The chemistry of American and African amber, copal, and resin from the genus *Hymenaea*

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Abstract

The comparison of the chemical composition of fossilized amber, copal, and resin is important for determining the botanic origin and original chemical composition of fossilized amber and copal, and for understanding the ecologic role of resin. Here we use solid phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS) to investigate the volatile and semi-volatile composition of amber, copal and resin from Africa and the Americas, produced by trees from the genus *Hymenaea*. We found there are four subgroups of *Hymenaea* resin, copal, and amber, based upon age and chemical similarity: African amber, American amber, African resin/copal (which also includes Colombian copal), and American resin/copal. This analysis allows us to narrow down the potential botanic origin of amber and copal samples, and also indicates that within this genus, resin similarity does not correspond closely with phylogenetic relationships. Therefore, resin chemistry may have been controlled by ecologic pressures, such as defence against herbivores, wood borers,

humidity, diseases, etc. and the original chemical composition of amber and copal could potentially be used to understand the role of resin in plant-insect interactions through time.

Keywords: Solid phase microextraction-gas chromatography-mass spectrometry, *Hymenaea*, Resin, Copal, Amber

1. Introduction

Trees in the genus *Hymenaea* (Fabaceae (=Leguminosae): Detarieae) (Fig. 1) have been prolific and important resin producers in Africa and the Americas for millions of years, producing extensive palaeontologically significant deposits of copal (semi-fossilized tree resin) and amber (fossilized tree resin) many of which contain exceptionally preserved fossils. The wealth of fossil and subfossil *Hymenaea* amber and copal is due primarily to the polylabdanoid macromolecular structure of the *Hymenaea* resin – which is characterized by ozic acid, ozol and *enantio* biformenes (Anderson et al., 1992) – that allows for rapid polymerization into amber and copal that is very durable over geologic time (Langenheim, 1995). We have investigated the volatile and semi-volatile chemical composition of known *Hymenaea* amber, copal and resin from a variety of sites, all described in more detail below.

The two most celebrated and best-studied deposits are the Miocene Dominican and Mexican Chiapas ambers (Penney, 2010; Solórzano Kraemer, 2010). Both contain diverse and abundant assemblages of exceptionally-preserved biological inclusions, providing a wealth of palaeobiological information about the small, soft-bodied, terrestrial fauna in a Miocene-age Neotropical forest ecosystem (Arillo and Ortuño, 2005; Penney, 2010; Solórzano Kraemer, 2010).

Dominican amber in particular is notable for its unusually high-quality preservation (Grimaldi et al., 1994; Grimaldi and Engel, 2005; Penney, 2010; McCoy et al., 2017). For example, of the approximately five percent of the amber pieces which contain biological inclusions (Lambert et al., 1985), 93 percent of these, when examined with tomography or dissection have internal soft tissues preserved (Grimaldi et al., 1994; Stankiewicz et al., 1998;

Heethoff et al., 2009; Greco et al., 2011; Van et al., 2014; McCoy et al., 2017), including details such as muscle fibres, myofibrils, and mitochondria (Henwood, 1992; Grimaldi et al., 1994; Labandeira, 2014). Chemical analyses (FTIR, C^{13}NMR, pyrolysis GC-MS) have highlighted similarities between Dominican amber and modern *Hymenaea* resin (Langenheim, 1969; Cunningham et al., 1983; Anderson et al., 1992; Langenheim, 1995; Martınez-Richa et al., 2000; Penney, 2010), and palaeobotanical investigations indicate that Dominican amber was produced by the extinct tree *H. protera* (Hueber and Langenheim, 1986; Poinar, 1991; Langenheim, 1995; Penney, 2010), a close relative of the extant *H. verrucosa* (Poinar, 1991). Amber attributed to *H. protera*, is also known from Cuba, Haiti, Puerto Rico and Jamaica (Iturralde-Vinent, 2001), but with such low abundance that it has never been studied in detail.

Mexican Chiapas amber, like Dominican amber, has exceptional soft tissue preservation of biological inclusions (Solórzano Kraemer, 2007, 2010) such that 55 percent of studied specimens have internal soft tissues (McCoy et al., 2017). Mexican amber is approximately the same age as Dominican amber, and is chemically very similar, but in some deposits has undergone more extensive thermal degradation (Bryant, 1983; Lambert and Poinar, 2002; Solórzano Kraemer, 2007, 2010). Chemical analyses (IR and C^{13}NMR) of Mexican amber from Chiapas shows many similarities with Dominican amber and modern *Hymenaea* resins, but, in addition, subtle differences indicate that the Chiapas amber was produced by one or more extinct tree species in the genus *Hymenaea* (Langenheim, 1966; Lambert et al., 1989; Lambert and Poinar, 2002), most likely *H. mexicana* (Brown, 2002) and *H. allendi*s (Calvillo-Canadell et al., 2010).

Another amber produced by *Hymenaea* is the less well known and recently described Ethiopian amber. Schmidt et al. (2010) described abundant well preserved arthropod inclusions and considered it as early Late Cretaceous in age (Cenomanian, ~93–95 Ma) although they did not comment on the botanic origin of the amber. Later Perrichot et al. (2016) re-evaluated the site based on additional amber material and associated sediment, which provided compelling evidence that Ethiopian amber is Cenozoic, likely Miocene. Moreover, these additional samples indicated that the amber was produced by the genus *Hymenaea*, similar to East African copals and Miocene ambers from Mexico or Dominican Republic.

In addition to the localities above we have also analysed samples from a recently discovered site in Venezuela which is of late Early Miocene to early Middle Miocene (Pérez et al., 2016) in age. The botanic origin of this amber has never been studied. In contrast to Dominican and Chiapas amber, no biological inclusions have been identified in Venezuelan amber (Pérez et al., 2016), and its botanic origin has not been investigated.

Copal deposits from, for example, Madagascar, Brazil, the Dominican Republic, Puerto Rico, and Colombia also offer abundant exceptionally preserved inclusions (Penney and Preziosi, 2010). The assemblages in copal have received little attention from palaeontologists because they are so young, but they are still an important resource for understanding the current biodiversity crisis and biases of preservation in amber (Penney and Preziosi, 2010). These copal samples are assumed to be produced by a species of *Hymenaea* based on chemical analyses and considerations of the major resin-producing trees in these regions (Schlüter and Von Gnielinski, 1986; Fearnside, 1989; Poinar, 1992; Langenheim, 1995; Clifford et al., 1997; Martınez-Richa et al., 2000; Lambert et al., 2002, 2005, 2014).

