# Analytical pyrolysis of proteins in samples from artistic and archaeological objects

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Abstract

The paper presents a study of proteins found in artistic and archaeological objects based on analytical pyrolysis. Proteins (mainly egg yolk and/or egg white, casein, animal glue and collagen) have been extensively used as paint binders, adhesives and varnishes in mural and easel paintings, and they can be found in archaeological findings, such as bones and skin tissues. In order to overcome limitations of wet chemical methods arising from the reduced solubility of aged proteins in samples of cultural heritage, a combination of analytical pyrolysis techniques was used to characterise reference materials, paint reconstructions and samples from different historical periods ($2^{nd}$ century BC-$20^{th}$ century AD) and geographical origins, which were collected from paintings and archaeological findings. In particular evolved gas analysis mass spectrometry (EGA/MS), pyrolysis coupled with gas chromatography/mass spectrometry (Py/GC/MS) and double shot pyrolysis/gas chromatography/mass spectrometry (DSP/GC/MS) were used. This analytical approach allowed us to characterise and differentiate the proteinaceous media, investigate their thermal behaviour and evidence changes occurring with ageing. Data clearly indicate that egg, casein and animal glue can be identified and distinguished in a sample of unknown composition using each of the analytical pyrolysis technique used. With time though differences tend to disappear to the extent that extremely degraded samples present pyrolytic profiles extremely similar to each other, irrespective of the nature of the proteins present. The data also indicate that proteins tend to become more thermally stable with ageing, suggesting that extensive intramolecular and intermolecular aggregation, and/or covalent cross-linking occur with time.

Keywords:
pyrolysis
proteins
protein degradation

cultural heritage

1. Introduction

Identification of proteins in samples from cultural heritage is often a difficult task [1,2]. From a technical point of view, paintings, polychromies, archaeological and paleontological objects are complex systems that often consist of multiple heterogeneous layers, in which pigments, fillers and other inorganic matter are mixed with organic materials. Egg yolk and/or egg white, casein, gelatin, animal glue and collagen are among the most common proteinaceous materials that can be encountered in this type of objects [1,3]. Research has evidenced that, as an effect of the long term exposure to the changeable and sometimes harsh environments where the object is displayed or stored, degradation phenomena take place. These include deamidation, hydroxylation, oxidation and carbonylation, partial hydrolysis, aggregation, cross-linking, formation of complexes with other organic binders and inorganic pigments and fillers [4-10].

Many analytical methods for the identification and characterisation of proteins in samples from artistic, archaeological and paleontological objects have been presented [1,2,11], based on staining, immunological, spectroscopic, chromatographic, and proteomics techniques.

Staining techniques are usually performed on paint cross-sections. The localisation of the proteins by stains is based on the use of dyes that may react with primary amines, or interact with specific portions of the proteins, to produce coloured and fluorescent products [12-21]. The localisation and identification of proteins by immunological methods are based on the antigen/antibody interaction. The antibody often requires to interact with 6-10 amino acids of the antigen to form the complex [21-24]. The specificity and sensibility of the immune techniques depend on the antibody availability and on their multiple epitopes. Spectroscopic techniques used to identify and localise binders are based on Infrared and Raman spectroscopy. The simultaneous presence of other organic and inorganic materials can strongly compromise the success of these techniques [25-32]. Chromatographic techniques using high-performance liquid chromatography (HPLC) and gas chromatography coupled to mass spectrometry (GC/MS) permit the identification of the material based on the determination of the amino acid composition evaluated after hydrolysis [3,33-38]. The thermal decomposition of the sample by pyrolysis coupled with GC/MS (Py/GC/MS) generates pyrolytic profiles which are useful for the identification of the materials [11,39]. Despite the potential of this technique, a relatively few examples in the literature discuss the identification of proteins in highly degraded samples, especially those from paintings and polychromies [39-45]. Proteomics techniques have been more recently adopted in the field of cultural heritage to identify proteinaceous materials by enzymatic digestion and peptides characterisation [46-54].

Each of these techniques has shown the potential of being able to successfully detect, and in some cases, identify, proteins in selected samples, but all of them present more or less drawbacks, which are seldom discussed and rarely linked to degradation phenomena.

The development of a robust and reliable analytical approach for the identification of proteinaceous materials in samples from artistic, archaeological and paleontological objects, as well as understanding its limitations, requires a better understanding of the physico-chemical changes undergone by proteins with time. Given the loss of solubility observed as a consequence of ageing [38,55-60], wet chemical methods may present insuperable difficulties, which may be, in part, overcome by analytical pyrolysis.

In this paper, we present a study, based on analytical pyrolysis, aimed at characterising and better understanding the ageing of proteinaceous materials, and how this reflects on the identification of proteins in paintings and archaeological objects. Pure reference materials, paint reconstructions and a set of samples from paintings and archaeological objects spanning from the 20$^{th}$ century AD to the 2$^{nd}$ century BC were investigated using evolved gas analysis-mass spectrometry (EGA/MS), pyrolysis-gas chromatography-mass spectrometry (Py/GC/MS) and double shot pyrolysis-gas chromatography-mass spectrometry (DSP/GC/MS).

## 2. Materials and methods

### 2.1. Reference materials

Casein (CAS), egg white (EGW) and animal glue (GLU) were purchased from Bresciani srl (Milan, Italy) and were used as reference materials [4].

### 2.2. Paintings and Archaeological samples

Easel and mural painting samples from artworks of different geographical origin and ages and one archaeological sample were investigated. Some of the samples are paint preparations which have been applied on a wooden support, and covered by paint layers. Others belong to mural paintings, which have, by definition, been more exposed to degrading environmental factors than easel paintings preparations. One sample is from an Egyptian mummy.

The sample code, the typology of the artwork and archaeological object, its age and geographical origin, as well as the nature of the proteinaceous materials present, as identified by GC-MS analysis[61](see Supplementary Material; Table S.1 and Figure S.1) are reported in Table 1.

All samples were analysed as powders. Samples of bigger dimension, as those from reference materials, were homogenised with an agate mortar previous analysis. When paint samples were available as flakes, these were pulverised with the aid of a scalpel.

### 2.3. EGA/MS

The instrumentation consists of a micro-furnace Multi-Shot Pyrolyzer EGA/Py-3030D (Frontier Lab) coupled with a gas chromatograph 6890 Agilent Technologies (Palo Alto, USA) equipped with a deactivated and uncoated stainless steel transfer tube (UADTM-2.5N, 0.15 mm i.d. × 2.5 m length, Frontier Lab). The GC was coupled with a 5973 Agilent Mass Selective Detector (Palo Alto, USA) single quadrupole mass. A program temperature was chosen for the micro-furnace chamber: initial temperature 50 °C; 10 °C/min up to

700 °C. Analyses were performed under a helium flow (1 ml/min) with a split ratio 1:20. The micro-furnace interface temperature was kept at 100 °C higher than the furnace temperature until the maximum value of 300 °C. The inlet temperature was 280 °C. The chromatographic oven was kept at 300 °C. The mass spectrometer was operated in EI positive mode (70 eV, scanning *m/z* 50–600). The MS transfer line temperature was 300 °C. The MS ion source temperature was kept at 230 °C and the MS quadrupole temperature at 150 °C. Samples, ranging from 30 to 500 µg, were placed into a stainless steel cup and inserted into the micro-furnace. The amount of sample used depended on the sample nature: samples relatively rich in organic materials, such as reference materials, and easel paintings were smaller in size compared to those coming from mural paintings. The sample underwent a thermal decomposition in inert atmosphere (He) over the chosen heating range, and evolved gaseous compounds were transferred to the mass spectrometer and directly ionised and analysed as a function of time.

### 2.4. Py/GC/MS

The instrumentation consists of a micro-furnace Multi-Shot Pyrolyzer EGA/Py-3030D (Frontier Lab) coupled to a gas chromatograph 6890 Agilent Technologies (USA) equipped with an HP-5MS fused silica capillary column (stationary phase 5 % diphenyl–95 % dimethyl-polysiloxane, 30 m × 0.25 mm i.d., Hewlett Packard, USA) and with a deactivated silica pre-column (2 m × 0.32 mm i.d., Agilent J&W, USA). The GC was coupled with an Agilent 5973 Mass Selective Detector operating in electron impact mode (EI) at 70 eV. Samples (30 µg for reference materials, easel paintings and polychromies and 100 µg for mural paintings and archaeological residues) were placed into a stainless steel cup and inserted into the micro-furnace. Stainless steel cups, after use were emptied and flame cleaned. Each sample was placed in a clean sample cup, which was previously analysed without sample, in order to ensure the absence of contaminants. The pyrolysis temperature was set at 600 °C for 1 min and interface temperature was 180 °C. The split/splitless injector was used with at 1:10 split ratio and it was interfaced with a liquid nitrogen cryogenic trap (Micro Jet Cryo-Trap MJT-1035E, Frontier Lab). The use of the micro Jet cryo-trap was not necessary but improved the quality of the peaks in the first region of the chromatograms, where highly volatile molecules were eluted. Chromatographic conditions were as follows: initial temperature 40°C, 2 min isothermal;10°C/min up to 140°C; 6°C/min up to 280°C; 10 C/min up to 300 C, 30 min isothermal. Carrier gas: He (purity 99.995%), constant flow 1.2 ml/min.

