

## Composition of Wax Made by the Australian Stingless Bee *Trigona australis*

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### Abstract

Analysis of the nest material of *T. australis* by gas chromatography/chemical ionization-mass spectrometry showed the wax to comprise a hydrocarbon fraction (90%), esters (6%) and free acids (4%). The major saturated hydrocarbons were C<sub>27</sub>, C<sub>31</sub> and C<sub>33</sub> with C<sub>25</sub> and C<sub>29</sub> being less abundant and C<sub>23</sub> and C<sub>35</sub> being present in small amounts. Traces of the intermediate, even-numbered homologues were also found. Hydrocarbons (C<sub>31</sub>, C<sub>33</sub> and C<sub>35</sub>) which contained one double bond were also present and traces of the diene C<sub>35</sub> were detected. The ester fraction did not contain compounds identical with those in beeswax made by the honeybee *Apis mellifera* and the acid fractions were also quite different. *T. australis* wax contained the following, saturated free acids: C<sub>10</sub> (trace), C<sub>12</sub>, C<sub>14</sub>, C<sub>16</sub>, C<sub>18</sub> and C<sub>20</sub>, monoenoic and dienoic C<sub>18</sub> and C<sub>20</sub> and traces of the trienoic C<sub>18</sub>. The wax of *T. australis* is colourless but the brown colour of the nest material derives from the inclusion of masses of pollen (*Eucalyptus* sp.) and solid material from the inside of the nest tree. The solid residue comprised between 12 and 30% by weight.

### Introduction

The stingless, native, Australian bees *Trigona carbonaria* (Friese) and *T. australis* (Smith) form small colonies which may survive for many years. Little is known of their behaviour and virtually nothing of their biochemistry. The combs of ovoid cells formed by both of these *Trigona* species are composed of evenly brown-coloured wax and are built in roughly ordered masses rather than in the sheets of precise, hexagonal cells that comprise the familiar honeycomb of the domestic honeybee (*Apis mellifera*). The *T. australis* and *T. carbonaria* hives are usually found in hollow *Eucalyptus* branches, the lumina of which are often partially filled with a decayed, digested wood and earth residue left by termites.

We have recently carried out an analysis of the beeswax made by *T. australis* and compared it with the well-defined composition of that made by *A. mellifera* (Tulloch 1971, 1972, 1980), using wax collected from a honeybee hive in Sydney as a chromatographic standard. The brown colour and soft texture of the *T. australis* wax suggested that it could have a composition quite different from that of *A. mellifera* beeswax. *T. australis* has a primitive hive and social structure and so it was of interest to examine the composition of its wax.

## Materials and Methods

All solvents were A.R. grade and the hexane and toluene were redistilled before use. The *T. australis* wax was from a colony kept near Richmond, N.S.W., the *A. mellifera* beeswax was from a hive in Gordon, N.S.W.

The wax of *T. australis* is less soluble in organic solvents [ $\text{CH}_2\text{Cl}_2$ ,  $\text{CHCl}_3$ , diethyl ether, hexane (0.2 g/ml)] and solid residue was removed by shaking the hexane solution (5 ml) with a small volume of water (1 ml) and centrifuging, whereupon the solid residue remained in the lower, aqueous phase and left a clear, colourless solution of wax in hexane.

Samples of hydrocarbon fractions of the waxes were centrifuged to the bottom of melting point tubes. The melting points are uncorrected.

The melting behaviour and rigidity of the *A. mellifera* and *T. australis* waxes at different temperatures were compared by placing parallel, thin rods of wax (3 mm in diameter) on a 600-mm strip of aluminium of which one end (100 mm) had been bent down into boiling water, and the other end (100 mm) into melting ice. The temperature at a number of points along the strip was measured with a thermister once the temperature had reached equilibrium.

Kieselgel 40 grade (Merck) was used for column chromatography of the wax (2 g wax, packed volume of silica 100 by 30 mm). Columns were eluted with toluene (300 ml) to give the hydrocarbon fraction, chloroform-hexane (80 : 20 v/v; 200 ml) to give the ester fraction and with acetone-acetic acid (1 : 1 v/v, 60 ml) to give the acid fraction.

The precoated t.l.c., plates (200 by 200 by 0.25 mm Silica gel 60F<sub>254</sub>) were from Merck and were developed three or four times with toluene to give clear separation of the ester components. A strip (30 mm wide) was cut from the edge, sprayed with 3.5 M  $\text{H}_2\text{SO}_4$  and heated to 120°C for 4 or 5 h. The wax components produced brown spots. The untreated zones could then be eluted, with acetone, from the remainder of the plate.

The mass spectrometer used was a Finnigan Model 3200 GCMS chemical ionization system equipped with a PPINICI attachment and interfaced to a Finnigan Incos 2300 Data acquisition and scan control system. Electron energy, emission current and electron multiplier voltage were, respectively, 90–100 eV, 100 mA and 1.2–1.3 kV. Methane (Matheson, Rutherford, New Jersey, U.S.A.) was used as the chemical ionization energy moderator (ion source pressure) and gas chromatograph carrier gas (flow rate 20 ml/min). The ion source was maintained at 110°C nominal.