98 *Hymenaea* currently comprises 15 species, 14 of which are well distributed in the
99 tropical and subtropical forests from Central America to Brazil and the West Indies.
100 *Hymenaea verrucosa* is the sole species found in East Africa and Madagascar and is
101 considered the most primitive species of the genus (Lee and Langenheim, 1975; Langenheim,
102 2003; Fougère-Danezan et al., 2010). However, a complete phylogenetic and molecular study
103 of all 15 species has not yet been carried out.
104 Thus far, chemical analyses have been very successful at constraining the botanic
105 origin of the amber and copal samples to the genus *Hymenaea*, but they have been less
106 successful at constraining it further to the species level. For example, some chemical
107 analyses find Dominican amber is very similar to *H. verrucosa* resin (Cunningham et al.,
108 1983), although most highlight similarities with *H. courbaril* resin (Langenheim, 1969;
109 Lambert et al., 1985, 2008, 2015). Others studies find very little chemical difference between
110 any *Hymenaea* species, both extant and extinct (Lambert et al., 2014). From the chemical
111 analyses alone, it is not clear that Dominican amber is produced by an extinct tree; chemical
112 differences with modern resin could be due to amberization (the chemical changes during
113 fossilization to transform resin into copal and then amber) or intraspecific resin variability
114 rather than to a different botanic origin. Determining the botanic origin of copal or amber
115 using chemical comparisons to modern resin requires either that the resin-producing species
116 is still extant (and included in the analysis), or that chemical similarity indicates phylogenetic
117 similarity. However, the chemical analyses to date do not consistently indicate whether
118 chemical similarity indicates a close phylogenetic relationship between the botanic producers.
119 Some analyses found that chemical similarity follows broad phylogenetic patterns (Lambert
120 et al., 2005), but others suggest that resin chemistry is more strongly controlled by

121 environmental factors, and that resin chemical variation is more likely to be functionally
122 rather than phylogenetically controlled (Langenheim, 1995).
123 The ecologic role of resin is not fully understood, but it includes defence against
124 insect herbivores, healing wounds (Langenheim, 1990, 1995, 2003; Pichersky and Raguso,
125 2016) and to prevent bacterial and fungal infections, or infestations by wood-boring
126 arthropods (McKellar et al., 2011; Beimforde et al., 2017).
127 Plant-insect interactions are a product of hundreds of millions of years of antagonisms
128 and co-evolution, and include some of the most complicated and important interactions in
129 modern ecosystems (Bryant et al., 1991; Labandeira et al., 1994; Howe and Jander, 2008;
130 War et al., 2012; Labandeira and Currano, 2013). By characterizing the chemical
131 components of amber, resin, and copal, and by precisely identifying the botanic producer, we
132 can better understand these interactions through time from the modern day and in the fossil
133 record. This is particularly interesting for amber fossil sites which preserve much of the
134 original insect herbivore fauna as inclusions in amber (Penney, 2010; Solórzano Kraemer,
135 2010; Labandeira, 2014; Peris et al., 2015), and also preserve some direct evidence of plant-
136 insect interactions in the form of leaves with herbivore damage (Labandeira, 2014).
137 The goal of this research is to use headspace solid phase microextraction-gas
138 chromatography-mass spectrometry (SPME-GC-MS) to elucidate the volatile and semi-
139 volatile components of various African and American resin, copal, and amber known to be
140 produced by the genus *Hymenaea*. Headspace SPME uses a coated fibre to extract
141 compounds from the headspace of a sealed vial containing a sample and transfer them to a
142 GC-MS for identification and quantification (Pawliszyn, 2011). This method has previously
143 been used to differentiate samples of modern resin (Hamm et al., 2003, 2005), identify small

amounts of resin in archaeological samples (Hamm et al., 2004), characterize the volatile components in Benzoin gum (Castel et al., 2006), identify two volatile degradation compounds of Baltic amber (Pastorelli, 2011), and differentiate Baltic and Romanian amber (van der Werf et al., 2014). These previous studies have also involved extensive methods testing (Hamm et al., 2003), which has informed our selection of SPME fibre, incubation temperature and time, and sampling time. Our analyses on *Hymenaea* resin, copal, and amber will provide a simplified, comparable chemical characterization of resin, copal, and amber that focuses on some of the most ecologically active compounds (Langenheim, 2003). This new source of chemical data from these samples will also complement previous analyses, to help better elucidate the botanic origin and ecologic role of these samples.

2. Materials and Methods

2.1. Samples

Samples of amber, copal, and resin were obtained from the Senckenberg Research Institute and Natural History Museum (SMF), from Alcaldía Municipio Urumaco, Colección Paleobotánica, Venezuela (AMU-PB) or collected from various locations around Africa and the Americas (table 1, Fig. 1): two samples of Ethiopian amber; two samples of Mexican Chiapas amber, one each from the Simojovel and Totolapa mines; one sample of Dominican amber; one sample comprised of various small pieces of Venezuelan amber, one sample of copal from the Dominican Republic; two samples of copal from Colombia; one sample of copal from either Puerto Rico or the Dominican Republic; nine samples of *Hymenaea verrucosa* resin, six of which were from the same tree; one sample of *H. courbaril* resin; one

sample of *H. parvifolia* resin; and two unknown samples, one from the collections of the Senckenberg Research Institute and Natural History Museum and one purchased from ebay under a listing for 'Dominican amber.' The pieces were selected to be homogenous and free of organic inclusions. Each sample was reduced to a fine powder using a ball mill, and 0.5 grams were sealed into a 20 ml headspace vial with a PTFE septa and magnetic screw top caps. Our sample sizes (0.5 g) are much larger than those used in previous SPME analyses of resin, copal, and amber, which range from 0.002 to 0.04 mg (Hamm et al., 2003; Hamm et al., 2004; Pastorelli, 2011). However, for headspace SPME, sample size is dependent upon headspace volume; we used 20 ml vials rather than the 2 ml vials used in the previous analyses (Hamm et al., 2003; Hamm et al., 2004; Pastorelli, 2011) and therefore increased our sample size accordingly. Moreover, we tested our method with various sample sizes of *Copaifera officinalis* resin purchased from an online supplier and found the results were essentially identical for samples sizes ranging from 0.01 g to 1 g (Supplementary Figs. 1 and 2).