A sample of GLU was analysed in triplicates by Py/GC/MS in order to estimate the reproducibility that can be expected when the same, homogenous, sample is analysed with the same technique. Extracted ion chromatograms were obtained for selected m/z: *m/z* 67–pyrrole, *m/z* 91-toluene *m/z* 107-phenol derivatives, *m/z* 117-indole and benzeneacetonitrile, *m/z* 131-methyl-indole, *m/z* 70 and 154-DKPs, *m/z* 186-diketodipyrrole (Figure 4). Areas where integrated, and normalised for their sum. The relative standard deviations (RSD) of the normalised areas and retention times were calculated, and resulted below 20% and 1%, respectively. Detailed data for each compound are reported in Supplementary Materials (Table S.2).

## 2.5. DSP/GC/MS

The analysis were conducted with the instrument illustrated in Py/GC/MS section above (2.4. Section). Double shot pyrolysis (DSP) entails the pyrolysis of the same sample at two different temperatures[62-65]. Each sample (from 100 to 30 μg) was placed into a stainless steel cup and inserted twice into the micro-furnace, to be pyrolysed once at 350°C and subsequently at 550°C (pyrolysis duration 1 minute). Gases evolved at the two different temperatures were eluted in the chromatographic column and detected by mass spectrometry. Temperatures were chosen based on the EGA/MS results as described in the following section.

# 3. Results

## 3.1. Reference materials

The proteinaceous materials examined in this study exhibit different amino acid compositions (Table 2). Casein in cow milk consists of four isoforms, such as $\alpha_{S1}$-casein, $\alpha_{S2}$-casein, β-casein and κ-casein [66]. Ovalbumin (OVA) is the most abundant protein in EGW, comprising about 55% of the total proteins in newly deposited eggs [67]. Animal glue (GLU) is obtained by boiling tissues such as bones, skins and cartilaginous parts of animals: it is mainly constituted of denatured and partially hydrolysed collagen [6,68].

The total ion thermogram (TIT) profile obtained by EGA/MS of CAS shows two non-well resolved peaks, ascribable to two main degradation steps (**Figure 1**) and the result is in accordance with the thermogravimetric profiles (TGA) reported in the literature [69-71]. The first peak has a maximum at 315°C and the second one at around 430°C. The thermal degradation of EGW and GLU occurs in a similar temperature range. EGW presents a curve with a maximum at 305°C and a shoulder at 420°C. GLU shows a curve peaking at 325°C with two main shoulders, one at about 380°C and another at about 430°C (**Figure 1**).

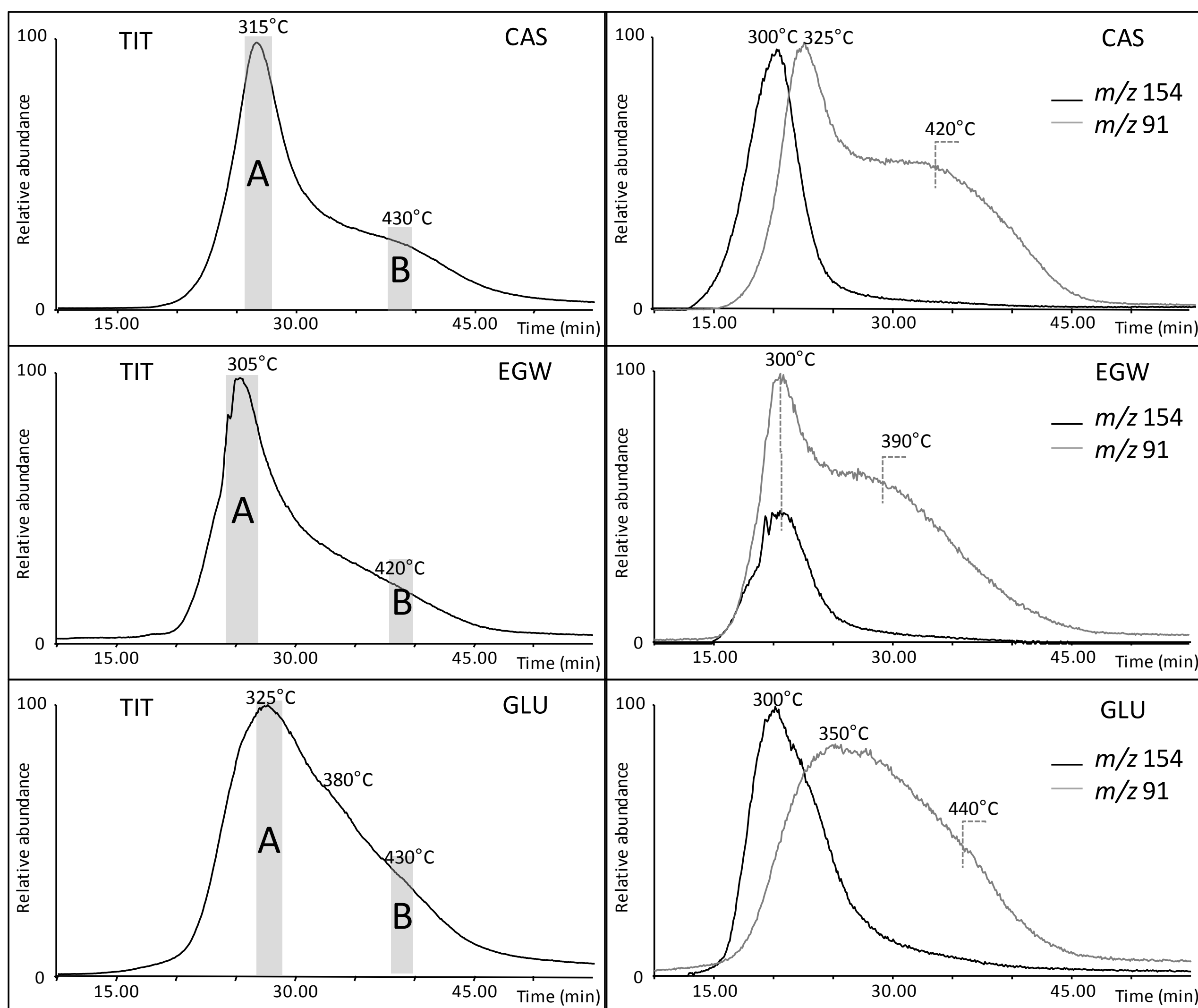
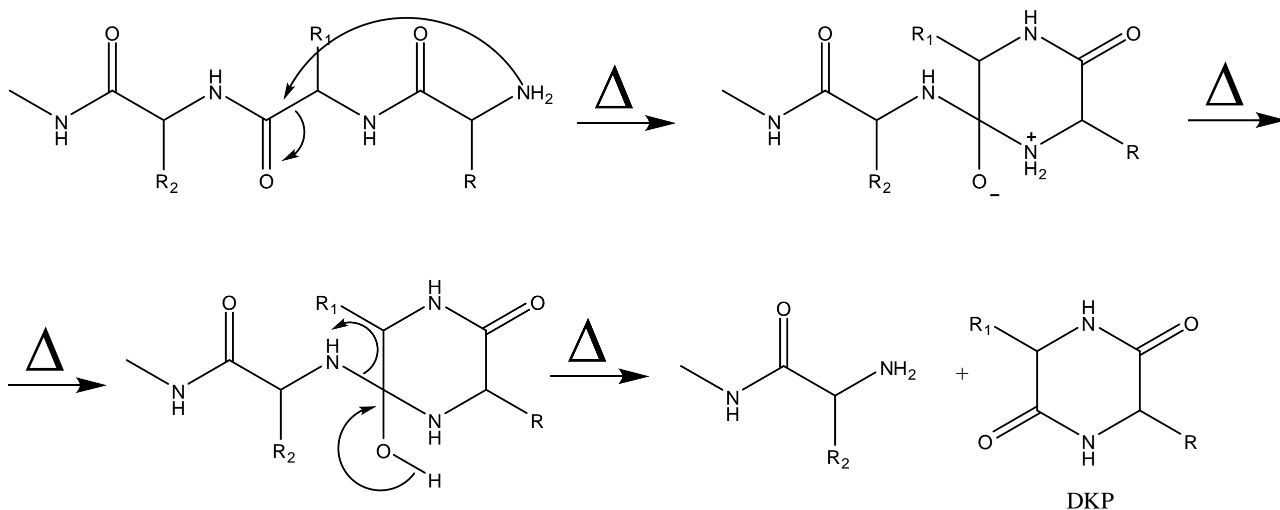



**Figure 1**: Total Ion Thermograms (TITs) (left) of CAS, EGW and GLU. Extract ion thermograms (right) of *m/z* 154 (DKPs; black line), *m/z* 91 (alkylbenzenes; grey line) for CAS, EGW and GLU.

