

1 **Study of thermal behavior and molecular composition of**
2 **mixtures of resinous materials and beeswax found as adhesives**
3 **in archaeological finds**

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12 **Abstract**

13 Beeswax and resinous materials have been used and combined since ancient times to obtain
14 adhesives. In this work, we studied mixtures of these two materials under heating to investigate
15 the effect of their interactions on the properties of the resulting adhesive. The results were then
16 compared with those of archaeological samples, to obtain new insights into ancient technological
17 knowledge. This study was performed by combining two different chemical-analytical techniques:
18 evolved gas analysis coupled with mass spectrometry and flow injection analysis-high resolution
19 mass spectrometry. Evolved gas analysis was used to investigate the thermal behavior of the
20 mixtures and obtain information on production techniques and ageing processes. The data were
21 processed by isoconversional method to estimate the activation energies associated with the
22 thermal degradation of the adhesives. The results suggested that archaeological mixtures were
23 subjected to a thermal pre-treatment before their use as adhesives and that heating led to a
24 material with new chemical features, due to the formation of hybrid species between resin
25 diterpenes and the beeswax compounds. The formation of these species was confirmed by high
26 resolution mass spectrometry.

27

28 **Keywords:** beeswax; pine resin; pine pitch; archaeological adhesives; mass spectrometry

29

30 **1. Introduction**

31 Beeswax and pine resin are among the most ubiquitous organic materials recovered as adhesives
32 from archaeological finds [1]. In particular, pine resin can be found either in its natural form or as
33 tar and pitch, products obtained by subjecting resinous wood to pyrolysis-like processes [2–4].

34 Adhesives found in archaeological objects are often constituted of mixtures of resinous substances
35 and other materials, such as beeswax or animal fats, which were added to improve the adhesive
36 properties of the formulation by modifying the physical-chemical properties of the resinous
37 substances [1,5–7].

38 Due to their complexity, the study of the composition of these materials requires the use of
39 instrumental techniques capable of molecular-level characterization, and mass spectrometry-
40 based techniques are particularly suitable for this purpose [3,6]. In addition, the use of such
41 techniques allows us to gain insights into the chemical transformations due to natural aging and
42 anthropogenic treatments [1,3,4,6,8].

43 In this view, the detection of key molecular species generated by the chemical interactions
44 between the mixture components is fundamental [16–20]. In a previous study, we showed that
45 flow injection analysis (FIA) coupled with high-resolution mass spectrometry through an ESI source
46 (ESI-Q-ToF) could be used to detect the presence of hybrid species resulting from reactions
47 between diterpenoids and fatty acids/hydroxy fatty acids from pine resin and beeswax,
48 respectively [17]. The formation of esters between the constituents of pine resin and beeswax was
49 attributed to the mixing and heating processes typically used to produce adhesive, suggesting not
50 only an intentional addition but also an advanced technological knowledge on the properties of
51 materials. Direct analysis by FIA-MS allowed us to overcome the limitations of chromatographic
52 techniques, such as the need for a hydrolysis or a thermal decomposition step which could lead to
53 a possible loss of information on the original composition of the organic residues and on the

54 structure of high molecular weight components. Correlating the molecular composition of
55 adhesives with their physical-chemical properties is also fundamental to understand the reasons
56 for their success in ancient history. In particular, the study of the thermal behavior is crucial to
57 understand the treatments underwent by the original materials and their preservation state.
58 Evolved gas analysis coupled to mass spectrometry (EGA-MS) is a powerful technique to study the
59 thermal behavior of complex organic samples and to determine the nature and amount of volatile
60 products formed during the thermal degradation of materials or the occurrence of cross-linking
61 phenomena [9,21]. Recently, EGA-MS has been used to estimate kinetic parameters of the thermal
62 degradation of organic materials through isoconversional methods [22–24]. Isoconversional
63 methods are a group of macroscopic modelling methods which provide kinetic data about
64 complex systems [22,25–27]. Model-free isoconversional methods are a sub-category of such
65 methods, that estimate the apparent activation energy of a reaction without any assumption on
66 its mechanism. An estimation of the activation energy of a pyrolytic process can provide useful
67 information on the energetics of the process.

68 In this work, we combined EGA-MS and FIA-HRMS to study both reference and archaeological
69 mixtures of resinous substance and beeswax, as well as mixtures of their characteristic single
70 components (abietic acid and hexadecyl- palmitate). EGA-MS was used to study the thermal
71 behavior and the thermo-chemistry of the mixtures. The obtained data were processed by
72 isoconversional method, to understand the variation of the thermal behavior of the mixtures
73 before and after heating, while FIA-HRMS was used to detect the formation of hybrid resin-
74 beeswax molecular species upon heating of the reference mixtures.

75

76

77 **2. Experimental**

78 **2.1. Chemicals**

79 Abietic acid (75%, Sigma-Aldrich) and hexadecyl palmitate (95% Sigma-Aldrich) were used to
80 prepare reference mixtures. Acetone (HPLC grade, Sigma-Aldrich) was used for homogenization of
81 the mixtures. Chloroform, hexane (HPLC grade, Sigma-Aldrich) and isopropanol (HPLC grade,
82 Fluka) were used as solvents for FIA-HRMS analyses. ~~Hexamethyldisilazane (HMDS, 99.9%, Sigma-~~
83 ~~Aldrich, USA) was used as derivatizing agent in UV/Py(HMDS)-GC/MS experiments.~~