Samples were injected into a 1.5 m (int. diam. 2 mm) U-shaped glass column [1.5% OV-1 on Gas Chrom Q (100–200 mesh)]. The GC injector, transfer line and interface oven were maintained at 280–300°C. The GC oven was held at 100°C for 1 min after injection and was usually programmed to rise at 10 degrees per minute to 300°C. Data acquisition began 1 min after the injection of 2–4  $\mu\text{l}$  of sample in hexane.

Hydrocarbon and fatty acid methyl ester samples were injected into a 30-m fused silica OV-17 capillary column (ext. diam. 0.26 mm, J. & W. Scientific Inc., Rancho Cordova, California, U.S.A.). The same temperature conditions were used except that the temperature ran at 100°C and then rose by 8 or 12°C per minute to 300°C. Data acquisition commenced after 1.5 min and 1–2  $\mu\text{l}$  of solution were injected.

## Results

### *Physical Properties of the Two Waxes*

When thin rods of *T. australis* and *A. mellifera* waxes were placed on a heated aluminium strip to measure their melting behaviour, several features rapidly became obvious. The *A. mellifera* wax melted, very few solid particles were visible, and the melted wax was pale yellow. It contained occasional fragments of plant tissues, pollen grains as well as unrecognizable materials. The *T. australis* wax, on the other hand, was colourless but left a dark-brown solid residue, comprising 12–36% by weight (Table 1).

The melting point of *T. australis* wax (58–60°C) was 2–4°C lower than that of *A. mellifera* wax (63–64°C); however, the latter wax was plastic over a 3°C range, slightly soft but rigid down to 55°C and hard below that temperature. *T. australis* wax was equally plastic over a similar short range but even down to 29°C it remained slightly soft.

The solid residues from *T. australis* wax were analysed by microscopy and histochemical staining and most of them consisted of aggregations of pollen grains of *Eucalyptus* sp. (Fig. 1a). Pieces of young adult bee exoskeleton (Fig. 1b) and fragments of xylem fibres and vessels (which stained with phloroglucinol HCl) could be distinguished and, under the crossed Nicol prisms of a polarizing microscope, numerous, very small, highly refractile particles, which were identified as fine sand grains, were also seen.

#### *Thin-layer and Column Chromatography*

Chromatography of *A. mellifera* wax on silica gel t.l.c. plates in benzene by Tulloch (1980) gave eight zones which he identified as hydrocarbon (I), near the solvent front, and, in order of increasing polarity mono-, di- and other esters, the zone at the origin being free acids (VIII). Toluene was used as the solvent in place of benzene (because of the carcinogenicity of the latter), and the pattern reported by Tulloch (1972) and White *et al.* (1960) was confirmed. Chromatography of

**Table 1. Composition of wax of *T. australis* and *A. mellifera***  
The amount of solid residue is expressed as a percentage of the total wax. The composition of soluble constituents is expressed as a percentage of the total soluble components. Data from Tulloch (1971)

Wax fraction	<i>T. australis</i> (% by weight)	<i>T. mellifera</i> (% by weight)
Solid residue	23.2 <sup>A</sup>	—
Hydrocarbon fraction	89.9	14
Ester fraction	5.9	67
Acid fraction	4.1	11.9
Unidentified material	—	7

