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Behaviour of phospholipids in analytical reactive pyrolysis

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Cultural Heritage Special Chapter

Abstract Checking for the presence of egg in a painting layer allows to decide whether or not it is a tempera. Several already assessed analytical techniques may be used to perform the chemical analysis for the detection of egg in paintings. As an advantageous and alternative methodology for the determination of egg, a new application of analytical pyrolysis, hyphenated with gas chromatography–mass spectrometry (GC–MS) system, in presence of hexamethyldisilazane (HMDS) and tetramethylammonium-hydroxide (TMAH), is reported here. The innovation lays mainly in the choice of new markers for the presence of egg. It is here demonstrated that in art diagnostic tris-TMS-ester and methyl ester of phosphoric acid, generated by the pyrolysis of standard phospholipids and synthetic painting layers containing egg as binding medium, may be used as new markers for identification of egg in tempera layers. The adoption of these new markers in analytical pyrolysis allows to obtain higher analytical performance with respect to classical markers (fatty acids), especially in terms of yield and, as a consequence, in terms of limit of detection.

Keywords Gas chromatography–mass spectrometry · Pyrolysis · Derivatisation · Phospholipids · Tempera · Pigments

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Introduction

Egg is a very complex matrix consisting of two separated phases: egg-white and egg-yolk. Egg-white is an aqueous colloidal solution of proteins, mainly albumins, and low quantities of fats, while egg-yolk can be considered as an emulsion, that is a colloidal dispersion of phosphorated proteins and lipids. The lipidic fraction is the most consistent in egg-yolk, and is made up of triglycerides (65% w/w), phospholipids (29% w/w) and cholesterol (5.2% w/w) [1, 2]. The phospholipids are triglycerides in which a phosphoric group esterifies one hydroxyl group of glycerol. This gives rise to a mono-glyceride of the tribasic phosphoric acid further combined with other compounds. When phosphoric acid is esterified with coline, the resulting groups of compounds are called *lecithin*, mainly contained in egg-yolk, whose general formula is reported in Fig. 1.

The concentration of elementary phosphorus is equivalent to 0.9% w/w of dried whole egg [1].

The presence of egg in a painting layer may be evidenced in analytical pyrolysis, as reported in previous articles [3–6], using as markers in the high concentration of palmitic acid and stearic acid, the presence in low concentration of indole and methyl indole and cholesteryl compounds in widely variable concentrations.

Low concentration of azelaic acid allows distinguishing egg from siccative oil painting layers, where the intensity of the pyrographic peak of this acid is usually higher: this marker it is not sometimes sufficiently reliable because the concentration of the dicarboxylic products may be low, when the oil layer is fresh.

Other articles in literature deal with pyrolysis of egg-painting layers [7–9].

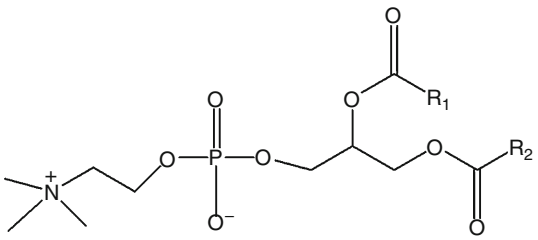

Fig. 1 Example of a phosphatidylcholine, a type of phospholipid in lecithin (R_x refers to a hydrocarbon group)

The detection of egg in painting layers is commonly performed by means of the characterisation of the amino-acid content and sometimes of the total fatty acids composition with gas chromatography–mass spectrometry (GC–MS) analysis [10–14].

Spectroscopic techniques, like for instance Micro Raman analysis [15], are also used for the characterisation of the ligands.

Van den Brick et al. used matrix-assisted laser desorption ionisation–Fourier-transform mass spectrometry to detect the alteration products from diacylphosphatidylcholines and triacylglycerols in egg tempera paintings for monitoring indoor museum conditions [16].

The determination of phospholipids is important in several fields: marine geochemistry [17], pharmaceutical research [18, 19], biology [20, 21] and studies concerning fat materials [22–27].

The phospholipids content has been taken into consideration in some articles to study egg-painting layers, considering for instance the concentration of phosphorus [28], but the elementary phosphorus can arise even from some pigments.

