Molecular and macromolecular fractions of geological ambers detected by evolved gas analysis-mass spectrometry

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ABSTRACT

We used evolved gas analysis-mass spectrometry (EGA-MS) to characterize a pool of ambers belonging to different geological varieties, ~~including ambers from central Italy which were not studied by mass spectrometric methods before,~~ with the aim ~~to obtain~~ of obtaining information on both their ~~molecular and macromolecular fractions~~ non-polymeric and polymeric components. Small molecular components can be trapped in amber during the fossilization process. Their characterization can provide insights into the maturation process of amber and help in determining its age and botanical origin. Most of the analyzed ambers showed two gas evolution regions, corresponding to the desorption of low-molecular weight compounds at low temperature, and to the pyrolysis of the macromolecular matrix at high temperature. We established characteristic *m/z* signals of the ~~molecular and macromolecular~~ non-polymeric and polymeric fractions, ~~useful to differentiate ambers,~~ and we were able to classify most of the samples in agreement with the available literature. We also performed principal component analysis of diterpenoid ambers using their average mass spectra. This allowed us to differentiate between succinite and rumanite ambers, which showed ~~apparently~~ very similar spectra. Ambers from central Italy were also successfully c~~a~~haracterized as diterpenoid ambers containing free ~~mono- and diterpenoids~~ tetrahydro- and octahydronaphthalenes and a poly-labdane polymeric network. The results demonstrate that EGA-MS constitutes a fast and reliable technique to obtain information on the molecular composition of ambers, and on their relative content of low- and high-molecular weight fractions.

Keywords: Amber; evolved gas analysis; mass spectrometry; terpenoids; succinite

1. INTRODUCTION

Amber is obtained by the fossilization of tree resin on a geological time scale. The fossilization process requires the resin to be incorporated in sediments under specific environmental conditions, such as a low availability of oxygen and closeness to a river delta or lagoon [1]. Such conditions can only be met in few places on the planet, making amber a rare, precious material whose value has been acknowledged since ancient times [2-4]. Fossilization of ~~tree~~ resin into amber is a complex phenomenon which involves two main processes: polymerization and maturation [5,6]. Polymerization involves the coupling of resin terpenoids, leading to the formation of the bulk macromolecular matrix of amber. This process is relatively fast, and can be even observed in fresh tree resin exposed to air and light. On the other hand, maturation is a slow process which involves isomerization and loss of functional groups [7]. Maturation depends not only on the composition of amber, but also on its geological age and thermal history [8].

As the macromolecular bulk of amber is formed and undergoes maturation, smaller, non-polymeric components of resin can either be lost in the environment, or trapped in the polymeric matrix. In the last decades, studies have shown that ambers at more advanced stages of maturation usually have a lower content of non-polymeric species [7]. Therefore, knowledge of the relative content of the two fractions could provide valuable insights into the degree of maturation of amber, as well as into the reaction pathways involved in the fossilization process. One of the main challenges in obtaining separate information on the molecular and macromolecular fractions of ambers is that they often share similar structural features, as the components of the non-polymeric fraction are the precursors of the polymeric one [9].

Common analytical techniques used to characterize amber are spectroscopies such as FTIR, Raman and NMR [10-13], and thermo-analytical techniques such as TGA and DSC [14,15]. ~~However, these~~ These techniques are used to characterize whole amber samples, and separating the contributions of the molecular and polymeric fractions is extremely challenging. The non-polymeric fraction of amber can be solubilized and characterized by derivatization followed by GC-MS analysis [16] or by SPME [17]. However, this approach can be time-consuming, and often requires considerable amounts of

sample, which are not always available. Analytical pyrolysis coupled to GC-MS (Py-GC/MS) is a powerful and widely used technique to characterize amber, providing molecular-level information on both the molecular and macromolecular fractions. Py-GC/MS is often carried out using *In situ* derivatization with a methylating or a silylating agent to improve the chromatographic performances of desorption/pyrolysis products with polar moieties [3,7,18-22]. The pioneering work by Anderson and co-workers with ~~this technique~~ Py-GC/MS led to the establishment of a well-known classification system in which ambers are categorized on the basis of the structure of their polymeric fraction [6]. Pyrolysis temperature in Py-GC~~-~~/MS experiments can be modulated to either desorb the non-polymeric components and leave the polymeric matrix unaltered, or to pyrolyze the macromolecular bulk [23]. ~~While this could constitute a promising approach for the separate characterization of the two fractions of amber, very few applications are available in the literature.~~ For instance, Anderson and co-workers reported that Py-GC/MS of succinite at different temperatures provided chromatograms containing different sets of terpenoid compounds [18]. Virgolîci and co-workers characterized amber by thermal desorption-GC/MS, heating the samples at 200 °C to desorb non-polymeric compounds without degrading the macromolecular bulk [24].

~~In addition, ambers~~ Ambers with different terpenoid compositions can show significantly different thermal behaviors. Establishing the optimal temperature to selectively achieve desorption or pyrolysis through Py-GC/MS requires preliminary studies, which can be very ~~time-consuming~~ time-intensive and consume significant amounts of sample.

Evolved gas analysis-mass spectrometry (EGA-MS) is a ~~fast and~~ versatile technique that can be used to monitor the evolution of desorption/pyrolysis products from a sample as a function of temperature. EGA-MS shares the advantage of Py-GC/MS of minimal sample preparation, and is often used to study ~~of~~ complex samples of both natural and synthetic origin~~, and~~. EGA-MS is also a powerful screening technique to establish the optimal desorption/pyrolysis temperatures before Py-GC/MS analysis [25-28], avoiding the need of preliminary Py-GC/MS experiments at different temperatures. To the best of our knowledge, however, no studies are available in the literature describing the thermal behavior of ambers using EGA-MS.

In the present work, we performed EGA-MS of a pool of geological ambers with different origins, with the aim of obtaining insights into the composition of both the ~~molecular~~ non-polymeric and polymeric fractions of amber and into their thermal behaviors. ~~Three ambers collected in central Italy are also analyzed here for the first time with a mass spectrometric method.~~

2. MATERIALS AND METHODS

2.1 Samples. 46 samples of geological amber were investigated in this study. A list of all samples is provided in **Table 1**Simetite is a characteristic amber deriving from a deposit close to river Simeto, in Sicily, which has been dated to Miocene [14]. Ambers from central Italy have been dated to Oligocene [31]. Information on the geological origin was not available for some of the samples. Before analysis, a small amount of each sample was grinded to a fine powder using a Pulverisette 23 laboratory scale ball mill (Fritsch, Germany). Cryo-milling with liquid nitrogen was not necessary, as all samples could be easily milled in less than 10 s, and no significant increase in temperature was observed.

2.2 Evolved gas analysis-mass spectrometry (EGA-MS). Experiments were performed with an EGA/PY-3030D microfurnace pyrolyzer (Frontier Laboratories, Japan) coupled to a 6890 gas chromatograph and a 5973 mass spectrometric detector (Agilent Technologies, USA). In each experiment, approximately 100 µg of sample were directly weighted in the sample holder and inserted in the pyrolysis furnace. The temperature of the furnace was then raised from 50 °C to 700 °C at 10 °C/min. The temperature of the pyrolysis interface was kept 100 °C above the furnace temperature, up to a maximum of 300 °C. Injection was performed in split mode at 280 °C with a 20:1 ratio. Desorption/pyrolysis products are directly sent to the MS detector through an UADTM-2.5N deactivated stainless steel capillary tube (Frontier Laboratories, Japan) at 300 °C. Helium (1 mL min^{-1}) was used as carrier gas. The mass spectrometer was operated in EI positive mode (70 eV)~~.~~, The observed *m/z* range was 50 – 800~~)~~. Values below *m/z* 50 are generally poorly diagnostic, and their detection was avoided. The ion source and quadrupole analyzer were at 230 °C and 150 °C, respectively.

