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Chemical preservation of plants and insects in natural resins

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The morphological preservation of fossils in amber is remarkable, but their chemical composition is largely unknown. The likelihood of DNA preservation in amber has been questioned but, surprisingly, the fate of more decay-resistant macromolecules such as ligno-cellulose in plants or the chitin–protein complex in insect cuticle has not been investigated. Here we report the results of investigations using pyrolysis-gas chromatography/mass spectrometry (py-GC/MS) of the tissues of insects and the plant *Hymenaea* from ancient and sub-fossil resins (2–20 ka) from Kenya, and from Dominican amber (25–30 Ma). The volatile components of the resin have penetrated even the internal tissues, resulting in the exceptional three-dimensional preservation of amber inclusions. Chitin is preserved in the bee and ligno-cellulose in the *Hymenaea* leaf from the Kenyan resins. There was no trace, however, of these macromolecules in tissues in Dominican amber. The presence of aliphatic polymer and sulphur-containing moieties in these tissues indicates that they have undergone diagenetic alteration; in view of this, the preservation in Dominican amber of a macromolecule as labile as DNA would be extraordinary.

Keywords: amber; fossil resin; ligno-cellulose; chitin; protein; py-GC/MS

'The bee is enclosed and shines preserved in amber, so that it seems enshrined in its own nectar'.
Marcus Martialis (AD 40–140)

1. INTRODUCTION

Inclusions in amber have been a subject of study for more than 200 years (Poinar 1992). Different resin components could account for the exceptional preservation of fossil inclusions, but the exact reason for their three dimensionality is still unknown. Amber inclusions may be rapidly dehydrated, protected from water and from microbial and fungal decay by entrapment (Poinar & Hess 1985; Langenheim 1995), although still susceptible to oxygen (Hopfenberg *et al*. 1988; Poinar 1994). Many reports have stressed the minute detail evident in insect tissues from various fossil amber deposits (Grimaldi *et al*. 1994*b*). Ultrastructural evidence at the cellular level indicated the presence of nuclei and mitochondria in insect tissues (Poinar & Hess 1982), and chloroplasts along with endoplasmic reticulum in plant tissues (Poinar *et al*. 1996*b*). The presence of non-racemized amino acids in insect tissues from ambers of various ages suggested that anhydrous conditions within the amber promoted long-term preservation (Bada *et al*. 1994; Wang *et al*. 1995). There have been seven reports to date of DNA retrieval from fossil insects and plants in amber (Higuchi & Wilson 1984; Cano *et al*. 1992, 1993; DeSalle *et al*. 1992; De Salle 1994; Henwood 1992; Poinar *et al*. 1993). In addition, there have been claims of viable bacteria from the guts of stingless bees in amber (Cano & Borucki 1995). Although it has been suggested that DNA cannot survive for more than 10 000 years in most environments (Pääbo & Wilson 1991; Lindahl 1993), recent studies have shown that the temperature of the depositional setting rather than the length of time is the crucial factor in DNA preservation (Höss *et al*. 1996; Poinar *et al*. 1996*a*). However, none of the claims of DNA isolation and bacterial resurrection from amber have been independently reproduced. Recent attempts to replicate these studies have failed to retrieve small fragments of DNA from preserved insect remains in both Quaternary resin from Africa and Dominican amber, leaving the question of DNA preservation in amber in controversy (Austin *et al*. 1997; Walden & Robertson 1997).

Why do fossils entombed in amber remain in such morphologically pristine condition (figure 1)? And is this morphological perfection matched by the state of preservation of the constituent biomolecules? To address these questions we used pyrolysis-gas chromatography/mass spectrometry (py-GC/MS) of microgram samples of tissue to assess the state of preservation of ligno-cellulose in leaves, and the chitin–protein complex in insects trapped in 2–20 ka resins from Kenya and 25–30 Ma Dominican amber. (Younger, incompletely polymerized resins are commonly

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referred to as copal; they contrast with amber, which is highly polymerized and cross-linked (Grimaldi *et al*. 1994*a*). The distinction between resin, copal and amber is not clear-cut, however, and we employ the terminology proposed by Anderson (1996) based on age. Thus resins from 250 to 5000 years old are 'ancient', and those from 5 ka to 40 ka are 'sub-fossil'. Older resins are termed 'amber'.) Clearly, if relatively decay-resistant, structural macromolecules such as ligno-cellulose and chitin have not survived in amber, the preservation of ancient DNA requires special explanation (Logan *et al*. 1993).

