Chapter 16
The Chemistry of Beeswax

Abstract Publications on the physical constants for the comb waxes of Asian and European beeswaxes first appeared a century ago. It was soon shown that carbon chain length was, on average, shorter in the Asian beeswaxes than in *A. mellifera,* which explains the lower melting points of the former. The Asian waxes are more similar to one another than to *A. mellifera.* In Asian beeswaxes, the amounts of C_{31} and C_{33} in the pool of free fatty acids are reduced, but C_{25} hydrocarbons are increased compared to that of *A. mellifera.* The major compound families in beeswax are alkanes, alkenes, free fatty acids, monoesters, diesters and hydroxymonoesters, while fatty alcohols and hydroxydiesters are minor constituents. There are notable species-specific differences in the beeswaxes among honeybee species, but all share a complex mixture of homologous neutral lipids. The amounts of acylglycerols are the same in scale and comb wax, but diacylglycerols dominate the former and monoacylglycerols the latter. There are more double-bonded fatty acids in comb than in scale wax, and a greater saturation of fatty acids in comb wax. Beeswaxes analysed with high temperature gas chromatography yielded a characteristic elution pattern for waxes of each honeybee species. A parsimonious, unweighted, pair-group analysis based on the distribution of the chemical constituents for 82 elution peaks of the derivatized comb waxes of six species of honeybees. The Euclidean distances of the beeswaxes present a picture very similar to those obtained from morphometric, behavioural and DNA sequence analyses. The wax glands and the products of their secretions were highly conserved features during honeybee evolution.

16.1 Introduction

In this chapter, discussions on the chemistry of beeswax are restricted entirely to honeybee wax scales and comb wax in a biological context. Investigations of both the chemical composition and physical properties of beeswaxes of *A. mellifera* have been pursued for centuries, and these earlier works have been documented by Grün and Halden (1929). Preparations for and practical uses of beeswax have also

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Table 16.1 Composition of beeswax derived from *A. mellifera* combs (Tulloch 1980)[a]

Constituent fractions	Number of components in fractions		
	Percentage %	Major	Minor
Hydrocarbons	14	10	66
Monoesters	35	10	10
Diesters	14	6	24
Triesters	3	5	20
Hydroxy monoesters	4	6	20
Hydroxy polyesters	8	5	20
Acid esters	1	7	20
Acid polyesters	2	5	20
Free acids	12	8	10
Free alcohols	1	5	?
Unidentified	6	7	?
Total	100	74	210

[a] Major components are those forming more than 1 % of the fraction; for minor components only estimates are given (Tulloch 1980)

been documented (Cowan 1908; Coggshall and Morse 1995), and the commercial industrial aspects of beeswax have been exhaustively monographed (Büll 1977); thousands of publications have appeared on these topics since then. However, the very first studies of Asian beeswaxes appeared only a century ago (Hooper 1904; Bellier 1906; Büchner 1906; Hooper and Büchner 1906; Ueno 1915; Roberts and Islip 1922; Ikuta 1931, 1934), who between them recorded the physical constants (specific gravity, melting point, acid and saponification values, etc.) of the comb waxes of *A. cerana, A. dorsata, A. florea* and *A. mellifera.*

As our knowledge of the hydrocarbon, alcohol and acid fractions of beeswaxes developed, two points of importance to honeybee biology emerged. Firstly, Phadke (1961) re-examined the physical constants of *A. cerana, A. dorsata, A. florea* and *A. mellifera* beeswaxes, and showed each to be extremely homogenous as evidenced by the very small standard deviations in the physical values of the samples measured. Shortly after, Narayana (1970) and Phadke et al. (1971) determined that carbon chain length was, on average, shorter in the three Asian beeswaxes than in *A. mellifera,* which accounts for the lower melting points of the Asian waxes. Progress in wax chemistry advanced with gradually improved analytical techniques of both thin-layer and gas-liquid methods of chromatography in the 1940 and 1950s (Touchstone 1993).

16.2 Chemical Composition

The composition and origin of *A. mellifera* comb beeswax has relatively recently been summarised by Tulloch (1980), and is shown in Table 16.1. The major components are defined as those exceeding more than 5 % of each fraction; those of lesser abundance are regarded as minor constituents. Tulloch regarded, as major

components, those which constituted more than 1 % of each fraction; those of lesser abundance were regarded as minor constituents. Nevertheless, if a particular fraction is itself small, then a given compound may well be 'major' in that fraction, but very minor with respect to the bulk composition of a beeswax sample. Tulloch (1980) regarded the large number of minor hydrocarbons as probably disproportionate, because of the relative ease with which they can be separated, vis-à-vis the seven groups of esters. The residue of some 44 % of beeswax is taken up entirely by minor constituents, to which Tulloch ascribed the relatively low melting point of intact beeswax and its plasticity.