2.3 Data processing. EGA-MS profiles were processed with MassHunter (ver. 10.0, Agilent Technologies, USA). Mass spectra were interpreted by comparison with those available in reference libraries (NIST20, NIST, USA) and in previous publications [8,18,32-39]. Reproducibility of the EGA-MS experiments was assessed by repeating the analysis of the same sample three times and calculating the error on the total integrated area of the thermograms divided by the sample weight. The relative standard deviation was 9% on average.

Principal component analysis (PCA) of succinite, rumanite, gedanite and gedano-succinite samples was performed with OriginPro 2018. The data matrix was built with the average mass spectra of all samples calculated through the whole thermal degradation range. For this data matrix, the *m/z* range was restricted to 50–350 to avoid contribution from the background noise. Two additional PCAs were performed using the average mass spectra calculated in two different temperature ranges: 50–300 °C for the ~~molecular~~ non-polymeric components, and 300–550 °C for the polymeric components. Principal components were always calculated using the covariance matrix.

Table 1. The samples were provided by nine different collections belonging to ~~European~~ museums and academic institutes. ~~Classification and geographical origins for all samples were taken as available by the curators of all collections.~~ Information on the classification and geographical origins of the samples are listed as provided by the institutes, when available. Eight samples derive from the amber deposit in Bitterfeld, the largest amber reservoir in Germany. Bitterfeld ambers have been dated to the Oligocene period, between 25 and 23 million years ago [12,16,29]. Three samples derive from different locations of the Pomeranian Voivodeship, including Gdansk and Mikoszewo. Ambers from the Baltic shores have also been dated to either Eocene or Oligocene [14,30]. All rumanite samples derive from the basins in Colti and Pătârlagele in Romania, which are dated to Oligocene [17]. Simetite is a characteristic amber deriving from a deposit close to river Simeto, in Sicily, which has been dated to Miocene [14]. Ambers from central Italy have been dated to Oligocene [31]. Information on the geological origin was not available for some of the samples. Before analysis, a small amount of each sample was grinded to a fine powder using a Pulverisette 23 laboratory scale ball mill (Fritsch, Germany). Cryo-milling with liquid nitrogen was not necessary, as all samples could be easily milled in less than 10 s, and no significant increase in temperature was observed.

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163 the error on the total integrated area of the thermograms divided by the sample weight. The relative
164 standard deviation was 9% on average.
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171 Principal components were always calculated using the covariance matrix.
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173

Table 1. Labels, geological classification, geographical origin~~s~~, and providers of all amber samples. 1 – Natural History Museum of Warszawa, Poland; 2 – Natural History Museum of Milano, Italy; 3 – The Louvre Museum in Paris, France; 4 – Getty Institute, Los Angeles, USA; 5 – University of Padova, Italy; 6 – Natural History Museum of Calci, Italy; 7 – University of Modena and Reggio Emilia, Italy; 8 – "G. Zannato" Museum of Archaeology and Natural Sciences, Montecchio Maggiore, Italy; 9 – Romanian National History Museum, Bucharest, Romania.

Label	Provider	Classification	Geographical origin	Aspect
Sg01	1	Siegburgite	Bitterfeld, Germany	
Gl01	1	Glessite	Hoyerwerda, Germany	
Gl02	1	Glessite	Not provided	
Gl03	1	Glessite	Not provided	
Ge01	1	Gedanite	Bitterfeld, Germany	
Ge02	1	Gedanite	Mikoszewo, Poland	
GS01	1	Gedano-succinite	Pomerania, Poland	
Go01	1	Goitschite	Bitterfeld, Germany	
Go02	1	Goitschite	Not provided	
BA01	1	Black amber	Bitterfeld, Germany	
BA02	1	Black amber	Bitterfeld, Germany	
BA03	1	Black amber	Ukraine	
BA04	1	Black amber	Poland	
Su01	1	Succinite	Bitterfeld, Germany	
Su02	1	Succinite	Bitterfeld, Germany	
Su03	1	Succinite	Klesow, Ukraine	
Su04	1	Succinite	Klesow, Ukraine	
Su05	1	Succinite	Jantarnyj, Russia	
Su06	2	Succinite	Not provided	
Su07	2	Succinite	Not provided	
Su08	2	Succinite	Not provided	
Su09	2	Succinite	Not provided	
Su10	2	Succinite	Not provided	
Su11	3	Succinite	Not provided	
Su12	3	Succinite	Not provided	
Su13	3	Succinite	Not provided	
Su14	3	Succinite	Not provided	
Su15	4	Succinite	Not provided	
Su16	4	Succinite	Not provided	
Su17	5	Succinite	Russia	
Su18	5	Succinite	Gdańsk – Stogi, Poland	
Su19	5	Succinite	Bitterfeld, Germany	
CI01	6	Unknown	Scannello, Italy	
CI02	7	Unknown	Loiano, Italy	
CI03	7	Unknown	Prignano, Italy	
Si01	8	Simetite	Sicily, Italy	
Si02	8	Simetite	Sicily, Italy	
Ru01	9	Rumanite	Colţi, Romania	
Ru02	9	Rumanite	Colţi – Pătârlagele, Romania	
Ru03	9	Rumanite	Colţi – Pătârlagele, Romania	
Ru04	9	Rumanite	Colţi – Pătârlagele, Romania	
Ru05	9	Rumanite	Colţi – Pătârlagele, Romania	
Ru06	9	Rumanite	Sibiciu de Jos, Romania	
Ru07	9	Rumanite	Colţi – Pătârlagele, Romania	
Ru08	9	Rumanite	Colţi – Buzău, Romania	
Ru09	9	Rumanite	Colţi – Buzău, Romania	

3. RESULTS AND DISCUSSION

3.1 Glessites and goitschites. EGA-MS profiles of a representative glessite and goitschite and corresponding average mass spectra are shown in **Figure 1**, along with proposed structures for the main *m/z* signals ~~in the average mass spectra~~. EGA-MS profiles of all samples are available in the Supplementary Material. The two amber types displayed similar thermal and mass spectral features. We identified three main gas evolution regions in all Gl and Go samples (see **Table 1** for compound labels): a first region between 100 and 200 °C, a second region between 200 and 300 °C, and a third region between 300 and 550 °C. The third region showed the highest signal intensity in all samples. High signals at *m/z* 202 and 204 were detected in all three regions of the thermograms. These signals can be ascribed to sesquiterpenoids. Sesquiterpenoids with cadinene-like structures were previously identified in glessite samples by Yamamoto et al. [16]. The main MS fragmentations for cadinenes are the loss of the methyl or the isopropyl group, generating the signals at *m/z* 161, 159, 189, and 187. The signal at *m/z* 408 can be ascribed to dimeric cadinene. The dimer undergoes the same MS fragmentations of the monomer, with loss of methyl or isopropyl groups giving *m/z* 365 and 393. Cleavage of the C-C bond between the two sesquiterpene units is also possible, generating *m/z* 203. The signal at *m/z* 203 can be observed in both the second and third region of the thermogram, indicating that higher cadinene oligomers and polymers are also present in Gl and Go samples. Oligomeric and polymeric cadinenes have been observed in both fresh and fossil triterpenoid resins [34,40].

