changes in their content. The values of the pa to st acid ratio (P/S) may thus be used for oil identification, while the value of az to pa acid ratio (A/P) can be used to estimate how

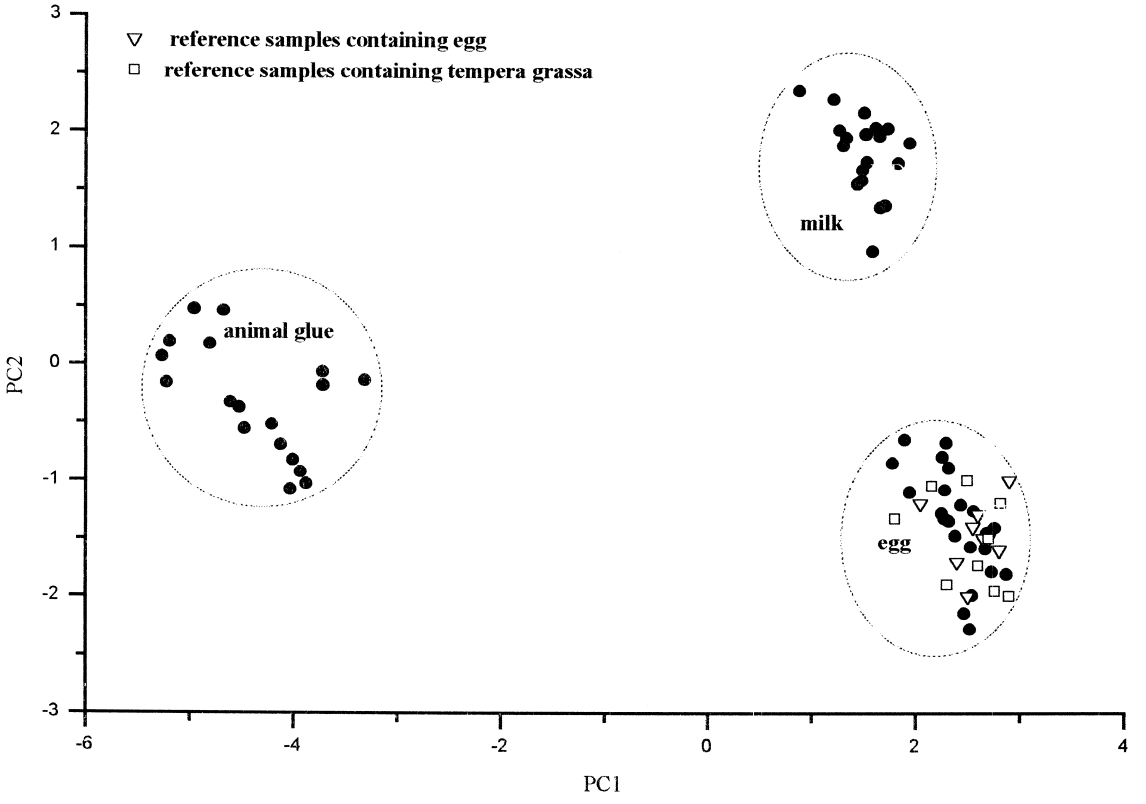

Fig. 3. PCA score plot of amino acid percentage data of reference samples containing egg, animal glue and milk binders (●) and of reference samples containing egg (▽) and ''tempera grassa'' (□) analysed according to the proposed procedure. Cumulative data variance on first two PCs 79%.