84 **2.2. Samples**

85 Beeswax and resin from *Pinus pinaster* were purchased locally, while archaeological pitch (P) was
86 taken from the bottom of an amphora found in the archaeological site of Medinet Madi Fayum
87 (Egypt), dating back to 2nd-3rd century BC [4]. Binary reference mixtures were obtained by mixing
88 abietic acid (AA), hexadecyl palmitate (HP), beeswax (B) and pine resin (R) (1:1 mass ratio).
89 Homogeneous mixtures were obtained by suspending the components in 1 mL acetone and then
90 sonicating the suspensions (Bandelin Sonorex Digitec, 15 min at room temperature). Additional
91 reference samples were also obtained by heating the mixtures at 200 and 300 °C in oven for 1 h
92 and 2 h. Table 1 summarizes the mixtures produced and the heating conditions. These heating
93 conditions were determined in a previous step of the analysis. For the temperature, the
94 experiments were performed starting from melting point of beeswax (62 °C - 64 °C) and pine resin
95 (70 °C - 135 °C) to the optimal temperatures, while for the time it was observed that results
96 obtained were not significantly different by heating the samples for one hour or two hours.
97 Six archaeological samples collected from the archaeological site of Antinoe (Egypt, 4th-5th century
98 AD) were used in this study. Their composition has already been determined in a previous work
99 [6]. Table 2 lists all archaeological samples and their composition.

100

101 **Table 1:** List of reference mixtures produced and their heating conditions. AA = abietic acid; HP =
 102 hexadecyl palmitate; B = beeswax; R = resin; P = pitch.

Mixture	Heating conditions
AA+HP	200 °C, 2 h
	300 °C, 1 h
AA+B	200 °C, 2 h
	300 °C, 1 h
R+HP	200 °C, 2 h
	300 °C, 1 h
R+B	untreated
	200 °C, 2 h
	300 °C, 1 h
P+B	untreated

103

104 **Table 2:** List of the archaeological samples and their composition. B = beeswax; P = pitch.

Sample	Composition
A3	P+B
C5	P+B
C6a	P+B
D1a	P+B
D2a	B
D2bs	P+B

105

106

107 **2.3. Evolved gas analysis-mass spectrometry (EGA-MS)**

108 **2.3.1. EGA-MS analysis**

109 All experiments were performed with an EGA/PY-3030D micro-furnace pyrolyzer (Frontier
 110 Laboratories, Japan) coupled to a 6890 gas chromatograph equipped with a split/splitless injector
 111 and a 5973 mass spectrometric detector (Agilent Technologies, USA). During each experiment, the
 112 furnace temperature was raised from 50 °C to 800 °C at six different heating rates (β = 15, 20, 25,
 113 30, 35, and 40 °C/min). The interface between the furnace and the GC injector was kept 100 °C
 114 above the furnace temperature, to a maximum of 320 °C. The GC injector was operated in split
 115 mode with a 30:1 ratio at 300 °C. The pyrolyzer and mass spectrometer were connected by an

116 Ultra-ALLOY deactivated and uncoated stainless-steel capillary tube (UADTM-2.5N, 2.5 m x 0.15
117 mm, Frontier Laboratories, Japan), which was kept at 300 °C. Helium (1.2 mL/min) was used as
118 carrier gas. The mass spectrometer was operated in EI positive mode (70 eV, m/z range 50–300).
119 The ion source and quadrupole analyzer were kept at 230 °C and 150 °C, respectively.

120 **2.3.2. Isoconversional method**

121 Conversion profiles were calculated from the EGA-MS profiles using equation (1), where S is the
122 total ion current of the mass spectrometer as a function of the temperature T , T_0 the initial
123 temperature, and A_{TOT} is the total area of the thermogram. The Kissinger-Akahira-Sunose
124 isoconversional method was used to calculate the apparent activation energy for the thermal
125 degradation of the samples. The equation for this method is (2), where α is the conversion, β is the
126 heating rate, E_a the apparent activation energy, R the gas constant and $r(\alpha)$ is a function of the
127 conversion. For a fixed value of α , the equation gives a linear trend of the left side as a function of
128 $1/T$. The slope of these functions can be directly related to the apparent activation energy [22,24–
129 26].

$$130 \quad (1) \quad \alpha = \frac{1}{A_{TOT}} \int_{T_0}^T S(\tau) d\tau$$

$$131 \quad (2) \quad \ln\left(\frac{\beta}{T^2}\right) = -\frac{E_a}{RT} + r(\alpha)$$

132 **2.4. Flow-injection analysis-high resolution mass spectrometry (FIA-HRMS)**

133 Approximately 0.1 mg of a reference mixture and archaeological sample were subjected to
134 ultrasound-assisted extraction at room temperature using 1 mL and 500 μ L of a 3:2 v/v
135 chloroform:hexane mixture for 15 and 20 min, respectively. In both cases, 20 μ L of the obtained
136 mixture were diluted in isopropanol (1:4000). The initial extraction step allowed us to increase the
137 amount of extracted material and avoid secondary transesterification of beeswax esters, which
138 could cause an alteration in the profile of the material [28,29]. Sonication was used to increase
139 efficiency and reduce extraction time [30,31].

140 The analysis was carried out using a 1200 Infinity HPLC coupled to a Jet Stream ESI interface with a
141 6530 Infinity Q-ToF quadrupole-time of flight tandem mass spectrometer (Agilent Technologies,
142 US). The eluents were methanol:water (85:15) and isopropanol; the flow rate was 0.2 mL/min and
143 the injection volume was 1 μ L. The mass spectrometer was calibrated using the HP0321 tuning mix
144 (Agilent Technologies, USA). The ESI operating conditions were: drying gas (N_2 , purity > 98%): 350
145 $^{\circ}C$ and 10 L/min; capillary voltage 4.5 kV; nebulizer gas 35 psig; sheath gas (N_2 , purity > 98%): 375
146 $^{\circ}C$ and 11 L/min. High resolution MS spectra were acquired in positive mode in the range 100–
147 3200 m/z . The acquisition time for full scan analysis was 1.5 min.