Additional signals were detected in the mass spectra of the second and third regions. Glessite samples showed high intensity of *m/z* 218, while goitschite samples showed high intensity of *m/z* 262. The even values indicate that the corresponding structures are obtained by rearrangement reactions. The most common rearrangement in terpenes is the retro-Diels-Alder (RDA) elimination. The formation of ions with *m/z* 218 and 262 through RDA of triterpenes has been documented in the ~~historic~~ papers by Djerassi and co-workers [32,33]. As triterpenes were detected in previous studies of glessites [16], we hypothesize that triterpenes are also present in our glessites and goitschites. The different *m/z* values indicate that Gl and Go samples contain triterpenoids with different structures. In particular, *m/z* 218

can be ascribed to triterpene hydrocarbons with the ursane and oleanane structures, such as α- and β-amyrin, while *m/z* 262 can be obtained from the methyl carboxylate equivalents of the same compounds. Note that additional MS fragmentations of these ion radicals can also generate *m/z* 189 and 203, as suggested in **Figure 1**.

The trends of the discussed *m/z* signals in the three gas evolution regions can be used to outline a general picture of the thermal behavior of Gl and Go ambers. The two amber types contain both monomeric and oligo/polymeric sesquiterpenes, most likely with the cadinene carbon skeleton. In addition, glessites also contain amyrin-like triterpenoids, while goitschites contain triterpenoids with ursane or oleanane structures bearing a methyl-carboxylate functional group, most likely located on carbon 28. These results allow us to ~~attribute~~ identify both Gl and Go ambers ~~to~~ as class II ambers ~~of the Anderson classification system~~ [6].

The results obtained for goitschites in this study are in disagreement with those of the study by Yamamoto and co-workers [16], which reported the presence of diterpenoids in a goitschite sample with the same geographical origin as sample Go01. This significant difference in results highlights ~~one of the main problems in amber classification: amber samples are often named after the mining sites from which they are excavated, and not on the basis of their molecular features. Therefore, ambers with very different botanical origin can often share the same geological denomination.~~ that the geological denomination of an amber sample should always be supported by information on its molecular composition. Different results obtained from the characterization of ambers with the same geological name are not necessarily ascribable to poorly conducted experiments or mislabeling of the samples. Rather, geological denomination of amber samples is often based on their geographical origin or physical and optical features, without information on its molecular composition. This is especially true for amber samples which entered museum and academic collections in the previous century, when instrumental analytical techniques were far less available.

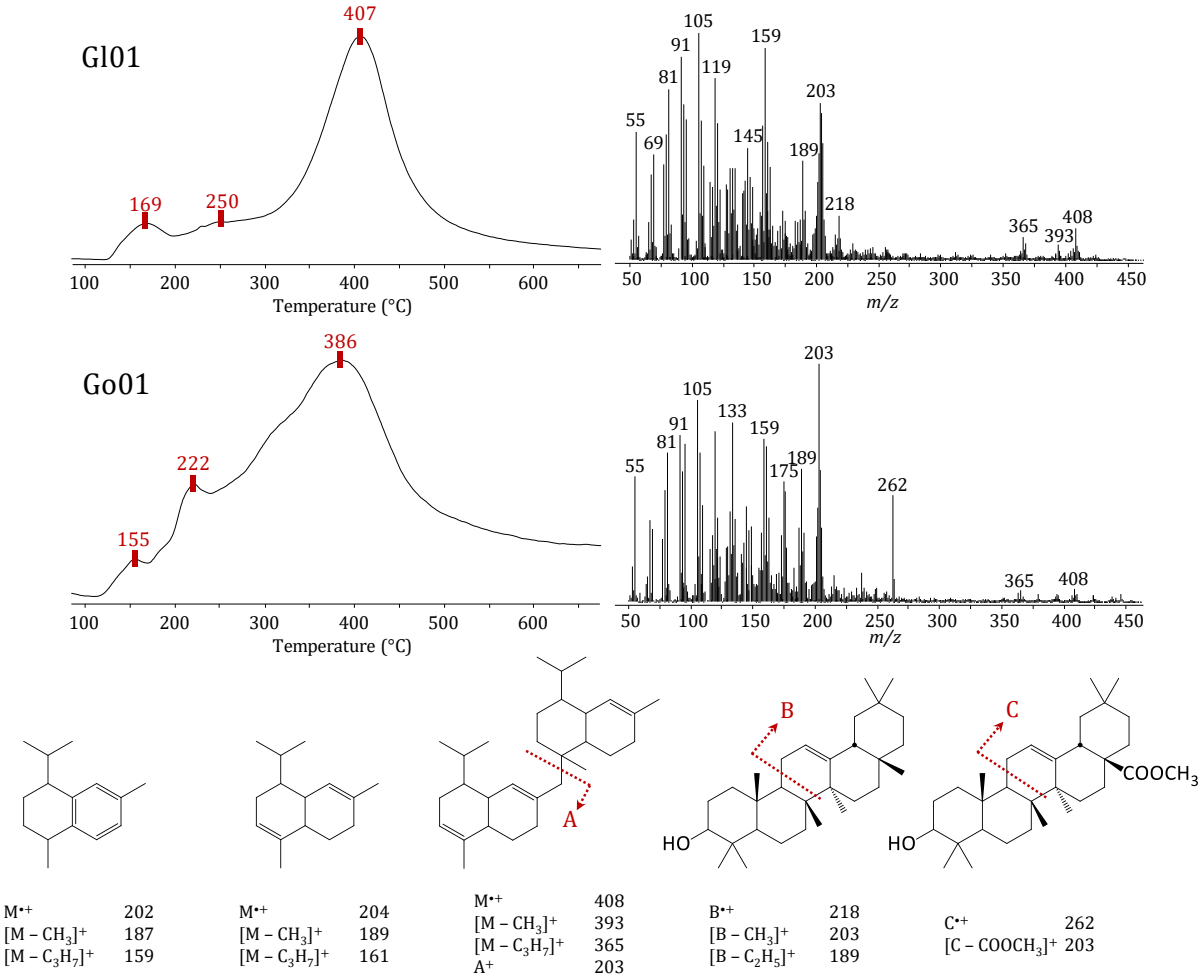

Figure 1: EGA-MS profiles and average mass spectra for samples Gl01 (glessite) and Go01 (goitschite). Proposed structures of ions for *m/z* signals of relevance are also shown.

3.2 Siegburgite and black ambers. EGA-MS profiles for sample Sg01 and a representative black amber sample are shown in **Figure 2**. Sample Sg01 was the only instance of polystyrene-like amber in the sample pool. Polystyrene-like ambers belong to class III ~~of the Anderson system~~ [10,41]. This peculiar type of amber has been documented since the 19th century [42]. Grimaldi and co-workers gave a thorough account of polystyrene-like amber slabs found in New Jersey, but this type of amber was also found in the Bitterfeld deposit [43,44].