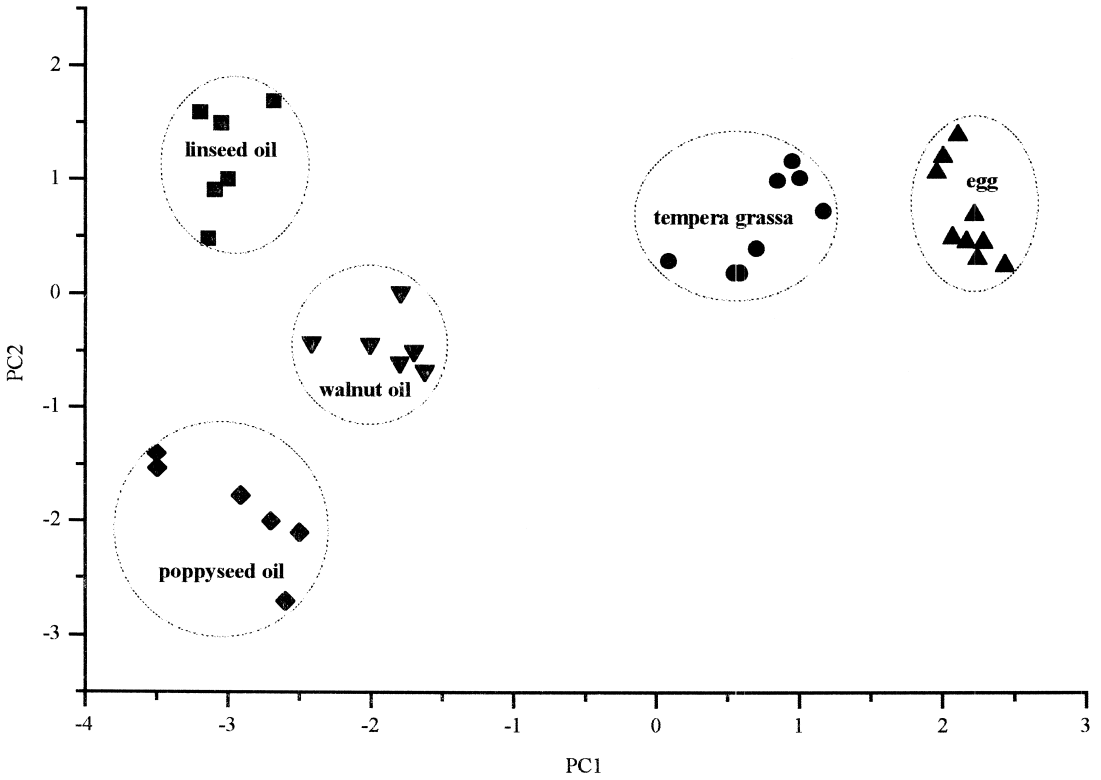

Fig. 4. PCA score plot of fatty acid percentage data of reference samples containing linseed oil (■), poppyseed oil (♦), walnut oil (▼), ''tempera grassa'' (●) and egg (▲). Cumulative data variance on first two PCs 81%.

much unsaturated fatty acids chain scission has occurred [1,13,14];

3. In the egg binder, the contents of pa and ole are about 34% and 53%; dicarboxylic acids are present in very low amounts (<2%) and their content may only increase a little with ageing, since egg contains low amounts of polyunsaturated acids. Thus, an A/P value lower than 0.05 together with the presence of cholesterol and the absence of significant amounts of dicarboxylic acids facilitates the identification of egg;
4. In the ''tempera grassa'', the content of fatty acids is very similar to that of egg binder except for an increase in dicarboxylic acids. P/S and A/P values depend on the relative amounts of binders used in the mixture. The identification of this binder is based on the presence of cholesterol and that of dicarboxylic acids, whose amounts in the sample have to be higher than 10%.

The PCA statistical approach, which is very useful for checking which cluster a sample belongs to and for highlighting *outliers,* cannot be used for identifying lipid binders in old samples. As mentioned above, the ageing changes the original composition of the paint layer due to the uptake of oxygen and the loss of volatile products with the disappearance of unsaturated acids and the formation of high amounts of dicarboxylic acids [13]. The different contents of these acids above all cause aged samples to fall outside the expected cluster. To get a reliable binder identification, P/S and A/P ratios together with the sum of azelaic, suberic and sebacic acid percentages (D) and the presence of cholesterol have to be taken into account. Table 5 summarises the values of these parameters for all the samples analysed. There is not much difference between P/S mean values of linseed and walnut oil but, since the relative standard deviation of P/S ratios is less than 12%, they can nevertheless be reliably distinguished. These data agree fairly well with those reported in

Table 5
Mean value of the palmitic to stearic acid ratios (P/S), azelaic to palmitic acid (A/P), and of the sum of azelaic, suberic and sebacic percentages (D) and the presence of cholesterol