148

149 3. Results and discussion

150 3.1 EGA-MS and isoconversional method

151 EGA-MS was used to study the thermal behavior and the thermo-chemistry of the mixtures
152 starting from the reference samples. Figure 1 shows the thermograms obtained for
153 R+B~~untreated~~_{untreated}, R+B~~300~~₃₀₀ and P+B~~untreated~~_{untreated} at a heating rate of 40 $^{\circ}C/min$, while the
154 relative average mass spectra are reported in Figure 2. Several experiments were carried out
155 varying the heating rate and Table 3 lists the temperatures of the signal maxima for each heating
156 rate for the various reference mixtures.

157

158 **Table 3:** Peak temperatures for each heating rate for the reference mixtures.

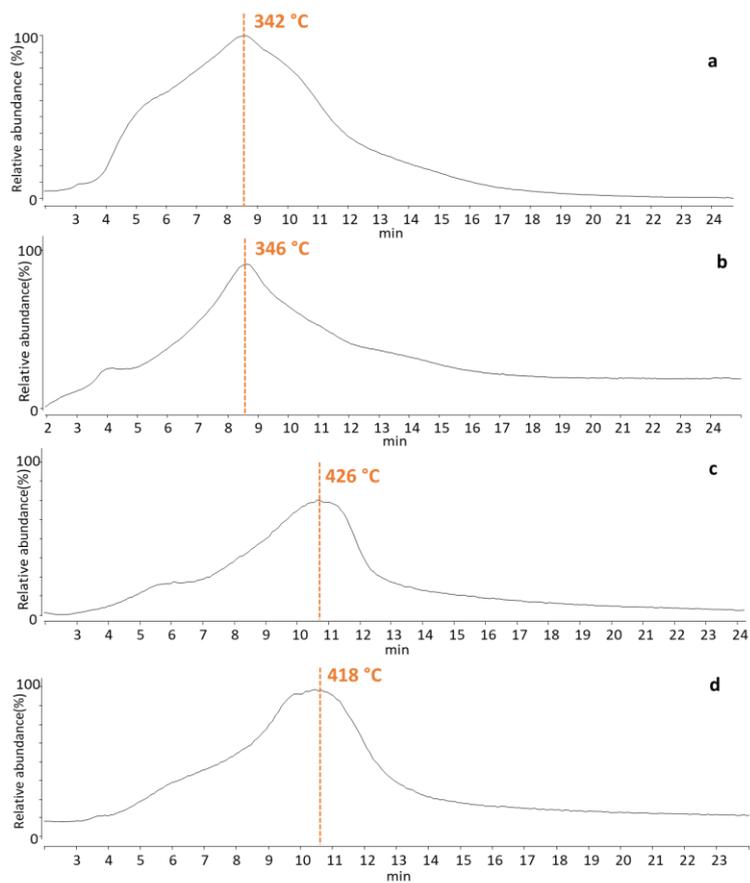
Heating rate	R+B untreated _{untreated}	R+B 300 ₃₀₀	P+B untreated _{untreated}
15 $^{\circ}C/min$	201	173	--
20 $^{\circ}C/min$	224	190	--
25 $^{\circ}C/min$	257	211	392
30 $^{\circ}C/min$	328	245	388
35 $^{\circ}C/min$	352	326	402
40 $^{\circ}C/min$	342	346	426

159

160 The signal peak in the mixture of pitch and beeswax was at higher temperatures than in the
161 samples that contained beeswax and pine resin, even after heating. In particular, the signal peak
162 for samples with pine resin was below 300 °C (not reported here), while it was higher than 300 °C
163 for sample P+B. 300 °C is the temperature to discriminate between thermal desorption and
164 pyrolysis; therefore, it is possible to assert that the pyrolysis of a polymerized/cross-linked fraction
165 took place in sample P+B~~untreated~~_{untreated}, while the thermal desorption of free molecular species
166 was the main phenomenon in samples R+B~~untreated~~_{untreated} and R+B~~300~~₃₀₀. In any case,
167 R+B~~untreated~~_{untreated} and R+B~~300~~₃₀₀ show different behaviors under heating. In fact, the
168 temperature of signal peak generally decreased going from R+B~~untreated~~_{untreated} to R+B~~300~~
169 ~~300~~(Table 3) or remained almost the same at the heating rate of 40 °C/min. These results may be
170 attributed to the fact that the species desorbed from the heated sample were more
171 defunctionalized and aromatized than those from the untreated ones.

172 As support, Figure 2 shows the average mass spectra of samples R+B~~untreated~~_{untreated} and
173 R+B~~300~~₃₀₀. The mass spectrum of sample R+B~~untreated~~_{untreated} (Figure 2a) was mainly
174 characterized by the peaks at m/z 241, 259, 287 and 302 ascribable to abietic acid, while the mass
175 spectrum of R+B~~300~~₃₀₀ (Figure 2b) showed peaks at m/z 197, 239, 285 and 300, and 298 and 237
176 ascribable to abieta-8,11,13-trienoic acid and abieta-6,8,11,13-tetraenoic acid. These compounds
177 will be addressed as dehydroabietic acid and didehydroabietic acid, respectively, in the remainder
178 of the paper [4]. Their presence, combined with the absence of signals ascribable to abietic acid,
179 confirms that the formation of unsaturated diterpenoid compounds was favored at the expense of
180 abietic acid when the mixture was heated during preparation. The signals at m/z values 71, 85, 99
181 and 69, 83, 97, 111 in each mass spectrum can be ascribed to the alkanes and alkenes,
182 respectively, deriving from the hydrocarbon chains and fatty acids and esters of beeswax [5]. The
183 average mass spectrum of the sample P+CB showed high intensities of the signals of unsaturated

184 diterpenoid compounds, but also showed a significant intensity of m/z 219, that can be ascribed to
185 retene, a molecular marker obtained from abietic acid and its derivatives during the heating of
186 resin to produce pitch.