The average mass spectrum for this sample showed the characteristic *m/z* signals ascribable to polystyrene [35]. The sample showed a single gas evolution region, with a signal peak at 405 °C. The high value for this signal peak indicates that the gas evolution peak corresponds to a thermal

degradation of the polymeric bulk of the material, and that sample Sg01 contains very small amounts of non-polymeric species. While polystyrene represents the bulk of the sample, the presence of oxygenated species in siegburgite has also been reported. These species are mainly cinnamic acid and its esters, which have been proven to act as cross-linkers in the polymeric network [41]. These compounds are most likely related to the original components of the resin. The most likely botanical origin for this amber is in fact the liquidambar plant (*Altingiaceae* family), whose resin has a high content of cinnamic acid and its esters [45]. Upon pyrolysis, these oxygenated species generate ~~characteristic compounds~~ cinnamic acid derivatives such as 3-phenylpropanylcinnamate, which has been proposed by Pastorova and co-workers as a marker to distinguish siegburgite from New Jersey class III amber and from synthetic polystyrene, which could be used to produce fakes [41]. ~~whose~~ The mass spectrum of 3-phenylpropanylcinnamate shows a signal at *m/z* 118 as base peak. The relative intensity of *m/z* 118 in sample Sg01 is indeed higher than what is usually observed for synthetic polystyrene ~~[32], suggesting that this sample contains cinnamic acid and its esters~~. However, *m/z* 118 can also be ascribed to methylstyrene, which is also a pyrolysis product of polystyrene [35]. Therefore, no definitive conclusion on the authenticity or the botanical origin of this sample can be drawn on this sample based on EGA-MS analyses alone.

Black amber is also known as stantienite [16]. Contrary to siegburgite, the EGA-MS profiles of all black ambers showed two main gas evolution regions: a first region in the range 100 – 300 °C, and a second region in the range 300 – 600 °C. Interpretation of the mass spectra of BA samples was not straightforward, as the highest signals throughout the thermogram were at low *m/z* values (below *m/z* 150), which are poorly diagnostic. However, three series of signals can be outlined, corresponding to saturated (*m/z* 57, 71, 85), mono-unsaturated (*m/z* 55, 69, 83, 97, 111), and di-unsaturated (*m/z* 67, 81, 95, 109, 123) linear aliphatic hydrocarbons. These signals were present with little variations in relative intensity throughout the EGA-MS profile, indicating that linear hydrocarbons are the main components of both the ~~molecular and macromolecular~~ non-polymeric and polymeric fractions of black ambers. This indicates that black ambers underwent extensive maturation, and are almost completely fossilized.

Yamamoto and co-workers [16] identified two homolog series of methyl esters of mono- and dicarboxylic fatty acids in dichloromethane:methanol extracts of a stantienite sample. Both categories of compounds share *m/z* 74 as a very intense signal in their mass spectra. However, this signal was not found in any of the BA samples of our study, suggesting that our black amber samples are either at a more advanced state of maturation, or that their content of these methyl esters is extremely low. The amount of methyl esters in the stantienite sample of Yamamoto is not known, as no information is provided on the amount of sample used for the extraction.

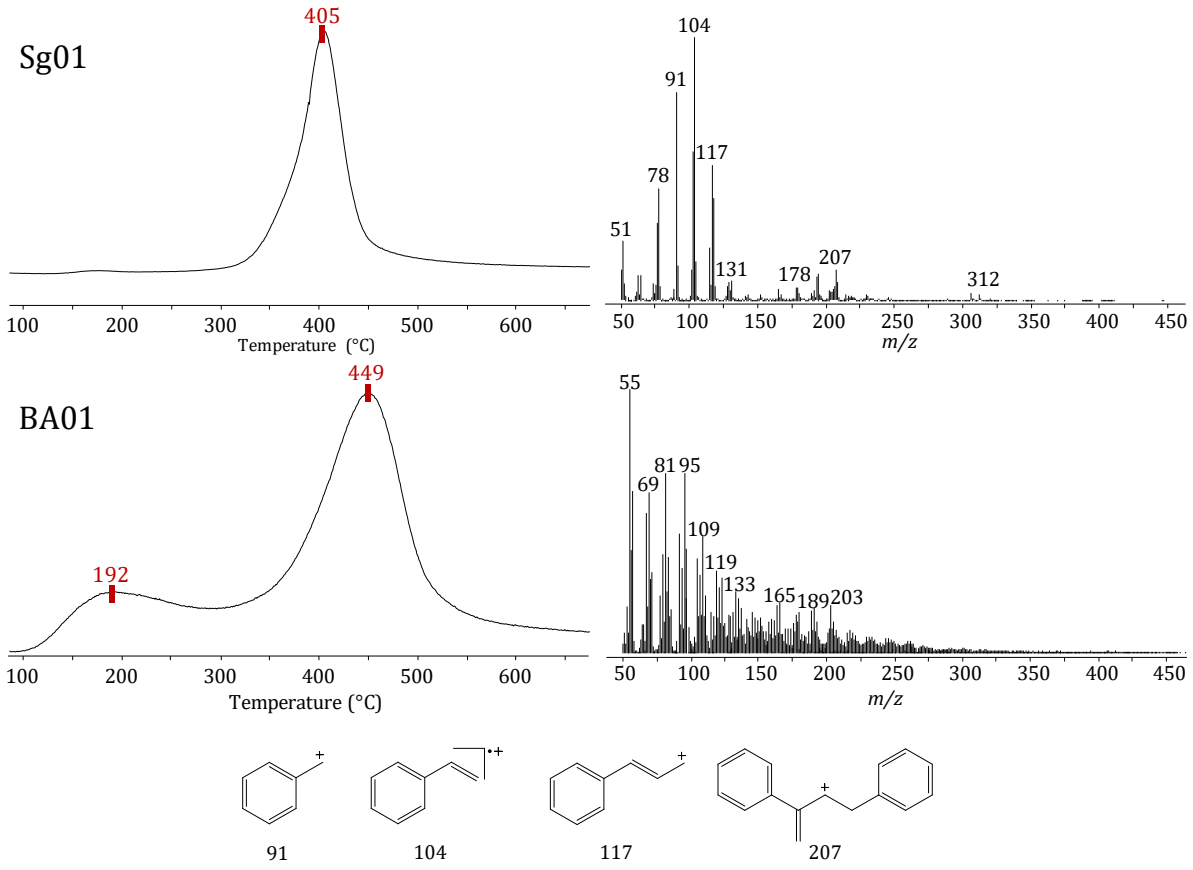

Figure 2: EGA-MS profiles and average mass spectra for samples Sg01 (siegburgite) and BA01 (stantienite). Proposed structures of ions for *m/z* signals of relevance in sample Sg01 are also shown.

3.3 Succinite, rumanite, gedanite and gedano-succinite. EGA-MS profiles and average mass spectra for these four types of amber are presented in **Figure 3**. All Su, Ru, Ge and GS samples showed two gas evolution regions in the temperature ranges 100 – 300 °C and 300 – 550 °C. As observed for