Py-GC/MS has been successfully applied to the study of macromolecules, including lignin, cellulose, proteins and chitin, in various biological tissues (Hatcher *et al*. 1988; Stout *et al*. 1988; de Leeuw *et al*. 1991; van Bergen *et al*. 1995; Stankiewicz *et al*. 1996, 1997*c*). Recently, it has been used to demonstrate the preservation of chitinous moieties in 25 Ma fossil beetles from Enspel, Germany (Stankiewicz *et al*. 1997*b*). Thus, it is a very powerful tool for investigating microgram amounts of tissues entombed in resins. It also provides a direct method of detecting several different macromolecules in a single analysis (i.e. the Class Ic resin itself (Anderson *et al*. 1992; Anderson 1995), lignin, polysaccharide, chitin and amino acids) based on identification of unique pyrolysis products (Pouwels *et al*. 1987; Hatcher *et al*. 1988; Anderson 1995; van Bergen *et al*. 1995; Stankiewicz *et al*. 1996, 1997*d*).

2. MATERIALS AND METHODS

Specimens of *Hymenaea* (leaf from 20 ka Kenyan sub-fossil resin; leaf, sepal and seed from Dominican amber), bees (*Hypotrigona* from 2 ka Kenyan ancient resin; four specimens of *Proplebeia dominicana* from Dominican amber) and chrysomelid beetles (figure 1; four specimens from Dominican amber) were investigated. The amber specimens were collected in La Toca mine in the Dominican Republic. The tissues of modern species closest to the fossils were also analysed (*Hymenaea verrucosa*, *Plebeia frontalis* and Chrysomelidae, respectively).

The specimens were washed with different solutions to remove contaminants, placed in Petri dishes, and cracked using liquid nitrogen. Sterile needles were used to remove the cuticle and internal tissues (figure 1), which are clearly distinguished from the surrounding resin by their dark colour. The samples were inserted directly into quartz sample holders for pyrolysis. The samples in quartz holders were pyrolysed (heating rate 20 °C ms^{-1}) in a flow of helium for 10 s in a platinum coil at 610 °C using a CDS (CDS Analytical Inc., Oxford, Pennsylvania) 1000 pyroprobe coupled to a Carlo Erba (Milan, Italy) 4130 GC, interfaced with a Finnigan (San Jose, California) 4500 MS. Compounds were separated using a Chrompack (Middleburg, The Netherlands) CP Sil-5 CB column (50 m × 0.32 mm internal diameter, film thickness 0.4 μm). The GC oven was operated as follows: isothermal for 5 min at 35 °C, temperature programmed at 4 °C min^{-1} to 310 °C and then isothermal for 10 min. The MS was operated in full scan mode (*m/z* 35–650 Da, 1 scan s^{-1}, 70 eV electron energy, 300 mA emission current and ionization source temperature of 170 °C). The pyrolysis interface was held at 250 °C and the GC injector maintained at 250 °C. Peaks were identified, based on their mass spectral characteristics and GC retention indices, by comparison with standards, and with reference to the literature (Stankiewicz *et al*. 1996, 1997*d*; Hatcher *et al*. 1988; van Bergen *et al*. 1995).