The conventional method for the determination of phospholipids is known as the acid digest/arseno-molybdate Barlett method and it consists in phosphorus analysis.

Several methods are available for the determination of phospholipids using thin-layer chromatography [17, 21, 23, 27], Fourier-transform infrared spectroscopy (FTIR) [22], high-performance liquid chromatography [19] and gas–liquid chromatography for the characterisation of the fatty acids composition [23], colorimetric methods for the analysis of organic phosphorus complexed with a chromogenic reagent [26].

In this article, pyrolysis–methylation [29] and pyrolysis–silylation [30–32] are described as useful techniques to identify the presence of phospholipids by the methyl ester and tris-TMS-ester phosphoric acid that these analytes produce when undergoing pyrolysis. The identification of this product of pyrolysis has been carried out and it has been stated that it can be a useful marker for the detection of egg in painting layers.

Materials and methods

The derivatising reagents used, tetramethylammonium-hydroxide (TMAH) 25% w/v water solution and Hexamethyldisilazane (HMDS), 98% w/v, were from Aldrich.

Standard phospholipids, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine and 1,2-dimyristoyl-rac-glycero-3-phosphocholine, were obtained from Aldrich.

Samples of painting standard layers, which are more than 10 years old, were prepared by the Opificio delle Pietre Dure (Florence, Italy) following ancient recipes.

Samples (0.5 mg) were scraped from the support, inserted into a quartz capillary tube and added with 5 μL of derivatising reagent prior to pyrolysis.

Pyrolysis was carried out at 600 °C for 10 s at the maximum heating rate using a CDS 1000 pyroprobe heated filament pyrolyser (Chemical Data System, Oxford, USA), directly connected to the injection port of a Varian 3400 gas-chromatograph coupled to a Saturn II ion-trap mass spectrometer (Varian Analytical Instruments, Walnut Creek, USA). A Supelco SPB5 capillary column (30 m, 0.25 mm I.D., 0.25 μm film thicknesses) was used with a temperature programme from 50 °C (held for 2 min) to 310 °C (held for 5 min) at 5 °C min^{-1} with helium as carrier gas. Temperatures of split/splitless injector (split mode) and Py–GC interface were kept at 250 °C. The Py–GC interface was kept at 250 °C and the injection port at 250 °C. Injection mode was split (1:50 split ratio) and gas carrier was helium at flow rate of 1.0 mL min^{-1}. Mass spectra were recorded at 1 scan s^{-1} under electron impact at 70 eV, scan range 45–650 *m/z*. Structural assignment of the pyrolytical fragments was based on match with the NIST mass spectra library or literature data; the principal products identified are listed in Tables 1 and 2.

Results and discussion

Pyrolysis–methylation

Analysis of standard phospholipids

Figures 2 and 3 show the pyrolytical patterns related to pyrolysis–methylation of standard phospholipids, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine and 1,2-dimyristoyl-rac-glycero-3-phosphocholine, and the chromatographic and mass spectrometric data of the main peaks are reported in Table 1.

Analytical pyrolysis in presence of TMAH is a powerful technique to characterise different kinds of organic compounds, but it has been pointed out that its strong alkalinity ($pK_b = 0.9$) [33] causes some secondary reactions such as degradation or isomerisation. It is likely that this behaviour

Table 1 Products arising from pyrolysis–methylation of the samples

Peak No.	Fragment	M_w	Characteristic ions *m/z* (relative abundance)[a]
1	Phosphoric acid, trimethyl ester	140	65 (7), 79 (43), 95 (30), 110 (100), **140** (15)
2	Octanoic acid, methyl ester	158	74 (100), 87 (43), 115 (23), 158 (2), **159** (12)
3	Nonanoic acid, methyl ester	172	74 (100), 87 (47), 143 (16), **172** (5), 173 (14)
4	Octanedioic acid, dimethyl ester	202	129 (44), 138 (39), 171 (77), **202** (8), 203 (100)
5	Dodecanoic acid, methyl ester	214	74 (100), 87 (60), 143 (16), 171 (13), **214** (13)
6	Nonanedioic acid, dimethyl ester	216	55 (77), 152 (100), 185 (86), **216** (10), 217 (67)
7	Tetradecanoic acid, methyl ester	242	74 (100), 87 (66), 143 (28), 199 (18), **242** (18)
8	9-Hexadecenoic acid, methyl ester	268	55 (100), 83 (45), 96 (52), 236 (28), **268** (7)
9	Hexadecanoic acid, methyl ester	270	74 (100), 87 (67), 143 (25), 227 (15), **270** (27)
10	9-Octadecenoic acid, methyl ester	296	55 (100), 69 (63), 83 (55), 264 (17), **296** (5)
11	Octadecanoic acid, methyl ester	298	74 (100), 87 (71), 143 (30), 155 (18), **298** (40)