289 the other amber types, these regions can be ascribed to desorption of ~~molecular~~ non-polymeric
290 compounds at low temperatures, and to the pyrolysis of the polymeric matrix above 300 °C. While this
291 behavior is shared by all samples, the two gas evolution regions displayed different profiles. For
292 instance, gedanites and gedano-succinites provided gas evolution regions with multiple signal peaks
293 both in the thermal desorption and the pyrolysis ranges, while most succinites and rumanites
294 provided a single peak in either range.
295 Interpretation of the mass spectra of these samples was based on the results of previous studies
296 dealing with succinite and rumanite [8,17,18,24], and on studies dealing with the interpretation of
297 mass spectra of diterpenes [36,37]. The thermal desorption region of all samples showed high signals
298 of *m/z* 95, 119 and 134. The signal at *m/z* 95 can be ascribed to camphene-like monoterpenoids, such
299 as borneol and camphor, ~~which~~ while those at *m/z* 119 and 134 can be ascribed to aromatic
300 monoterpenes such as *p*-cymene. ~~The main aromatic monoterpene found in succinite is *p*-cymene,~~
301 ~~whose structure is~~ Structures for these compounds are shown in **Figure 3**. All these compounds were
302 detected in the volatile fraction of rumanite and succinite samples by SPME [17]. ~~All gedanite~~ Gedanite
303 and gedano-succinite samples showed additional signals at *m/z* 241, 257, 287, 302, with peaks at
304 approximately 240 °C. These signals can be ascribed to diterpenoid acids. A survey of the mass spectra
305 of diterpenoid acids available in the literature and in the NIST library showed that the abietane,
306 pimarane and isopimarane structures are the most likely to be present in Ge and GS samples. The
307 signal at *m/z* 302 corresponds to the molecular ion of abietic/pimaric/isopimaric acid, while the other
308 signals can be ascribed to ions obtained by characteristic fragmentation patterns, such as the loss of
309 methyl radical or decarboxylation.
310 The second gas evolution region showed very complex mass spectra in all samples. Discussion of this
311 region of the thermogram is particularly challenging, as the signals with the highest intensities were at
312 relatively low *m/z* values, which are poorly diagnostic. Compared to the first gas evolution region,
313 higher relative intensities were observed for *m/z* 173, 175, 177, 189 and 191. These signals can be
314 ascribed to the bicyclic pyrolysis products of the poly-labdanoid polymeric network which is known to
315 be the main constituent of the bulk of succinite and rumanite ambers [8]. The same signals were found

316 in gedanite and gedano-succinite, indicating that these samples also share a similar composition of the
317 polymeric fraction. The mass spectra of both gas evolution regions demonstrate that Su, Ru, Ge and GS
318 samples all share very similar compositional profiles. This is in agreement with results available in the
319 literature, which ascribe all these amber varieties to a common botanical source [8,39], and allows us
320 to assign all ~~our~~ samples to class I ~~of the Anderson system~~. Class I contains a range of subclasses with
321 significantly different molecular composition. A pivotal discrimination criterion among these
322 subclasses is the presence and amount of succinic acid, which can be found for instance in subclass Ia,
323 and Id ambers, but not in subclasses Ib and Ic [6]. Succinic acid in amber can be found in amounts as
324 high as 8% [39], and can be both a component of the non-polymeric fraction, and part of the polymeric
325 matrix, forming ester bonds with hydroxy groups on polymerized terpenoids [46]. Free succinic acid
326 can be converted in the corresponding anhydride during heating [47,48]. However, both succinic acid
327 and succinic anhydride show high signals at low *m/z* values, which are poorly diagnostic as they can
328 also be ascribed to numerous fragmentations of the terpenoid components. Detection of the terpenoid-
329 succinic acid esters is also challenging, as their main fragment ions are the same of non-esterified
330 terpenoids [46]. Therefore, detection of succinic acid or its derivatives in EGA-MS profiles was not
331 possible, and we avoided assigning the samples to specific subclasses.
332 As Su, Ru, Ge and GS ambers all provided similar gas evolution profiles, principal component analysis
333 (PCA) was performed to highlight differences between the four types. Average mass spectra in the
334 range *m/z* 50 – 300 and 100 – 550 °C were used as data matrix. The results are presented in **Figure 4**.
335 The first two principal components accounted for approximately 75% of the total variance. The most
336 interesting discrimination of the samples was along the first principal component, in which all
337 rumanite samples were at negative values, and all succinite, gedanite and gedano-succinite samples
338 were at positive values. The loading plot of the first principal component, also shown in **Figure 4**,
339 indicates that *m/z* 55, 57, 69 and 83 are the main signals pulling samples towards negative values.
340 While not diagnostic, these *m/z* signals can be ascribed to small hydrocarbon fragment ions deriving
341 from the terpenes of both the ~~molecular and macromolecular~~ non-polymeric and polymeric fractions
342 of amber. The results of the PCA indicates that rumanite samples have higher relative intensities of

these signals. This suggests that rumanite has a higher content of defunctionalized terpenes, and a lower content of oxygenated functional groups. This result is consistent with the findings of Stout and co-workers [8], who identified rumanite as a variety of succinite amber which underwent a partial thermal degradation, accelerating the maturation process of amber and favoring defunctionalization and the loss of hydroxy and carboxy groups. Exposure to high temperatures also likely favored the loss of low-molecular weight components from the bulk of the amber. This hypothesis is supported in our results by the loading of *m/z* 119, which pulls samples towards positive values of the PC1. As *m/z* 119 can be ascribed to monoterpenes, the results of the PCA suggest that succinite, gedanite and gedano-succinite samples have a higher content of low-molecular weight compounds than rumanite.

PCA was also repeated using average mass spectra obtained either from the thermal desorption region (100 – 300 °C) or from the pyrolysis region (300 – 550 °C) of the thermogram. The resulting score and loading plots are available in the Supplementary Material. Interestingly, both additional PCAs provided very similar results to those obtained with the first one. This indicates that the compositional differences between rumanite and succinite/gedanite/gedano-succinite involve both the ~~molecular and macromolecular~~ non-polymeric and polymeric fractions. When considering MS signals from the thermal desorption region, a high load of *m/z* 119 was obtained pulling samples towards negative values of PC1, and Su, Ge and GS samples were found at negative PC1 scores. This suggests that these samples have a higher content of cymene-like monoterpenoids, which are the main responsible for the signal at *m/z* 119 in the thermal desorption region. This is in agreement with the results obtained by van der Werf and co-workers [17], in which PCA of SPME chromatograms also showed that Baltic amber contains more cymene terpenoids than rumanite.

Note that succinite, gedanite and gedano-succinite could not be separated from each other, even along the other principal components. This indicates that the compositional differences between these three types of amber are small in comparison with the differences from rumanite samples. We avoided repeating PCA on Su, Ge and GS ambers as the number of samples is not sufficient to draw statistically significant conclusions. To attempt discrimination of gedanite and gedano-succinite, principal

component analysis was also repeated using normalized total ion profiles instead of the average mass spectra, but no further discrimination was obtained.