The average mass spectra associated to the temperature ranges 310-320°C for first peak of CAS, 300-310°C for EGW and 320-330°C for GLU (mass spectra A, as indicated in **Figure 1**) and temperature ranges 425-435°C for the second peak of CAS, EGW and GLU (mass spectra B, as indicated in **Figure 1**), are reported in **Figure 2**. Based on the mass spectra presented in the literature obtained by classic Py/GC/MS [42,72,73], it is possible to observe that the MS spectra of the first peak for CAS, EGW and GLU (A) is characterised by fragment ions which can be ascribed to nitrogen-containing cyclic compounds, such as 2,5-diketopiperazines -DKPs - (*m/z* 70, 111, 125, 154, 196)[74-77]. The formation of DKPs in pyrolysis [78-80] is suggested to involve the cyclisation of neighbouring amino acids in a polypeptide chain [81] as shown in the scheme below (**Scheme 1**).




**Scheme 1**: Formation of DKPs by pyrolysis [81].

The fragment ions at *m/z* 70 and 154 have been described in the mass spectra of pyrolysates of dipeptides and they are markers of diketopiperazines from proline [72,74,78,80]. Several proline-amino acids combinations in DKPs give, in fact, as principal fragment ions an immoniun ion from proline at *m/z* 70, and a fragment ion, via McLafferty rearrangement, at *m/z* 154 (**Scheme 2**)[77].

$-C_3H_6$

DKP from pyrolysis of Pro-Val/Val-Pro
*m/z* 196

*m/z* 154

1-pyrroline
*m/z* 70

*m/z* 84

-HCN

*m/z* 125

$-H_2C{=}OH$

*m/z* 111

*m/z* 98

*m/z* 94

**Scheme 2**: Most characteristic fragment ions (m/z 196, 154, 125, 111 and 70) in the MS spectra of the first peak of TITs[77].

Fragment ions observed in the average MS spectra in the temperature range 425-435°C in CAS, EGW and GLU (B), such as *m/z* 67, 80, 91, 94, 107 and 117, can be ascribed to aromatic and nitrogen-containing compounds, such as pyrrole and alkyl-pyrrole (*m/z 67* and *80)*, indole and alkyl-indole (*m/z 91* and *117 )*, phenol and alkyl-phenol (*m/z* 94 and 107), toluene and methyl-benzene (*m/z* 91), styrene and benzeneacetonitrile (*m/z* 117), whose formation has been attributed to the thermal degradation of the of amino acids' lateral chains, as well as of aromatic amino acids [73,82-84].

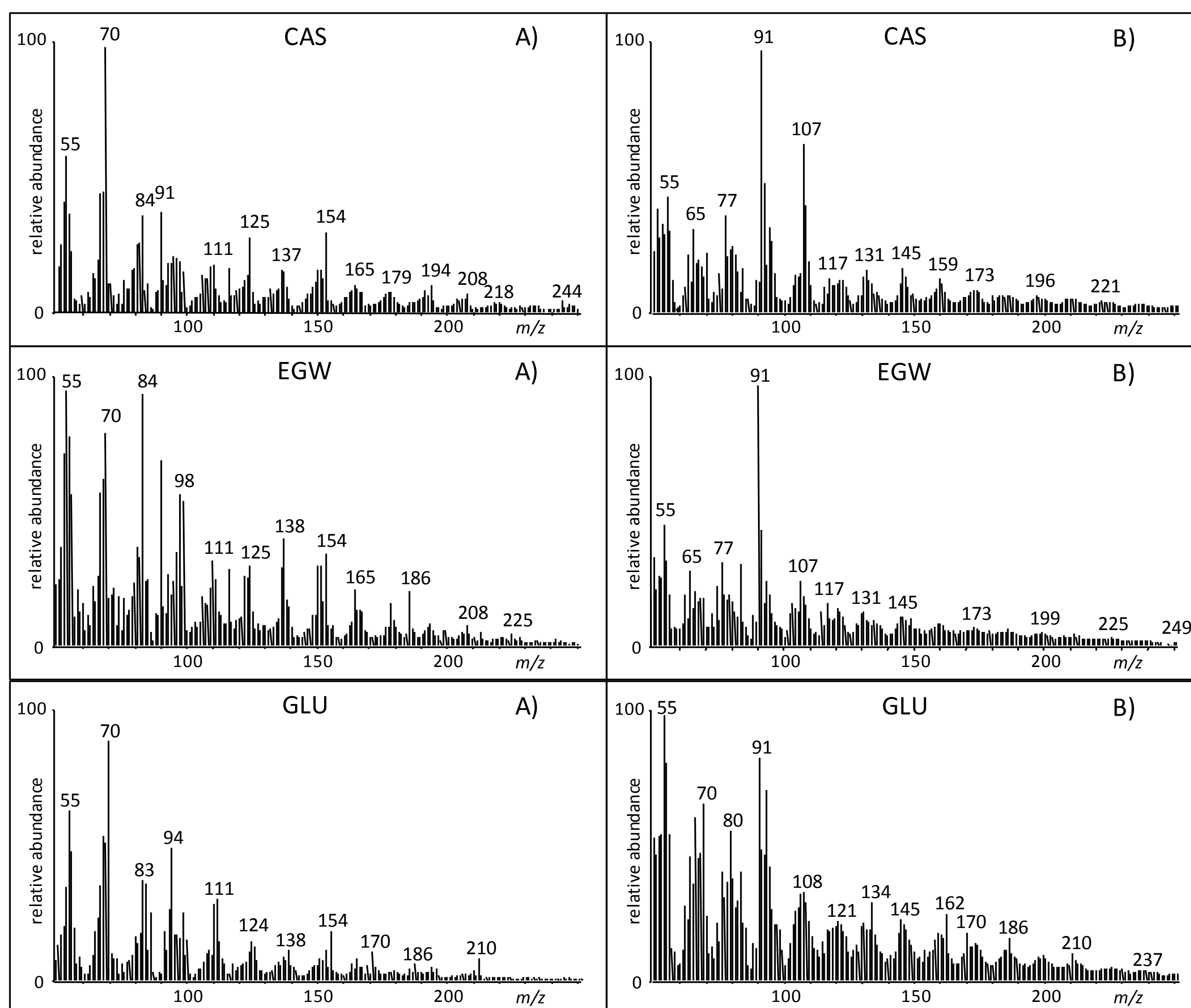



**Figure 2**: Average mass spectra of CAS, EGW and GLU obtained in the temperature ranges indicated in the text and highlighted in Figure 1 as areas A and B, respectively.

In order to identify in detail the main products of thermal decomposition at the different temperatures, and thus better understand the EGA/MS spectra, we analysed CAS, EGW and GLU by double shot pyrolysis/gas chromatography/mass spectrometry (DSP/GC/MS) at the temperatures of 350°C and 550°C (**Figure 3** and Table 3). Despite the EGA/MS spectra are quite similar, the chromatograms obtained by flash pyrolysis at

350°C of the three proteinaceous binders revealed significant differences. The most intense peaks in the pyrogram of CAS are ascribable to DKPs, among which cyclo(Pro-Val)(#6,8), cyclo(Pro-Leu)(#10) and cyclo(Pro-Ile)(#7,9) were the most abundant. The profile observed is in agreement with the amino acid composition of casein (Table 2; [85]) and the observation that proline favours the formation of DKPs, while more hydrophilic amino acids, such as aspartic and glutamic acids, asparagine, arginine, lysine, inhibit DKPs formation [77,86,87]. The pyrogram at 350°C of EGW shows the typical profile of a globulin protein [74]. It presents a imidazole derivative(#17), as well as DKPs (cyclo(Pro-Leu)(#10), cyclo(Pro-Ile)(#9) and cyclo(Pro-Val)(#6) are the most abundant). The pyrogram at 350°C of GLU is dominated by diketodipyrrole(#20), cyclo(Pro-Gly)(#21), cyclo(Pro-Pro)(#22) and cyclo(Pro-HyP)(#23 and 24) in accordance to the fact that Pro, its hydroxylate form - Hyp- and Gly are the most abundant amino acids in collagen (Table 2**Errore. L'origine riferimento non è stata trovata.**). At 550°C, pyrrole(#34), toluene(#1), dimethyl-benzene(#27), styrene(#28), phenol(#19), methyl-phenol(#29), ethyl-phenol(#30) and indole(#3) are the most abundant peaks observed the pyrograms of CAS, EGW and GLU, thought they show different relative abundances.