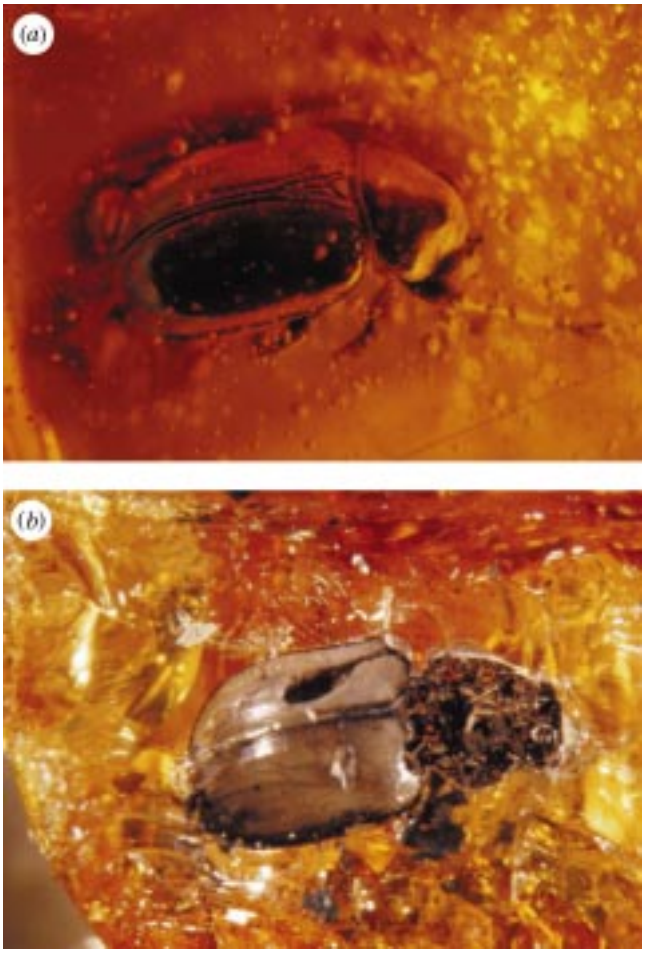

Figure 1. Chrysomelid beetle in *ca*. 25–30 Ma Dominican amber: (*a*) intact, and (*b*) after removal of top part of enclosing amber using liquid nitrogen. Note elytra, and internal tissue exposed after removal of the pronotum.

3. RESULTS AND DISCUSSION

(a) *Plant* (Hymenaea) *inclusions*

The leaf of the modern tropical legume *Hymenaea* (figure 2*a*), like other modern leaf tissues, yields a pyrolysate dominated by C_{16} mono- and diunsaturated, and saturated fatty acids, which are characteristic pyrolysis markers of cutin (Tegelaar *et al*. 1989). Lignin and polysaccharide markers are also apparent, although relatively less abundant (figure 2*a*). The lignin pyrolysis products are 2-methoxyphenol (G, guaiacyl) and 2,6-dimethoxyphenol (S, syringyl) moieties, recognized building blocks of angiosperm lignin (Stout *et al*. 1988; van Bergen *et al*. 1995).

The *Hymenaea* leaf preserved in sub-fossil resin (*ca*. 20 ka) yields relatively more pronounced lignin components than the modern specimen (figure 2*b*), with 4-methylphenol, 4-ethenyl-2-methoxyphenol, 2-methoxy-4-(2-(*E*)-propenyl)phenol and their 2,6-dimethoxyphenol equivalents as dominant markers. Abundant markers with a C_3 side chain, and minor components with ketone functionality, indicate unequivocally that the lignin polymer is very well preserved (Stout *et al*. 1988; Stankiewicz *et al*. 1997*d*). Polysaccharide markers (e.g. furaldehyde and furan-type components, and anhydrosugars such as levoglucosan) are also prominent in the *Hymenaea* in the