2.2 Headspace SPME-GC-MS

The vials containing the powdered samples were randomly loaded in a Triplus RSH autosampler and placed in an agitator where they were equilibrated at 80°C for one hour. The SPME fibre, a 65 μm Polydimethylsiloxane/Divinylbenzene (PDMS/DVB) fibre initially conditioned at 250°C for 30 minutes per the manufacturer's instructions, was automatically introduced and exposed to the head space for one hour. After sampling, the fibre was inserted into the injection port of a Thermo Scientific Trace 1310 GC, which had a Thermo TG-5MS

30 m column with 0.25 mm ID and 0.25 μm film thickness, coupled to an ISQ QD single quadrupole mass spectrometer, where it was desorbed for 2 minutes at 250°C. Splitless injection (1 minute) was used. The injector temperature and transfer line temperature were 250°C, and the GC program had an initial temperature of 40°C, held for five minutes, that ramped up to 280°C at a rate of 10°C per minute, where it was held for 5 minutes. A liquid injection of a standard mixture containing a series of *n*-alkanes was used to calibrate retention indices to aid in identifying the peaks.

2.3 Peak identification

The chromatograms for each sample were imported into the program AMDIS, which automatically deconvolutes the data to extract the pure component spectra, allowing for more accurate identification. The major peaks in each chromatogram were identified through a National Institute of Standards and Technology (NIST) MS database search, including information from both the MS fragmentation patterns and the retention indices. We found 126 compounds, which we compiled into a search library in AMDIS. We then used the analysis function in AMDIS to automatically compare all peaks in each chromatogram to this search library, so that we had comparable data for each sample.

2.4. Semi-quantitative analysis

The relative amounts of each compound were calculated as the percent of the total peak area of the 126 selected compounds, and these data were analysed with principal components analysis (PCA) (following van der Werf et al., 2014) using the program R. These data quantitatively represent the SPME chromatograms, and therefore provide a way to

quantitatively compare the chromatograms. However, the SPME chromatograms do not quantitatively represent the samples, because SPME extracts different compounds with differing levels of completeness (Hamm et al., 2003). Nonetheless, the results for identical samples are completely reproducible because the use of an autosampler results in exactly comparable timings and temperatures; these are two factors that play a major role in the efficiency of the equilibrium-based extraction. Therefore, these analyses allow reproducible, semi-quantitative comparisons of the samples (van der Werf et al., 2014).

3. Results

Components 1 and 2 of the PCA (which encompass ~ 49% of the variation in the dataset (Fig. 2)) indicate that the volatile and semi-volatile chemical constituents of resin, copal, and amber vary based on age and location (Fig. 3). Components 3-6, although they together encompass another 31% of the variation, do not separate the samples in any meaningful way (Supplementary Fig. 3) and so are not considered. Component 1 (~38% of variation (Fig. 2)) separates the ancient amber from the recent resin/copal (Fig. 3). Component 2 (~11% of variation (Fig. 2)) separates the African samples from the American samples (Fig. 3). The exception to this is Colombian copal, both samples of which group with the African resins, rather than with the other American samples (Fig. 3). Within these groups, we see large variation within species, and overlap between species. The nine samples of *H. verrucosa* resin all cluster within the same group (the African resin/copal group) in the PCA, but they span the entire range of variation of that group, overlapping with the Colombian copal samples, which were almost certainly produced by one of the American *Hymenaea* species, rather than *H. verrucosa* which is restricted to Africa (Fig. 3). Multiple

samples from one *H. verrucosa* tree have a restricted chemical composition relative to the entire range of *H. verrucosa*, but they still show some variation (Fig. 3). Similarly, the two samples of Mexican amber from Chiapas (both produced by *H. mexicana* or *H. allendis)* fall within the American amber group in the PCA, but span the entire range of variation of the group and overlap with the Dominican amber sample (produced by *H. protera*).

The four groups in the PCA (Fig. 3) are primarily determined on the basis of 12 of the 126 chemical compounds (Fig. 4). All of the ambers (in contrast to the resin and copal samples) have high amounts of 1,1,4,5,6-pentamethyl-2,3-dihydro-1H-indene, 4,8,11,11-tetramethyl-tricyclo[7.2.0.0(3,8)]undec-4-ene, trimethylphenyl- butanone, caryophyllene isomer, and tetrahydro-tetramethyl-naphthalene (Figs. 4 and 5). American amber (see Supplementary Fig. 4 for chromatograms) is distinguished from African amber (see Supplementary Fig. 5 for chromatograms) by the relative proportions of these compounds (Figs 4 and 5): American amber is dominated by1,1,4,5,6-Pentamethyl-2,3-dihydro-1H-indene and African amber by the other four compounds. All of the resin and copal samples have high abundances of 13-epimanool, caryophyllene, biformene, and α-curcumene. However, American resin/copal (Figs. 4 and 5, see Supplementary Fig. 6 for chromatograms) also has high amounts of humulene-1,2-epoxide, α-humulene, and β-bisbolene. Colombian copal (see Supplementary Fig. 7 for chromatograms) has relatively small amounts of these three compounds (Fig. 6) and so it clusters with the African resin/copal (see Supplementary Fig. 8 for chromatograms) in the PCA (Fig. 3).

The analysis also includes two samples of unknown geographic and botanic origin (see Supplementary Fig. 9 for chromatograms). The first unknown was suspected to be *H. courbaril* resin from Mexico or Colombia. In our analysis, this sample (labelled 'unknown')

groups with the recent American resin/copal samples but not with the Colombian copal samples, suggesting it was not from Colombia (Fig. 3). However, there is not sufficient differentiation between different American resins and copals to discriminate any further the exact provenance of this sample. The second unknown sample was purchased from ebay and was sold as Dominican Amber (labelled 'Dominican amber?') (Fig. 3). Our analysis strongly suggests that this sample is from the Americas, however, it is more likely to be resin or copal than amber.

4. Discussion

Our results support four conclusions regarding the volatile/semi-volatile composition of known *Hymenaea* resin/copal/amber: (i) there is extensive intraspecific variation and interspecific overlap in resin chemistry; (ii) chemical similarity does not correspond to phylogenetic similarity at the species level, which has implications for using resin chemistry to determine the botanic origin of amber, copal, or an unknown resin sample; (iii) environmental factors might be important for controlling chemical composition; and (iv) resin chemistry changes during the process of amberization, but despite this it is may still be possible to gain some understanding of the original resin chemistry of an amber sample.

