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An evaluation of the impact of urban air pollution on paint dosimeters by tracking changes in the lipid MALDI-TOF mass spectra profile

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ABSTRACT

We evaluated the impact of urban air pollution on egg yolk tempera paint dosimeters (binary mixture samples made with historic artist´s blue, red and white pigments) by tracking changes over time in their lipid matrix-assisted laser desorption ionization time-of-flight mass spectra (MALDI-TOF-MS) profiles. We studied triacylglycerols (TGs), phospholipids (PLs) and their oxidation by-products from paint dosimeters that had been exposed outdoors for six months to the polluted atmosphere in the city center of Granada (Spain). Four types of chickens' eggs were also analyzed to find out whether their lipid mass spectra (lipid fingerprints) varied significantly. The ultimate goal of this research is to provide a precise analytical protocol to show whether the changes in the egg yolk identified in paint dosimeters are due to pigment-binder interactions. The Bligh-Dyer (BD) method was optimized for the extraction of the lipids. This innovative procedure included a washing-step prior to the mass spectrometric analysis, which proved crucial for obtaining higher quality lipid fingerprints. A novel interpretation of the results is proposed by applying the BD method, which suggests that transesterification processes occurred in the lipid fractions that were catalyzed by the pigments in the paint dosimeters. In blank dosimeters specific ions produced by oxidative cleavage of PLs and/or TGs may be used as markers of the presence of egg yolk binders. The composition and structure of the specific lipid compounds are also tentatively proposed. In aged dosimeters the intact content of the TGs and PLs decreased; however, we propose that short-chain oxidative products arising from TGs and PLs are present in all the samples, except for the white lead based dosimeter. We end with a new explanation as to why this dosimeter behaves differently from the others.

1. Introduction

One of the greatest hazards for open-air paintings on the buildings that form part of our architectural heritage is the darkening and degradation of colors caused by urban pollution, aggressive marine atmospheres and climate change. This problem is intensified in hot Mediterranean coastal cities with heavy traffic, such as those in southern Spain. Color changes can be caused by chemical or mineralogical transformation of pigments, oxidation of organic binders, precipitation of new mineral phases (including salts) and dust or pollen accumulation on the surface of the paintings, often producing irreversible aesthetic damage [1]. Preventive conservation of these ancient paintings is becoming increasingly important nowadays, requiring complete characterization of the artwork, its microclimatological and atmospheric environment, and of the interactions at the artwork-atmosphere interface using a multi-analytical and multidisciplinary approach. However, and in spite of the clear financial savings associated with preventive conservation as compared to restoration once damage has been done, few studies have addressed the effects of atmospheric aerosols (gas and particulate matter) and/or climate change on the surface of outdoor paintings [1–5]. Moreover, up to now, preventive conservation to deal with the impact of atmospheric pollution has focused almost exclusively on indoor research in museums, churches, art galleries, etc. where the environmental conditions (temperature, relative humidity and luminosity) and air quality are closely controlled, and any color changes in the paintings can be evaluated [3,6,7].

The characterization of historical paintings is quite challenging as they often have a complex composition and structure, in which painting materials such as inorganic/organic pigments, mineral fillers and organic binders are closely mixed, in addition to the small amount of sample permitted for use in research. These limitations mean that scientific research can only advance with the

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aid of model paint samples, known as paint dosimeters or mock-ups. Various studies have focused on the use of naturally aged paint dosimeter inside museums, aimed at characterizing organic compounds and varnish [3,6,7]. However, to our knowledge there are no papers on the use of paint dosimeters to evaluate the damage caused by outdoor exposure to urban air pollution.

The simultaneous characterization of pigments and organic binders in the same paint (layer) sample, either original or laboratory prepared, is also unusual. Only recently have researchers begun to describe the processes of interaction between pigments and binders in both historical paintings and dosimeters –artificially aged under UV irradiation-, examining the role of inorganic pigments in the weathering process of organic binders [8–14]. While pigments can be characterized quickly and accurately, the identification of organic binders and their ageing in paint samples is quite problematic for various reasons: (i) they appear in smaller amounts than inorganic pigments in the paint layers (the normal pigment to binder ratio is ca. 3:1), thus the occurrence of a high quantity of pigments, mineral fillers and desiccants could complicate accurate identification of the binder, and (ii) organic binders are less stable than mineral pigments and hence deteriorate faster, making identification more complex.

The identification of the organic binders used in paintings is crucial when it comes to identifying the technique used by the artist and deciding on the most suitable conservation/restoration treatments. Although numerous studies have addressed the question of how to identify the different organic binders (i.e. oils, proteins, waxes or gums), less research has been done on the in-depth characterization of their ageing processes, particularly for proteinaceous binders as a result of their intricate composition and structure [8,14–19].

To date, proteinaceous binders have been studied using an array of analytical techniques such as Fluorescence Spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), Raman Microscopy (RM), Gas Chromatography (GC) and Liquid Chromatography (HPLC) [6,8,14,18,20,21]. Mass spectrometry (MS) also plays an important role in their characterization [14,22–25]. Of the various ionization techniques used in the analysis of proteinaceous binders, Matrix-Assisted Laser Desorption Ionization (MALDI) coupled with Time of Flight Mass Spectrometers (MALDI-TOF-MS) has become increasingly popular over the last decade in the characterization of organic binders used in artwork [12,14,26]. Pioneering research applying this technique to paint samples used specific enzymatic cleavage (trypsin) to break the bonds of the amino acid compounds of those proteins, so obtaining peptides. The peptides were then compared using the relevant databases to discover the source of the proteins found in the paint samples [12,27]. More recent authors applied MALDI-TOF-MS to analyze various kinds of binders, for instance rabbit blue, in paint dosimeters artificially aged with UV radiation [14] and oils from real easel paint samples [28].