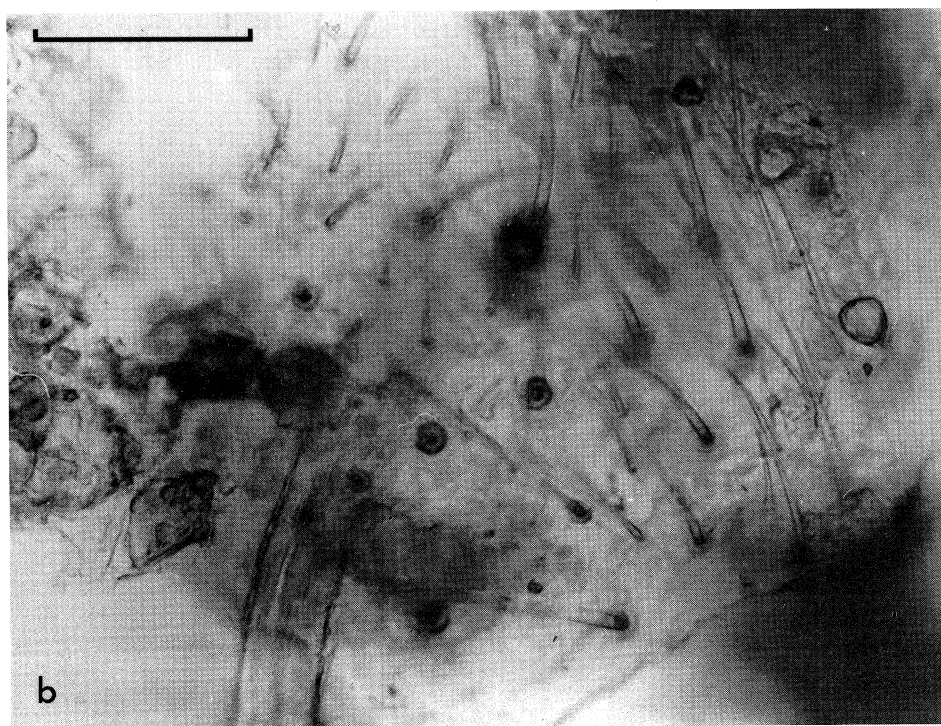
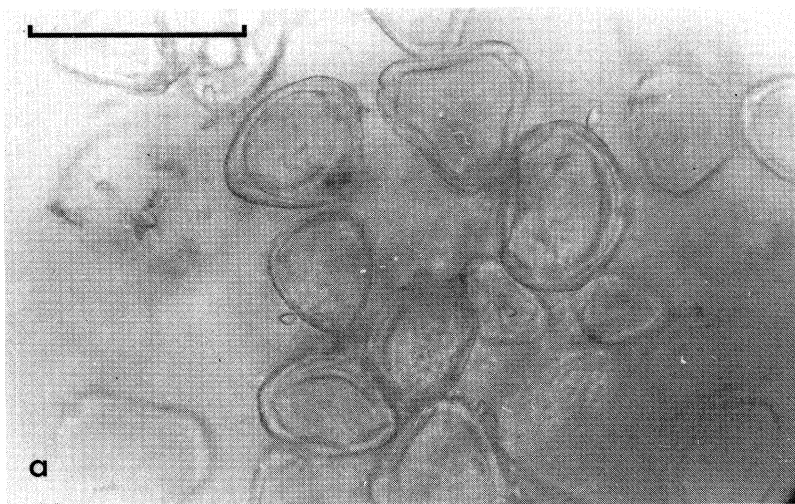
<sup>A</sup> Mean of three determinations (18.8, 12.3, 35.7).

*T. australis* wax, in contrast, was difficult. Most of the material is hydrocarbon which ran at the solvent front and hindered the movement of the solvent so severely that only very small quantities of the wax could be chromatographed adequately by t.l.c. The method of detection used, following Tulloch, was charring by heating after a spray of 3 M H<sub>2</sub>SO<sub>4</sub>. It is a relatively insensitive method and only three of the minor, more polar constituents of the whole *T. australis* wax could be detected by this means. They did not coincide with any of the zones obtained with *A. mellifera* wax. Consequently the further investigation of *T. australis* wax was carried out after column chromatography using hexane-dichloromethane (1 : 1 v/v) on silica gel to give a hydrocarbon fraction, an ester fraction and a free acid fraction (Table 1). Thin-layer chromatography of the ester fraction of *T. australis* wax showed five minor zones and one major zone after charring, all of which remain to be characterized. The free acid fraction of *T. australis* wax was slightly more polar than the analogous zone of *A. mellifera* wax and it also constituted a slightly larger proportion, by weight, of the non-hydrocarbon fraction of the wax.

### Gas-Liquid Chromatography/Mass Spectrometry

#### Hydrocarbons

The pattern and relative proportions of the materials present in wax in Australian samples of *A. mellifera* by gas-liquid chromatography/mass spectrometry (g.l.c./m.s.) were similar to those reported for Canadian wax by Tulloch (1971, 1972).



**Fig. 1.** Photomicrographs of components of the solid residue from wax of *T. australis*. (a) Masses of pollen grains, entirely *Eucalyptus* sp. (b) Fragments of integument identified as young imago. Scales 100  $\mu$ m.

*T. australis* wax (Fig. 2) was similar in that a family of large peaks of hydrocarbons which emerged from the g.l.c. column first, was followed by a series of smaller peaks which had similar retention times to the peaks identified as esters and hydroxyesters in *A. mellifera* wax. The hydrocarbons were identified as the odd-numbered  $C_{23}$ ,  $C_{25}$ ,  $C_{27}$ ,  $C_{29}$ ,  $C_{31}$ ,  $C_{33}$  and small quantities of  $C_{35}$ ; presumably they were formed by decarbonylation (Cheesbrough and Kolattukudy 1984) of the even-numbered aldehydes (Figs 2, 3a–3e). In addition there are very small peaks (c. 1%) of the even-numbered hydrocarbons occurring between the major peaks of the homologues with odd numbers of carbon atoms.

The  $C_{23}$  and  $C_{35}$  peaks were the smallest as judged by integrals of their ion current,  $C_{25}$  and  $C_{29}$  were intermediate,  $C_{27}$  and  $C_{31}$  were larger and  $C_{33}$  the largest (Figs 2, 3a). No  $C_{21}$  or  $C_{37}$  could be detected. No obvious reason for the pattern of relative abundance of the hydrocarbons can be suggested. A very similar pattern is found in *A. mellifera* wax although that of other species of the genus differs slightly (Tulloch 1980).