4.1. Intra- and inter- specific variation

Previous analyses of *Hymenaea* resin chemistry have encompassed both leaf/primary stem resin (Martin et al., 1971, 1974; Langenheim et al. 1978) and trunk resin (Lambert et al., 1985, 1989; Cunningham et al., 1983; the current study). These two sources of resin vary because trunk resin has a polymeric macromolecular structure as well as volatile and semi-

282 volatile components whereas leaf/primary stem resin lacks the polymeric structure
283 (Langenheim, 1995).
284 Previous studies of the macromolecular chemical composition of *Hymenaea* resin
285 using C^{13} NMR and infrared (IR) spectroscopy have highlighted intraspecific resin variation
286 (Martin et al., 1971, 1974; Langenheim et al., 1978). These studies primarily focused on *H.*
287 *courbaril*, which is a highly variable species including multiple subspecies, and which may
288 actually represent up to three species (Souza et al., 2014). Our analyses focused on the
289 variation within *H. verrucosa* resin, and yet still found extensive chemical variation (Fig. 3),
290 suggesting that, regardless of whether a consensus phylogenetic grouping for *H. courbaril* is
291 met, variable resin chemistry does occur within species and individuals of *Hymenaea*.
292 Analyses of Dominican (Lambert et al., 1985) and Mexican amber (Lambert et al., 1989) also
293 show significant chemical variation, suggesting intraspecific chemical variation within *H.*
294 *protera* and *H. mexicana*/*H. allendis* resin.
295 Previous studies comparing the resin chemistry of different species of *Hymenaea* have
296 reached mixed conclusions. The most common comparison is between *H. verrucosa* and *H.*
297 *courbaril*, which typically can be distinguished using C^{13} NMR and IR spectroscopy, which
298 elucidates the macromolecular structure (Cunningham et al., 1983). In contrast, analyses of
299 the macromolecular structure of resin from multiple species of *Hymenaea* (including both *H.*
300 *verrucosa* and *H. courbaril*), using C^{13}, ^{1}H and COSY NMR and IR find that they are
301 chemically very similar (Martin et al., 1976; Lambert et al., 2014).
302 Our results suggest that the volatile and semi-volatile compositions of *Hymenaea*
303 resins follow the same broad patterns as the macromolecular structures of these resins: *H.*
304 *verrucosa* and *H. courbaril* have very different volatile and semi-volatile compositions; but

305 there is also significant overlap between different *Hymenaea* resins. *H. courbaril* and *H.*
306 *parvifolia* (as well as the tree(s) which produced most of our American copal samples) cannot
307 be distinguished, and *H. verrucosa* and the Colombian copal tree cannot be distinguished.
308
309 *4.2. Phylogenetic similarity and botanic origin*
310 Large scale chemical analyses suggest that resin chemical groups do follow broad
311 taxonomic patterns: resin chemistry can often be used to identify families, sometimes to
312 identify genera, but rarely to identify species (Lambert et al., 2005; Sonibare et al., 2012). As
313 such, chemical analyses are useful for identifying the botanic origin of an unknown sample
314 (including amber samples) to higher taxonomic levels, but species-level identification of an
315 amber-producing tree requires palaeobotanical investigation to supplement the chemical
316 analyses.
317 Our analyses, which are restricted to one genus, cannot address the utility of SPME-
318 GC-MS for family or genus level identification of the botanic source of an unknown sample.
319 We have found that resin chemical similarity for species within the genus *Hymenaea* does not
320 correlate to close phylogenetic similarity, and therefore, as with other analyses, is not
321 sufficient for species-level identification of the botanic origin of a fossil sample or an
322 unknown recent sample. As previous chemical analyses have suggested (Lambert et al.,
323 2015), the volatile and semi-volatile composition of Dominican amber is more similar to
324 American resins and copal, such as *H. courbaril* resin, than to the more closely related *H.*
325 *verrucosa* resin. Both samples of Colombian copal (which were almost certainly produced by
326 one of the American *Hymenaea* species) fall within the range of variation of the less closely
327 related African *H. verrucosa* resin. However, Martínez-Richa et al. (2000) have also

328 previously noted that Colombian samples were very similar to African samples. Moreover,
329 some modern species (*H. courbaril* and *H. parvifolia;* and *H. verruscosa* and the Colombian
330 copal tree) overlap in the SPME-GC-MS PCA and therefore cannot be distinguished at all.
331 These analyses can be used to rule out potential botanic producers of an unknown sample (for
332 example if it clusters with the American resin/copal samples it was not produced by *H.*
333 *verrucosa*), but cannot be used to identify it definitively to the species level.
334 The sample of amber from Venezuela clusters nicely with the Chiapas and Dominican
335 amber, confirming that the botanic origin of this newly discovered amber is very likely also a
336 species of *Hymenaea*. An alternative hypothesis is that the Venezuelan amber samples are
337 produced by a species of *Copaifera*. This genus also includes prolific resin producing trees,
338 has a very similar distribution as *Hymenaea* in the Americas, and *Copaifera* resin has
339 previously been very difficult to distinguish from *Hymenaea* resin using ^{13}C, ^{1}H, and COSY
340 NMR spectroscopy (Lambert et al., 2009; Lambert et al., 2014). However, all the *Hymenaea*
341 samples in this analysis, and the Venezuelan amber samples, were clearly distinct from the
342 *Copaifera officinalis* samples used for methods testing (Supplementary Fig. 2), suggesting
343 that the Venezuelan amber samples were more likely produced by a species of *Hymenaea*.
344
345 *4.3. Environmental factors*
346 SPME-GC-MS analysis separates known *Hymenaea* resin/copal/amber into four
347 distinct subgroups on the basis of chemical similarity. As discussed above, these groups are
348 not based upon phylogenetic similarity, which suggests chemical variation in *Hymenaea* resin
349 is more strongly influenced by environmental variation (e.g. biotic factors such as herbivore
350 pressures and abiotic factors such as temperature and aridity) than by phylogenetic

351 constraints. Most of the research on the ecologic role of resin (including for *Hymenaea* resin)
352 focuses on leaf resins, which may have very different composition than the trunk resins, even
353 from the same tree (Langenheim, 1995, 2003). However, some of the general conclusions
354 about specific compounds are still applicable to trunk resin. The non-volatile compounds
355 (which are not considered in this analysis) generally affect the viscosity and the
356 polymerization of the resin, and provide physical defences such as trapping attackers and
357 coating and sealing wounds (Langenheim, 2003; Martínez-Delclòs et al., 2004). The volatile
358 compounds (which include those measured in this analysis) generally provide chemical
359 defences (Langenheim, 2003). Some are directly toxic to herbivores or fungi (Langenheim et
360 al., 1980; Arrhenius and Langenheim, 1983; Welker et al., 2007), and others attract predators
361 or parasites of attacking herbivores (Dicke et al., 1990; Langenheim, 1994). Compositional
362 variation in *Hymenaea* resin has been linked to selection in response to the types and
363 quantities of attacking pests (Langenheim, 2003). However, the efficacy of a resin chemical
364 compound against a specific attacking organism varies based on abiotic environmental
365 factors suggesting that abiotic factors may have an indirect effect on resin chemical
366 composition (Langenheim, 1995).
367 The two resin/copal subgroups defined in our study (the American resin group and the
368 African resin group, Fig. 4) therefore most likely indicate two distinct biotic environmental
369 pressure regimes, including attacks on the trees by herbivores, wood-infesting arthropods,
370 pathogens, and fungi. Most of the seven compounds that distinguish these two groups have
371 been linked to defensive functions, although they have only been investigated in a few tree
372 species and against a few types of attackers (Table 3). African resins are characterized by
373 four chemical compounds, of which three have been subject to an investigation of their