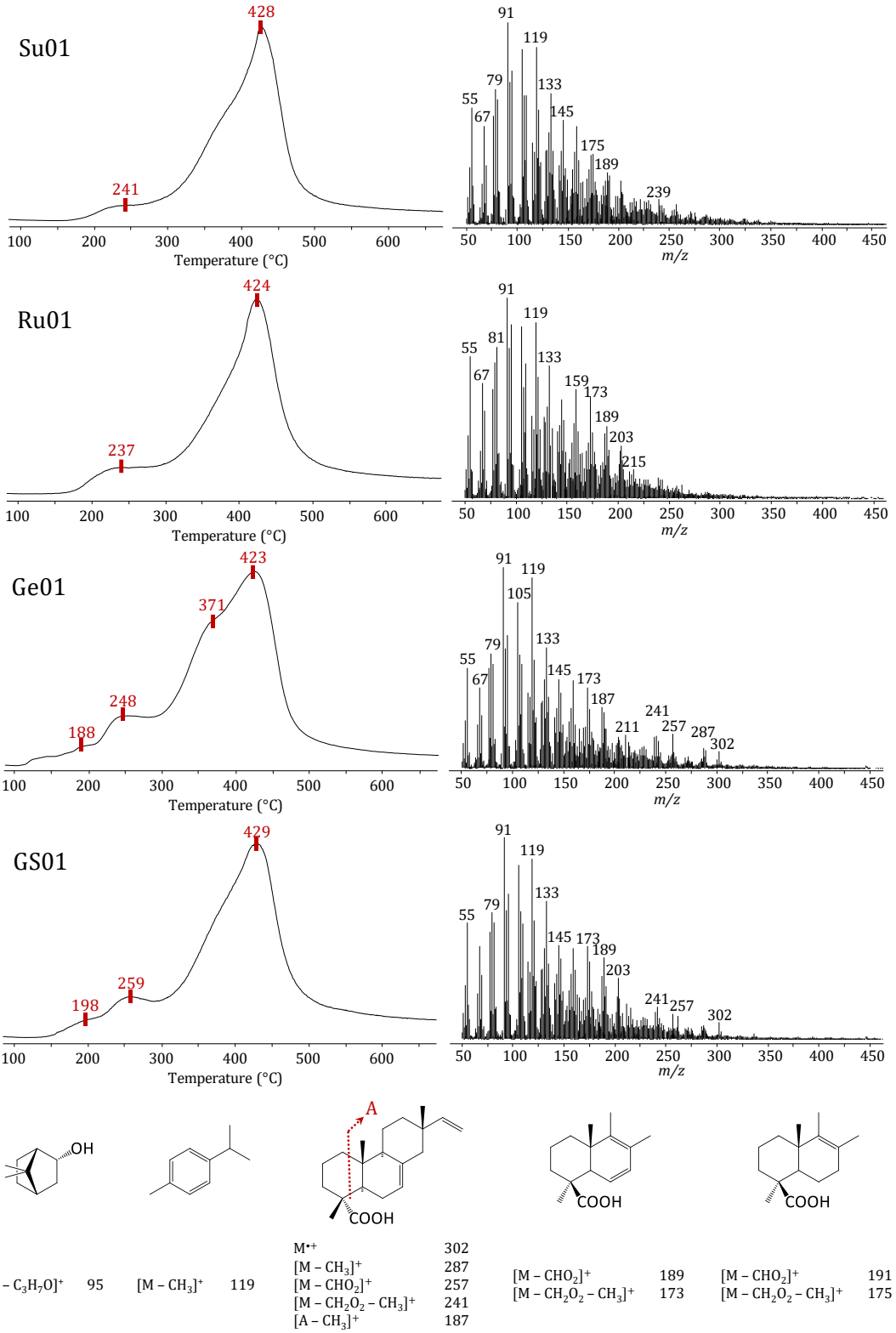

Figure 3: EGA-MS profiles and average mass spectra for samples Su01 (succinite), Ru01 (rumanite), Ge01 (gedanite) and GS01 (gedano-succinite). Proposed structures of ions for *m/z* signals of relevance are also shown.

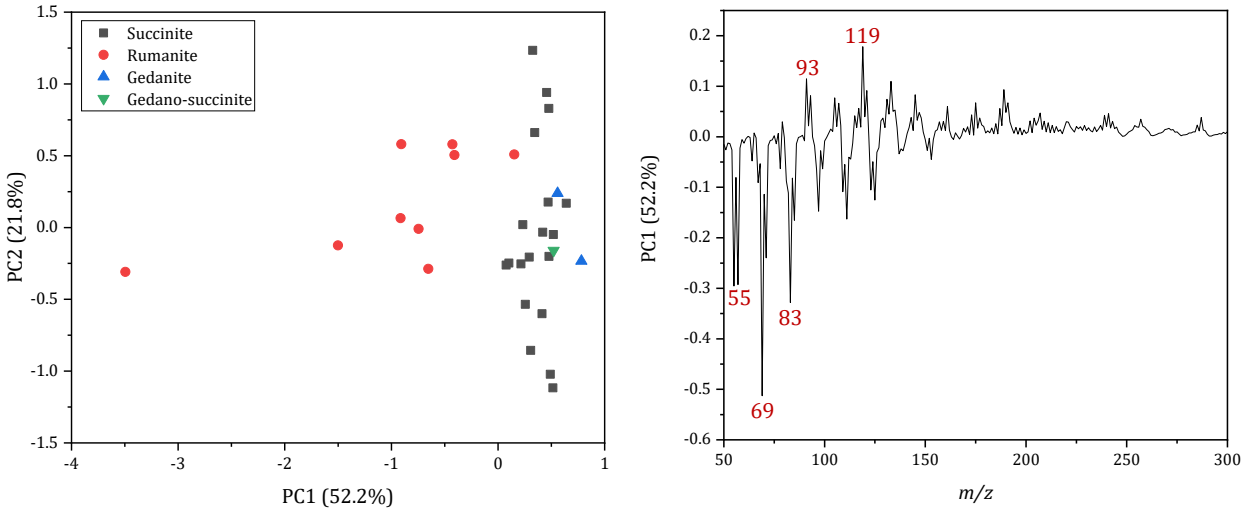

Figure 4: Left – Score plot of the first two principal components of ~~all~~ Su, Ru, Ge and GS samples. Right – Loading plot of the first principal component.

3.4. Italian ambers. EGA-MS profiles for samples Si01 and CI01-03 are shown in **Figure 5**. Sicilian amber has been documented in a previous study by van der Werf and co-workers [38]. Py-GC/MS analysis of this amber provided mostly diterpenes with the enantio-biformene carbon skeleton, as well as bicyclic compounds deriving from the pyrolysis of these diterpenes. The characteristic *m/z* signals of these compounds were also detected in the mass spectra of both simetite samples in this study. High relative intensities of *m/z* 245 and 260 were observed in the thermal desorption region. These signals can be ascribed to biformene-like diterpenes with 19 carbon atoms, which were found in significant amounts in the study by van der Werf and co-workers. ~~The most abundant biformenes found in the study by Werf and co-workers display a saturated aliphatic chain with a terminal carboxy group~~. ~~The structure~~ A representative structure for one of these compounds is shown in **Figure 5**. ~~Note that *m/z* 245 and 260 correspond to decarboxylated forms of this compound. The same signals could be obtained from biformene structures with no functional groups.~~ Interestingly, the relative intensity of *m/z* 119 in these samples was lower than those observed in the other diterpenoid ambers, indicating a low content of aromatic monoterpenes.

Pyrolysis of the polymeric fraction of Sicilian ambers showed very similar signals to those observed for the ambers described in Section 3.3, leading us to the conclusion that the macromolecular network

is mainly constituted of polymeric labdane terpenoids. Based on the findings of van der Werf [38], we can hypothesize that ~~T~~this polymeric network is ~~most likely~~ composed of biformene-like labdane, reflecting the composition of the non-polymerized fraction. These results allowed us to conclude that Sicilian ambers can also be ascribed to class I ~~of the Anderson system~~. More specifically, we can hypothesize that these samples belong to class Ic, due to the presence of *enantio*-biformene terpenoids, but confirmation of this hypothesis would require chromatographic separation of the pyrolysis products.