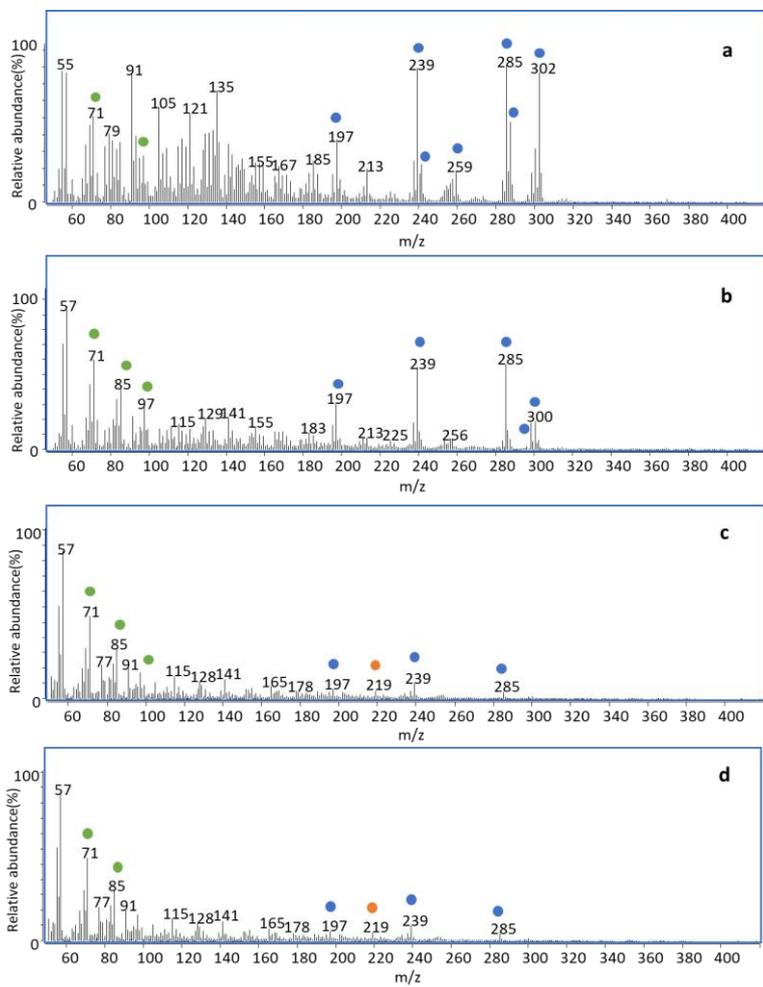


187

188 **Figure 1** Thermograms obtained at the heating rate of 40 °C/min for references mixtures a)

189 R+B~~untreated~~_{untreated}, b) R+B~~300~~₃₀₀ and c) P+B~~untreated~~_{untreated} and d) archaeological sample C6a.

190 The temperature of signal maximum is shown for each thermogram.



191

192 **Figure 2:** average mass spectra of previous thermograms: a) R+Buntreated_untreated, b) R+B300_300, c)

193 P+B300_untreated and d) C6a. In each mass spectrum, the green dots (•) indicate the m/z signals

194 ascribable to desorption/pyrolysis products of beeswax, the blue dots (•) indicate signals of pine

195 resin, and the orange dots (•) indicate signals of pine pitch.

196

197

198 By applying the isoconversional method to the data obtained from EGA-MS we obtained the
199 following values of apparent activation energy values: $1.68 \pm 0.3 \cdot 10^4 \text{ J mol}^{-1}$ and $2.26 \pm 0.4 \cdot 10^4 \text{ J}$
200 mol^{-1} for samples R+B~~untreated~~_{untreated} and R+B~~300~~₃₀₀, respectively, and $3.89 \pm 0.7 \cdot 10^4 \text{ J mol}^{-1}$ for
201 P+B~~untreated~~_{untreated}. The apparent activation energies of heated references were higher than that
202 of untreated samples, highlighting that the thermal pre-treatment and the ageing led to the
203 removal of the more volatile components, promoting the formation of higher molecular weight
204 species through cross-linking reactions. As a further confirmation, the final part of the
205 thermogram of R+B~~300~~₃₀₀ shows a higher signal intensity than the thermogram of
206 R+B~~untreated~~_{untreated}, while the opposite is true for the signals at lower temperatures. Figure 1 and
207 Figure 2 shows also the thermograms and the resulting mass spectrum obtained for the
208 archaeological sample C6a (Figure 1d) a representative archaeological mixture of pitch and
209 beeswax, sample C6a (Figure 1d). EGA-MS results for the other archaeological samples are
210 available in the Supplementary Material.

211 The thermograms obtained for the archaeological samples are consistent with the thermal
212 behavior highlighted for the reference mixture P+B~~untreated~~_{untreated} rather than the two mixtures
213 containing pine resin.