ecologic role: 13-epimanool is associated with resistance to vole browsing in the bark of larch trees (Sato et al., 2009; Seki et al., 2012); caryophyllene in pine trees has been shown to inhibit complete needle destruction by caterpillars (although it is also associated with a higher frequency of caterpillar attacks) (Petrakis et al., 2005), and to discourage attacks by ants and fungi (Barnola et al., 1997), in *Hymenaea* to discourage ant, and caterpillar attacks (Langenheim et al., 1980; Hubbell et al., 1983), and in Dipterocarps to discourage termite attacks (Messer et al., 1990); α-curcumene has been found to repel whiteflies in tomatoes (Bleeker et al., 2011). Finally, the effects of biformene on attackers has not been investigated. The American resins and copals are defined on three compounds, two of which have been studied: α-humulene discourages termites in dipterocarps (Messer et al., 1990) and insect herbivores in *Hymenaea* (Langenheim et al., 1980, 1986); humulene-1,2-epoxide deters caterpillar herbivores in *Hymenaea* (Langenheim et al., 1980); and β-bisabolene has not been studied. More research on the effects of these chemicals against a wider range of attackers, and which organisms typically attack the different *Hymenaea* species, is necessary to determine what selective pressures promote the production of one chemical compound over another, and therefore influence the chemical composition of *Hymenaea* resin. However, it is interesting to note that the African resins have compounds that deter mammalian attackers and fungi as well as arthropods. Moreover, in many analyses, caryophyllene (characterizing the African resins and copals) is identified as one of the most prominent and effective anti-herbivore defence chemicals (Langenheim et al., 1980, 1986; Welker et al., 2007). It may be that the African (and Colombian) species of *Hymenaea* are subject to attack by a more diverse and persistent fauna than the American species.

397 *4.4. Amberization and original volatiles*

398 As resin fossilizes into copal or amber ('amberization'), it undergoes a complex
399 process of maturation including oxidation, oligomerization, and cross linking that changes the
400 molecular structure (Grimalt et al., 1988; Anderson and Winans, 1991; Anderson et al., 1992;
401 Anderson and Crelling, 1995; Tonidandel et al., 2008). The SPME method used in this
402 research only captures small molecular weight compounds and so we observe two particular
403 consequences of amberization: a decrease in the original low molecular weight volatile and
404 semi-volatile compounds (Tonidandel et al., 2008), and an increase in low molecular weight
405 degradative compounds (Pastorelli, 2011). Although we did observe fewer peaks (which in
406 this analysis all represent low molecular weight volatile and semi-volatile compounds) in the
407 amber chromatograms than in the resin and copal chromatograms (Fig. 5), the amber was
408 separated from the resin/copal in the PCA primarily by the presence of degradative
409 compounds, which have aromatic rings and only very short side chains (Fig. 4). These
410 variations allow us to distinguish amber samples from more recent resin/copal samples,
411 which is useful for determining if unidentified samples are amber or not (Fig. 3). However,
412 this analysis is not very precise, and could not be used to get relative ages for two samples
413 unless they are very different in age: e.g. Chiapas amber and Dominican amber are very close
414 in age (Penney, 2010; Solórzano Kraemer, 2010) but cover a wide range in component 1 in
415 the PCA (Fig. 3), and we cannot distinguish between resins and copals (Fig. 3). Previous
416 attempts to use NMR, FT-Raman spectroscopy, thermogravimetric analyses, and atmospheric
417 pressure photoionization (Brody et al., 2001; Ragazzi et al., 2003; Kimura et al., 2006;
418 Tonidandel et al., 2008; Lambert et al., 2015) have been more effective at determining the

age of a resin, copal or amber sample; for more recent samples, ^{14}C dating can also be effective (Burleigh and Whalley, 1983).

In order to understand the role of resin in plant insect interactions in the fossil record, it is necessary to know the chemical composition of the original resin, rather than the fossilized amber, which is often obscured by the amberization process. Based on our analyses, the amber does retain some of the original volatile compounds (e.g. Ethiopian amber 2 contains α-pinene). However, we can also make assumptions about the original volatile and semi-volatile composition based on similarities to modern resins. For example, the Dominican and Chiapas amber samples clustered with the American resins/copals, and were therefore most likely originally characterized by 13-epimanool, α-curcumene, biformene, and caryophyllene. In contrast, the Ethiopian amber is most similar to the African resin and copal group, and therefore may have been originally characterized by α-humulene, humulene-1,2-epoxide, and β-bisabolene isomer.

5. Conclusions

SPME-GC-MS can distinguish four chemical subgroups within *Hymenaea* resin, copal and amber: American amber; African amber; American resin/copal; and African resin/copal (which also includes Colombian resin). Both amber groups are defined based on degradative chemical compounds produced during the process of amberization, and the differences between the groups can be explained by different original chemical compounds (which are then influenced by amberization). The resin/copal groups are defined on the basis of original volatile and semi-volatile compounds that all play a role in defence against herbivores, fungi, and pathogens. Variations in the chemical composition of different

Hymenaea species do not follow phylogenetic patterns, and are most likely due to selection pressures from different herbivore fungi, and pathogen assemblages. More research is required to determine which herbivores, fungi or pathogens exert most selective pressure on the chemical composition of resin. However, the current knowledge of the defensive role of the key volatile compounds suggests that the African resin/copal group is defined by more effective and more broadly applicable defensive chemicals, and therefore they may need to defend against a more diverse fauna. The chemical similarity between the amber and resin, in combination with some remnants of original volatile compounds, may help infer the original volatile and semi-volatile composition of the amber samples. This, in combination with more research on the defensive role of specific resin chemicals and the preserved arthropod herbivore fauna in the amber fossil assemblages, may provide insights on the role of resin in plant-insect interactions through geologic time.