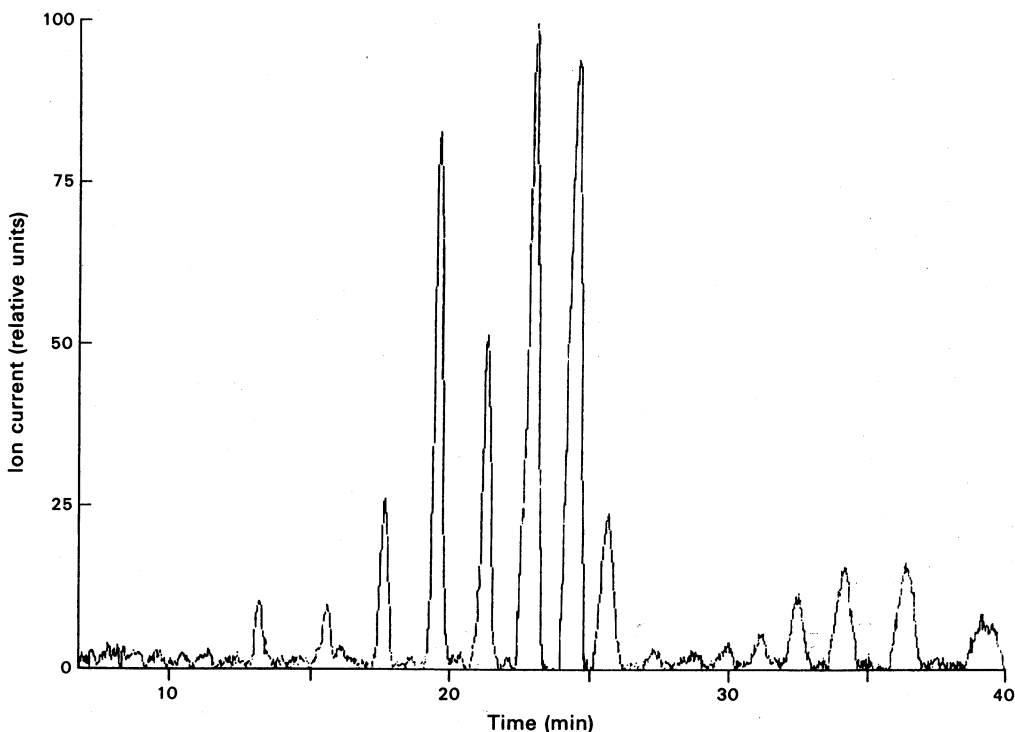
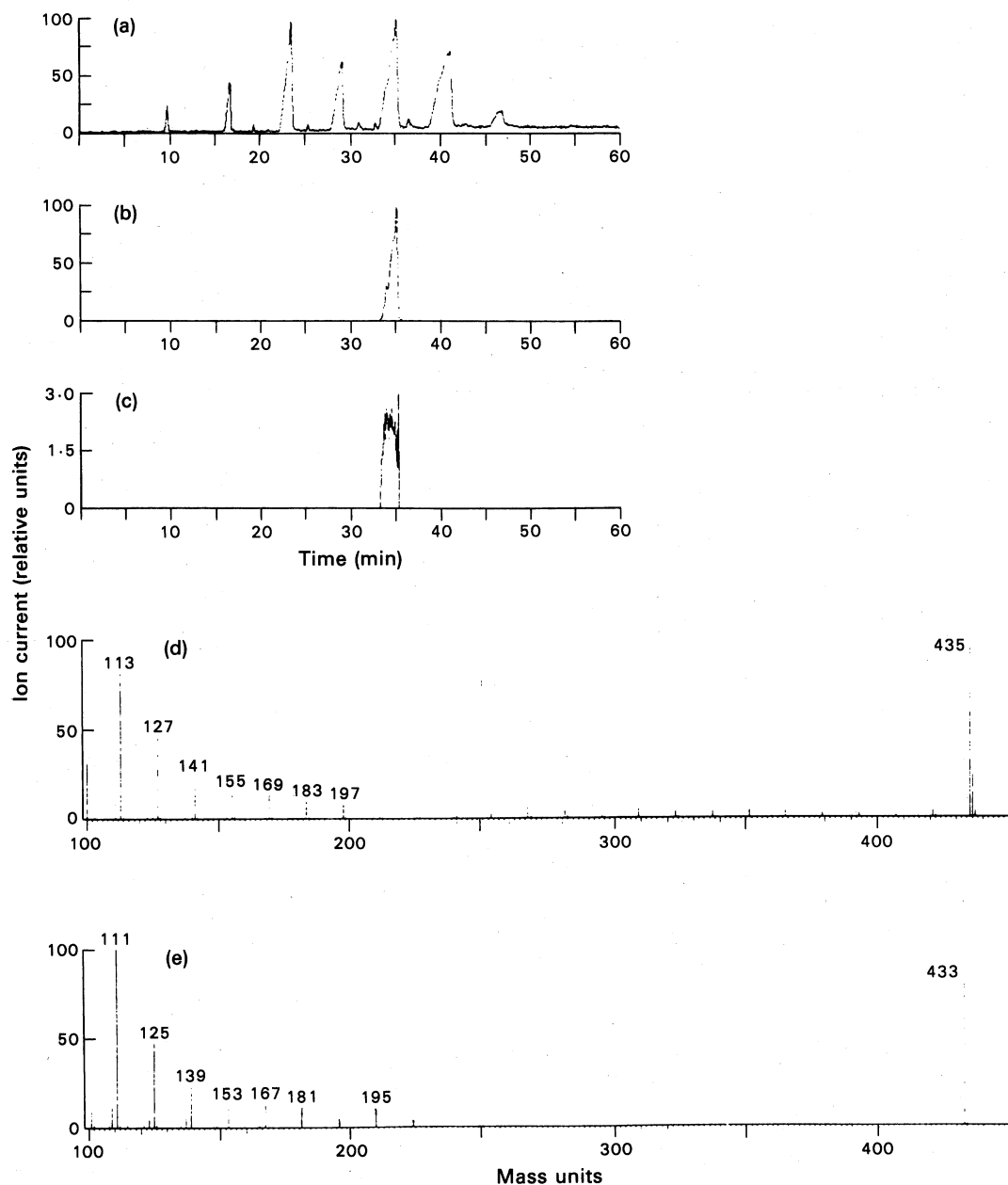