A common organic binder used by artists for many centuries is egg yolk, which is about 70% lipid and 30% protein [29]. The correct characterization of egg-yolk therefore involves analyzing both components. Specialists in this field have proposed several analytical protocols based on different extraction methods followed by MALDI-TOF-MS analysis to investigate egg yolk-based (tempera) paintings [22,23,30]. In the framework of two Research Projects aimed at analyzing pigment-binder interactions in real polychromes and paint dosimeters exposed at open and semi-open monuments in the polluted urban atmosphere of Granada (southern Spain), we applied these analytical procedures to typify tempera dosimeters (binary mixtures of pigments mixed with pure egg-yolk) that we had made in the laboratory. Replication of published protocols was only possible when the linear mode of MALDI-TOF-MS was applied, but this provided limited resolution spectra leading to low mass accuracy. In complex paint mixtures it is better to perform in-depth analyses of organic binders using higher resolution MS data in reflectron mode for more accurate, more reliable peptide mass fingerprinting (PMF) [14,27].

The aim of this paper is therefore to present an optimized extraction method for MALDI-TOF mass spectrometry in order to analyze the lipid fraction of blank egg-yolk-based paint dosimeters and to track changes when they are naturally aged for long periods in an urban atmosphere. We also analyzed four types of chickens' eggs to find out whether their lipid fingerprints varied significantly. The final goal is to provide a precise accurate analytical protocol, which proves that the changes in the egg yolk identified in the paint samples are due to pigment-binder interactions.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were of analytical reagent grade unless stated otherwise. Reverse-osmosis quality water (purified with a Milli-RO plus Milli-Q station from Millipore Corp., Madrid, Spain) was used throughout. Methanol, chloroform, acetonitrile, ammonium phosphate dibasic and diammonium citrate were supplied by Panreac (Barcelona, Spain). α-cyano-4-hydroxycinnamic acid (A-CHCA) matrix, sinapinic acid (SA) matrix, 2,5-dihydroxybenzoic acid (DHB) matrix and trifluoroacetic acid (TFA) were all from Sigma Aldrich (Barcelona, Spain).

2.2. Paint dosimeters

Twenty paint dosimeters were prepared according to'Old Master recipes' to obtain egg yolk tempera painting standards similar to those used by medieval artists [31]. Tempera is a painting technique in which finely ground pigments are first mixed with water and later blended with a solidifying proteinaceous binding agent, in our case egg yolk [32]. The first nine model samples were pure pigments and the tenth was the pure egg yolk binder. The ten remaining model samples were binary mixture paint samples composed of each of the pure pigments mixed with the egg yolk binder. We selected three blue pigments (azurite, malachite and lapis lazuli), three red pigments (cinnabar, minium and hematite), and three white pigments (lead white, calcite and gypsum) for analysis. All pigments were purchased from Kremer Pigments GmbH & Co. KG (Madrid, Spain).

To prepare the binder, the egg yolk was separated from the white using the usual method of pouring it back and forth in the half shells. It was then rolled onto a paper tissue to remove the layer of clinging egg white and most of the chalazae. The skin was punctured at the bottom using a pin and the liquid content poured into a jar. The preparation of the tempera paint dosimeters was as follows: approximately 0.5 g of each pigment powder was placed in a small bowl and several drops of beaten egg yolk (different amounts for each pigment) were added to form a fluid paste. This procedure was adapted to emulate real paint layers with variable pigment concentrations as found in ancient paintings. Then, we used a paintbrush to spread several layers of the paste in a fine coat on a glass slide. To obtain the pure binder model sample, the beaten egg yolk was spread directly onto the glass slide [33]. Four different types of eggs were included in the study, i.e. brown, white, organic and free-range eggs, all of them from the domestic market. Paint dosimeters were prepared as explained above for pure binder model samples.

2.3. Natural ageing test

The paint dosimeters were placed in the courtyard of the church of "San Juan de Dios" in the city (historic center) of Granada (Spain), where they were exposed to environmental pollution for six months, including heavy construction works, heavy traffic and extreme sun, heat and cold, in addition to rain and wind conditions.

2.4. Lipid extraction procedure

The lipid fraction in the paint dosimeters was extracted using optimized Bligh-Dyer (BD) method (Fig. 1 supplementary material) [6,22]. Briefly: a small amount of sample was scraped off the paint dosimeters, crushed and placed in an Eppendorf. 150 μL of $CHCl_3$/MeOH (1:2) were added to 150–200 μg of sample followed by vigorous vortex-mixing (IKA Vortex 4 digital, USA) and 20 min ultrasonication (Ultrasons-HD Model, Spain; Power 120 W/frequency 40 kHz). Then, 50 μL of $CHCl_3$ were added, followed by 50 μL of H_2O and the mixture was vortexed and ultra-sonicated again (20 min) after each addition [2]. To facilitate phase separation, the mixture was centrifuged (10 min at 3000 × g). The bottom organic layer where the lipid fraction was expected to be extracted was collected, concentrated under a gentle nitrogen stream and reconstituted into 0.5 mL of a CH_3CN/H_2O/TFA (50:49.9:0.1) solution for later analysis.

2.5. MALDI-TOF-MS and data analysis

The lipid extracts were mixed with matrix solution in volume ratio 1:1 (2 μL) and applied on a stainless steel MALDI target (Bruker Daltonik GmbH, Bremen, Germany). The matrix was a solution of A-CHCA (25 mM in a solution of ammonium phosphate dibasic 8 mM), 1 μL of the lipid fraction/matrix mixture was deposited on the spot of the MALDI plate and left to dryness (Fig. 1 supplementary material)[34]. Before recording the MALDI-TOF mass spectra, the MALDI samples were spot-washed to suppress the A-CHCA matrix cluster and to reduce the chemical noise in the subsequent spectra [34]. To this end, 1.5 μL of ammonium washing buffer was dropped on the spot containing the dry extracted sample. After 3–5 s, the supernatant retained on the spot was removed using a 2 μL pipet. The MS analysis was performed on this washed MALDI sample as explained below.