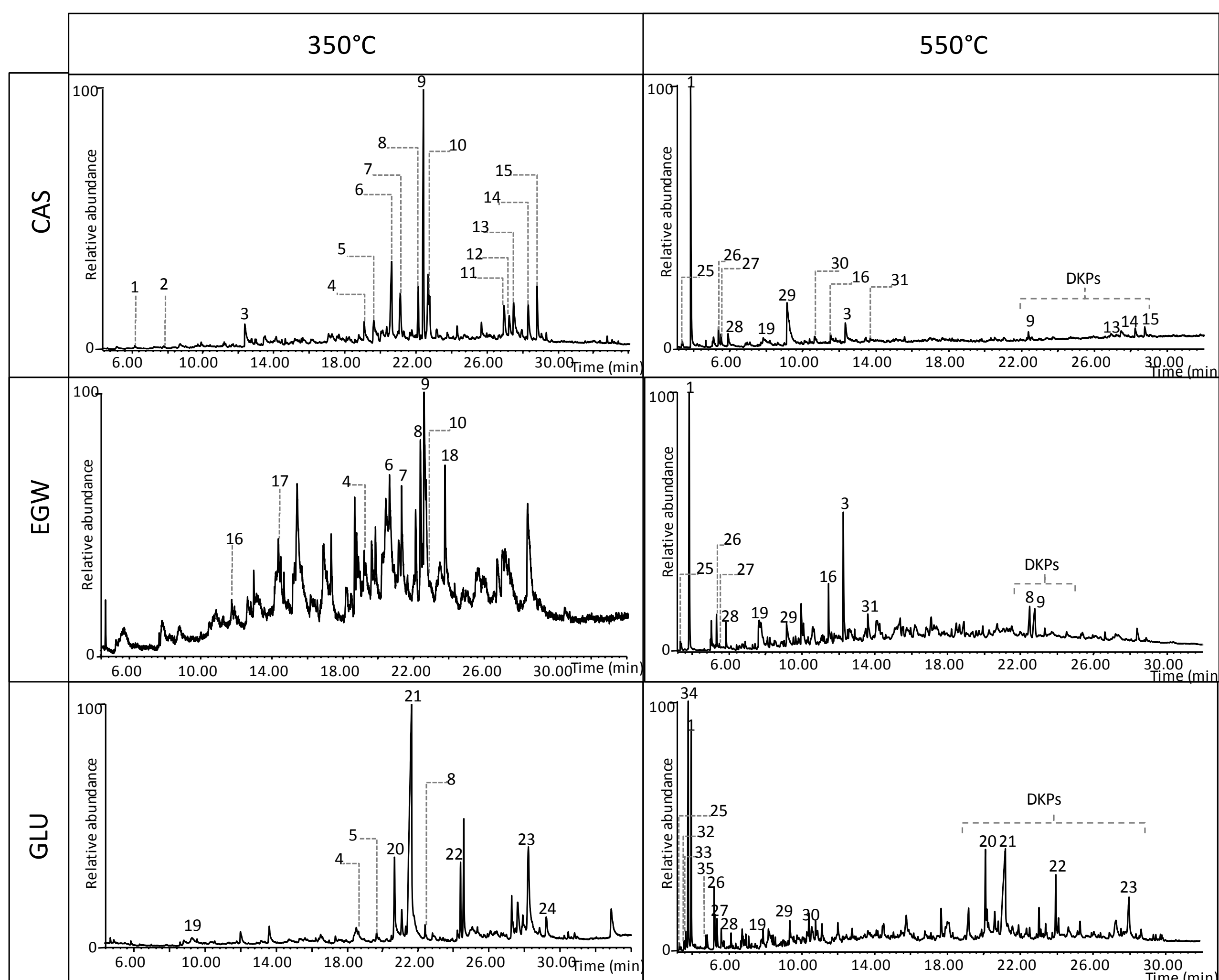



**Figure 3**: DSP/GC/MS chromatograms at 350 and 550°C of CAS, EGW and GLU. Attribution of peak numbers is reported in Table 3.

Py/GC/MS at 600°C of EGW, OVA and GLU give pyrograms that appear as the sum of the two double shot pyrograms at 350°C and 550°C, where, at lower retention times, aromatic molecules are found, and, at higher retention times, DKPs are eluted (pyrograms are reported in Supplementary Material Figure S.2– peak assignment Table 3).

Py/GC/MS data clearly indicate that the three proteinaceous binders can be easily distinguished on the basis of pyrolytic profiles of DKPs as well as nitrogen and oxygen containing aromatic molecules, in agreement with literature observations [41,43,44,88]. Pyrrole(#34) is the most abundant compound in GLU, but it is also present in CAS and EGW. Toluene(#1) and methyl-phenol(#29) are the main pyrolysis products of CAS, followed by indole(#3), benzenepropanenitrile(#16), phenol(#19), benzeneacetonitrile(#36), methyl-indole(#31) and diphenyl-ethylene(#37). Toluene(#1), benzeneacetonitrile(#36), phenol(#19) and indole(#3) are the most abundant peaks in the pyrogram of EGW, followed by diphenyl-ethylene(#37), methyl-indole (#31) and methyl-phenol(#29). The peaks of benzeneacetonitrile(#36), phenol(#19) and indole(#3) are quite low in the pyrogram of GLU, whereas diethyl-pyrazine(#41) is more abundant. More diagnostic are DKPs, as they are associated to the amino acidic sequences in the protein: cyclo(Pro-Ile)(#9) and cyclo(Pro-Ala)(#4 and 5) dominate the DKPs in CAS, cyclo(Pro-Val)(#6) and cyclo(Pro-Ile)(#9) in EGW, and cyclo(Pro-Gly)(#21), cyclo(Pro-Hyp)(23 and 24) and diketodipyrrole(#20) prevail in the DKPs region of GLU.
The extracted ion chromatograms of ions with *m/z* 67, 91, 107, 117, 131, 70, 154 and 186 for the three proteinaceous binders are shown in **Figure 4**, highlighting the differences described above. The extracted ion thermograms of the same ions are reported in Supplementary Material Figure S.3

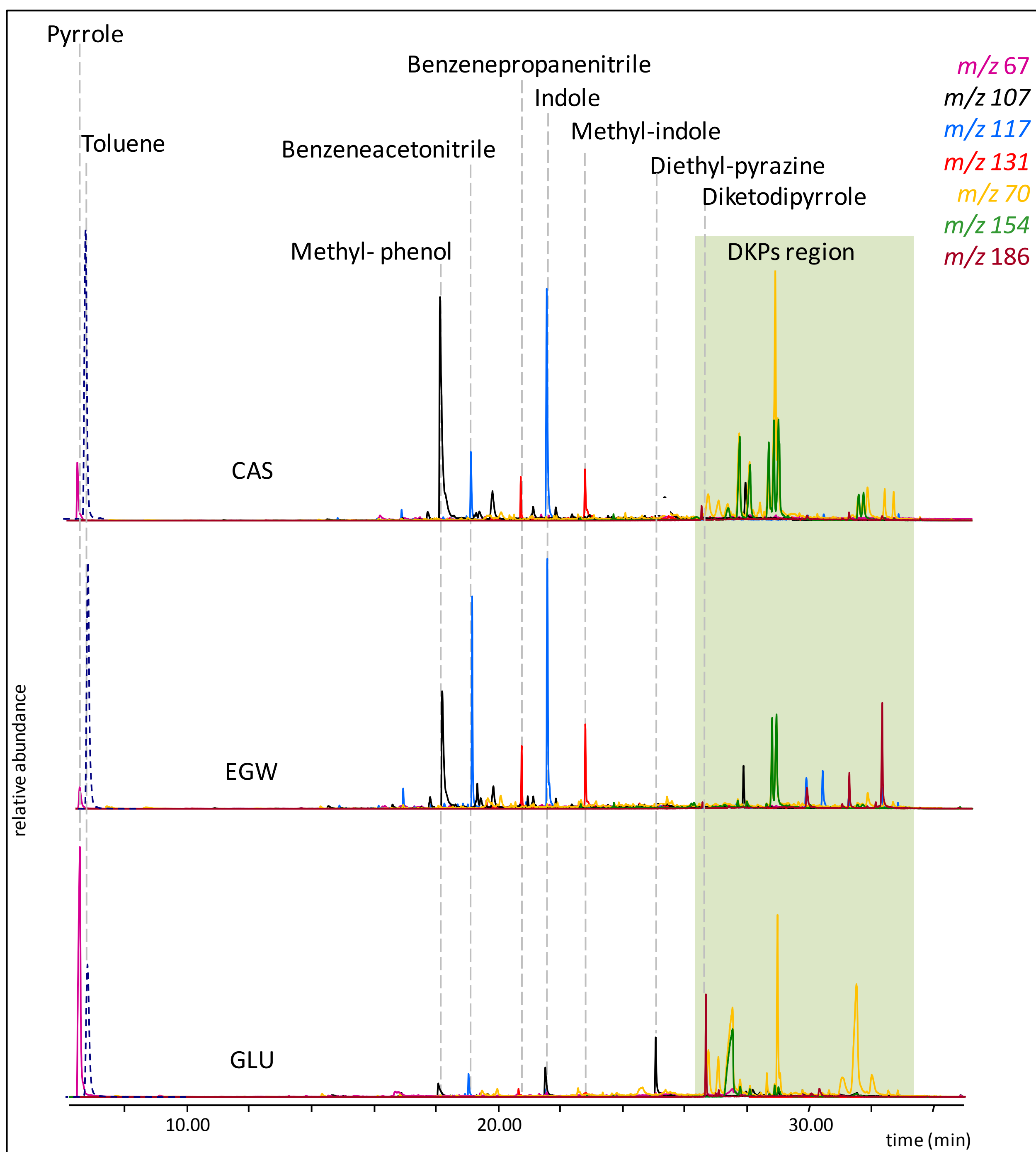



**Figure 4**: Extract ion chromatograms of flash Py/GC-MS at 600°C of CAS, EGW and GLU. Fragment ions: *m/z* 67–pyrrole, *m/z* 91-toluene *m/z* 107-phenol derivatives, *m/z* 117-indole and benzeneacetonitrile, *m/z* 131-methyl-indole and benzenepropanenitrile, *m/z* 70 and 154-DKPs, *m/z* 186-diketodipyrrole.