By combining both gasliquid and thin-layer methods of chromatography Tulloch (1973, 1974, 1975, 1980) also studied the composition of waxes from different honeybee species. He found that the waxes from different *A. mellifera* races were very similar as a group, but the unsaturated C_{31} hydrocarbon peak was smaller and the C_{35} hydrocarbon peak larger in the African bee, *A. m. scutellata,* than in the European races of *A. mellifera*. By contrast, he reported that waxes of the Asian bees, *A. cerana, A. dorsata* and *A. florea*, resemble each other more closely than any of them do to *A. mellifera* waxes as previously reported by Narayana (1970) and Phadke et al. (1971). In the Asian waxes there is a smaller pool of free fatty acids (analysed as methyl esters), reduced amounts of C_{31} and C_{33}, but increased C_{25} hydrocarbons compared to *A. mellifera* waxes. The recordings from the gas-liquid chromatography analyses by Tulloch are shown in Fig. 16.1.

Despite the assiduous efforts of numerous chemists who have sought to analyse the composition of beeswax, we have very few observations on the chemistry of newly secreted wax scales. Huber (1814) investigated the solubility properties of wax scales and of fragments of newly fashioned white comb wax. He observed that the wax scales readily dissolved in turpentine (presumably comprising then, as now, a pot-pourri of terpenes, but mainly the monoterpenes $\acute{\alpha}$- and β-pinene), but that comb wax left a white residue. When scale and comb wax samples of equivalent weight were placed in vessels of sulphuric ether (probably diethyl ether), the former became opaque but did not dissolve, while the latter dissolved leaving a white residue in the vessel.

When Huber allowed the ether to evaporate from the vessels, he always obtained a recoverable layer of scale wax residue, which led him to conclude that if the scales were indeed crude wax, then the bees must impregnate them with some additional substance to obtain the whiteness and ductility of newly constructed comb wax. To this we can add the observations of Young (1963), who analysed wax scales for the presence of (2-^{14}C)-acetate that had been injected into wax-producing bees. He found that the label was incorporated in the free acid and ester fractions of wax scales. Finally, Lambremont and Wykle (1979) performed a thin-layer chromatographic separation of scale wax and found the resulting chromatographic pattern similar to that obtained by Tulloch (1970) from cappings wax, with the exception that their chromatograms lacked activity at the diester position.

Subsequently, Davidson and Hepburn (1986) compared the glycerols of scale and comb wax. Their assays showed that the monoacylglycerol and diacylglycerol

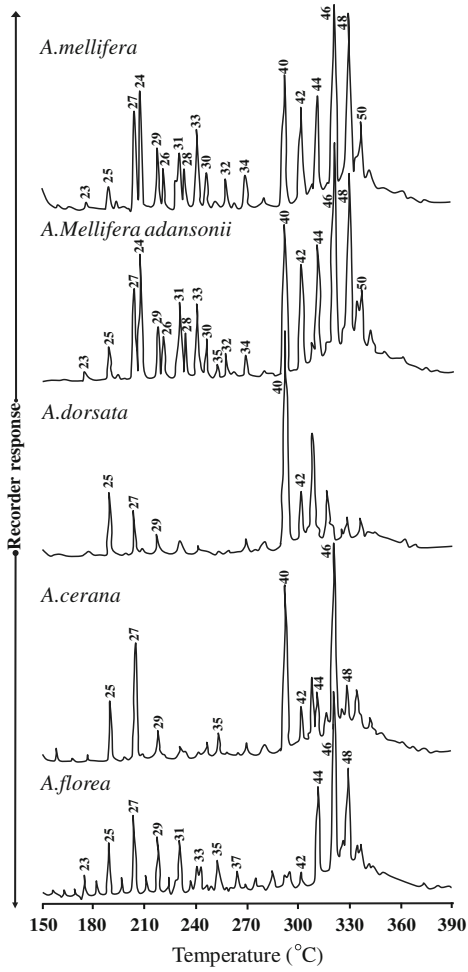

Fig. 16.1 The spectra obtained from gas–liquid chromatographic analyses of *A. mellifera, A. m. scutellata* (= *adansonii*), *A. dorsata, A. cerana* and *A. florea* comb waxes. Hydrocarbons are indicated by odd-numbered peaks (23–35), free acids by even-numbered peaks (24–34) and monoesters by even numbers (40–50) (Tulloch 1980)

fractions comprised about 91 % of the total glycerol in scale and comb wax. While the total level of the acylglycerols were the same in scale and comb wax, the diacylglycerols dominated the scale wax glycerol pool, and the monoacylglycerols the comb wax glycerols. Within the acylglycerol fractions there were substantially more double-bonded fatty acids in scale than in comb wax. Although about 50 % of the fatty acid fractions were the same in the two waxes, there was a significantly greater degree of saturation in the fatty acids of comb wax.