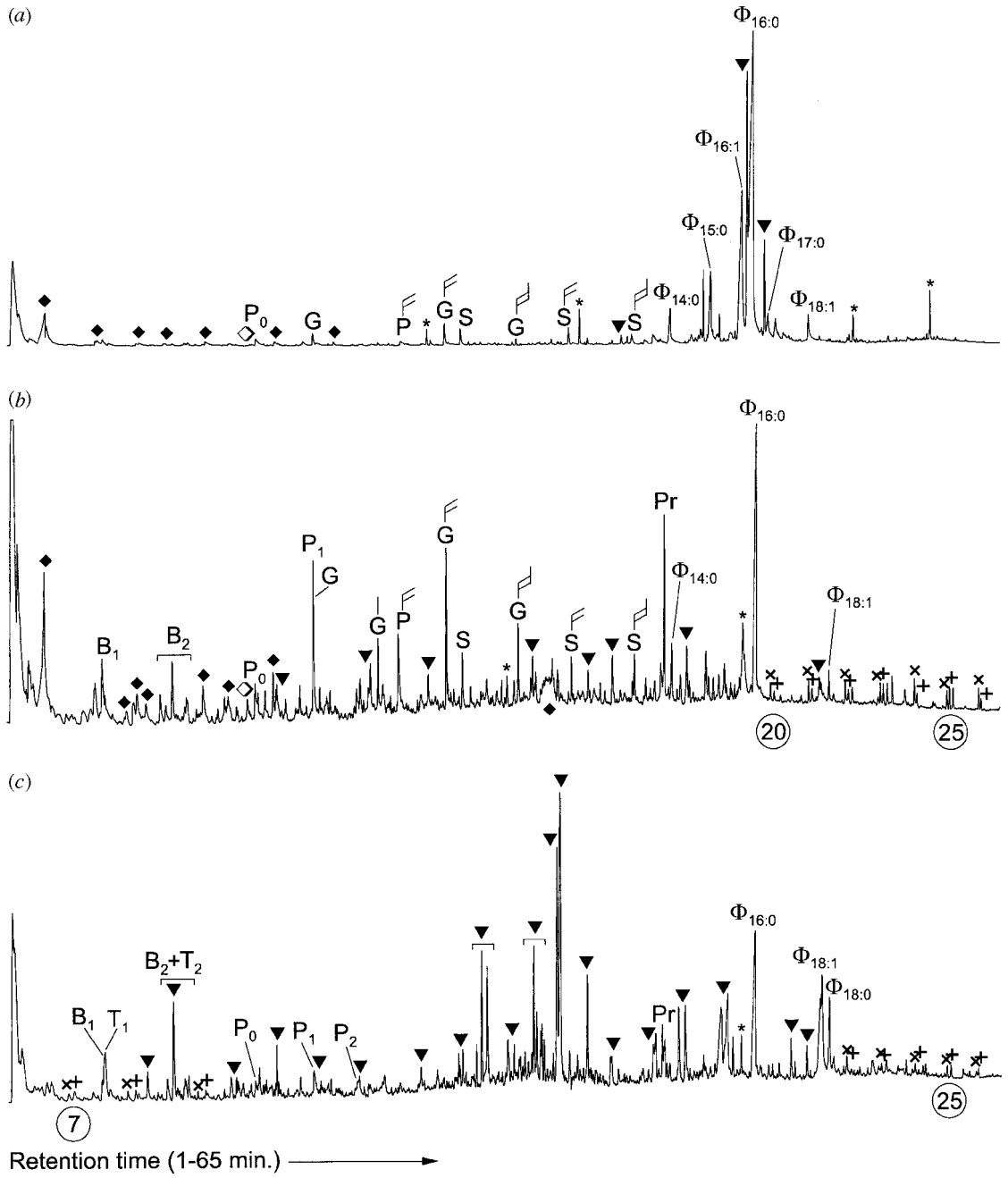

Figure 2. Total ion chromatograms (pyrolysis at 610 °C for 10 s) of leaf tissue of (*a*) modern *Hymenaea verrucosa* from Madagascar; (*b*) *Hymenaea* extracted from *ca*. 20 ka Kenyan sub-fossil resin; and (*c*) *Hymenaea* extracted from *ca*. 25–30 Ma Dominican amber. Key to the peaks: filled triangles, pyrolysis products directly related to resin polymer; filled diamonds, polysaccharide pyrolysis products; empty diamonds, hemicellulose marker; ×, *n*-alk-1-enes; +, *n*-alkanes. P_n, alkylphenols; B_n, alkylbenzenes; T_n, alkylthiophenes, where *n* indicates the extent of alkyl substitution (0, none; 1, methyl; 2, ethyl or dimethyl, etc.). G, 2-methoxyphenol; S, 2,6-dimethoxyphenol; Pr, prist-1-ene; *, contaminants. $\Phi_{x:y}$, fatty acids, where *x* indicates a carbon number and *y* refers to degree of unsaturation (0, fully saturated; 1, monounsaturated; 2, diunsaturated). Circled numbers indicate the carbon number in the aliphatic chains. Side chains of the G and S components are attached at carbon position 4 of the aromatic ring.