## 3.2. Samples from paintings and archaeological objects

### 3.2.1. Samples containing collagen and collagen based materials

EGA profiles of artistic and archaeological samples containing animal glue and collagen are reported in **Figure 5**. The EGA/MS spectra are reported in Supplementary Materials (see, Figure S.4). The TIT of EP1997 and EP15$^{th}$AD show a maximum at 330°C, and sample EP15$^{th}$AD also clearly shows a shoulder at

410°C. The EGA/MS spectra of EP1997 and EP15th AD at 330°C are dominated by the fragment ions of DKPs (*m/z* 70, 111, 124, 154), as well as that of diketodipyrrole(#20) (*m/z* 186). Fragment ions of nitrogen and oxygen containing aromatic molecules are identified at *m/z* 67, 107, 117, 131, in the second region of the TIT.

The TIT of the wall painting sample, MP2nd BC, is markedly different. The first peak at 290°C is due to the thermal decomposition of the Hg containing pigment (vermilion, HgS). The signal associated to the thermal degradation of animal glue presents only one peak at high temperatures (peak maximum: 450°C). The MS spectrum associated presents fragment ions ascribable to nitrogen containing aromatic molecules at *m/z* 67 and 131. The EGA profile of the archaeological sample (M1st AD) presents three peaks. The first peak at 240°C is associated to the evaporation of free fatty acids [89] originating from the hydrolysis of the fats of the skin, the second peak at 330°C to the thermal decomposition of cellulose [90], deriving from the linen of the bandage. The third peak, presenting a maximum at 460°C can be ascribed to the thermal decomposition of proteins (*m/z* 67 and 131).

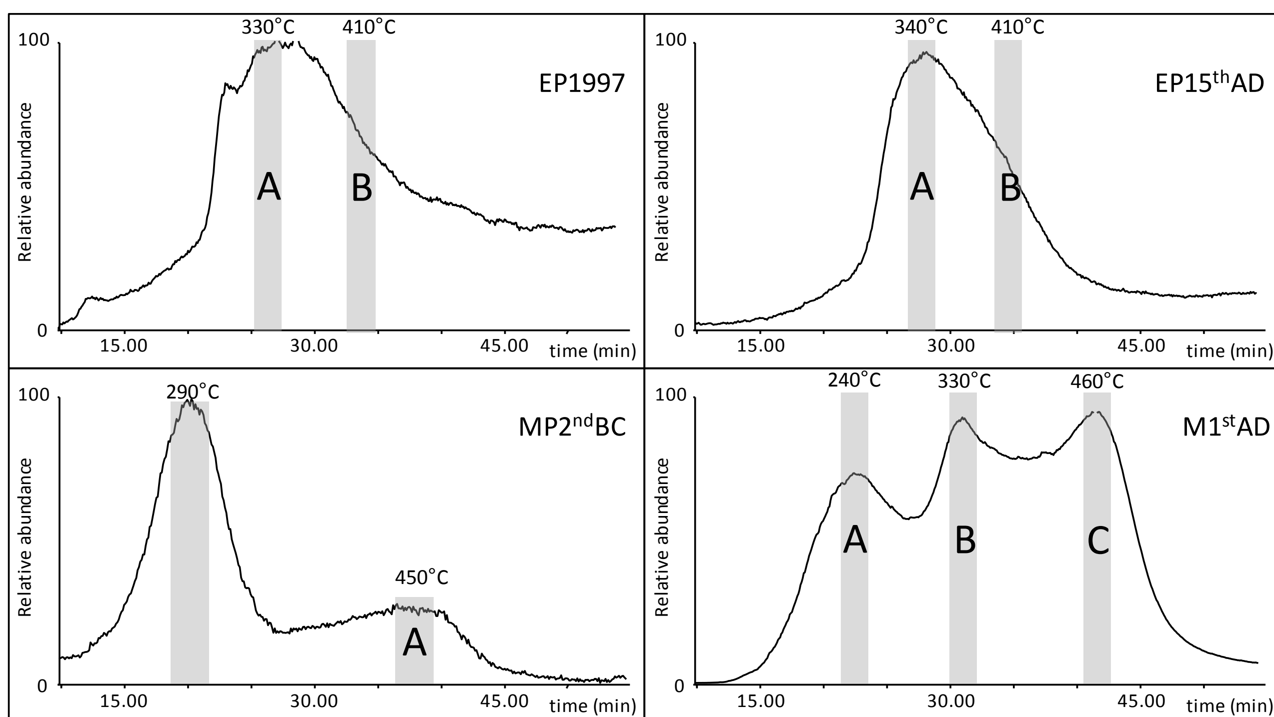



**Figure 5**: Total ion thermograms (TITs) of EP1997, EP15th AD, MP2nd BC and M1st AD.

The extracted ion chromatograms obtained by Py/GC/MS of these samples are reported in **Figure 6** and the peak assignation is reported in Table 3. Two distinct regions could be discerned from Py/GC/MS analysis of EP1997. In the first region of the chromatogram (time range 4.00-8.00 min) aromatic hydrocarbons (toluene(#1), ethyl-benzene(#26) and styrene(#28)) and nitrogen containing aromatic rings, such as methyl-pyrrole(#32) and pyrrole(#34), are present. In the second region (time range 20.00-26.00 min), diketodipyrrole(#20), cyclo(Pro-Ala)(#4), cyclo(Pro-Gly)(#21) and cyclo(Pro-Pro)(#22) dominate the DKPs

region of the chromatogram. In the pyrogram of EP15$^{th}$AD, there are the same aromatic hydrocarbons found in EP1997, and the nitrogen containing aromatic rings are pyrrole(#34), methyl-pyrrole(#32) and benzonitrile(#42). Diketodipyrrole(#20), cyclo(Pro-Gly)(#21) and cyclo(Pro-Pro)(#22) showed lower relative abundances in the DKPs region. In the pyrogram of the MP2$^{nd}$BC, toluene(#1), dimethyl-benzene(#27), styrene(#28), methyl-styrene(#43), as well as the polycyclic aromatic hydrocarbons indene(#44), acetophenone(#45) and biphenyl(#46) constitute the aromatic hydrocarbon fraction of the pyrolysis products. Pyrrole(#34) is the only nitrogen containing molecule identified and DKPs are absent. Saturated hydrocarbons (marked in the chromatogram with an asterisk (*)) are due to an external contamination. The chromatogram of M1$^{st}$AD is more complex than the other samples because it is a mixture of different organic materials: lipids, proteins and cellulose. The extracted ion chromatograms of M1$^{st}$AD shows numerous peaks in the elution region between 7.00 and 20.00 min due the pyrolysis of the bandage and the fats. Pyrrole(#34) is the only nitrogen containing molecule present and DKPs are absent.