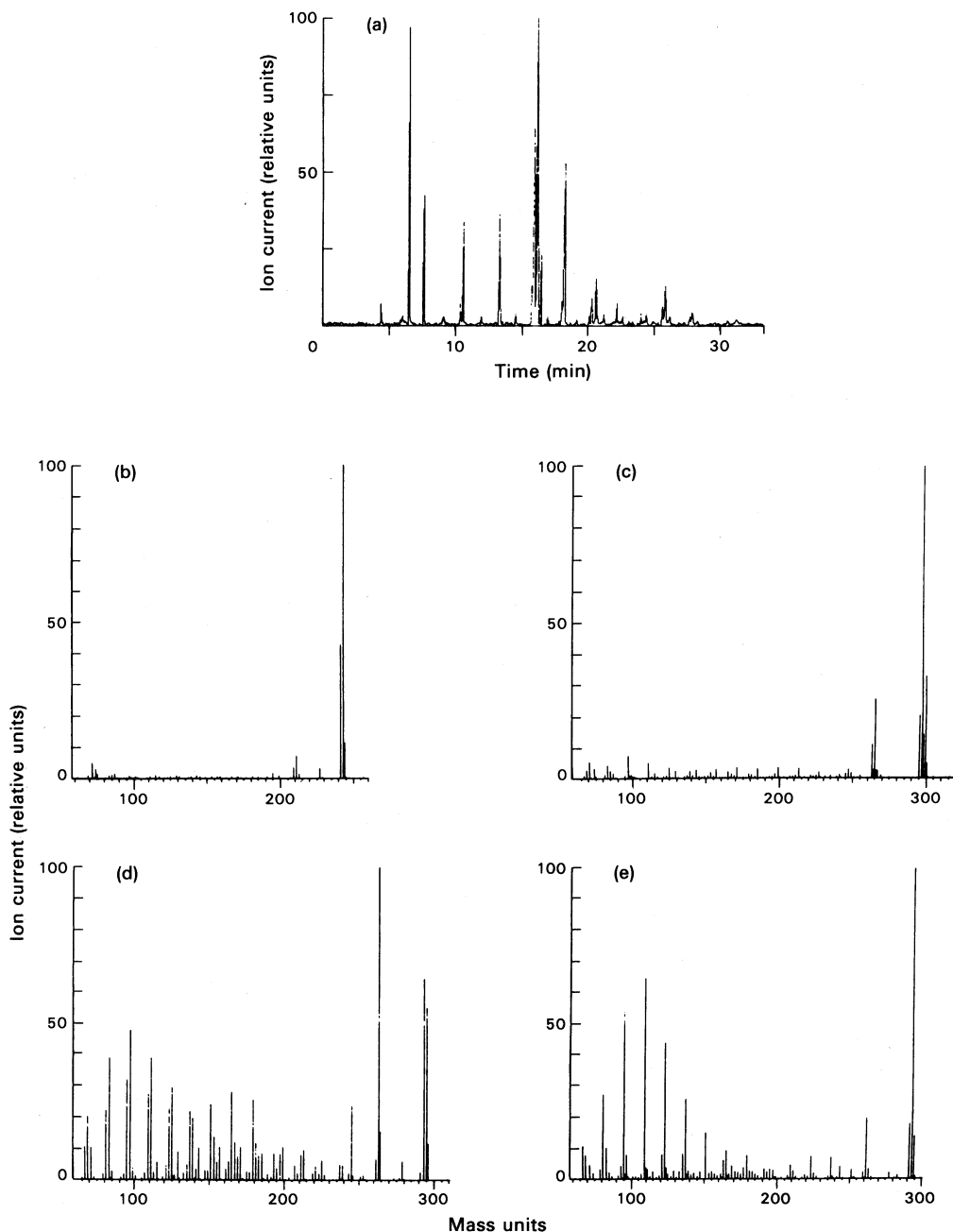
Fig. 2. G.l.c./m.s. trace of *T. australis* wax. The soluble wax components were methylated with ethereal diazomethane. 1.0 m 1.5% OV-1 column, 80°C at 1 min then 8°C/min to 325°C. Data collection began 1 min after injection. The peak at retention time 13 min is  $C_{18}$  methyl esters (see Fig. 4). The large peaks between 15 and 26 min are the  $C_{23}$ ,  $C_{25}$ ,  $C_{27}$ ,  $C_{29}$ ,  $C_{31}$ ,  $C_{33}$  and  $C_{35}$  hydrocarbons, small intermediate peaks are those of the even-numbered homologues. The peaks after 26 min are unidentified long-chain esters.