The MALDI-TOF-MS spectra were obtained using a Bruker Biflex MALDI-TOF mass spectrometer (Bruker-Franzen Analytik GMBH, Bremen, Germany). The instrument used a nitrogen laser at 337 nm (OEM VSL-337i, Laser Science, Inc., Newton, MA, USA) attenuated to about 75% of its maximum power (250 μJ), with a 3 ns pulse width for desorption/ionization. Positive MALDI spectra were obtained by averaging 50 individual spectra, recorded using delayed extraction, the reflector mode and an accelerating voltage of 19.6 kV, unless otherwise stated. Angiotensin II $[M+H]^+$ (m/z=1046.5), angiotensin I (m/z=1296.6), Substance P $[M+H]^+$ (m/z=1347.7), Bombesin $[M+H]^+$ (m/z=1619.8) and Renin substrate $[M+H]^+$ (m/z=1758.9) mixtures with an average molecular weight of 400 and 2000, respectively, were used for internal calibration.

For the data analysis, the registered mass spectra were exported as txt files and edited with mMass software [35]. This program was used for the sequential data processing performed on the mass spectra before the lipidomic analysis. Fig. 2 supplementary material shows the work-flow of these procedures. Smoothing using the Savitzky-Golay method was applied with a window size of 0.05 m/z and 5 cycles. The baseline was corrected with approximately 75% accuracy. The deisotoping step was also executed with this program and was followed by the peak-picking procedure, thereby obtaining the corresponding lipid mass spectra profile of the paint dosimeters by MALDI-TOF- MS in the interval between 500 and 2000 Da (m/z) with an accuracy of 0.6 Da [8]. Afterwards the mass spectra were exported in txt format with data pairs representing the m/z and its relative intensity signal. The lipid assignment in the mass spectrum was performed using international lipidomic databases, i.e. www.lipidmaps.org., using a command included in the mMass software [35]. The particular criteria for accepting the lipid assignment are discussed below in the "results and discussion" section.

3. Results and discussion

3.1. Optimization of the lipid extraction procedure

The first step in this research was the optimization of the overall lipid extraction protocol, which includes solution of the solid dosimeter sample, extraction and isolation from other components, bearing in mind that the extracted samples will be further analyzed by MALDI-TOF-MS. Although several dissolving procedures were checked (agitation, sonication, mixtures of organic solvents, etc) that described by Bligh and Dyer [22] showed the best results. Therefore the solubilization of the samples was achieved using the BD method, i.e. with a mixture of chloroform/methanol 1:2 (v/v), with sonication in an ultrasonic bath for 20 min with a further addition of chloroform and water in a proportion of 1:1 (v/v). This method is widely used for the extraction of organic compounds with different polarities, and in our case for the extraction of the lipids and proteins from the organic fraction of the paint dosimeters. Fig. 1 supplementary material sets out the procedure.

The concentration of the analytes in the solid sample taken from the paint dosimeters is another important issue in that high intensities in the signals could saturate the mass spectra and/or affect the ionization process. It is therefore necessary to control the concentration in the samples. To this end, different concentrations of the lipids content ranging over 5.0 mg/L to 5000.0 mg/L were tested. These concentrations were adjusted by the volume used to reconstitute the samples in the step prior to adding the matrix for MALDI-TOF-MS analysis. The best results were obtained with a concentration of 500 mg/L [25]. Once we had optimized both the composition of the solution used to dissolve the solid sample and the amount of sample to be used, we then extracted the lipids fraction using a modification of the Bligh and Dyer method [22]; the most common method for separating lipids and proteins. The proposed modification is the use of acetonitrile/water in a 1:1 (v/v) ratio instead of chloroform and methanol in a 1:1 (v/v) ratio to reconstitute the sample after being taken to dryness with a gentle stream of nitrogen. The purpose of this change is to allow the further use of Zip TIPC18® filters, widely used to clean the samples immediately before analysis by MS. This type of filter does not support media with chloroform levels of over 1%. After experiments using these filters showed that they had no significant effects on the mass spectra, we decided not to use them. However we decided to continue using the mixture of acetonitrile/water in a 1:1 (v/v) ratio, due to its excellent results for dissolving lipids with different grades of polarities.

3.2. Optimization of the MALDI-TOF-MS procedure

The optimization of the MALDI-TOF-MS analysis focused above all on the selection of the most suitable matrix for the ionization of the samples and on optimizing the spot-washing step, which had been shown to improve the quality of the mass spectra [13,22,36]. The ionization of the compounds to be analyzed by MALDI-TOF-MS

depends to a large extent on the matrix, which means that correct selection is crucial. In this research, we selected the three matrices most commonly used in the bibliography, namely DHB (2,5 dihydroxybenzoic acid), SA (sinapinic acid) and A-CHCA (alpha cyano - 4 -hydroxy – cinnamic acid) [37]. All these matrices were prepared with acetonitrile and H_2O at different percentages, and trifluoroacetic at 0.1%. The concentration of the matrix was close to the level of saturation of the organic medium. Of these three, we opted for A-CHCA because it provided the best mass spectra based on the number and quality of the signals obtained (*m/z*). The selected conditions of this matrix were: A-CHCA at a concentration of 25 mM in 50% acetonitrile, 49.9% H_2O and 0.1% TFA (trifluoroacetic). Phosphate dibasic ammonium (8 mM) was added to the matrix to produce a final concentration ranging from 8 mM to 10 mM.