Sample	P/S	A/P	D (%)	Cholesterol
Linseed oil	1.5	1.0	24	No
Walnut oil	2.2	0.9	23	No
Poppyseed oil	3.8	1.1	33	No
Egg	3.0	<0.05	1.5	Yes
''Tempera grassa''	2.1	0.3	12	Yes

the literature [1,11–14] and confirm the constancy of P/S ratios for oils from different sources and different stages of ageing. Thus, the following general criteria for binder recognition may be adopted:

(a) If $A/P>1$, $D>20\%$ and cholesterol is absent, a drying oil alone is present, and its identification can be based on the P/S values reported in Table 5

(b) If $A/P<0.1$ and $D<2\%$ the organic binder is a protein. If cholesterol is absent there is probably either some animal glue or egg white or a casein binder; if it is present, the binder is egg yolk or whole egg. These binders need to be confirmed by amino acid determination and PCA analysis;

(c) If $A/P>0.3$, $D>10\%$ and cholesterol is present, the organic binder is probably a ''tempera grassa''. The kind of oil used in ''tempera grassa'' should again be identified from the P/S value, which for the emulsion of linseed oil and egg used in reference sample is 2.1. Since this value depends on the relative amounts of egg and oil used by artists, a great variability of the ratio is to be expected.

3.3. Characterisation of binders in old wall paintings

The overall procedure was applied to four samples from ''*The Legend of the True Cross*'' wall painting and the results for amino and fatty acid distribution are reported in Table 6.

As far as amino acids determination is concerned, it should be stressed that in samples 4 and 8 most of the amino acids were detected near the detection limit, thus implying a very low protein content. The PCA analysis performed on amino acid relative percentages, shown in Fig. 5, highlights that the old samples are located on the upper left part of the cluster of egg binder: this behaviour reflects the small changes in amino acid pattern due to ageing [25]. Cholesterol was also found in all the samples, confirming the identification of the egg binder. A total proteinaceous content of 0.1 (0.01%, w/w), 5 (0.5%, w/w), 0.1 (0.01%, w/w) and 25 μg (4%, w/w) was estimated in samples 4, 7, 8 and 9 respectively. Table 7 reports the data for lipid identification, and following the pattern recognition schema, ''tempera grassa'' may be identified and linseed oil hypothesised in the mixture. Sample 9, which contains a higher amount of egg, also presents a higher P/S value and oleic acid content; on the other hand, samples 4 and 8, with an egg binder content less than 0.01% (w/w), show values more similar to linseed oil.

In conclusion, Piero della Francesca used ''tempera grassa'' as a dispersing binder of lead white as hypothesised by art historians [17], and he probably used varying amounts of egg and oil to obtain his bright, shimmering effects.

Table 6
Amino acid and fatty acid percentage distribution in samples from ''*The Legend of the True Cross*''

	Sample			
	4	7	8	9
Amino acids				
Ala	5.6	9.4	4.2	9.1
Gly	7.4	5.2	5.9	7.5
Val	3.7	11.7	6.8	9.8
Leu	6.9	13.7	7.3	14.3
Ile	9.4	7.5	5.5	8.6
Met	0	4.3	0.9	0.2
Ser	10.4	5.5	6.8	7.5
Pro	5.0	5.1	3.9	2.3
Phe	3.7	7.4	4.4	6.9
Asp	20.1	11.1	23.2	14.0
Glu	24.6	13.0	24.9	13.3
Lys	0	4.3	4.2	3.8
Hyp	0	0	0.0	0
Tyr	3.3	1.8	2.0	2.6
Fatty acids				
La	0.8	1.1	1.7	0.3
My	4.7	3.6	3.8	1.1
Pa	24.6	38.1	23.9	34.0
St	17.2	16.3	21.4	10.1
Ol	7.5	5.8	6.9	36.3
Su	13.5	10.8	12.0	4.0
Az	27.1	22.5	25.5	13.1
Se	4.6	1.8	4.8	0.9