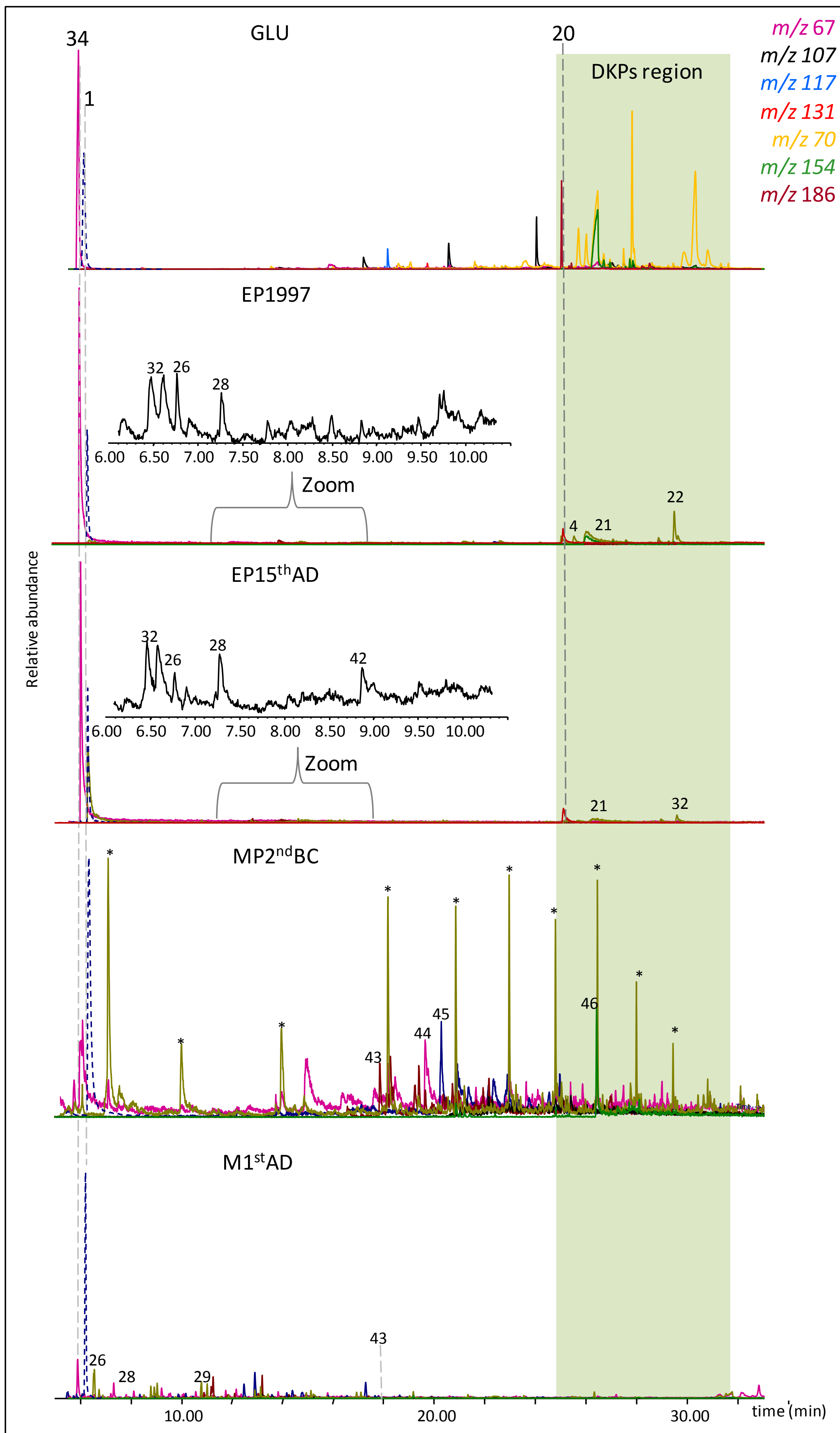

GLU
EP1997
EP15thAD
MP2ndBC
M1stAD
DKPs region
m/z 67
m/z 107
m/z 117
m/z 131
m/z 70
m/z 154
m/z 186
Zoom
Relative abundance
time (min)
10.00
20.00
30.00

**Figure 6**: Extract ion chromatograms of Py/GC/MS at 600°C of GLU, EP1997, EP15$^{th}$AD, MP2$^{nd}$BC and M1$^{st}$AD . Fragment ion: : *m/z* 67–pyrrole, *m/z* 91-toluene *m/z* 107-phenol derivatives, *m/z* 117-indole and benzeneacetonitrile, *m/z* 131-methyl-indole and benzenepropanenitrile, *m/z* 70 and 154-DKPs, *m/z* 186-diketodipyrrole.

### 3.2.2. Samples containing casein and egg

Py/GC/MS results of P6$^{th}$AD (containing egg) and MP4$^{th}$BC (containing milk) are reported in **Figure 7** and the peaks interpretation is shown in Table 3. In the pyrogram of P6$^{th}$AD nitrogen containing aromatic compounds – pyrrole(#34) and benzonitrile(#42) - are detected as well as other aromatic molecules, i.e. toluene(#1), ethyl-benzene(#26), styrene(#28), indene(#44) and benzaldehyde(#47), including polycyclic aromatic hydrocarbons, such as naphthalene(#48). DKPs are absent. Heptadecanenitrile(#49) and octadecanenitrile(#50) are detected, which have been identified as markers of egg yolk [39].

Pyrrole(#34), toluene(#1), dimethyl-benzene(#27), styrene(#28), trimethyl-benzene(#51) and indene(#44) are detected in the pyrogram of MP4$^{th}$BC, together with acetophenone(#45), methyl-indene(#52). Polycyclic aromatic hydrocarbons, such as naphthalene(#48), methyl-naphthalene(#53), biphenyl(#46) and phenanthrene(#54) are detected. DKPs are absent.

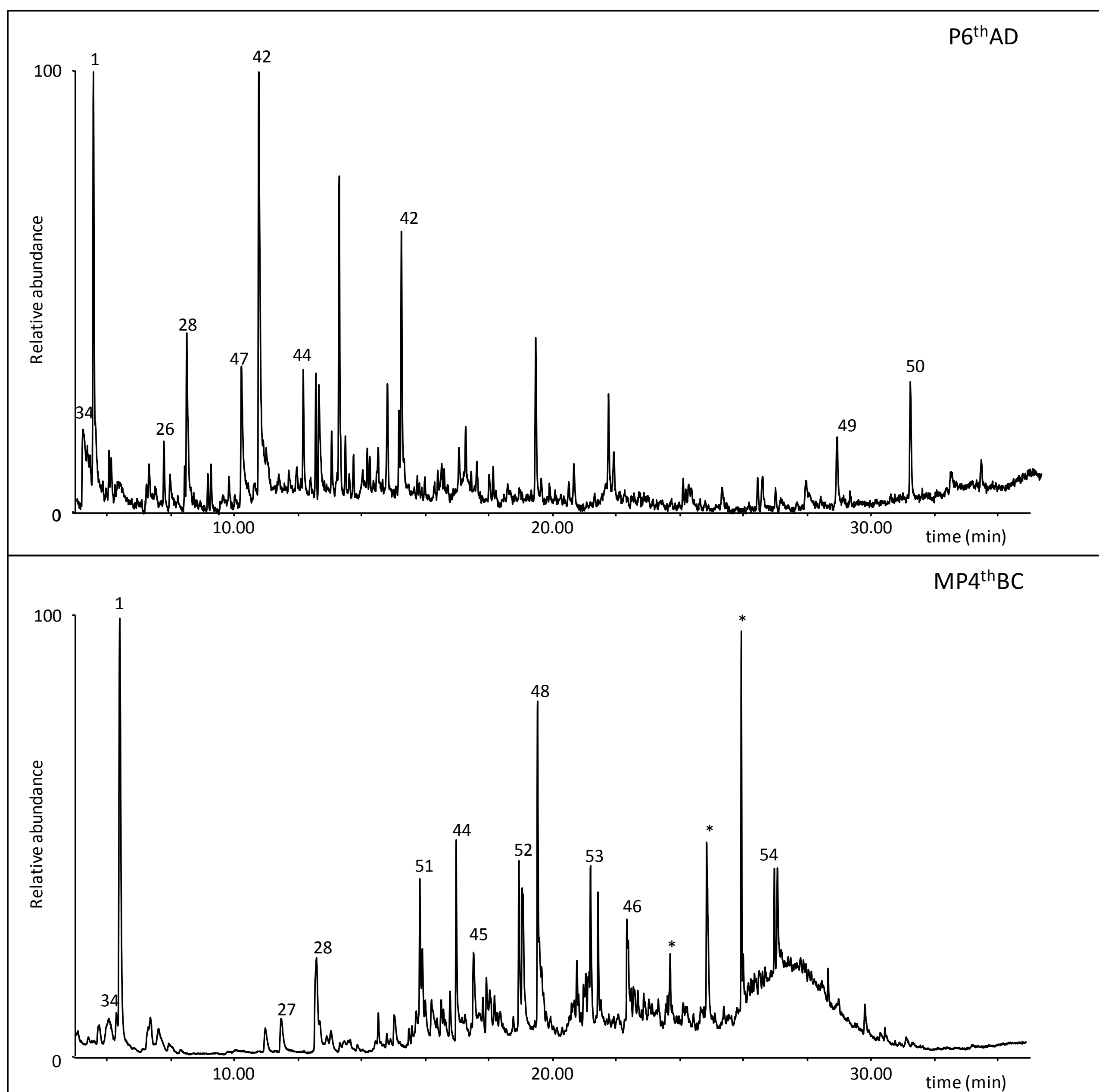



**Figure 7**: Py/GC/MS at 600°C of P6$^{th}$AD and MP4$^{th}$BC.

3.2.3. Samples containing proteins of unknown origin

The pyrogram of MP2$^{nd}$AD is shown in **Figure 8** and the peaks assignation is reported in Table 3. In the pyrogram pyrrole(#34) is the only nitrogen containing aromatic compound detected. Toluene(#1) and indene(#44), together with several polycyclic aromatic hydrocarbons, such as azulene(#55), naphthalene(#48), methyl-naphthalene(#53), fluorene(#57), phenanthrene(#54), methyl-fluorene(#58), methyl-phenanthrene(#59) and fluoranthene(#60) are the most abundant peaks in the chromatogram.