Samples CI01-03 were obtained from central Italy. ~~A single literature reference is available for these samples, in which characterization was performed by infrared spectroscopy [49], but no references were found reporting mass spectral data. Therefore, interpretation of their mass spectra was based on the results obtained for the other samples. The main differences in these samples were found within the thermal desorption region of their gas evolution profiles. Sample CI01 showed a very high signal of *m/z* 119, indicating the presence of free aromatic monoterpenes such as cymene. On the other hand, while samples CI02 and CI03 showed high signal intensity at *m/z* 95. This *m/z* value was not observed for other samples, but it can be ascribed to monoterpenes with the bornane or camphene carbon skeleton, such as borneol and camphor. Additional signals can be found that could be ascribed to monoterpenes, such as *m/z* 109 in CI02 and *m/z* 121 in CI03.~~ Two literature studies are available describing ambers from this region [31,49], including a work by van der Werf in which Py-GC/MS was performed on a sample with the same geological origin as sample CI03. As most of the other samples, CI ambers showed two thermal degradation regions. In the first region, all samples showed high signals at *m/z* 119, 159, and 173. Samples CI02 and CI03 also showed high intensities of *m/z* 95. Signals at *m/z* 95 and 119 can be ascribed to fragment ions of octahydronaphthalenes, while signals at m/z 159 and 173 can be ascribed to tetrahydronaphthalenes with one aromatic and one aliphatic ring. These species are found in class I ambers as a result of defunctionalization and loss of the aliphatic side-chain of labdanoid diterpenes [50]. Octahydronaphthalenes were indeed found in the study by van der Werf [31]. Representative structures of tetrahydro- and octahydronaphthalenes are shown in **Figure 5** to account for these signals. ~~These structures have been chosen among those detected in Py-~~

~~GC/MS of other diterpenoid ambers [17,18]. As monoterpenes are highly volatile compounds, their presence suggests that these samples are less mature than the other ambers analyzed in this study.~~ Sample CI02 also showed the same signals at *m/z* 245 and 260 that were found in the Sicilian ambers. This led us to the conclusion that sample CI02 is also constituted of biformene terpenoids. A high relative intensity was also observed for *m/z* 231 in CI02. While this signal is not characteristic of biformene-like terpenoids, unidentified compounds with this signal were detected in Py-GC/MS of Sicilian amber [38].

Finally, all three samples provided very similar mass spectra in the pyrolysis region. These mass spectra were like those obtained for Sicilian ambers and the other diterpenoid ambers, suggesting the presence of a labdanoid-based polymeric network. For this reason, all CI samples were also ascribed to

class I ~~of the Anderson system~~. This also agrees with the results obtained by van der Werf [31].

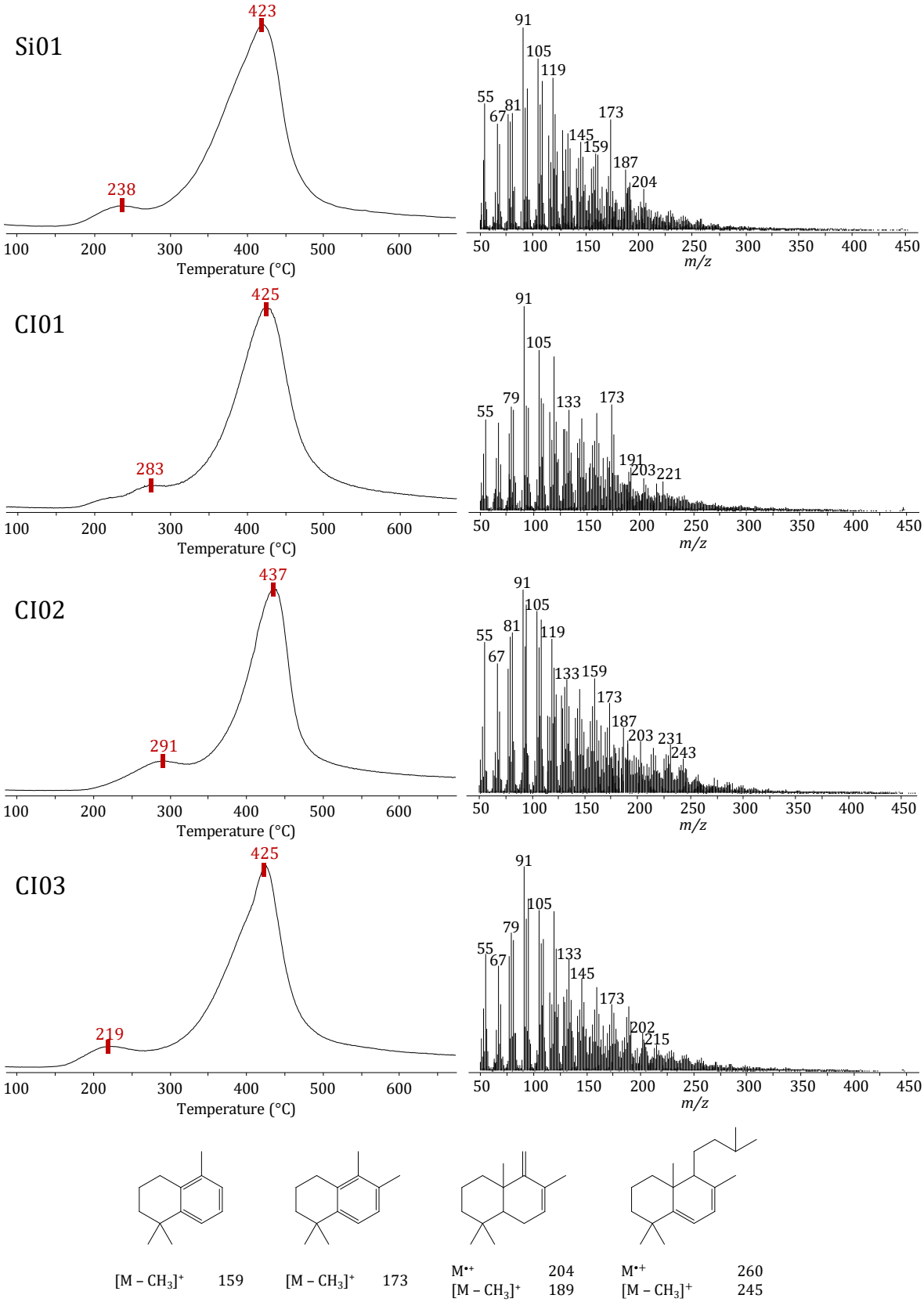

Figure 5: EGA-MS profiles and average mass spectra for samples Si01 (simetite) and CI01-03 (ambers from central Italy). Proposed structures of ions for *m/z* signals of relevance are also shown.

438

439 **Table 2 Table 2** provides a synthetic report of the ~~Anderson~~ classes and main components of both

440 ~~molecular~~ non-polymeric and polymeric fractions for all amber samples in this study.