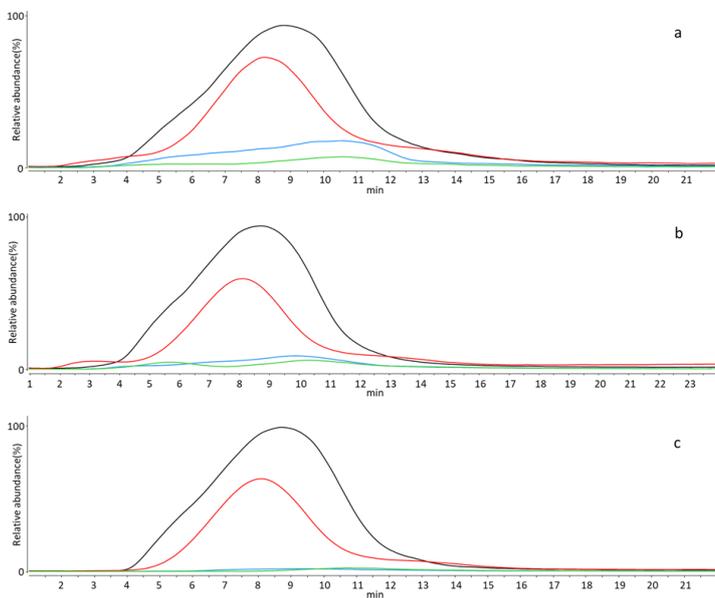
214 The average mass spectra associated with the thermograms are dominated by signals ascribable
215 to beeswax components and by m/z 316 and 330, ascribable to oxidized terpenes such as 7-
216 hydroxyabieta-8,11,13-trienoic acid (7-hydroxydehydroabietic acid) and 15-hydroxy-7-oxoabieta-
217 8,11,13-trienoic acid (15-hydroxy-7-oxodehydroabietic acid), together with dehydroabietic acid
218 and 7-oxodehydroabietic acid. The m/z values 219 and 234, ascribable to retene, were also found
219 in these samples.

220 The thermograms of the archaeological samples showed a peak above 300 °C, similar to that
221 observed for sample P+B~~untreated~~_{untreated}, and, also in this case, in the final part of thermograms

222 the ion current remained high. In addition, the activation energy values increased further, to
223 approximately $6.00 \cdot 10^4 \text{ J mol}^{-1}$. This highlights that, in a similar way to heating, aging promoted
224 the increment of cross-linking reactions and the formation of high molecular weight species in
225 archaeological samples.

226 Further ~~confirmation of the increment of cross-linking reactions is~~ information on the generation
227 ~~of a macromolecular network can be~~ obtained by ~~comparison of comparing~~ the extracted ion
228 thermograms of different samples obtained from distinct m/z values, characteristic of
229 dehydroabietic acid. Figure 3 compares the extracted ion thermograms for m/z 197 (a), 239 (b)
230 and 285 (c) of reference samples R+B~~untreated~~_{untreated}, R+B~~300~~₃₀₀ and P+B~~untreated~~_{untreated}, and
231 archaeological sample C6a. ~~The shift of the peak in the more aged and heated samples~~
232 ~~(P+Buntreated and C6a) confirms the increment of cross-linking reactions and the formation of~~
233 ~~high molecular weight species in archaeological samples.~~ The temperature of signal maximum for
234 these ions was at significantly higher values for the two samples containing archaeological pitch.
235 This indicates that the presence of pitch is the main responsible for the higher thermal stability.
236 Interestingly, sample R+B₃₀₀ did not show any significant shift of the temperature of signal
237 maximum compared to R+B_{untreated}, indicating that the formation of the cross-linked network is
238 mostly formed during the ageing of pitch, rather than during its preparation.

239



240

241 **Figure 3:** Extracted ion thermograms of a) m/z 197, b) m/z 239 and c) m/z 285 respectively. Black
 242 profiles (•) correspond to R+B~~untreated~~_{untreated}, red profiles (•) to R+B~~300~~₃₀₀, blue profiles (•) to
 243 P+B~~untreated~~_{untreated}, and green profiles (•) to archaeological sample C6a.

244

245 3.2 FIA-HRMS

246 Flow-injection experiments of heated reference mixtures provided complex mass spectra, as
 247 shown in Figure 4 (a) and (b). These mass spectra showed clusters of signals that could be ascribed
 248 to resinous and beeswax components, both as free molecules and as oligomers [17]. In
 249 comparison to sample R+B~~200~~₂₀₀, sample R+B~~300~~₃₀₀ showed a higher relative intensity of the
 250 signals corresponding to free fatty acids of beeswax and monomeric diterpenoids of resin
 251 compared to the intensities of the clusters of oligomers. This indicates that heating of the mixtures
 252 favors the cleavage of ester bonds of higher-molecular weight oligomers to give lower-molecular
 253 weight ones. Moreover, the clusters corresponding to beeswax oligomers in sample R+B~~300~~₃₀₀

254 showed a greater number of m/z signals with high relative intensities than the corresponding
255 clusters in sample R+B~~200~~₂₀₀. This indicates that the oligomers are not only degraded to smaller
256 aggregates, but also chemically modified. The most likely hypothesis in this case is a partial
257 oxidation of the oligomers taking place during heating. This is highlighted in Figure 5, showing the
258 portion of the mass spectra of samples R+B~~200~~₂₀₀ and R+B~~300~~₃₀₀ characteristic of beeswax
259 monoesters and resin dimers. As can be observed, the same species responsible for some of the
260 signals in sample R+B~~200~~₂₀₀ can be found in sample R+B~~300~~₃₀₀ at different m/z values, due to
261 either the addition of oxygen or the loss of H₂ after the more intense heating.

262 Finally, sample R+B~~300~~₃₀₀ also showed a small number of m/z signals that could be ascribed to
263 hybrid compounds, generated by the formation of ester bonds between beeswax and resin
264 components. These compounds were also detected in the mass spectra of the mixtures of
265 reference abietic acid and beeswax heated at 300 °C. Proposed structures for these hybrid
266 compounds are shown in Figure 6 (a) and (b). Confirmation of the proposed structures was based
267 on their MS/MS fragmentation patterns. Fragmentation examples are presented in Figure 7, which
268 shows the tandem mass spectra of the hybrid species identified in sample AA+B~~300~~₃₀₀. Samples
269 heated at lower temperatures did not show these signals, indicating that the threshold
270 temperature required for their formation is between 200 °C and 300 °C.