As mentioned earlier, a major problem affecting the quality of the mass spectra is the presence of sodium and potassium salts. These salts have to be removed to avoid complex mass spectra that are difficult to interpret due to the presence of adducts and also to avoid potential deletion of important signals. Smirnov [34] proposed a method for removing adducts involving a change in the matrix composition and washing with ammonium citrate. For this reason, we decided to add ammonium hydrogen phosphate to the matrix at a concentration of 8 mM, keeping the final concentration of the A-CHCA at 25 mM. This procedure proved decisive when the reflectron mode was used, because it improved the quality of the mass spectra. If the MALDI spot had not been washed prior to the analysis, only the linear mode could have been used, since the number of signals in the mass spectra was very poor in reflectron mode. This may be due to the suppression of signals in reflectron mode caused by the formation of adducts by the salts.

3.3. Characterization of the blank paint dosimeters

In order to assess whether the kind of egg used in the paint dosimeters affected the results, egg yolks from eggs with different colored shells were analyzed, i.e. brown shell and white shell, and from different origin, i.e. organic and free-range eggs (both with brown shells). The results indicated that the mass spectra were closed both in terms of the signals (*m/z*) and the intensities, and no important differences were detected. Even the paint dosimeters prepared using only binder media produced mass spectra similar to those. As an example of this, Fig. 1 shows the mass spectra of the lipid fraction of three different kinds of eggs (i.e. white and brown shells, and free range eggs) and the mass spectra of a blank egg yolk based paint dosimeter) where can be corroborated close lipid mass spectra profile. We detected phospholipids and triglycerides that were very close in class but with differences in their fatty acids composition. This shows that the particular kind of egg used in the dosimeter did not affect the mass spectra and could not, therefore, be considered a discriminate parameter. This meant that further analysis of the mass spectra for the dosimeters had to be based on the pigment.

The next stage was to analyze the mass spectra in greater depth to find out more about the particular composition of the paint dosimeters. Peak assignment in mass spectrometry is a difficult task. As indicated above, in this research initial lipid assignment was performed using the most frequently used international lipidomic data base [35]. This was followed by a specific and meaningful analysis of the results. Lipid assignment of the *m/z* signals started from a survey analysis of the most important classes of lipids in egg yolk. Table 4 supplementary material shows the lipid classes that previous researchers considered most likely to be present in the egg yolk [29]. We also specifically looked for the most common fatty acids in egg yolk: 14:0 myristic acid, 16:0 palmitic acid, 16:1 palmitoleic acid,18:0 stearic acid, 18:1 oleic acid,

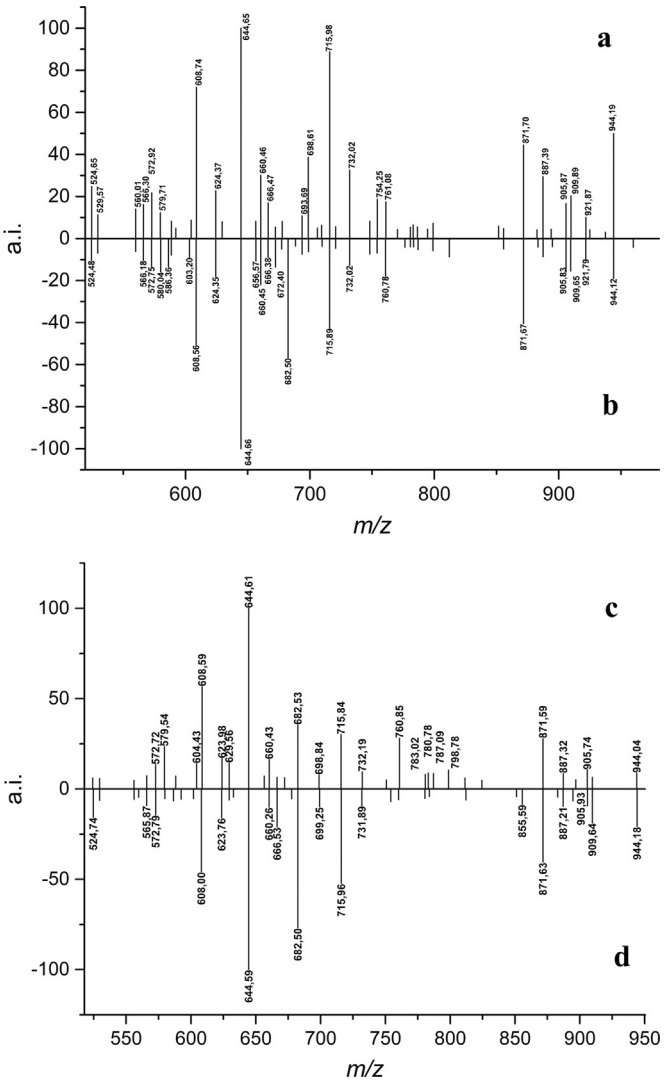

Fig. 1. MALDI-TOF mass spectra of the lipid fractions in the paint dosimeters (a) Free-range egg yolk (b) White shell egg yolk (c) Brown shell egg yolk (d) Egg yolk-based paint dosimeter.

18:2 linoleic acid, 18:3 linolenic acid, 20:4 arachidonic acid, behenic acid 22:0 (only sphingomyelin) and 22:6 docosahexaenoic acid (DHA), all of them in positive ionization mode [22,29,30,36,37]. We will now discuss the particular results of the application of this procedure for accepting the lipid assignment in all the paint dosimeters.