6. Acknowledgements

Thanks to Armando Arroyo Gomez for *H. parvifolia* sample Jorge Domingo Carrillo Briceño, and Marcelo Sánchez-Villagra (Paleontological Institute and Museum,Switzerland) and Carlos Jaramillo (Smithsonian Tropical Research Institute) for Venezuela amber samples and Instituto del Patrimonio Cultural de Venezuela to allow the collection of them, Ravaka Ravelomanana for the collection of some Madagascar resin, Claudia Franz (Senckenberg Research Institute, Germany) for preparing samples of copal and amber, and Enrique Pañalver (Instituto Geológico y Minero de España, Spain) and Xavier Delclòs (Barcelona University, Spain) for important discussion. This work was supported by a Royal Society Newton International Fellowship to VEM, and AMBERIA 2015-2017 [grant number

465 CGL2014-52163] and VolkswagenStiftung [grant number 90 946] to MSK. The manuscript
466 was also improved by the comments of two anonymous reviewers.
467
468

469 **Figure Captions**

470 Figure 1: Resin *in situ* on a *Hymenaea verrucosa* tree in the northwest of Madagascar.

471 Figure 2: The percent of variation that is controlled by each component of the PCA.
472 Components 1 and 2 control ~ 49% of the variation.

473 Figure 3: PCA of all samples of resin, copal, amber, including two unknowns. Red squares
474 are amber samples, orange circles are copal samples, yellow stars (and the yellow polygon)
475 are resin samples, and black plus signs are unknown samples. Component 1 separates the
476 ancient amber samples (on the negative side of dimension1) from the recent resin and copal
477 samples (positive side of dimension 1). Component 2 separates the American samples
478 (positive side) from the African samples (negative side), with the exception of Colombian
479 copal which clusters with the African resins. Each of the four groups is delineated by a
480 convex hull polygon.

481 Figure 4: Variable loadings for components 1 and 2 of the PCA. The 12 specifically
482 identified variables (chemical compounds) are most important for defining components 1 and
483 2; the other 114 variables cluster near the origin. The one variable identified with a grey star
484 defines the American amber group, the four variables identified with green squares define the
485 African amber group, the four variables identified with blue circles define the African
486 resin/copal group, and the three variables identified with purple hexagons define the
487 American amber/copal group. Key variable groupings are also indicated by convex hull
488 polygons.

489 Figure 5: Representative chromatograms from the African amber group (A,B), the African
490 resin/copal group (C,D), the American amber group (E,F) and the American resin/copal
491 group (G,H). (A,C,E,G) Full chromatographs. (B,D,F,H) Proportionate peak area for selected

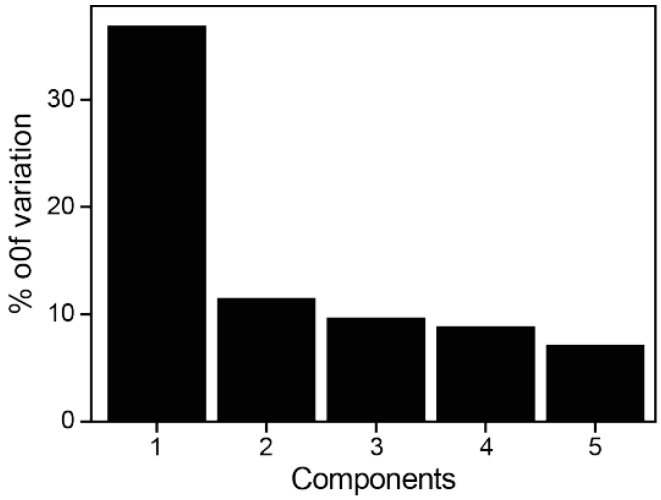
compounds that are most important for defining the groups; colours and shapes are as in Fig. 4. Peaks of selected compounds are labelled to identify the compounds, and correspond to the numbers in Fig. 4: 1 is 1,1,4,5,6-pentamethyl-2,3-dihydro-1H-indene; 2 is 4,8,11,11-tetramethyl-tricyclo[7.2.0.0(3,8)]undec-4-ene; 3 is trimethylphenyl- butanone; 4 is caryophyllene isomer; 5 is tetrahydro-tetramethyl-naphthalene; 6 is 13-epimanool; 7 is caryophyllene; 8 is biformene; 9 is and α-curcumene.; 10 is α-humulene-1,2-epoxide; 11 is humulene; and 12 is β-bisabolene isomer.

Figure 6: Figure 5: Colombian copal samples illustrated as in figure 5. Full chromatograms (A,C), and proportionate peak area for selected compounds that are most important for defining the groups (B,D); colours and shapes are as in Fig. 4. Peaks of selected compounds are labelled to identify the compounds, and correspond to the numbers in Fig. 4: 1 is 1,1,4,5,6-Pentamethyl-2,3-dihydro-1H-indene; 2 is 4,8,11,11-tetramethyl-tricyclo[7.2.0.0(3,8)]undec-4-ene; 3 is trimethylphenyl- butanone; 4 is caryophyllene isomer; 5 is tetrahydro-tetramethyl-naphthalene; 6 is 13-epimanool; 7 is caryophyllene; 8 is biformene; 9 is and α-curcumene.; 10 is humulene-1,2-epoxide; 11 is α-humulene; and 12 is β-bisabolene isomer. Notice how the highest peaks of the selected compounds (B, D) correspond to those that are enriched in African resins.