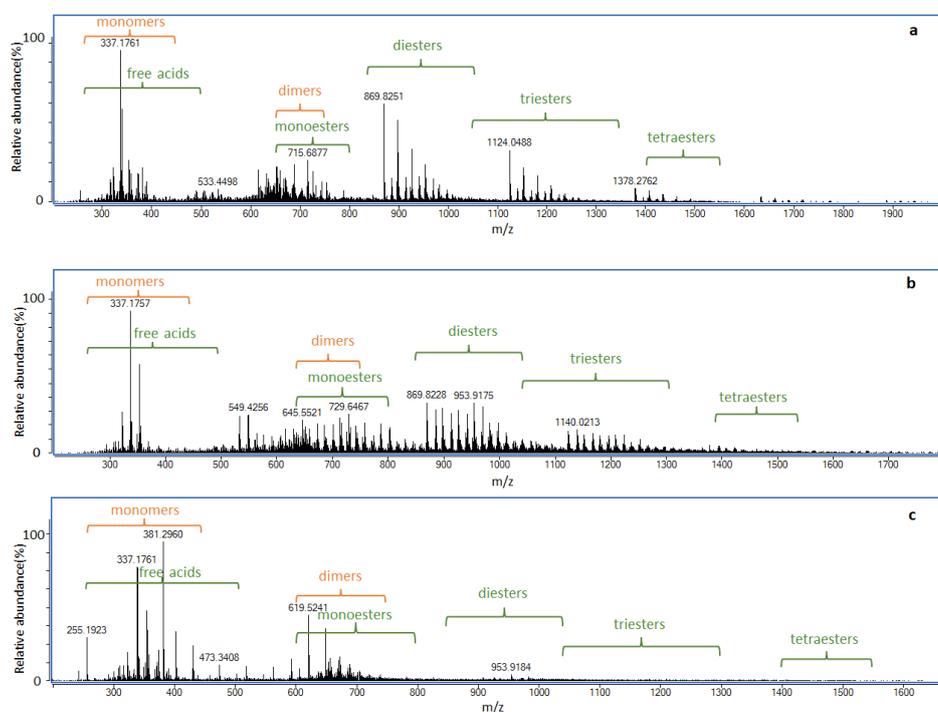
271 Signals ascribable to hybrid species were also detected in the mass spectra of archaeological
272 samples, as shown in Figure 6 (c). A complete list of all hybrid compounds is presented in Table 5.
273 The archaeological sample with the highest number of hybrid compounds was D2bs, while no
274 hybrid compounds were detected in samples C5, D1a and D2a. Archaeological samples were only
275 partially solubilized in the injection solvent used for flow-injection analyses. This is likely due to the
276 formation of a polymeric network during the ageing of these samples, as already hypothesized
277 from the EGA-MS results. This is consistent with the higher apparent activation energies obtained

278 for the archaeological samples compared to the reference mixtures. The low solubility of
279 archaeological samples is the most likely explanation for the lower signals obtained in their mass
280 spectra, especially for the beeswax oligoesters, and for the absence of signals ascribable to hybrid
281 compounds.

282 It is interesting to notice that the ester bonds in the hybrid compounds of the reference mixtures
283 mostly involve the carboxy group of **abietanic abietane** acids and long chain alcohols of beeswax.

284 As free alcohols are almost absent in fresh beeswax [1], we can conclude that the most likely
285 reaction mechanism leading to the formation of hybrid compounds is trans-esterification of the
286 beeswax esters with **abietanic abietane** acids. It is also worth noting that the hybrid compounds
287 obtained in the reference mixtures involve both oxidized and non-oxidized abietic acid. This
288 indicates that oxidation of abietic acid and formation of hybrid compounds have similar reaction
289 rates under the heating conditions adopted in this study.

290



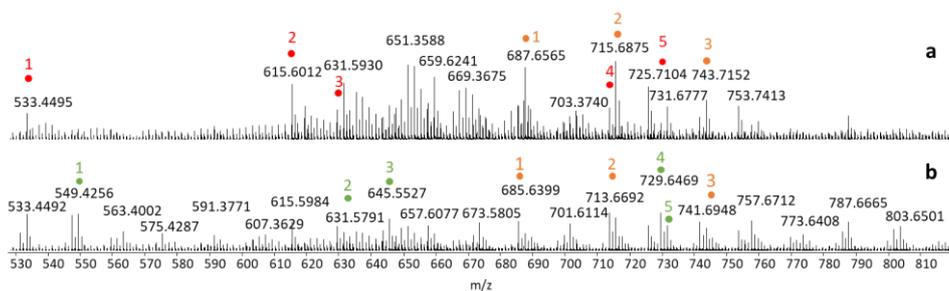
291

292 **Figure 4:** FIA-HRMS average mass spectra of sample a) R+B₂₀₀₂₀₀, b) R+B₃₀₀₃₀₀ and c) sample
 293 D2bs. In each spectrum, clusters highlighted in green indicate *m/z* signals ascribable to ionization
 294 and fragmentation of beeswax components, while clusters highlighted in orange indicate those of
 295 pine resin.