Each paint dosimeter has a unique fingerprint, which is different from the fingerprint of the egg yolk (Fig. 5 supplementary material). We suggest that the differences in the spectral profiles for each paint dosimeter are due to the interaction between the binder and the particular pigment in the paint dosimeter since, as indicated above, the binder itself is not a discriminate parameter. Although the fingerprint for each blank paint dosimeters is unique and depends on the pigment composition, the inspection of the entire mass spectra revealed the grouping of the *m/z* signals into three main areas. Radicals from lipids and lysophospolipids were detected from 524 *m/z* to 700 *m/z*; intact phospolipids and sphingomyelins from 700 *m/z* to 850 *m/z*; and intact triglycerides from 850 to 1000 *m/z*. Consequently, we suggest the most characteristic lipids that we found in these mass ranges.

The results obtained for the paint dosimeters after applying the criteria for lipid signal assignment in the 524-700 *m/z* interval are shown in Table 1 supplementary material. Previous authors [22]

identified radicals from phospholipids and triglycerides in this zone. We suggest here for the first time that these *m/z* signals are the results of transesterification reactions or alcoholysis [38–40] with methanol, because the latter was used in the extraction process (see Fig. 1 supplementary material). As shown in supplementary material Fig. 3, the transformation of triglyceride (TG) into diglyceride occurs due to breakage of the ester bond and subsequent protonation. In addition, we propose that the differences we observed between the lipid mass spectra profiles for the blank paint dosimeters are due to the particular pigments in the dosimeter that may act as catalysts, favoring (or not) the transesterification reactions, therefore acting throughout this reaction. This behavior was not observed in the mass spectra from the blank paint dosimeter prepared using only the binder, i.e. egg yolk, in which mass spectra profiles were analogous and unaffected by this reaction.

The outcomes of the lipidomic approach (Tables 1–3 supplementary material) were further investigated to justify the proposed identification of each lipid selecting one target for each lipid class. The signal at 524.73 *m/z* was assigned to LPC 18:0/0:0 as indicated in the bibliography [36], which is known to come from PC. The signal at 556.12 *m/z* was assigned to LPC 18:3(6Z,9Z,12Z)/0:0 $K^+$and the signal at 566.46 *m/z* to LPC 20:4 (5Z,8Z,11Z,14Z)/0:0 Na^+, both of which are LPC with different fatty acids forming potassium and sodium adducts respectively. These LPC were detected in all the blank paint dosimeters, including the dosimeters with only binder, as can be seen in Table 1 supplementary material.

The application of the lipidomic approach also identified several ceramides even though they are not present in egg yolk, nor have they been previously identified or proposed in samples from historic paintings. We propose that the ceramides (Cer) could come from sphingomyelin (SM) [41] by a rupture of the ether linkage between the phosphate and the group head during the extraction step through the transesterification reactions with methanol. For example, signals at 586.57 *m/z* and 598.51 *m/z* can be attributed to Na^+ and $K^+$of Cer d18:1/18:1(9Z), respectively. Other lipid compounds identified, but again not typically found in egg, are diglycerides (DG). These lipids come from triglycerides by the rupture of the ester linkage in one of the three fatty acids promoted by the aforementioned transesterification reaction (see supplementary material Fig. 3). As triglycerides are the most abundant lipids in egg yolk (see supplementary material Table 4), DG are the most abundant signals in this first interval, i.e. 524 *m/z* to 698 *m/z* (see Table 1 supplementary material), in all the blank paint dosimeters, both with and without pigment. Also in this zone, i.e. 524–698 *m/z*, we suggest that radicals from phospholipids (usually detected at around 700–837 *m/z*) are detected as results of the loss of the group head caused by the transterification reaction followed by protonation. If we accept this procedure, the following signals can be explained: 644.59 *m/z* PA 16:1(9Z)/16:1 (9Z) or PA 14:0/18:2(9Z,12Z) and 693.85 m/z PA 14:0/22:6 (4Z,7Z,10Z,13Z,16Z,19Z) or PA 18:3(9Z,12Z,15Z)/18:3(9Z,12Z,15Z). These compounds appear with great intensity in all the spectra.

In addition to ceramides, we also propose the identification of another class of lipids that have never been described in cultural heritage samples, namely plasmalogens [29,42]. These were detected in the 524–698 *m/z* zone at low intensity values (see Table 1 supplementary material). We propose the presence of two types of plasmalogens: the'O' prefix, which is used to indicate the presence of an alkyl ether substituent, and the 'P' prefix, which is used for the 1Z-alkenyl ether. For example at 687.45 *m/z* PA P-18:0/18:1(9Z) and PA O-18:0/18:2(9Z, 12Z) are the results of the loss of the corresponding group head of the plasmalogens, similarly as explain above but for the loss of the phsopholipids group head (see supplementary material Fig. 4). Intact phospholipids were also detected in the size window between 700 and 837 *m/z*. (see Table 2 supplementary material). The first type of this class of lipids identified in the blank paint dosimeters were sphingomyelins at 700.11 *m/z*, i.e. SM (d18: 2/16: 0), and at 702.90 *m/z* SM d18: 1/16:0. An ion at 740.03 *m/z* matched the potassium adduct SM d16:1/18:0. In this *m/z* interval, PC and PE were also detected in the blank paint dosimeters, such as mass at 754.37 *m/z* PC 18:3 (6Z,9Z,12Z)/16:1(9Z) and 760.17 *m/z* PE 18:3(9Z,12Z,15Z)/18:2 (9Z,12Z) or PE 16:1(9Z)/20:4(5Z,8Z,11Z,14Z) Na^+. As expected, TGs were detected in the higher mass interval, between 837 and 1000 *m/z* (see Table 3 supplementary material). These lipids are the most abundant in the blank samples, as they are the most abundant in the egg yolk binder. Of special interest was the signal at 871.68 *m/z*, which was detected with high intensity in all the blank samples; this signal is assigned to the TG 16: 1 (9Z)/18: 0/18:0) K^+or TG 16: 0/18:1(9Z)/18:0 K^+.