[a] The value in bold indicate the molecular ion

Table 2 Products arising from pyrolysis–silylation of the samples

Peak No.	Fragment	M_w	Characteristic ions *m/z* (relative abundance)[a]
1	Phosphoric acid, bis-TMS-monomethyl ester	256	73 (18), 133 (16), 211 (14), 241 (100), **256** (15)
2	Phosphoric acid, tris-TMS-ester	314	73 (60), 283 (8), 299 (100), 300 (24), **314** (16)
3	Octanedioic acid, bis-TMS-ester	318	75 (100), 169 (36), 187 (50), 303 (63), **318** (3)
4	Nonanedioic acid, bis-TMS-ester	332	73 (47), 201 (27), 317 (100), **332** (2), 333 (23)
5	Tetradecanoic acid, TMS-ester	300	73 (68), 117 (100), 129 (39), 285 (59), **300** (8)
6	9-Hexadecenoic acid, TMS-ester	326	73 (100), 117 (72), 129 (59), 311 (41), **326** (8)
7	Hexadecanoic acid, TMS-ester	328	73 (49), 117 (100), 129 (38), 313 (46), **328** (22)
8	9-Octadecenoic acid, TMS-ester	354	73 (100), 117 (64), 129 (57), 339 (30), **354** (6)
9	Octadecanoic acid, TMS-ester	356	73 (65), 117 (100), 129 (40), 341 (57), **356** (22)

[a] The value in bold indicate the molecular ions

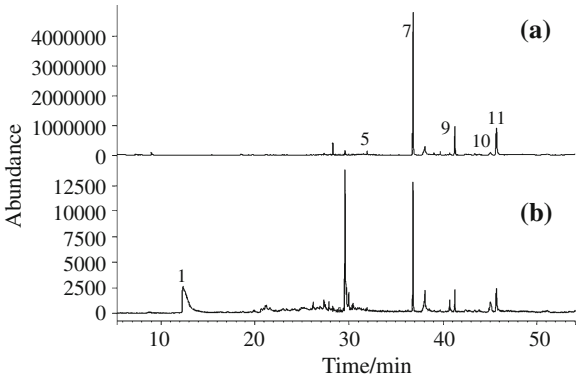

Fig. 2 **a** Reconstructed ion chromatogram arising from pyrolysis–methylation of 1,2-dimyristoyl-rac-glycero-3-phosphocholine. **b** Single ion monitoring for *m/z* (110 + 140) the ions deriving from phosphoric acid, trimethyl ester

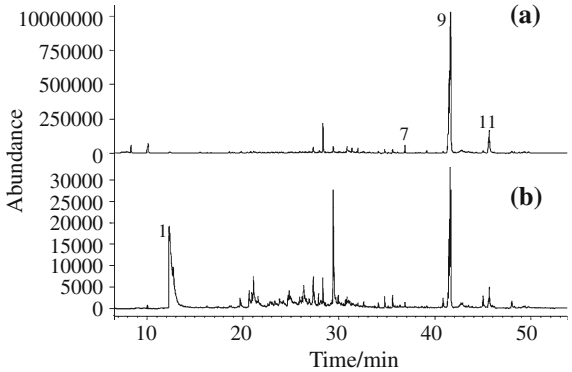

Fig. 3 **a** Reconstructed ion chromatogram arising from pyrolysis–methylation of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine. **b** Single ion monitoring for *m/z* (110 + 140) the ions deriving from phosphoric acid, trimethyl ester

leads to a low yield in trimethyl phosphate, so that it is advisable to use the Single Ion Monitoring (SIM) relative to the characteristic ions of the trimethyl phosphate to detect its presence in a sample.