Figures

Figure 1

514 **Figure 2**



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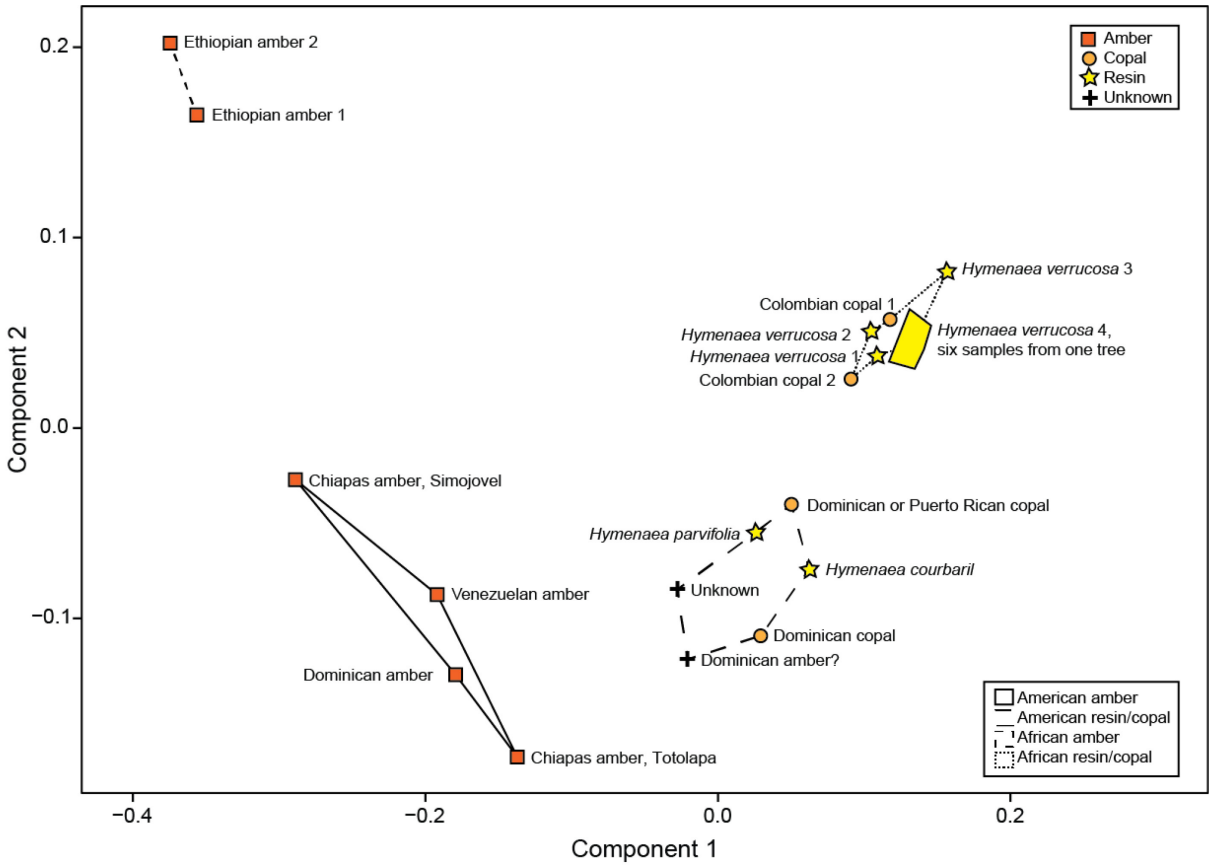
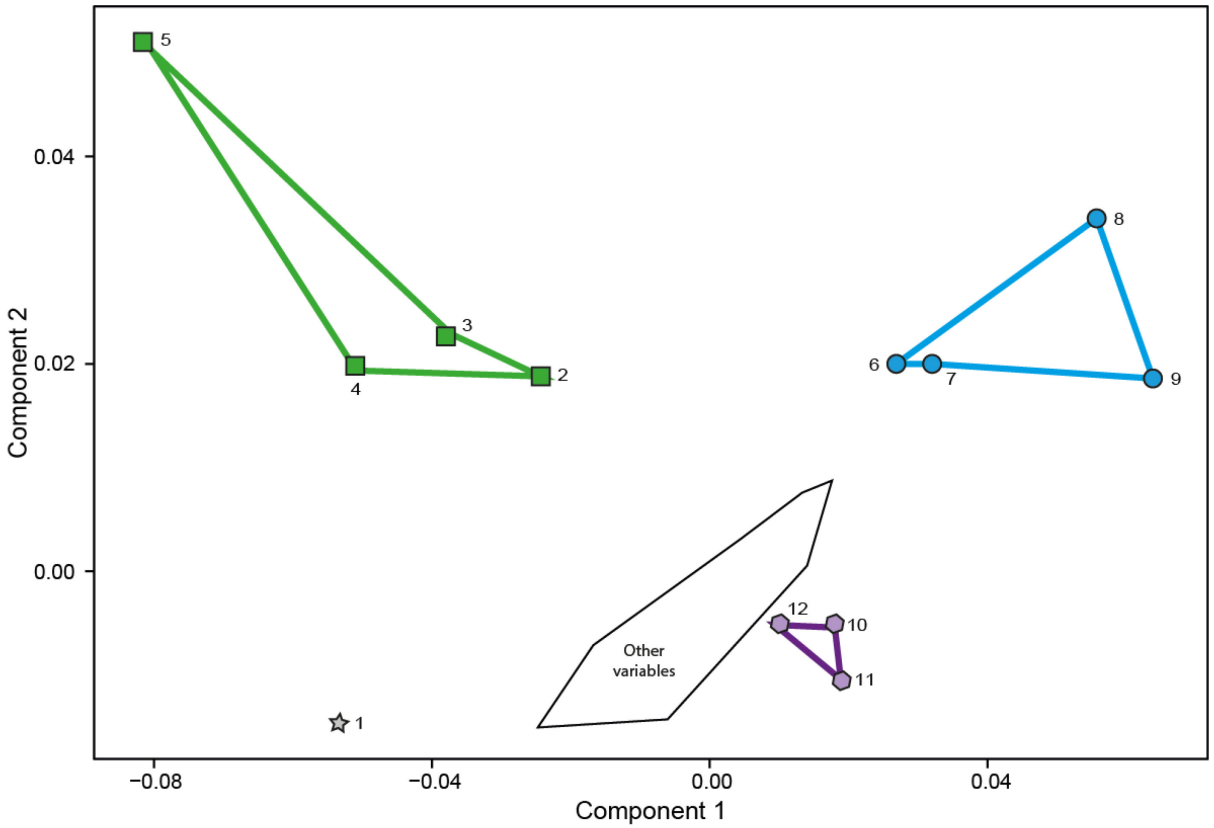
518 **Figure 3**