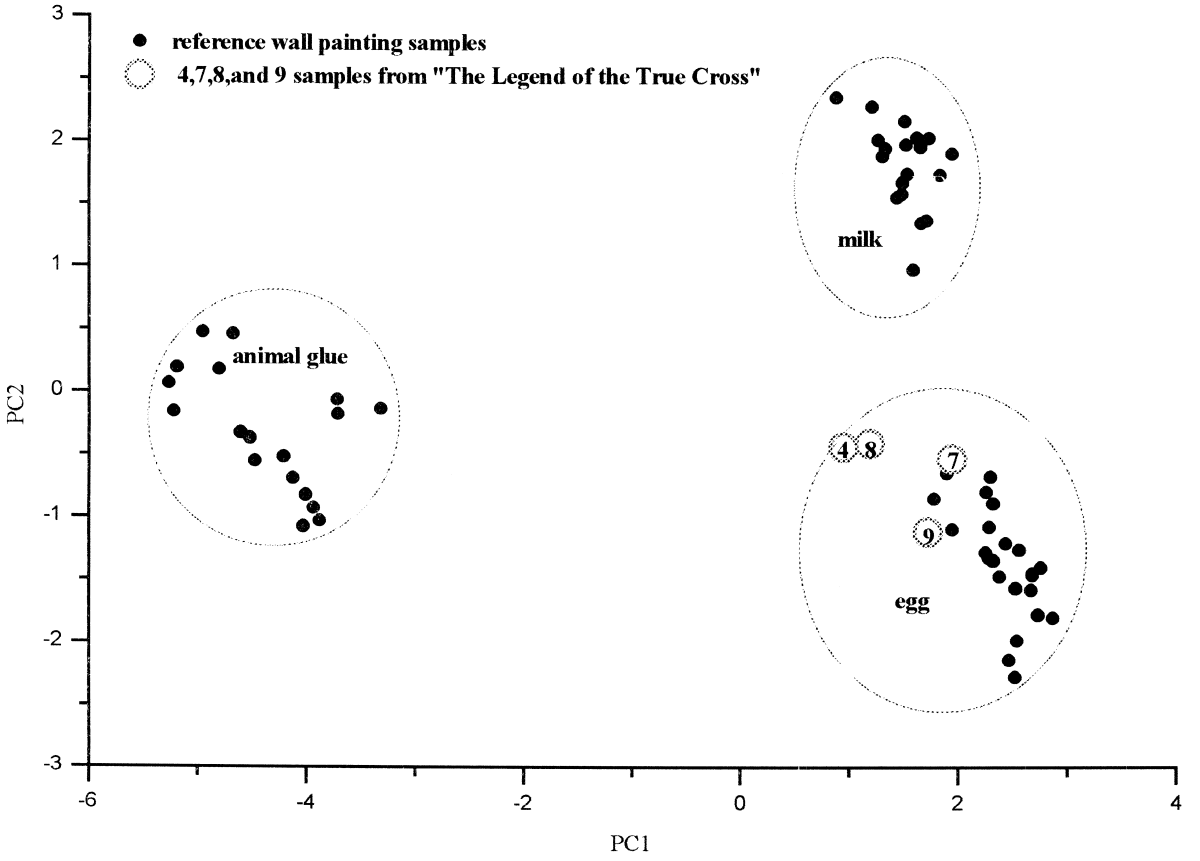

Fig. 5. PCA score plot of amino acid percentage data of reference samples containing egg, animal glue and milk binders (●) and of samples Nos. 4, 7, 8 and 9 from ''*The Legend of the True Cross*''.

Table 7
Values of parameters for the lipid identification in samples from ''*The Legend of the True Cross*''

Sample	*P*/*S*	*A*/*P*	*D* (%)	Cholesterol
4	1.3	0.9	45	Yes
7	2.3	0.6	36	Yes
8	1.1	1.1	42	Yes
9	3.4	0.4	18	Yes

4. Conclusions

The use of the proposed procedure allows the characterisation of proteinaceous binders and drying oils present in the same painting sample from any work of art. Reliable results in terms of accuracy, repeatability and sensitivity are obtained for the determination of amino acids and fatty acids. The method provides satisfactory recoveries for the considered binders, overcoming the problems connected with equilibrium and solubility of lipids in the acid hydrolysate. Moreover, the silyl reagent (MTBSTFA) used is able to silylate amino acids, mono- and dicarboxylic acids, and partially cholesterol. The analysis of a wide collection of reference wall painting samples (this kind of artistic samples contains the lowest amounts of organic material) allows one not only to optimise the analytical procedure, but also to collect data for developing a binder identification pattern. Since the content of amino acids does not change significantly with ageing, PCA analysis of their contents is a valuable tool for the characterisation of the proteinaceous binder. The identification of drying oils has to be based on *P*/*S* values and dicarboxylic acid contents,

whose concentrations depend on the state of conservation of the artistic object. A pattern recognition scheme based on selected values of A/P, P/S and D, and on the presence/absence of cholesterol, reliably allows one to distinguish between proteinaceous binders, ''tempera grassa'' and drying oils. The identification of the oil used in ''tempera grassa'' may be doubtful because artists may have prepared emulsions with an egg to oil ratio different from the 3:1 such as used in the reference samples.