3.4. Outdoor aged paint dosimeters characterization

The last step in this research was the evaluation of the mass spectra lipid profile of the paint dosimeters exposed to atmospheric long-term natural ageing in an urban atmosphere. Our aim here was to find out more about the ageing process due to exposure and assess the role of the different pigments in this process. With this in mind, we began by evaluating the mass spectra for several paint dosimeters, which considered possible short-chain products and more likely oxidative β-cleavage of bonds in the lipids previously proposed for the blank dosimeters. To this end we conducted a comparative study between blank and naturally aged paint dosimeters in the three mass spectral intervals described for the blank paint dosimeters.

The lowest degree of similitude in the paint dosimeter mass spectra was in the mass interval between 524 and 698 *m/z*, which displays transesterification and alcoholysis reaction compounds that originated in the lipid extraction processes. However, there are two masses at 527.22 *m/z* and at 685.64 *m/z* which are detected in all the mass spectra, except for white lead, which will be discussed later because it is unusual. The identification of these two peaks was very complicated as they resulted from degraded products yielded by the particular lipid extraction procedure. Nevertheless, they were always detected and with a significant level of intensity in all the naturally aged paint dosimeters (less for Pb-based dosimeter). Although still unidentified, these two masses can therefore be proposed as markers of degradation.

In the mass range from 698 to 1000 *m/z*, where intact lipids were found in the blank paint dosimeters, (see Table 4), beta oxidative cleavage and fragmentation from those proposed in the blank sample were suggested for the naturally aged paint dosimeter. As shown in Table 4, in this mass spectral range, five signal masses were detected in all the naturally aged paint dosimeters (except white lead): 719.52 *m/z*, 725.15 *m/z*, 913.78 *m/z*, 948.58 *m/z*, 954.90 *m/z*. We suggest that the signal at 719.52 *m/z* comes from the TG 14:0/16:1(9Z)/22:6 (4Z,7Z,10Z,13Z,16Z,19Z) Na^+ (871.68 *m/z* in blank paint dosimeters) as a sodium adduct [$M+Na^+$]. This TG was detected in all the blank paint dosimeters. As shown in Fig. 2a, there is short-chain and subsequent oxidation at the carbon 11 in the docosahexaenoic acid, obtaining the corresponding aldehyde, due to instability of the aldehyde and its tendency to continue to oxidize. It is located in its most stable form, carboxylic acid, which was found without a sodium adduct in the form [$M+H^+$]. A similar breakup pattern is proposed to explain signals at 725.15 *m/z* and at 851.71 *m/z*; in this case they could come from TG 16:0/16:0/18:3 (9Z,12Z,15Z)Na^+ which undergoes beta oxidation and subsequent oxidation to its most stable form, as shown in Fig. 2b. We suggest that the signal at 913.78 *m/z* comes from 921.69 *m/z* TG (16:1(9Z)/18:3(9Z, 12Z, 15Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)Na^+), in

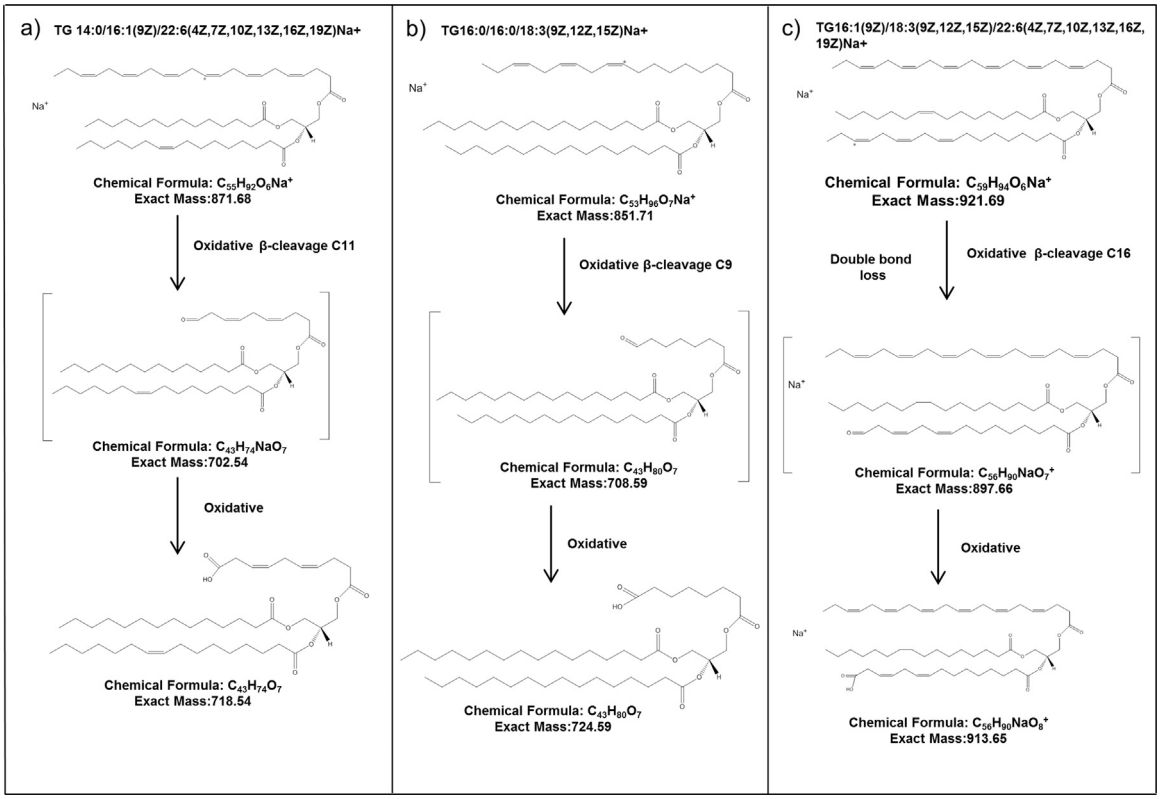

Fig. 2. Proposed formation of short-chain products from triacylglycerols after oxidative β-cleavage.

which a fatty acid loses a double bond followed by beta oxidation, with further oxidation occurring at carbon 16 of linolenic acid.