In spite of this situation, the recognition of the phosphate is still possible thanks to a good specifity of the mass spectrum. The mass spectrum of trimethylphosphate (Fig. 4) shows the peak at 110 *m/z* as the main peak derived from the molecular ion by the loss of a neutral formaldehyde molecule.

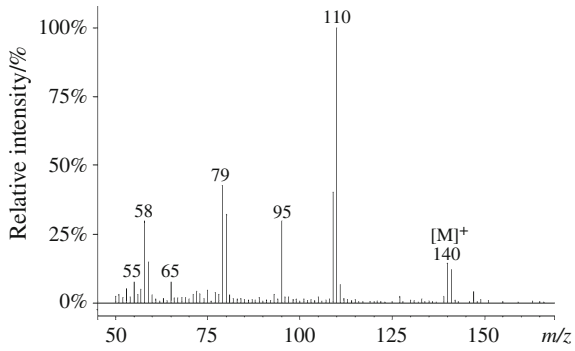

Fig. 4 Mass spectrum of phosphate, trimethyl ester

Analysis of standard painting layers

The result, obtained with the pyrolysis–methylation of a standard painting layer prepared from whole egg (Fig. 5, Table 1), has confirmed the trend highlighted for the standard phospholipids: the presence of the peak corresponding to the phosphoric acid, trimethyl ester, is evident only in the single ion monitoring.

In Fig. 6, the single ion monitoring for *m/z* (110 + 140) arising from pyrolysis–methylation of standard painting layers prepared from whole egg with different pigments such as malachite [$Cu_2(CO_3)(OH)_2$], smalt blue [SiO_2,-K_2O,Al_2O_3,CoO], azurite [$Cu_3(CO_3)_2(OH)_2$] and cinnabar [HgS] are reported.

The presence of a pigment changes the yield of trimethyl phosphate, in particular regarding smalt blue and cinnabar. This behaviour can be explained considering the fact that some inorganic salts, like some painting pigments, can affect the formation of some pyrolysis products or lead to secondary pyrolysis reaction [34, 35]: the ability of the powder pigment to influence the pyrolitic behaviour could not be excluded, and can justify the difficulty to detect the presence of phosphate when this technique is used for recognition of egg in tempera layers.

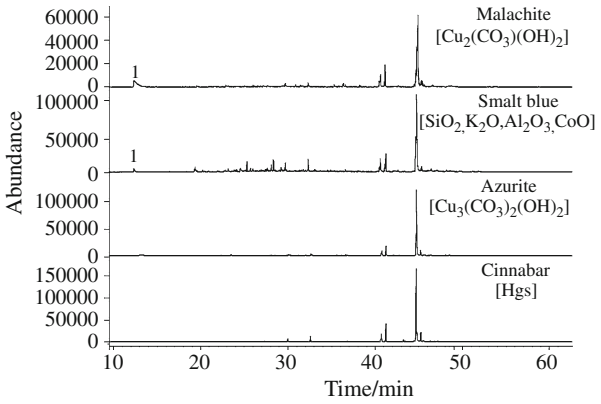

Fig. 6 Single ion monitoring for *m/z* (110 + 140) arising from pyrolysis–methylation of standard painting layers prepared from whole egg with different pigments: malachite, smalt blue, azurite and cinnabar

Pyrolysis–silylation

Analysis of standard phospholipids

In Figs. 7 and 8 (Table 2) the pyrolytical patterns related to the pyrolysis–silylation of the standard phospholipids, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine and 1,2-dimyristoyl-rac-glycero-3-phosphocholine are reported.

The chromatographic profile obtained in both cases shows that the use of a derivatising reagent less aggressive than TMAH leads to an increased yield of tris-TMS-phosphate, which is clearly visible in the total ion chromatogram: the peak 2 (Fig. 9) has a molecular peak corresponding at 314 *m/z*, which is the molecular weight of tris-TMS-phosphate with base peak 299 *m/z* obtained from the loss of a methyl group.