sub-fossil resin. The presence of 4-hydroxy-5,6-dihydro-(*2H*)-pyran-2-one (open diamond in figure 2*b*) testifies to the high-quality chemical preservation of hemicellulose (Pouwels *et al*. 1987; Stankiewicz *et al*. 1997*d*). Fatty acids are much less pronounced (as evidenced by their lower abundance relative to G and S compounds, and a lack of unsaturated C_{16} components) in the fossil leaf compared with the modern specimen. This clearly indicates decomposition of the cutin polymer, a symptom of the early stages of plant decay (Tegelaar *et al*. 1989, 1991). Thus, ligno-cellulose is well preserved in *Hymenaea* leaves from sub-fossil resin, while the cutin polymer is partly degraded.

Specimens of *Hymenaea* from Dominican amber yield none of the polysaccharide or guaiacyl and syringyl lignin markers evident in the modern leaves, or those from sub-fossil resin. The pyrolysate is overwhelmingly dominated by products derived from labdanoid diterpenes (Anderson 1995) (figure 2*c*). The only detectable markers of lignin are (alkyl)phenols (P_0 to P_2), which indicate

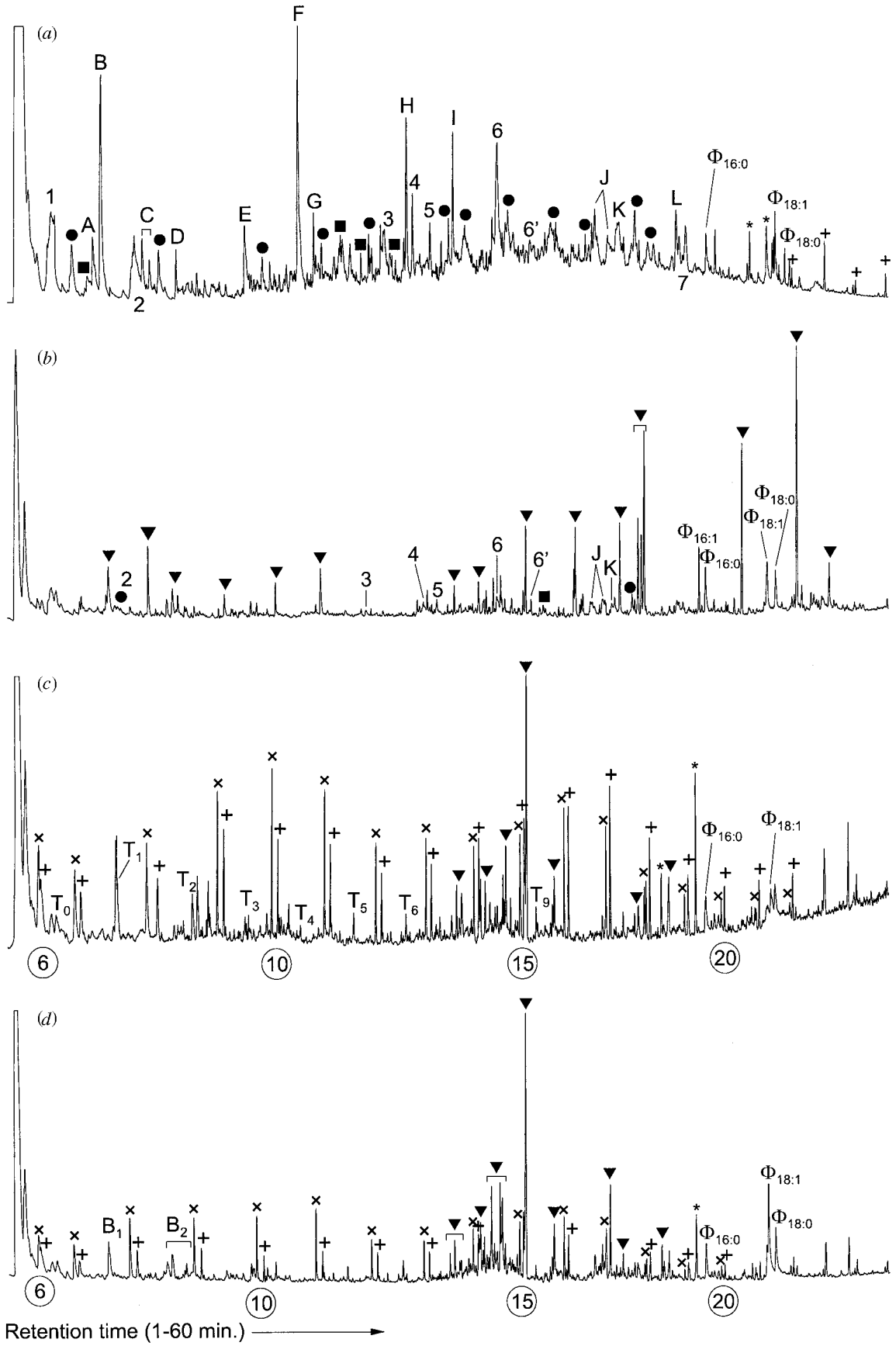

Figure 3. Total ion chromatograms (pyrolysis at 610 °C for 10 s) of tissue from (*a*) abdomen of modern stingless bee, *Plebeia frontalis*; (*b*) bee *Hypotrigona* extracted from *ca*. 2 ka Kenyan ancient resin; (*c*) stingless bee, *Proplebeia dominicana*, extracted from *ca*. 25–30 Ma Dominican amber; and (*d*) chrysomelid beetle extracted from *ca*. 25–30 Ma Dominican amber. Numbers indicate major pyrolysis products derived from chitin: 1, acetic acid; 2, acetamide; 3, 3-acetamidofuran; 4, 3-acetamido-5-methylfuran; 5, 3-acetamido-4-pyrone; 6, 6′ and 6″, oxazoline derivatives; 7, 1,6-anhydro-2-acetamido-2-deoxyglucose; filled squares, other important components directly related to chitin polymer. Letters indicate products derived from amino acids: A, pyrrole; B, toluene;

extensive decomposition and diagenetic alteration (Hatcher *et al*. 1988; van Bergen *et al*. 1995; Stankiewicz *et al*. 1997*d*). The other products released from the amber *Hymenaea* leaf are prist-1-ene, C_{16} and C_{18} fatty acids and a series of *n*-alk-1-enes and *n*-alkanes (figure 2*c*), which are also evident in *Hymenaea* from sub-fossil resin (figure 2*b*). The lack of methoxyphenols and polysaccharide pyrolysis products in the total pyrolysate confirms that even the ligno-cellulose macromolecule has undergone extensive decomposition, despite its enclosure by resin. This is surprising in the light of reports of the preservation of ligno-cellulose in Miocene and Oligocene plant remains in sediments (Stout *et al*. 1988; van Bergen *et al*. 1995; Stankiewicz *et al*. 1997*d*) and of partly modified lignin in Mesozoic coalified logs (Hatcher *et al*. 1988).

(b) *Insect inclusions*