In the absence of hard analytical knowledge as to the total composition of beeswax scales vis-à-vis that of newly built comb, a rather circuitous route must be taken to assess the possible differences among European, African and 'africanized' (*A. m. scutellata*) subspecies. Of equal importance, what exactly is it that a honeybee worker does when she chews scales in the process of comb construction? To this end Eckert (1922, 1927) repeated the basic experiment of Dumas and Edwards

(1843) to assess the effects of cane sugar versus honey on the composition of wax. He compared the fresh, white wax of newly constructed combs built by bees fed sugar, with the yellowish wax produced by a colony given nectar and honey, and found no differences between them. The dimension of age was added to composition studies by Jordan et al. (1940), who compared old comb wax, wax newly secreted by young bees and new wax produced by bees of more than a month old. Replicate and parallel measurements were made on cleaned combs, but no significant differences were found between the waxes of young and old bees. These two waxes did, however, differ from old comb wax in that the latter had an iodine number twice that of the former. This they attributed to a greater contamination of the old wax by carotenoids derived from pollen.

16.3 Chemometrics

Titschack (1969) analysed and tabulated the acid, saponification and ester values for *A. mellifera* African waxes, ranging in origin from Morocco and Ethiopia through the Ivory Coast and south to Mozambique. Because these data were sorted by countries, individual results cannot confidently be ascribed to any particular honeybee subspecies (Hepburn and Radloff 1998). Nonetheless, there were statistically significant differences in composition between several African waxes from different sources, pointing to possible genetic differences among the races. This approach was extended by Tulloch (1980) who showed that the waxes of Asian honeybees were chemically different from those of *A. mellifera*, and that the African and European subspecific profiles of *A. mellifera* waxes also differed.

With the development of high resolution capillary gas chromatography, this work has been extended, particularly by Brand-Garnys and Sprenger (1988). They characterised the waxes of different *A. mellifera* races on the basis of unique hydrocarbon and ester profiles, and recognised 16 subspecific waxes, ten of which are of African geographical origin (Table 16.2). Unfortunately no information is given as to the origin of these waxes, or of variations between the samples, so these data elude chemotaxonomic analysis. Recently, Beverly et al. (1995) showed that the pyrolysis-mass spectral peaks obtained from European and African beeswaxes differed in their relative intensities, but no unique molecules peculiar to any specific wax were obtained. Nonetheless, this approach might be a useful line of further inquiry.

With even more sophisticated gas-chromatographic methods than previously available Aichholz and Lorbeer (1999) and Aichholz et al. (2000) re-examined the comb waxes of the Asian honeybees, *A. andreniformis, A. cerana, A. dorsata, A. florea* and *A. laboriosa* as well as *A. mellifera,* and showed that they are complex mixtures of homologous neutral lipids containing a range of 20–64 carbon length molecules. Aichholz et al. (2000) investigated beeswaxes with high temperature gas chromatography and obtained a characteristic elution pattern for the waxes of each honeybee species, confirming and extending the earlier analyses of Tulloch (1980) and Brand-Garnys and Sprenger (1988).

Table 16.2 Wax characteristics of different *A. mellifera* races (Brand-Garnys and Sprenger 1988)[a]

Races	R1	R2	R3	R4	R5	R6	Type
adansonii	0.181	0.267	0.079	1.314	0.76	1.238	II
anatolica	0.261	0.341	0.019	0.908	0.721	0.905	III
capensis	0.257	0.222	0.055	1.121	1.095	1.54	III
carnica	0.184	0.351	0.017	0.921	0.678	0.937	II
caucasica	0.237	0.274	0.003	1.178	0.725	0.914	III
iberica	0.26	0.155	0.01	1.401	0.706	1.012	II
intermissa	0.213	0.285	0.076	0.958	0.768	1.163	II
jemenitica	0.235	0.328	0.027	0.883	0.893	0.846	I
lamarckii	0.215	0.262	0.168	0.952	0.943	1.329	IV
ligustica	0.264	0.257	0.015	1.124	0.685	0.975	II
litorea	0.261	0.212	0.048	1.324	0.748	1.281	II
mellifera	0.323	0.167	0.009	1.282	0.785	0.981	III
monticola	0.269	0.212	0.052	1.082	1.001	1.438	II
nubica	0.218	0.255	0.087	1.19	0.829	1.256	II
scutellata	0.228	0.247	0.063	1.13	0.891	1.358	II
unicolor	0.211	0.254	0.101	1.191	0.689	1.1	II