### Esters

The g.l.c. retention times of the esters of *T. australis* wax do not coincide exactly with those of *A. mellifera* nor do the t.l.c.  $R_F$  values. The ester fraction



**Fig. 3.** (a) G.l.c./m.s. trace of the hydrocarbon fraction of *T. australis* wax. Capillary OV-17 column, 100°C at 1.5 min then 8°C/min to 300°C. Data collection began at 1.5 min. (b) Ion current carried by a fragment ion of the saturated component of the unresolved  $C_{31}$  peak. (c) Fragment ion of the singly unsaturated  $C_{31}$  hydrocarbon. The first part of the peak of ion current (unsaturated hydrocarbon predominates) was subtracted by the computer from the latter part of the peak to give the mass spectrum of the saturated hydrocarbon (d). The reverse procedure gave (e), the fragmentation pattern of the singly unsaturated  $C_{31}$  hydrocarbon.



**Fig. 4.** (a) G.I.c./m.s. trace of the ion current carried by methyl esters of the free fatty acids in *T. australis* wax. The acid fraction from silica gel column chromatography was methylated and injected into an OV-17 capillary column (50 m), 100°C at 1.5 min, then 8°C/min to 300°C. Data collection and time markers begin 1.5 min after injection. The peak at 6.5 min is an impurity from the diazomethane, that at 4.5 min is  $C_{10}$  (methyl caprate), 8 min  $C_{12}$ , 10.5 min  $C_{14}$ , 13 min  $C_{16}$ , 15.5 min  $C_{18:0}$ ,  $C_{18:1}$  and  $C_{18:2}$  (major), 16 min  $C_{18:3}$ , 18 min  $C_{20:0}$ ,  $C_{20:1}$  and  $C_{20:2}$  (not resolved). None of the higher peaks showed fragmentation patterns of methyl esters. The small peaks between the major, even-numbered homologues are of acids with an odd number of carbon atoms. (b) Fragmentation pattern of the  $C_{14}$  saturated acid. (c)  $C_{18:1}$  which was imperfectly separated from  $C_{18:0}$ , hence the ion at  $m/z$  299. (d)  $C_{18:2}$ . (e)  $C_{18:3}$ .

of *T. australis* wax can be separated into six minor and one major zones by t.l.c. on silica gel in toluene.

#### *Free acids*

The acid fraction eluted from silica gel columns in acetone-acetic acid (1:1 v/v) was methylated and g.l.c./m.s. showed the presence of methyl esters of C<sub>10</sub>, C<sub>12</sub>, C<sub>14</sub>, C<sub>16</sub>, C<sub>18</sub> and C<sub>20</sub> fatty acids (Figs 4a-4c). Trace amounts of the odd-numbered fatty acids were found between the even-numbered homologues. The fragmentation patterns of the methyl esters of even numbered acids were compared with those of authentic, commercial standards to provide unambiguous identification. Monoenoic C<sub>18</sub> (Fig. 4c) and C<sub>20</sub> acids and dienoic C<sub>18</sub> (Fig. 4d) and C<sub>20</sub> acids were also present, the octadecadienoic acid being the most abundant. Octadecatrenoic acid was also found (Fig. 4e).

Separation of long-chain, free fatty acids from *T. australis* wax was attempted by partitioning the wax between 1 M NaOH and hexane. The acids were extracted with ether after acidification (pH 2) of the aqueous phase with H<sub>2</sub>SO<sub>4</sub>. No fatty acids of more than 20 carbon atoms were detected after methylation and analysis by g.l.c./m.s. Methylation of a sample of unseparated wax followed by g.l.c./m.s. also failed to detect the characteristic fragment ions or the parent ions of fatty acids with more than 20 carbon atoms. We conclude, therefore, that long-chain acids (C<sub>22</sub>-C<sub>34</sub>), as found in *A. mellifera* wax by Tulloch (1972), are not present in wax made by *T. australis*.