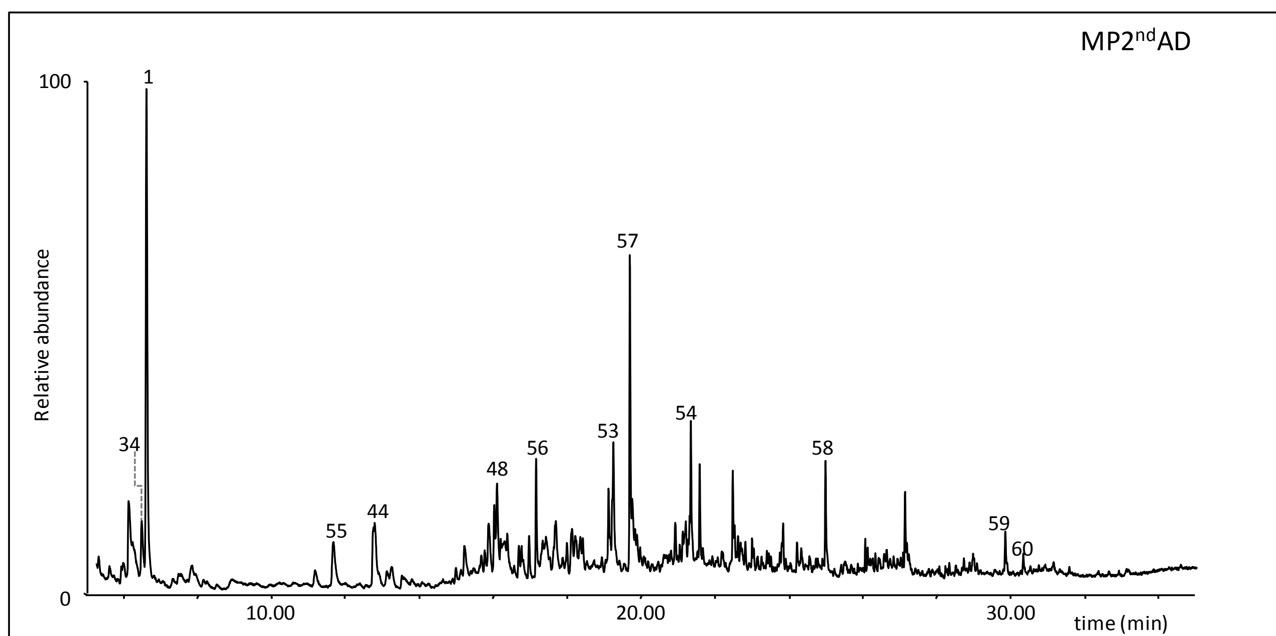



**Figure 8**:Py/GC/MS at 600°C of MP2$^{nd}$AD.

4. Discussion

Despite the differences at the molecular level between the different proteins, the TITs curves of CAS, EGW and GLU are quite similar. The temperatures at which thermal degradation is observed cannot be associated to the molecular weight of the proteins (degradation temperatures of CAS I/II steps, 315/430°C and MW of bovine β-casein 25107.33 Da; EGW 305/420°C MW of chicken ovalbumin 42881.24 Da and GLU 325/380-430°C MW of bovine collagen α1(I) 138938.49 Da). We believe that the shape of the TIT curve, the temperatures at which thermal degradation is observed, as well as the nature of the gases that are evolved, is, in part, due to the primary structure of the proteins - different proteins produce different DKPs – and, in part, to their secondary, tertiary and quaternary structures.

The ion at *m/z* 154 can be used to monitor in the TITs curves the evolution of DKPs from the sample as it is heated up ( **Figure 1** ; black line). At the lowest temperatures (200°C) "depolymerisation" starts, with formation of DKPs. DKPs are thus evolved by the thermal decomposition of the portion of the proteins which is less thermally stable. Based on the peak area, it can be estimated that more than 60% of the DKPs is evolved before 350 °C. The ion at *m/z* 91 can be used to monitor the evolution of some of the aromatic compounds (methyl-phenol, benzenepropanenitrile, indole, alkylbenzenes). The shape of the extracted ion curve of *m/z* 91 clearly shows, at least, two maxima ( **Figure 1**; grey line). When the temperature increases, amino acids' lateral chains are pyrolysed, leading to the formation of alkylbenzenes, benzenepropanenitrile, ethyl-cyanobenzene and styrene [42] - the first peak observed in the extracted ion curve of *m/z* 91, peaking at 325°C, 300°C and 350°C, for CAS, EGW and GLU, respectively. The pyrolysis of the polypeptide chain remaining after the loss of lateral groups, occurs at higher temperatures, and most likely leads to the formation non-specific oxygen and nitrogen containing aromatic molecules, explaining the second peak in

the extracted ion curve of *m/z* 91[1].It is also important to stress that at the solid state, proteins, although not in the native form[2], will have portions which are stabilised by hydrogen and ionic bonding, as well as van der Waals' interactions, which could be intramolecular and/or intermolecular [4]. These portions of the protein are more stable than others where such bonds and interactions are absent, or are less frequent. We expect that these stabilised portions of the protein require higher temperatures to degrade. We thus believe that the relative intensity of the first portion versus the second portion of the TITs curves is, at least in part, related to the secondary, tertiary and quaternary structure of the proteins, and in particular the second part of the curve is due, in part, to the thermal degradation of the portions of the proteins which are associated, aggregated and/or cross-linked.

Data relative to case studies that contained collagen and collagen based materials (EP1997, EP15thAD, MP2ndBC and M1stAD) seem to support the interpretation discussed above, as that the older and the more degraded the sample, the higher is the temperature at which the peak at *m/z* 91 presents a maximum (from 430 to 460°C), and the smaller is the relative amount of DKPs that are formed during heating (**Figure 6** and **Figure 9**). These results are in agreement with published studies on collagen from archaeological bones, showing that, the lower the amount of DKPs in the pyrogram, the higher the degradation [78,79,99,100]. When a protein is highly aggregated and cross-linked, it is reasonable to hypothesise that the formation of DKPs cannot occur at a significant extent, as it involves that the consecutive cyclisation of two amino acids.

This result is very interesting, as protein aggregation and cross-linking (both intramolecular or intermolecular) are to be considered responsible for several drawbacks that may be encountered in the analysis of proteins in samples from artistic, archaeological and paleontological objects. Aggregation and cross-linking:

- may make the protein binding site unavailable for the stain;

[1] This behaviour is the same observed for materials such as poly(vinyl acetate), which shows in EGA a first peak, due to deacetylation of the side chains, and a second peak, ascribable to the chain scission reactions of a highly regular unsaturated polymer (such as polyene), which is obtained after the loss of the lateral chain [91] B. Rimez, H. Rahier, G. Van Assche, T. Artoos, M. Biesemans and B. Van Mele, *Polymer Degradation and Stability*, 93, (2008) 800.

[2] The 3-D structure of native *β*-casein is best described by the term rheomorphic [92] C. Holt and L. Sawyer, *Journal of the Chemical Society, Faraday Transactions*, 89, (1993) 2683, [93] Y.D. Livney, A.L. Schwan and D.G. Dalgleish, *Journal of Dairy Science*, 87, (2004) 3638.: in other words, as was indicated by Kumosinski et al.[94] T.F. Kumosinski, E.M. Brown and H.M. Farrell, ibid., 76, (1993) 931., CAS is not a fixed structure, as might be inferred from the static model, and on the other hand, it is not a random coil, as it is often referred to in the literature. Rather, it is a structure with several more definable and rigid parts, and others that are more flexible and dynamic, allowing the molecule to transform between several energetically favourable conformations under the influence of environmental forces, such as shear or thermal motion. OVA possesses the typical tertiary structure called serpin fold. The native serpin does not have a fixed structure but has a highly flexible peptide loop. The native fold is not the most thermodynamically favourable conformation and the central loop have an exceptional mobility leading to metastable folding intermediates [95] J.A. Huntington and P.E. Stein, *Journal of Chromatography B: Biomedical Sciences and Applications*, 756, (2001) 189, [96]P.E. Stein, A.G.W. Leslie, J.T. Finch, W.G. Turnell, P.J. McLaughlin and R.W. Carrell, *Nature*, 347, (1990) 99.. Articular cartilage collagen in its native form has a non-homogenous and layered structure [97] A. Jeffery, G. Blunn, C. Archer and G. Bentley, *Journal of Bone & Joint Surgery, British Volume*, 73-B, (1991) 795, [98] W.J. Landis, K.J. Hodgens, M.J. Song, J. Arena, S. Kiyonaga, M. Marko, C. Owen and B.F. McEwen, *Journal of Structural Biology*, 117, (1996) 24.. The layers of the intermediate and superficial zones themselves are composed of a fine meshwork of fibrils and adjacent layers merge or are closely linked by bridging fibrils. In the superficial layers, the fibrils of collagen are smaller and the network is finer than the deeper and calcified zones [97] A. Jeffery, G. Blunn, C. Archer and G. Bentley, *Journal of Bone & Joint Surgery, British Volume*, 73-B, (1991) 795.