The evaluation of the lipid mass spectra in the higher values of the 850-1000 *m/z* showed the presence of two signals at 948.58 *m/z* and at 954.90 *m/z* in all the naturally aged paint dosimeters (Fig. 2c). We tried to interpret these two signals according to the mechanism of degradation cited above for the compounds previously identified in the blank paint dosimeters. Neither of these compounds matches these signals. It is possible however that these signals are the same as those detected in the blank paint dosimeters with the addition or subtraction of sodium. These compounds would therefore be resistant to natural ageing by exposure to the urban atmosphere. If 22 mass units are subtracted from the signal at 948.58 *m/z* detected in all the naturally aged paint dosimeters, the resulting value matches the signal at 926.52 *m/z* from the blank paint dosimeters; this last signal was assigned to TG 18:1(9Z)/18:3(9Z,12Z,15Z)/22:6 (4Z,7Z,10Z,13Z,16Z,19Z) (see Table 4 supplementary material). We therefore suggest that what we are witnessing is the breakup of the sodium adduct and that no alteration process occurs. Something similar occurs in the case of the signal at 954.90 *m/z* albeit in the opposite direction. In this case, the signals correspond to the protonated form while in the blank paint dosimeters the sodium adduct was detected at 976.05 *m/z* TG 18:1(9Z)/20:4(5Z,8Z,11Z,14Z)/22:6 (4Z,7Z,10Z,13Z,16Z,19Z)Na^+.

In general there are significantly fewer signals (*m/z*) in the lipid mass spectra of the naturally aged paint dosimeters than in those for the blank paint dosimeters. This finding could be attributed to the degradation of many lipids resulting from exposure of the paint dosimeters to the polluted urban atmosphere, so demonstrating the serious damage that this can cause. In addition to the degradation patterns described above, there may be others that could not be detected due, for example, to low ionization properties.

3.5. White lead-based paint dosimeter as an exceptional case

White lead-based dosimeters behave differently from all the other paint dosimeters in that the number of signals (*m/z*) in the lipid mass spectrum increases with pollution exposure while a fall is detected in the other paint dosimeters (Fig. 3). It is well known that lead is a catalyst which accelerates oxidation, and tends to form lead soaps. We suggest that this increase in the number of signals (*m/z*) occurs due to an increase in β-oxidative cleavage at the double bonds of the PL and TG that contain unsaturated fatty acids. We based this suggestion on our analysis of the PL or TG identified in the blank paint dosimeters, to which we applied the

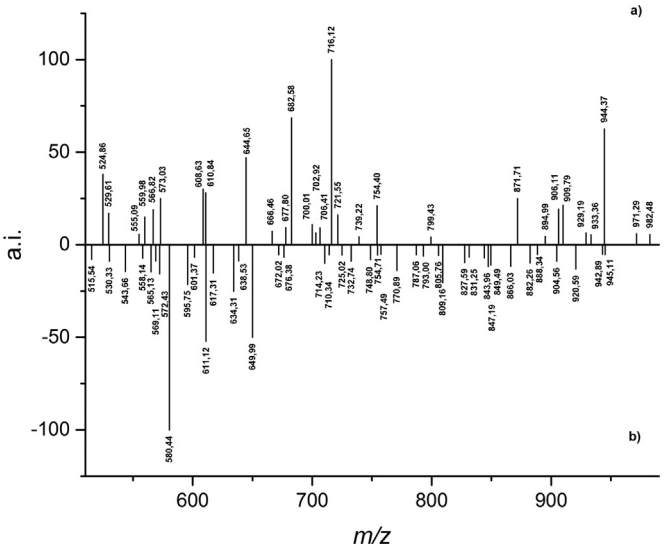

Fig. 3. MALDI-TOF mass spectra of the lipid fractions in the white lead-based paint dosimeters (a) Naturally aged (b) Blank dosimeter.