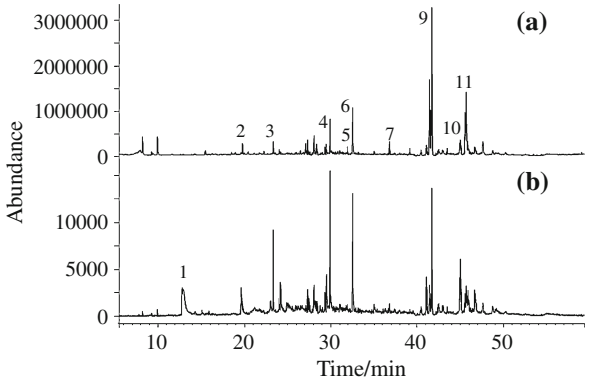

Fig. 5 **a** Reconstructed ion chromatogram arising from pyrolysis–methylation of standard painting layer prepared from whole egg. **b** Single ion monitoring for *m/z* (110 + 140) the ions deriving from phosphoric acid, trimethyl ester

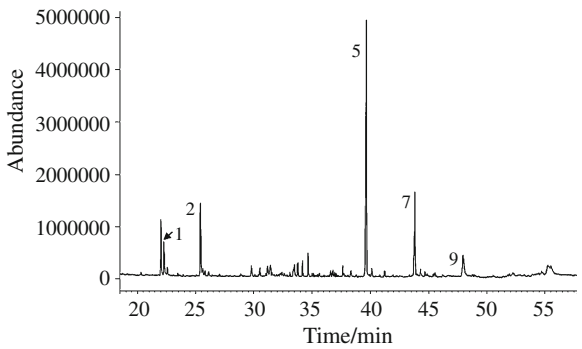

Fig. 7 Reconstructed ion chromatogram arising from pyrolysis–silylation of 1,2-dimyristoyl-rac-glycero-3-phosphocholine

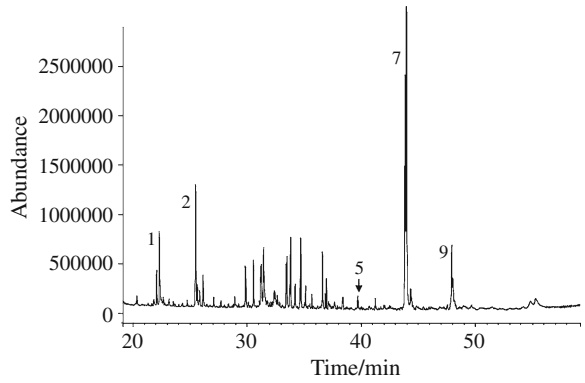

Fig. 8 Reconstructed ion chromatogram arising from pyrolysis–silylation of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine

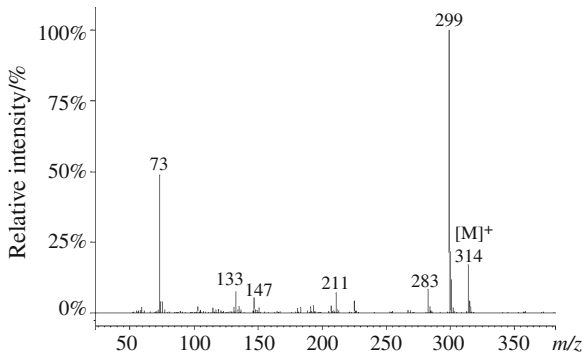

Fig. 9 Mass spectrum of phosphoric acid, tris-TMS-ester

Analysis of standard painting layers

Figure 10 reports the reconstructed ion chromatogram arising from pyrolysis–silylation of a standard painting layer prepared from whole egg.

The result confirms the hypothesis expressed in the case of standard phospholipids: analytical pyrolysis performed in the presence of HMDS leads to the formation of a greater amount of phosphate. The corresponding peak (Fig. 10, peak 2) is the most intense and gives the mass spectrum reported in Fig. 11:

The consistent peak at 387 *m/z* may be attributed to an association ion $M(TMS)^+$: we have found that the TMS^+ gives such type of association with the molecular compound, working with ion-trap detector.