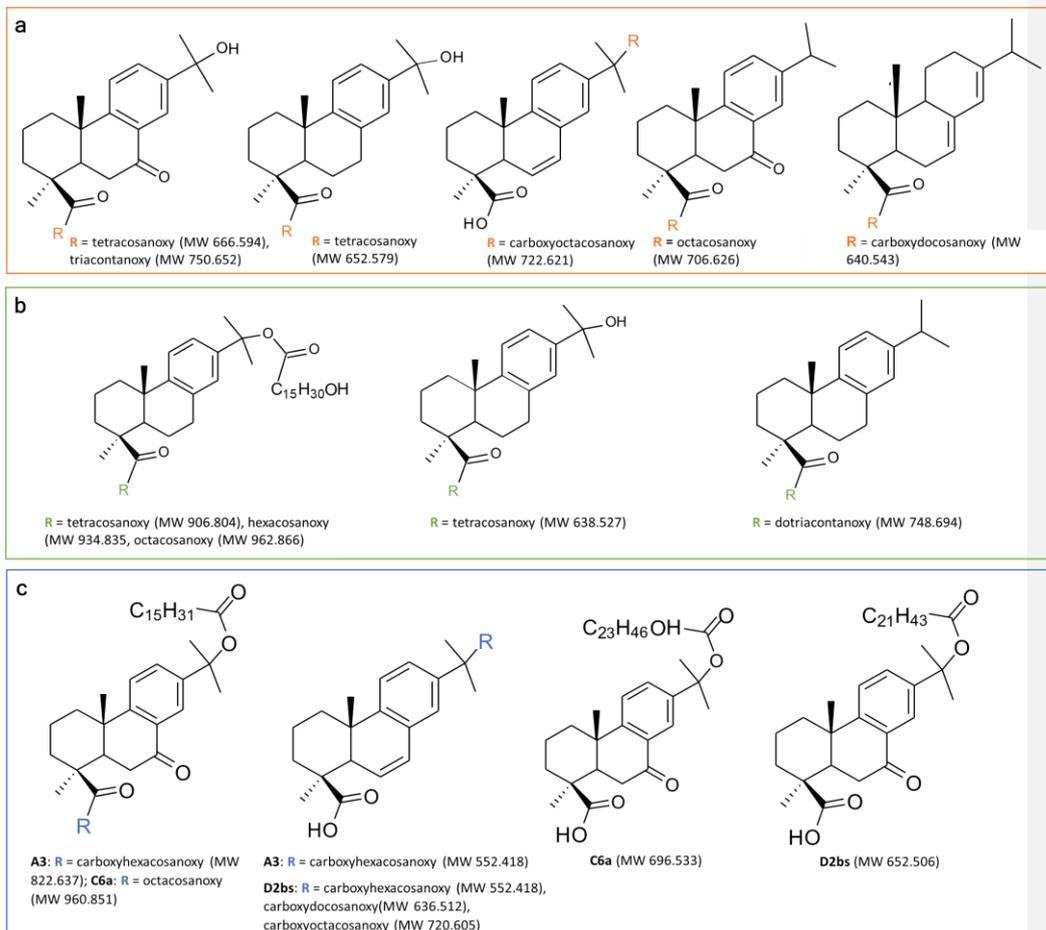
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297 Contrary to the reference samples, the ester bonds in archaeological samples mostly involve free
 298 fatty acids from beeswax and the hydroxy group on carbon number 15 of the **abietanic abietane**
 299 acids, which is a result of the oxidation of resin compounds. This indicates that, for the
 300 archaeological samples, oxidation of **abietanic abietane** acids took place before trans-esterification
 301 reactions. The reason for this is most likely that pitch, rather than fresh resin, was used to prepare
 302 the archaeological mixtures, as pitch is richer in oxidated **abietanic abietane** acid.

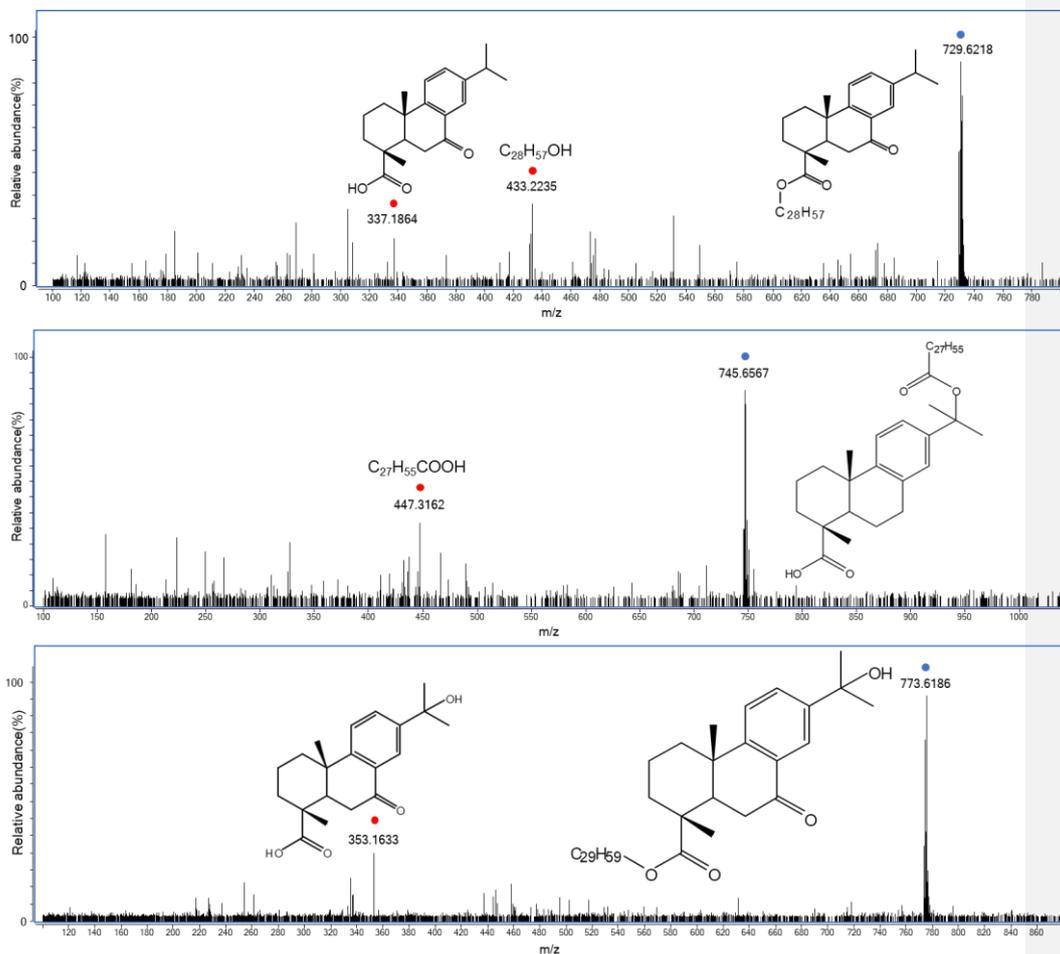
303 The presence of ester bonds involving the hydroxy group on carbon number 15 of **abietanic**
 304 **abietane** acids should not hinder the formation of other ester bonds involving the carboxy group.
 305 However, such ester bonds were not detected in the hybrid compounds of archaeological samples.
 306 This leads us to conclude that trans-esterification reactions induced by heating of the mixtures at
 307 300 °C were not favored during the preparation of the archaeological mixtures, meaning that the
 308 mixtures were heated at lower temperatures.
 309 With this conclusion, a question remains on how the ester bonds could have been formed in the
 310 archaeological adhesives. The most likely explanation is that the hybrid species are not a result of
 311 heating, but rather of ageing. During ageing, the esters of beeswax undergo hydrolysis to give free
 312 carboxylic acids, which can then generate the hybrid species with oxidized **abietanic abietane** acids
 313 without the need of high temperatures.
 314