#### *Turpentine Tree Resin*

When the inner bark of the turpentine tree (*Syncarpia glomulifera*) is damaged it exudes a resin which attracts *T. australis* and *T. carbonaria* worker bees and they collect it in their pollen baskets. Consequently, turpentine tree resin is a second possible source of wax components. G.l.c./m.s. of the resin, which is readily soluble in hexane, shows the presence of some four major and 20 minor components,  $\alpha$ -pinene being the most abundant and the only component identified so far. It has been suggested in the past that the propolis in the hives is largely comprised of resinous materials collected by the bees. However, no compound from the resin was detected in the wax. Therefore, either the turpentine gum was unavailable to the *T. australis* hive whose wax was investigated or the resin is not mixed with the wax of which the combs are composed or the resin components had reacted to become non-volatile. However, some days after a turpentine tree, 20 m from a nest of *T. carbonaria*, was cut for gum collection the entrance of the nest and the bark immediately surrounding the hole was observed to have become covered with a shiny, resinous deposit. A sample of this deposit dissolved readily in methylene chloride to give a yellow solution while resin from other *Eucalypts* was almost insoluble. Resin freshly exuded from turpentine trees also dissolved readily in methylene chloride to give a yellow solution and the g.l.c./ms of the two samples were very similar (Figs 5a and 5b) with peaks at the same retention time showing identical fragmentation patterns. Thus, at least the resinous material placed in and around the nest entrance by *T. carbonaria* can be derived from the turpentine tree.

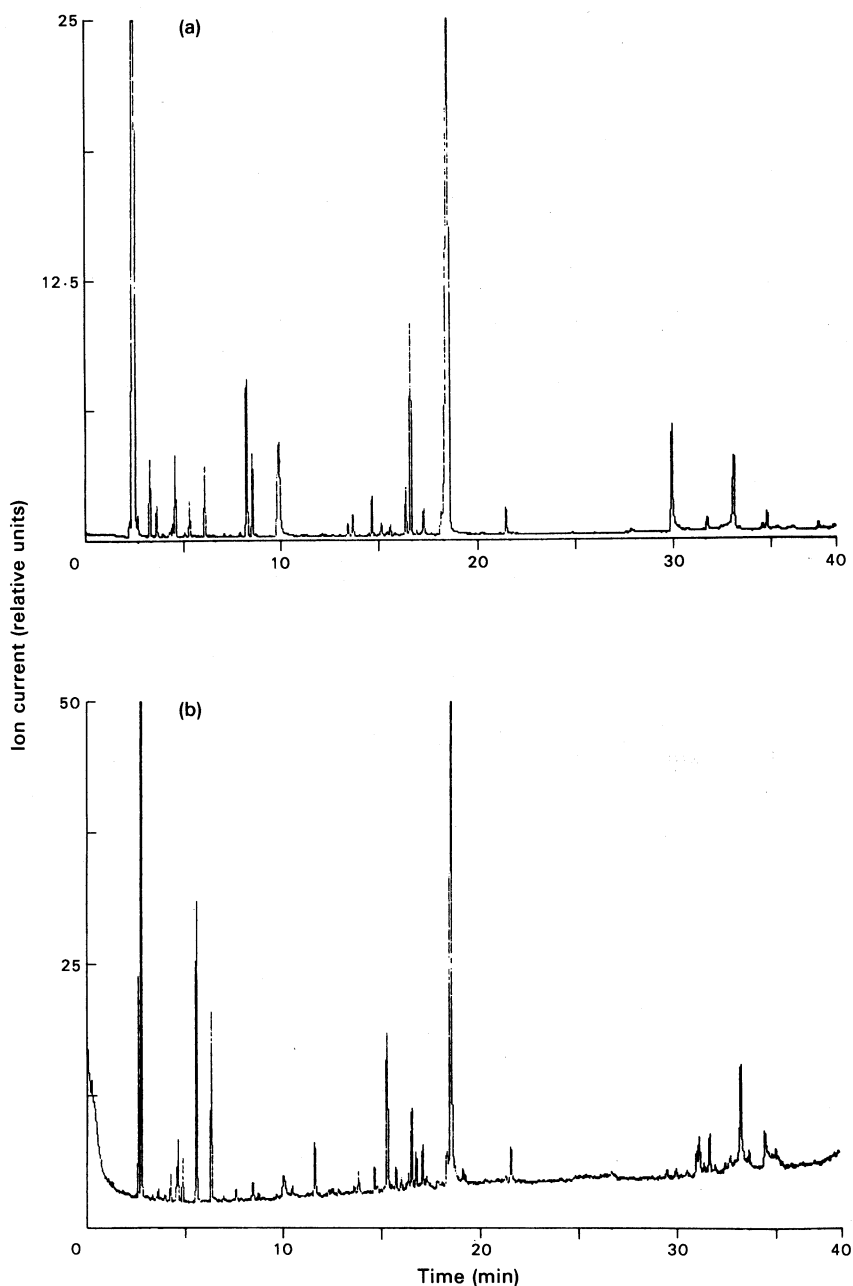
#### **Discussion**

##### *Solid Material in Waxes*

All samples of *T. australis* wax examined contained solid material which was mixed uniformly with it, and gave it its brown colour. Bumble bees (*Bombus*



*terrestris*) make a wax with a low melting point (30–40°C), and have also been reported to mix pollen into the wax when it is used as a structural material (Alford 1975). All of the solids in *T. australis* wax identified so far, other than pollen