441

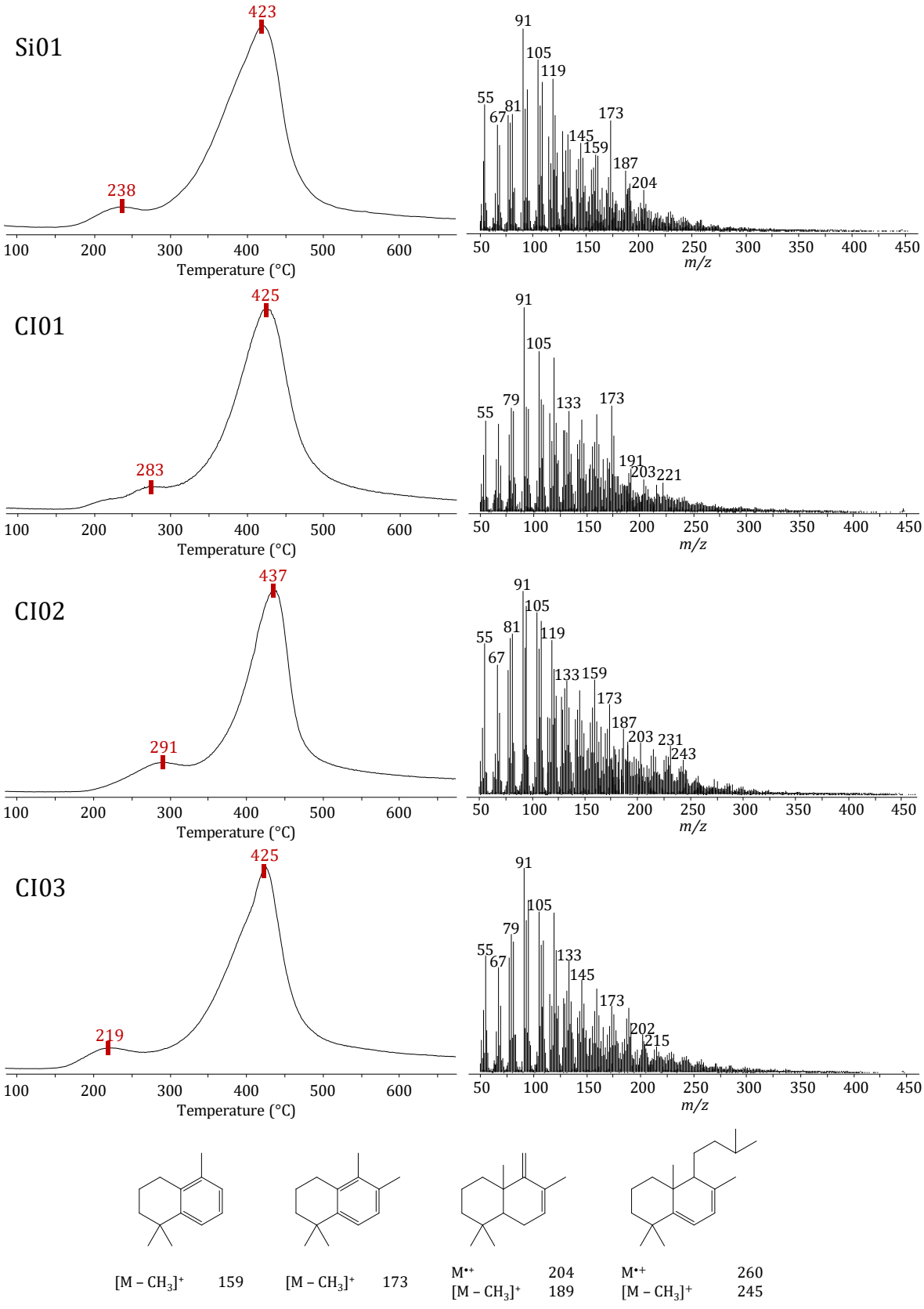

Figure 5: EGA-MS profiles and average mass spectra for samples Si01 (simetite) and CI01-03 (ambers from central Italy). Proposed structures of ions for *m/z* signals of relevance are also shown.

446 **Table 2:** ~~Anderson class~~ Classes and composition of the ~~molecular and macromolecular~~ non-polymeric

447 and polymeric fractions of all amber samples in this study, based on EGA-MS results.

Amber type	Class	~~Molecular~~ Non-polymeric fraction (< 300 °C)	Polymeric fraction (> 300 °C)
Glessite	II	Cadinene-like sesquiterpenes Ursane/oleanane-like triterpenes	Polymeric cadinenes
Goitschite	II	Cadinene-like sesquiterpenes Ursane/oleanane-like triterpenes with methyl-carboxylates on C28	Polymeric cadinenes
Siegburgite	III	-	Polystyrene, possibly with oxygenated cross-linkers
Black amber	-	Aliphatic hydrocarbons	Aliphatic hydrocarbons
Succinite	I	~~Aromatic monoterpeniods~~ Camphene-like monoterpenoids Cymene-like monoterpenoids	Polylabdene
Rumanite	I	~~Aromatic monoterpenoids~~ Camphene-like monoterpenoids Cymene-like monoterpenoids	Polylabdene
Gedanite	I	~~Aromatic monoterpenoids~~ Camphene-like monoterpenoids Cymene-like monoterpenoids Pimarane-like diterpenoid acids	Polylabdene
Gedano-succinite	I	~~Aromatic monoterpenoids~~ Pimarane-like diterpenoid acids	Polylabdene
Simetite	I	Biformene-like diterpenoids	Polylabdene ~~(likely based on *enantio*-biformene)~~
CI01	I	~~Aromatic monoterpenoids~~ Tetrahydronaphthalenes	Polylabdene
CI02	I	~~Bornane/cambphene-like monoterpenoids~~ Tetrahydronaphthalenes Octahydronaphthalenes Biformene-like diterpenoids	Polylabdene
CI03	I	~~Bornane/cambphene-like monoterpenoids~~ Tetrahydronaphthalenes Octahydronaphthalenes	Polylabdene

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4. CONCLUSIONS

Gas evolution profiles of almost all ambers showed two main regions, which could be ascribed to the desorption of low-molecular weight compounds at low temperatures, and to the pyrolysis of the polymeric network above 300 °C. Samples with different geological origin generally showed different desorption/pyrolysis temperatures and different number of desorption peaks, highlighting the usefulness of EGA-MS in providing information for the development of Py-GC/MS experiments targeting different fractions of specific amber samples. ~~The results agree with previous literature studies and show that EGA-MS can be used to obtain information on both the molecular and macromolecular fractions of geological amber samples with different compositions and geographical origins. EGA-MS profiles could be used to differentiate between two types of class II ambers, glessite and goitschite, based on the different constituting triterpenoids. Distinction of succinite and rumanite ambers was also possible by principal component analysis of their average mass spectra.~~

~~The results obtained for ambers with documented molecular compositions were also used to characterized three previously unreported amber samples deriving from central Italy. This demonstrates that EGA-MS analyses can be used not only to evaluate the content of molecular and macromolecular components of ambers with known composition, but also to characterize ambers with unknown origin.~~

EGA-MS provided valuable information on the thermal behavior of all amber samples, and on their content of non-polymeric and polymeric components. The results ~~could be used~~ allowed us to attribute a class to most of the samples. These classes were in agreement with those attributed in previous literature studies dealing with ambers with same geological origins, with the only exception of the goitschites, which were identified as triterpenoid ambers and not diterpenoid. ~~to assign most of the samples to an Anderson class. However, further classification was not possible~~ The two types of class II ambers, glessite and goitschite, could be distinguished based on the different constituting triterpenoids. Distinction of succinite and rumanite samples was also possible by principal component analysis of their average mass spectra.

For ambers of class I, subclasses could not be attributed due to the inability to detect the presence of succinic acid or to pinpoint specific terpenoid structures. ~~Succinic acid is a minor component of amber at best, with a net content of 8% in the richest samples. In addition, the mass spectrum of succinic acid only presents high signals at low *m/z* values, which are difficult to isolate from the signals of all other components in the complex mass spectra. This means that, while EGA-MS can provide valuable information on the thermal degradation profile of an amber sample, other techniques such as Py-GC/MS or spectroscopic techniques should also be used to obtain a complete picture of the molecular composition.~~ This highlights a limitation of EGA-MS results, which is the lack of chromatographic separation of the desorption/pyrolysis products. For this reason, additional characterization with Py-GC/MS or spectroscopic techniques should be performed on the sample pool. A particularly promising approach would be the use of step-wise Py-GC/MS, which could be achieved by sequentially heating an amber sample at two different temperatures, in order to achieve the desorption of the ~~molecular~~ non-polymeric fraction, followed by the pyrolysis of the residual polymeric fraction. For such a study, the choice of desorption/pyrolysis temperatures would be guided by the EGA-MS results obtained in this work.

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