Modern insect tissues yield pyrolysates dominated by products of protein (pyrroles, phenols, indoles, cyanobenzenes and 2,5-diketopiperazines) and chitin (acetamide, acetamidofurans, acetamidopyrones and oxazoline derivatives) (figure 3*a*; Stankiewicz *et al*. 1996). C_{16} and C_{18} fatty acids are also present as free components. Tissue from the fossil bee *Hypotrigona* from *ca*. 2 ka-old ancient resin yields derivatives that clearly indicate the presence of chitin and protein moieties (figure 3*b*), even though the pyrolysate is dominated by bicyclic terpenoids. The cuticle and internal tissues of the stingless bee *Proplebeia dominicana*, and chrysomelid beetles from Dominican amber yield total ion chromatograms with prominent resin markers (i.e. cyclic terpenoids). These diterpenoids are relatively more abundant in the cuticle than in the internal tissues. In contrast to modern insects, and those in the ancient resin, no markers characteristic of either chitin or proteins are present (figure 3*c*,*d*). (When a mixture of amber and modern beetle cuticle (in a ratio of 10:1) was pyrolysed, resin pyrolysis products were dominant, but chitin markers were still evident in characteristic mass chromatograms for individual components. Thus, the absence of chitin markers in the amber specimens is not considered to be a masking effect of the amber.) Straight chain hydrocarbons (C_6 to C_{20} *n*-alk-1-enes and *n*-alkanes) were relatively abundant in all eight specimens studied (figure 3*c*,*d*). These components were not present in the pyrolysate of the resin alone and must derive from the fossil insect tissue. Similarly abundant homologous series of alkenes and alkanes have also been reported in the pyrolysates of the cuticle of Palaeozoic and Mesozoic arthropods (Stankiewicz *et al*. 1997*a*, 1998), where chitin or protein markers were likewise absent. The alkenes and alkanes are characteristic pyrolysis products of an aliphatic polymer (Tegelaar *et al*. 1989), which does not occur in modern insects. The encapsulation of the insects in resin precludes an exogenous source for the aliphatic polymer. Hence we interpret it as a diagenetic product resulting from *in situ* polymerization of cuticular waxes and internal body lipids (Stankiewicz *et al*. 1997*a*, 1998). The presence of long-chain alkylated thiophenes (T) in the Dominican amber insects (figure 3*c*) is the result of incorporation of sulphur (possibly from amino acids such as methionine and cysteine) into functionalized lipids (e.g. fatty acids) during early diagenesis (Sinninghe Damsté *et al*. 1989). Although both the Kenyan resins and Dominican amber belong to Class Ic (Anderson 1995) certain differences in the distribution and abundance of their pyrolysis products are apparent (figures 2 and 3). These can be explained by the higher degree of polymerization (i.e. maturity) of the Dominican amber.