**Fig. 5.** Composition of the resin from a turpentine tree (a) and of the material in the entrance of a *T. carbonaria* nest (b). Gas chromatography/chemical ionization-mass spectrometry trace of the relative ion current. Where peaks from two samples occurred at the same retention times they had identical fragmentation patterns. Capillary column 30 m bordered phase OV-101, 6°C/min from 80°C to 300°C, data acquisition from 1 min.

grains, can be accounted for by material from within the tree and so need not have been collected and brought to the nest. Examination by the same procedures of the dark-brown material lining the hollow branch of the tree (*Eucalyptus* sp.) from which the nest was obtained revealed that small particles had been mixed with the wax, larger ones had not. The clearest evidence of this was provided by the silica particles, those from the wax were very small (up to 0.01 mm), some showed Brownian movement, while sand grains in the material left by termites measured up to 0.5 mm. Pieces of *T. australis* integument carrying bristles were also present in the solid residue (Fig. 1). They were identified by comparison with the structure and colour of young adult bees in their pupae.

### Hydrocarbons

The hydrocarbons showed a parent ion one mass unit less than the molecular weight. This is commonly observed with hydrocarbons in chemical ionization-mass spectrometry (Field 1968) and is attributed to the uptake of a proton from the ionizing gas, followed by the loss of H<sub>2</sub>.

Fragment ions at  $m/z$  73, 85, 97 and 111 mass units, just below the major ions 75, 87, 99 and 113, indicated the presence of analogues containing one double bond. Parent ions two mass units less than the saturated compounds were detected in hydrocarbons with 29 and 31 carbon atoms and the peaks of the unsaturated C<sub>33</sub> and C<sub>35</sub> compounds comprised almost half and slightly more than half, respectively, of the ion current of the parent ion region. The ion current carried by the unsaturated compound may not be in exactly the same relationship to the number of molecules present compared with the ion current carried by the saturated hydrocarbons. Consequently the proportion of saturated to unsaturated compounds cannot be determined exactly by this means without recourse to pure standards. Comparison of tetradecene and tetradecane under our conditions showed that the monoene was detected with exactly twice the sensitivity compared with the saturated compound. The difference in sensitivity between the C<sub>33</sub> alkane and monoalkene can be expected to be much less.

All samples in which unsaturated hydrocarbons occurred showed the presence of parent ions two mass units less than those of the saturated analogues. Thus one double bond is present per molecule (Field 1968). Only the C<sub>35</sub> hydrocarbon parent ion peak showed the presence of a hydrocarbon molecule with two double bonds (5% of the ion current of the monoene).

The composition of *T. australis* wax differs considerably from that of *A. mellifera* wax in its content of free acids. C<sub>10</sub>, C<sub>12</sub> and C<sub>14</sub> were not detected in *A. mellifera* wax (Tulloch 1972) while most (96%) of the acid fraction was composed of the saturated C<sub>24</sub>, C<sub>26</sub>, C<sub>28</sub>, C<sub>30</sub>, C<sub>32</sub> and C<sub>34</sub> acids which were not found in the samples of *T. australis* wax, either by isolation of the fatty acid fraction or by g.l.c./m.s. of the whole wax after methylation. The presence of the 18:3 acid (Fig. 4e) may indicate that it is obtained from the diet. Linolenate (Z,Z,Z-octadeca-9,12,15-trieneoic acid), as an ester, is an abundant constituent of the membranes of plastids and hence the acid could be linolenic acid and derived from pollen. The positions of the double bonds in these unsaturated acids from wax, as in the hydrocarbons, have not yet been determined.

The production of wax hydrocarbon is costly in energy terms (a molecule of C<sub>31</sub> hydrocarbon requires about 12 glucose molecules). Thus the increase in bulk

of wax produced by the incorporation of solid faecal matter and termite debris present in hollow trees may constitute a considerable saving in energy. Analyses show that the amount of solid material present in the wax varies from 16 to 30% but even the lower values still represent a considerable saving in sugar. The structural effects of the solid residue have not yet been examined.

Overall, the composition of *T. australis* wax is close to that of *A. mellifera* but the former contains a smaller proportion of hydroxylated esters and a much larger proportion of hydrocarbons, in general, and unsaturated hydrocarbons in particular. This agrees with the high iodine number (32) reported for *T. carbonaria* wax by Rayment (1935) compared with *A. mellifera* (10).

The high proportion of unsaturated alkyl chains in *T. australis* wax would contribute to its plasticity at low temperature. The greater plasticity of the *T. australis* wax over a wide temperature range may be advantageous to a small bee and the combs do not have to be as strong and rigid as those of *A. mellifera* because they do not contain a heavy load of honey.

### Acknowledgments

We thank Dr A. M. Duffield and Mr R. Lidgard for the mass spectra, Miss K. Alexander for help with the chromatography and Mr G. Parossean of Ku-Ring-Gai Council for his help in obtaining turpentine resin. We are very grateful to Dr Ann Ashford for the photomicrography.

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