In Fig. 12, the total ion chromatograms arising from pyrolysis–silylation of standard painting layers prepared from whole egg with different pigment—malachite [$Cu_2(CO_3)(OH)_2$], smalt blue [SiO_2,K_2O,Al_2O_3,CoO], azurite [$Cu_3(CO_3)_2(OH)_2$] and cinnabar [HgS]—are reported.

As in the case of pyrolysis–methylation, the effect of the pigment on the formation of tris-TMS-phosphate is evident; in particular, it is clear that the presence of smalt blue as that of cinnabar is associated with the lowest intensity of the peaks.

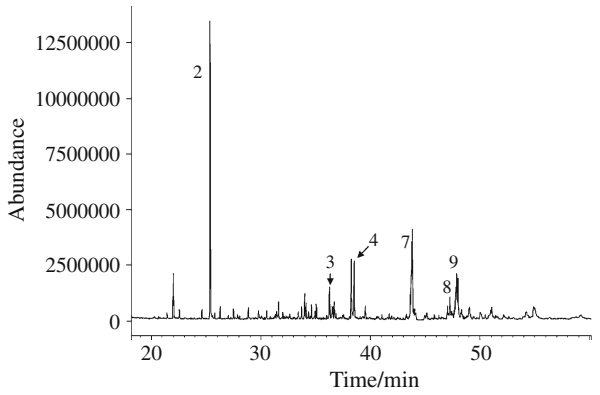

Fig. 10 Reconstructed ion chromatogram arising from pyrolysis–silylation of standard painting layers prepared from whole egg

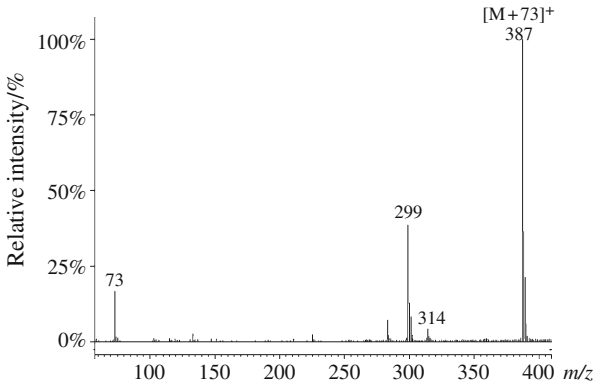

Fig. 11 Mass spectrum of compound corresponding to the peak 2

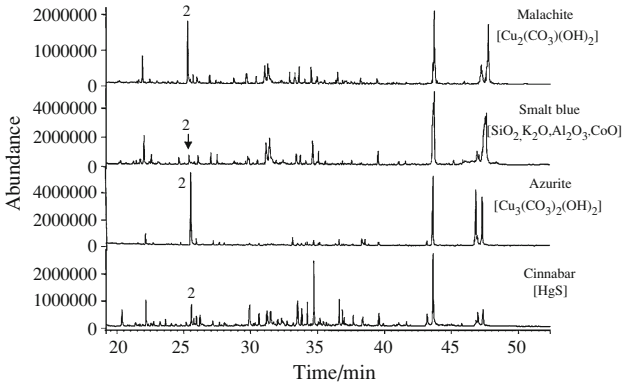

Fig. 12 Reconstructed ion chromatogram arising from pyrolysis–silylation of standard painting layers prepared from whole egg with different pigments: malachite, smalt blue, azurite and cinnabar

Conclusions

The study here presented shows a new application of analytical pyrolysis in the characterisation of complex

organic compounds such as the egg-yolk largely used in the old painting technique as ligand.

We have compared two of the reagents usually used in pyrolysis for the speciation of high polar compounds: TMAH as methylating reagent and HMDS as sylilating reagent. Both the reagents give a positive recognition of phosphoric acid esters present in lipidic structures with the formation of trimethyl phosphate and tris-TMS-phosphate, offering a further potential marker for the analysis of painting ligands.

However, the silylation appears to be more useful, thanks to the higher yields obtained.

Considering the positive results obtained with OPD tempera layers, the next objective is the application to the real painting cases but, until now, we have obtained scarce results probably because of the difficulties to find reliable ancient tempera sample and the high degradability that distinguishes this kind of compounds.

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