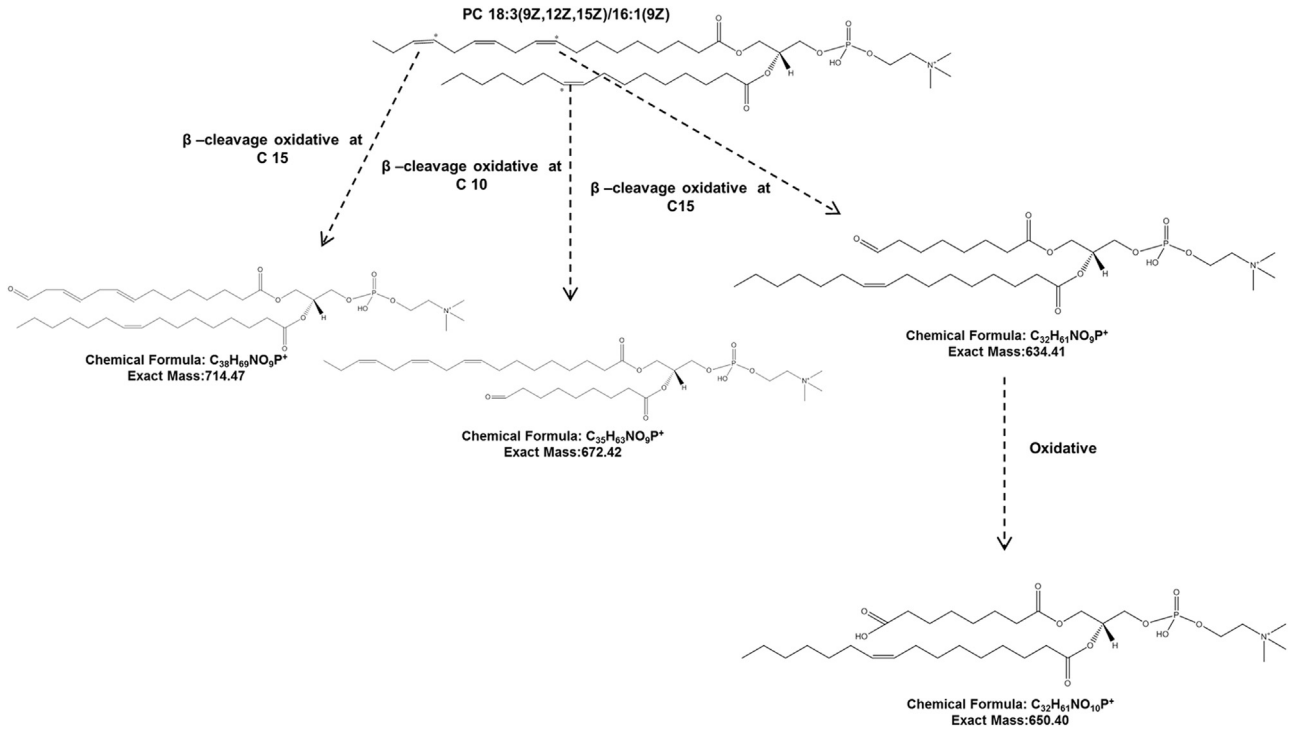

Fig. 4. Proposed formation of short-chain products from PC 18:3(9Z,12Z,15Z)/16:1(9Z) after oxidative β-cleavage.

oxidative reactions catalyzed by lead in search of the compounds resulting from the oxidative reactions on the naturally aged paint dosimeters. For example, in Fig. 4, the ion at *m/z* 754.38 corresponding to PC 18:3(6Z,9Z,12Z)/16:1(9Z) is one of the most abundant phospholipids, (see Table 2 supplementary material) and is present in almost all the blank paint dosimeters we studied. When a β-cleavage oxidative reaction was performed at the C15 of the linolenic acid, an aldehyde with *m/z* 714.47 was obtained, which was detected only in the lipid mass spectrum of the naturally aged lead white based paint dosimeters.

Likewise when the same oxidative reaction was performed at the C9 of the same linolenic acid, a signal at 634.41 *m/z* was observed, which continued to oxidize until it reached its more stable form, i.e. carboxylic acid, corresponding to a signal at 650.40 *m/z*. This signal was also detected in the naturally aged white lead paint dosimeter. None of these signals were detected in the other naturally aged paint dosimeters. Finally, in the other fatty acid of this phospholipid, palmitic acid, we propose β-cleavage oxidative at C9, obtaining the corresponding aldehyde 9, matching the observed signal at 672.42 *m/z* in the naturally aged white lead paint dosimeter. According to these reactions, the increase in the number of signals in the lipid mass spectra for the naturally aged white lead-based dosimeter seems logical because from a phospholipid detected in the homologous blank paint dosimeter, up to four different signals (*m/z*) could be suggested (and detected) in the lipid mass spectra of the naturally aged dosimeter. It is well-known that unsaturated fatty acids oxidate from double bonds to carboxylic acid (with the aldehyde before the carboxylic compound). Thus the aldehyde proposed at this ageing stage will probably be detected as carboxylic acid due to the oxidation effect of the urban atmosphere.

4. Conclusions

In this research we applied and optimized the Bligh-Dyer (BD) method, one of the most commonly used for the extraction and separation of protein and lipid material. This included a new step in which the adducts were removed. The results suggest that a transesterification process occurs due to the organic solvent (methanol) used in the extraction step. The products of this reaction are detected above all in the mass interval between 500 and 700 *m/z*. Results can therefore be erroneous if this reaction is not taken into account. We propose that the signals detected in the mass range (500-700 *m/z*) come from transterification reactions between reactants, lipids and pigments. As a result, and in order to interpret the changes in the lipid fraction of the paint dosimeters, we decided to focus the study on the mass region between 700 and 1000 *m/z*, evaluating the changes caused by urban pollution in the paint samples, and not those occurring during the extraction method.

The lipid fingerprints for the different kinds of eggs were very similar. From this, we deduced that the differences in the spectral profiles of the blank paint dosimeters are mainly due to the presence of the particular pigment that promotes a characteristic interaction between the binder and the pigment. We propose that these interactions (binder-pigment) depend on the particular pigment in each binary paint dosimeter, as they each had a unique fingerprint.

The naturally aged paint dosimeters also have a unique fingerprint, but we propose that they have similar degradation processes (β-cleavage oxidative and short-chain) because a decrease in the *m/z* signals is observed in a similar range. The mass spectrum for the white lead-based dosimeter by contrast showed an increased number of *m/z* signals. We suggest that this is due to the fact that oxidation in white lead is slower than in the other pigments studied, and that these reactions occur in almost all the double bonds of unsaturated fatty acids.

Another important conclusion is that five *m/z* signals, i.e. 719.52 *m/z*, 725.15 *m/z*, 913.78 *m/z*, 948.58 *m/z* and 954.90 *m/z*, were detected in all the paint dosimeters (except the white lead-based dosimeter). We propose these signals as markers for the presence of egg yolk as a binder in both blank and aged paint dosimeters. Finally, we propose the presence of several

compounds in our egg yolk-paint dosimeters that have hitherto never been detected in paint samples from the cultural heritage field. These include plasmalogens, ceramides and sphingomyelin.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2016.04.006.

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