The most important insight into the mechanism of preservation of the amber inclusions came from investigations of the internal tissues of the insects. Bicyclic resin products (especially those corresponding to molecular weights of 190, 192, 204, 206 and 220 Da) were present in high abundance even in those tissues not directly in contact with the amber. Thus, although the protective coating of amber may prevent collapse of the tissues, the interactions must be more intimate. It is likely that resin molecules formed chemical cross-links involving biochemical components connected via sulphur and/or other terminal olefinic groups of the diterpenoid units. Such molecular interactions explain both the retention of the three-dimensional morphology and the preservation of the finest details of the internal tissues.

4. CONCLUSIONS

Although resistant macromolecules such as lignin, cellulose and chitin are evident in the plant and insect specimens from 20 ka and 2 ka Kenyan resins, there is no evidence from the molecular data that they survive in Dominican amber. On the contrary, pyrolysis of the amber inclusions demonstrates the presence of aliphatic polymers and sulphur-containing moieties in bee and beetle specimens, and to a lesser extent in *Hymenaea*, indicating advanced diagenetic transformation during 25–30 million years of burial. Similar diagenetic changes have been observed in fossil cuticles and kerogen preserved in sediments (Sinninghe Damsté *et al*. 1989; Stankiewicz *et al*. 1997*a*, 1998). The degradation and diagenetic alteration of relatively resistant macromolecules such as lignin, cellulose and chitin appear incompatible with reports of the survival of DNA in plant and insect tissues in Dominican amber. It is unlikely that DNA would be selectively preserved while ligno-cellulose and chitin are extensively degraded. This is borne out by the failure of

Figure 3 (*continued*) C, methylpyrroles; D, ethenylbenzene; E, phenol; F, 4-methyl phenol; G, ethylcyanobenzene; H, indole; I, methylindole; J, 2,5-diketopiperazine of Pro-Ala; K, 2,5-diketopiperazine of Pro-Gly; L, 2,5-diketopiperazine of Pro-Pro; filled circles, other important components related to proteins. Filled triangles, pyrolysis products directly related to resin polymer; ×, *n*-alk-1-enes; +, *n*-alkanes; *, contaminants; T_n, alkylthiophenes, B_n, alkylbenzenes, where *n* indicates the extent of alkyl substitution (0, none; 1, methyl; 2, ethyl or dimethyl, etc.). $\Phi_{x:y}$, fatty acids, where *x* indicates a carbon number and *y* refers to degree of unsaturation (0, fully saturated; 1, monounsaturated; 2, diunsaturated). Circled numbers indicate the carbon number in the aliphatic chains.

comprehensive attempts to replicate amplifications of DNA not only from amber-preserved insects (Austin *et al*. 1997; Walden & Robertson 1997), but also from bees in Quaternary resin from Africa (Austin *et al*. 1997).

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