- may control the degree of flexibility of the polypeptides chains, and may form deleterious changes of the protein conformation, compromising the antigen/antibody interaction [15,101];
- cause loss of solubility, making the protein extraction necessary for chromatographic and proteomics techniques a difficult task [3,34,102];
- may affect the degree of access of cleavage enzymes.

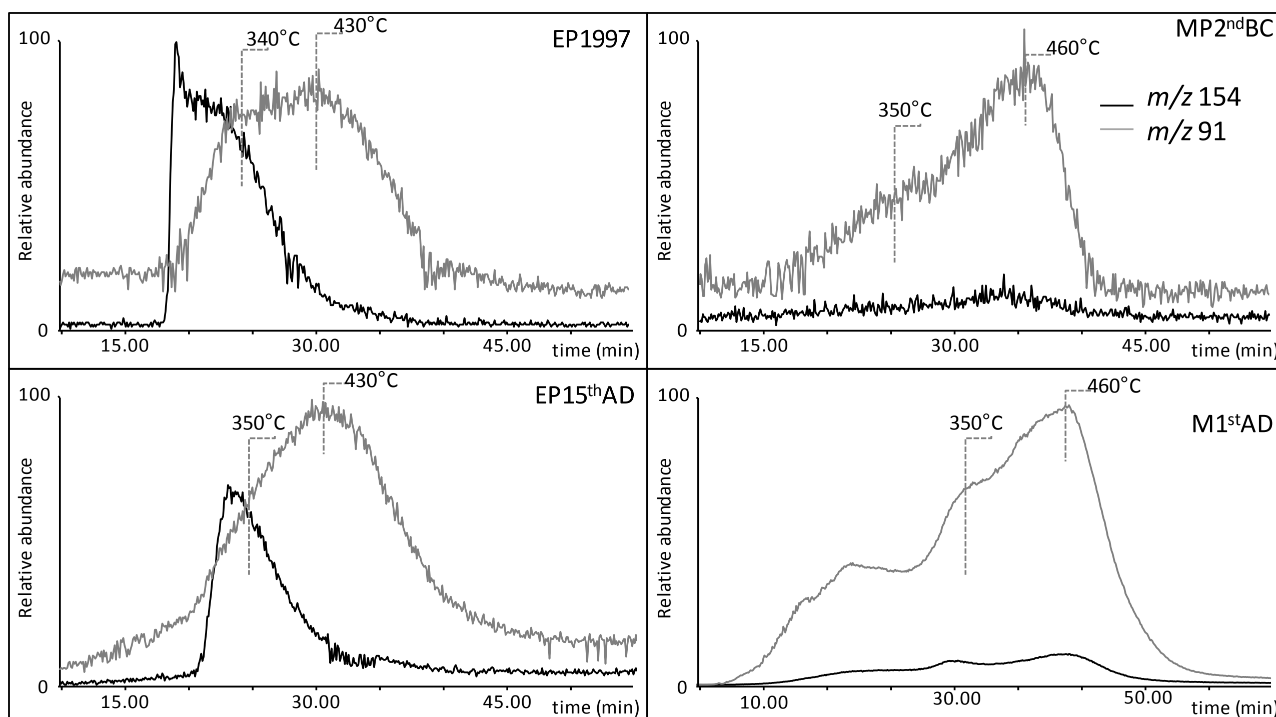



**Figure 9**: Extract ion thermograms of *m/z* 154 (DKPs; black line), *m/z* 91 (alkylbenzenes; grey line) for EP1997, EP15$^{th}$AD, MP2$^{nd}$BC and My1$^{st}$AD.

Another interesting result is that identification of a protein in a sample from artistic and archaeological object is strongly compromised by the level of degradation. Py/GC/MS of pure materials showed that EGW, GLU and CAS can be distinguished by evaluating their chromatographic profiles, as discussed in the Results section. The pyrolytic profiles of degraded samples lack DKPs, and nitrogen containing aromatic molecules lose their specificity: pyrrole is obtained from the pyrolysis of GLU, EGW and CAS (P6$^{th}$AD, MP4$^{th}$BC, MP2$^{nd}$BC, MP2$^{nd}$AD), indole, benzeneacetonitrile, benzenepropanenitrile, and methyl-indole are not detected any longer in the pyrograms. Moreover a relatively high number of polycyclic aromatic molecules are produced, which could well be the result of the pyrolysis of highly aggregated/cross-linked proteins. The proteins present in the sample that showed the highest amount of polycyclic aromatic molecules among its pyrolysis products - MP2$^{nd}$AD - could not be identified by GC/MS, because of their high insolubility (See Supplementary Material, Table S.1). Interestingly, despite the absence in the pyrogram of indole, methyl-phenol, benzeneacetonitrile, benzenepropanenitrile and methyl-indole, egg can still be identified in sample

P6$^{th}$AD thanks to the presence of hexadecanenitrile and octadecanenitrile. These molecules have been reported in the pyrograms of egg yolk reference samples, and their presence in the pyrogram could be ascribed to the pyrolysis of the product of a strong chemical interaction that had been formed between the carbonyl moieties of egg lipids[3] and the primary ammine group of Lys and possibly Arg in egg proteins. Interestingly, such compounds are not observed in the pyrogram of M1$^{st}$AD, despite the fact that proteins and lipids are simultaneously present. Although more research is necessary to prove this point, the data seem to suggest that the strong chemical interactions that are responsible of the formation of hexadecanenitrile and octadecanenitrile during pyrolysis of egg are originated in the lipoproteins of the yolk, and do not take normally place when lipids and proteins are simultaneously present.

## Conclusions

Analytical pyrolysis was used to characterise proteinaceous materials in reference samples, ancient paintings and archaeological findings. Evolved gas analysis coupled with mass spectrometry (EGA/MS), pyrolysis coupled with gas chromatography/mass spectrometry (Py/GC/MS) and double shot pyrolysis gas chromatography/mass spectrometry (DSP/GC/MS) were used as analytical pyrolysis techniques. EGA/MS allowed us to investigate the thermal behaviour of proteinaceous materials. DSP/GC/MS was used to support the interpretation of the EGA/MS data. Both Py/GC/MS and EGA/MS were used to characterise the different proteinaceous materials and highlight changes occurring with ageing.

The data show that, under anoxic heating, all proteinaceous materials investigated, egg white, casein and animal glue, that diverge for amino acid sequences, molecular weights and spatial conformations, undergo the same three main thermal degradation mechanisms. The first leads to the formation of 2,5-diketopiperazines DKPs, cyclic compounds, whose formation has been suggested to be consequence of progressive cyclisation of neighbouring amino acids in a polypeptide chain. The second thermal degradation mechanism is related to the pyrolysis of amino acids' side chains, and the third is due to the pyrolysis of the residual polymer, as well as the most thermally stable portions of the proteins. Despite the thermal degradation phenomena are the same, the three proteinaceous materials show different pyrolytic profiles at the molecular level, as a consequence of the different amino acid sequences: cyclo(Pro-Ile) and cyclo(Pro-Ala) dominate the DKPs in CAS, cyclo(Pro-Val) and cyclo(Pro-Ile) in EGW, and cyclo(Pro-Gly), cyclo(Pro-Hyp) and diketodipyrrole prevail in the DKPs region of GLU. Pyrrole is the most abundant aromatic compound in GLU, but it is also present in CAS and EGW. Toluene and methyl-phenol are the principal pyrolysis products of CAS. Toluene, benzeneacetonitrile, phenol and indole dominate the aromatic compounds in the pyrogram of EGW, while diethyl-pyrazine is most abundant aromatic marker in GLU.

By analysing samples from artistic and archaeological samples dating from the 2$^{nd}$ century BC to the 20$^{th}$ century AD, it is possible to conclude that, the older and the more degraded the sample, i) the smaller is the relative amounts of DKPs produced by pyrolysis, ii) the higher is the temperature at which the third thermal degradation step occurs, iii) the smaller is the number of nitrogen containing compounds that are detected in

[3] Hexadecanoic and octadecanoic acids are the most abundant fatty acids in egg lipids

the pyrograms, and iv) the higher is the relative amount of non-specific polycyclic aromatic hydrocarbons produced by pyrolysis. As a result, in highly degraded samples, pyrograms tends to be similar to one another, due to the absence of DKPs and the loss of aromatic markers. The presence of a proteinaceous material can be ascertained based on the detection of pyrrole. Moreover it seems that egg yolk can still be identified in aged samples, thanks to the presence of hexadecanenitrile and octadecanenitrile in the pyrograms.

Data also indicate that proteins tend to became significantly more thermally stable with ageing, suggesting extensive intramolecular and intermolecular aggregation, and/or covalent cross-linking, offering the first insight into the correlation between the degree of aggregation and/or cross-linking of the proteins and ageing. Although more research is still necessary to better understand what is the role played by aggregation and covalent cross-linking in determining the thermal behaviour of proteinaceous materials in artistic and archaeological samples, the data presented clearly highlight that analytical pyrolysis has the potential of contributing to our understanding of the ageing and degradation of proteinaceous materials by overcoming solubility limitations affecting wet chemical sample pre-treatments.

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