519

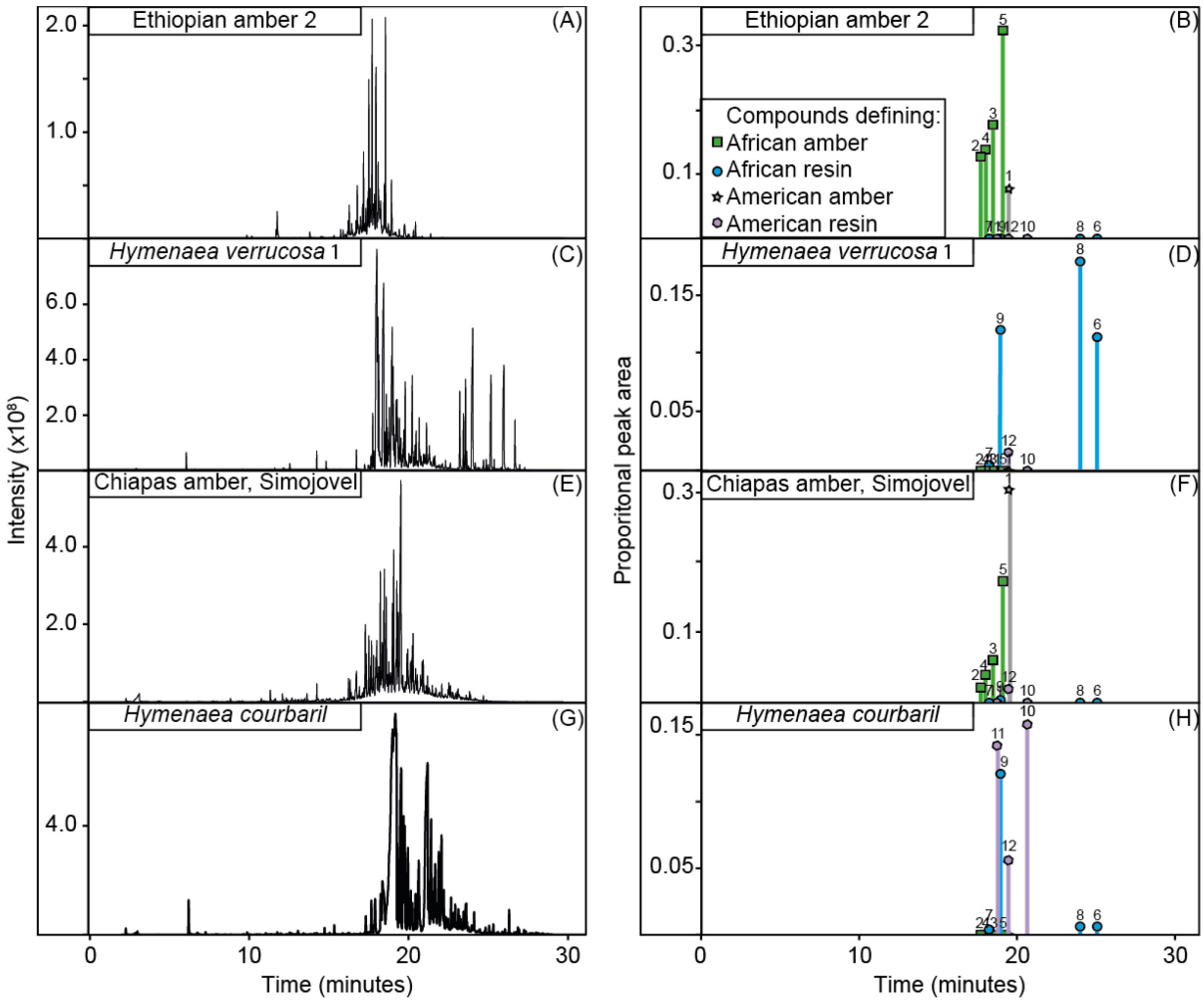
520

Figure 4



1	1,1,4,5,6-Pentamethyl-2,3-dihydro-1H-indene	AMERICAN AMBER
2	4,8,11,11-Tetramethyl-tricyclo[7.2.0.0(3,8)]undec-4-ene	AFRICAN AMBER
3	Trimethylphenyl-butanone	
4	Caryophyllene isomer	
5	Tetrahydro-tetramethyl-naphthalene	
6	13-Epimanool	AFRICAN RESIN AND COPAL
7	Caryophyllene	
8	Biformene	
9	α-Curcumene	
10	Humulene-1,2-epoxide	AMERICAN RESIN AND COPAL
11	α-Humulene	
12	β-Bisabolene isomer	

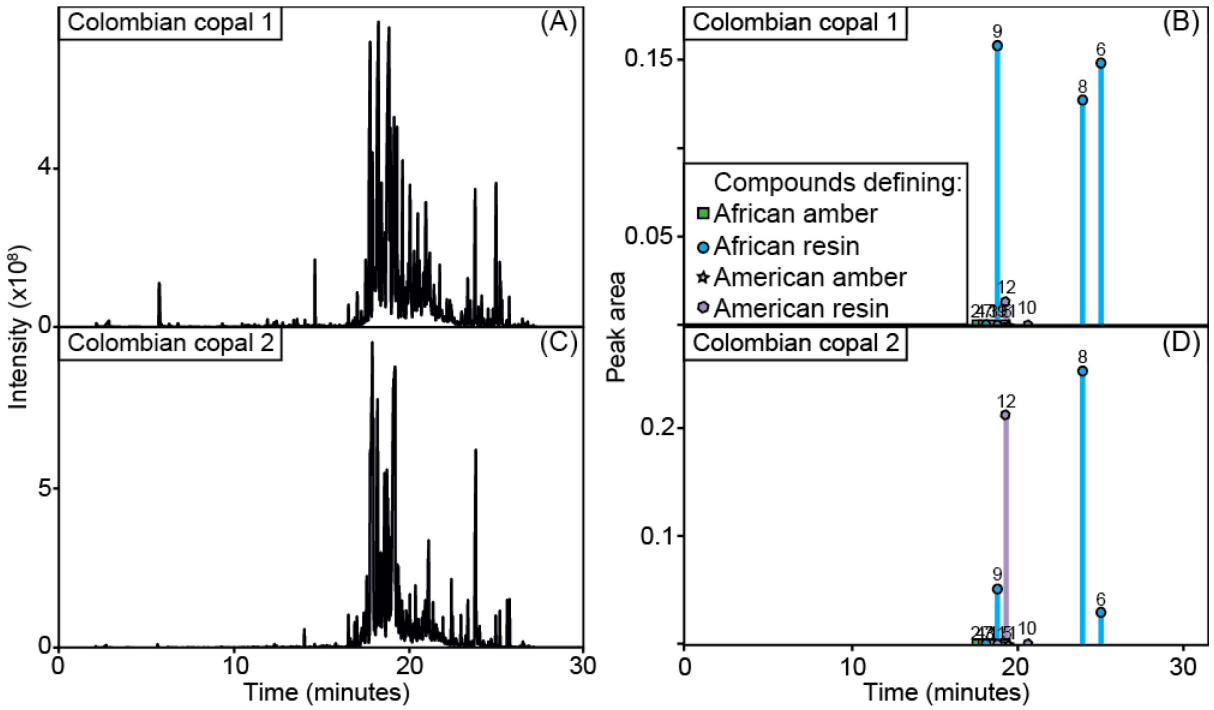
524 **Figure 5**



525

526

527 **Figure 6**



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