The identification of ''tempera grassa'', most probably made by linseed oil and egg, in samples from ''*The Legend of the True Cross*'' wall painting confirms the hypotheses made by art historians and provides restorers with valuable information for planning conservation.

Acknowledgements

The authors gratefully acknowledge the National Research Council (Committee for ''Science and Technology for the Cultural Heritage'' and the project ''Safeguard of Cultural Heritage'') along with the University of Pisa for financial support.

References

[1] J.S. Mills, R. White, The Organic Chemistry of Museum Objects, Buttersworth, London, 1994.
[2] P. Bensi, in: C. Danti, M. Matteini, A. Moles (Eds.), Le Pitture Murali, Centro Di, Firenze, 1990, p. 73.
[3] M. Matteini, A. Moles, La Chimica nel Restauro, Nardini, Firenze, 1989.
[4] R. White, J. Kirby, Nat. Gallery Techn. Bull. 15 (1994) 64.
[5] S.L. Vallance, Analyst 122 (1997) 75R.
[6] M.P. Colombini, R. Fuoco, A. Giacomelli, B. Muscatello, Stud. Cons. 43 (1998) 33.
[7] M.P. Colombini, R. Fuoco, A. Giacomelli, B. Muscatello, N. Fanelli, Sci. Technol. Cultural Heritage 7 (1998) 1.
[8] M.P. Colombini, R. Fuoco, F. Modugno, in: Proceedings of 2nd National School of Chemistry for Cultural Heritage, Pisa, 1997, p. 67.
[9] M.P. Colombini, R. Fuoco, F. Modugno, accepted by J. Chromatogr. A (1999) in press.
[10] M.R. Shilling, H.P. Kanjian, L.A.C. Souza, J. Am. Inst. Conservation 35 (1996) 45.
[11] J. Mills, Stud. Cons. 11 (1966) 92.
[12] M.R. Shilling, H.P. Kanjian, in: 11th Triennial Meeting, Edinburgh, ICOM Committee for Conservation Preprints, Vol. 1, James and James, London, 1996, p. 220.
[13] M.R. Shilling, H.P. Kanjian, D.M. Carson, Techne 5 (1997) 71.
[14] N. Khandekar, A. Phenix, J. Sharp, Conservator 18 (1994) 62.
[15] W. Nowik, Stud. Cons. 40 (1995) 120.
[16] A. Casoli, P.C. Musini, G. Palla, J. Chromatogr. A 731 (1996) 237.
[17] R. Bellucci, C. Frosinini, in: A. Roy, P. Smith (Eds.), Painting Tecniques History, Materials and Studio Practice, The International Institute for Conservation of Historic and Artistic Works, London, 1998, p. 89.
[18] M. Matteini (Opificio delle Pietre Dure Scientific Laboratory, Florence, Italy), personal communication.
[19] Y. Ghoos, B. Geypens, M. Hiele, P. Rutgeerts, G. Vantrappen, Anal. Chim. Acta 247 (1991) 223.
[20] R.R. Allen et al., in: D. Swern (Ed.), Bailey's Industrial Oil and Fat Products, Vol. 2, Wiley, New York, 1992.
[21] P. Cok, B. de Bernard, O. Radillo, M.P. Francescato, Synoptic Food Composition Tables, Piccin, Padova, Italy, 1987.
[22] M.I. Aveldano, L.A. Horrocks, J. Lipid Res. 24 (1983) 1101.
[23] J.M. Halket, in: K. Blau, J.M. Halket (Eds.), Handbook of Derivatives for Chromatography, 2nd ed, Wiley, Chichester, 1993, p. 297.
[24] R.W. Kelly, P.L. Taylor, Anal. Chem. 48 (1976) 465.
[25] A. Karpowicz, Stud. Cons. 26 (1981) 153.