315
 316 **Figure 5:** Mass spectra of a) R+B~~200~~₂₀₀ and b) R+B~~300~~₃₀₀. Red dots (•) indicate the precursor, while
 317 green dots (•) and orange dots (•) indicate the specie after the addition of oxygen and the loss of
 318 H₂, respectively.



319 **Figure 6:** Structures of resin-wax esters detected in a) AA+B300₃₀₀, b) R+B300₃₀₀ and c)
 320 archaeological samples, correlated to Table 5. Molecular weights are also shown for each
 321 compound.



322 **Figure 7:** tandem mass spectra of hybrid esters found in reference sample AA+B~~300~~₃₀₀. Blue dots

323 (•) represent the precursor ion, while red dots (•) correspond to the identified fragments.

Commentato [ER1]: Le masse non sono le stesse riportate in tabella e poi non ci sono nello schema sopra.

324

325

326 **Table 5:** *m/z* signals **predicted** and **detected** in FIA-HRMS spectra and corresponding compounds.

Sample	<i>m/z</i> [M+Na] ⁺ Predicted	<i>m/z</i> [M+Na] ⁺ Detected	Compound
AA+B ₃₀₀ ₃₀₀	689.548	689.551	15-Hydroxy-7-oxodehydroabietic acid + tetracosanol
	729.616	729.622	7-oxodehydroabietic acid Oxodehydroabietic acid + octacosanol
	773.642	773.645	15-Hydroxy-7-oxodehydroabietic acid + triacontanol
	663.533	663.532	Abietic acid + hydroxydocosanoic acid
	675.569	675.567	15-Hydroxydehydroabietic acid + tetracosanol
	745.611	745.657	15-Hydroxydehydroabietic acid + octacosanol
R+B ₃₀₀ ₃₀₀	661.517	661.513	15-Hydroxydehydroabietic acid + tetracosanol
	771.684	771.706	Didehydroabietic acid + dotriacontanol
	929.794	929.793	Hydroxypalmitic acid + 15-Hydroxydehydroabietic acid + tetracosanol
	957.825	957.825	hydroxypalmitic-Hydroxypalmitic acid + 15-Hydroxydehydroabietic acid + hexacosanol
	985.856	985.863	hydroxypalmitic-Hydroxypalmitic acid + 15-Hydroxydehydroabietic acid + octacosanol
A3	575.408	575.404	15-Hydroxydidehydroabietic acid + palmitic acid
	845.627	845.615	15-Hydroxy-7-oxodehydroabietic acid + palmitic acid + hydroxypalmitic acid
C6a	719.523	719.520	15-Hydroxy-7-oxodehydroabietic acid + hydroxytetracosanoic acid
	983.841	983.843	15-Hydroxy-7-oxodehydroabietic acid + palmitic acid + octacosanol
D2bs	575.408	575.406	15-Hydroxydidehydroabietic acid + palmitic acid
	659.502	659.500	15-Hydroxydidehydroabietic acid + docosanoic acid
	675.496	675.497	15-Hydroxy-7-oxodehydroabietic acid + docosanoic acid
	743.595	743.592	15-Hydroxydidehydroabietic acid + octacosanoic acid

Commentato [ER2]: Attenzione, non c'è corrispondenza con la struttura riportata in figura

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329 **4. Conclusions**

330 The formation of new hybrid species between diterpenoids of the resinous and beeswax
331 components has been confirmed both by EGA-MS and FIA-HRMS. Structures of these hybrid
332 components hypothesized from the FIA-HRMS spectra strongly suggest that trans-esterification
333 reactions are the main mechanisms responsible for their formation. In EGA-MS analyses, the shift
334 of the signal peak temperature from reference samples containing pine resin to mixture
335 containing pitch and archaeological samples suggested the formation of species with high
336 molecular weights and reticulated fractions. This was confirmed by the calculated values of
337 apparent activation energy. The formation of high molecular weight species, including resin-
338 beeswax hybrid esters, was also highlighted by data obtained from FIA-HRMS. Flow injection
339 analyses suggested that the formation of high molecular weight species occurred after heating the
340 reference mixture at 300 °C. Hybrid species were also found in archaeological samples, but with
341 different structures, suggesting that their formation is more likely due to hydrolysis of beeswax
342 esters during ageing, and that the archaeological adhesives were heated at lower temperatures
343 than the ones used for the references.

344

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