[a] R1 is defined as the quotient of the quantity of hydrocarbons and 27 carbon atoms out of the total hydrocarbon pool and so on. Types are defined as the sequence of the absolute quantity of straight chain esters 40, 42 and 44 carbon atoms

In another analysis of beeswaxes Puleo (1991) published gas chromatograms of the comb waxes of African *A. m. scutellata* and European *A. m. ligustica* honeybees, and demonstrated striking differences in both their hydrocarbon and straight chain monoester fractions. In the former, the percentage of C_{33}:1 unsaturated hydrocarbon is greater than the concentrations of C_{29} and C_{31} saturated hydrocarbons, while the converse occurs in the latter subspecies. Also, the percentage of C35:1 unsaturated hydrocarbon is ten times greater in *A. m. scutellata* ($\sim$1.2) than in *A. m. ligustica* ($\sim$0.2). Likewise, there is a lower percentage concentration of C_{48} relative to the C_{46} esters in *A. m. scutellata* than in *A. m. ligustica* (Puleo 1991). He also reported that there are also minor components associated with the hydrocarbon fraction, in that the even-numbered, straight chain hydrocarbons vary in length from C_{22} to C_{34} and may constitute 0.02–0.2 % of the total.

Following Tulloch (1980), Aichholz et al. (2000) defined the major compound families as those exceeding 5 % of the total, so that alkanes, alkenes, free fatty acids, monoesters, diesters and hydroxymonoesters are the major compound families, while fatty alcohols and hydroxydiesters are minor constituents (Table 16.3). There are notable species-specific differences in the waxes among honeybee species (Table 16.3), but all share a complex mixture of homologous neutral lipids: C_{25}–C_{29} alkanes, C_{40}–C_{54} monoesters, C_{42}–C_{52} hydroxymonoesters, and C_{56}–C_{58} diesters (Aichholz and Lorbeer 1999; Aichholz et al. 2000). Presently our knowledge of the composition of the waxes of all honeybee species is nearly equal; however, pathways of synthesis remain available only for *A. mellifera* (Hepburn et al. 1991). Given what is known of species-specific composition

Table 16.3 The major compound families of *A. andreniformis*, *A. florea*, *A. cerana*, *A. mellifera*, *A. dorsata* and *A. laboriosa* comb waxes (Aichholz and Lorbeer 1999)

Compound family	*A. andreniformis*	*A. florea*	*A. cerana*	*A. mellifera*	*A. dorsata*	*A. laboriosa*
Alkanes total	18.5	12.5	11.4	12.8	10.8	10.8
Alkenes total	5.9	7.5	7.4	2.9	0.6	5.3
Diene total	3.4	–	–	–	–	–
Hydrocarbons total	27.8	20	18.8	15.7	11.4	16.1
Fatty acids total	2.6	0.8	3.6	18	4.9	4.3
Fatty alcohols total	–	0.4	1.8	0.6	–	–
Monoesters total	27.5	41.1	33.4	40.8	36.9	37.5
Hydroxymonoesters total	13.6	9.1	18.1	9.2	23.3	23.6
Diesters total	12.9	15.7	12.2	7.4	11.9	8.8
Hydroxydiesters total	3.9	2.3	3	–	1.4	1.1
Esters total	57.9	68.2	66.7	57.4	73.5	71
Total	88.3	89.4	90.9	91.7	89.8	91.4

(Table 16.4), there is considerable opportunity for biochemical studies of beeswaxes in future.

16.3.1 Chemometric Classification of Beeswaxes

For any experimental study into the numerous interactions between pheromones and comb and/or cuticular waxes known to occur (Breed et al. 1995a, b, 1998), it is essential to know the chemical composition of the waxes involved and to be able to classify them. The chemical compositions of comb and cuticular waxes of honeybees have been extensively investigated (Blomquist and Ries 1979; Blomquist et al. 1980; Lockey 1985; Hepburn 1986; Francis et al. 1989), but with different methods. In a seminal paper, Frölich et al. (2000) established objective and quantitative chemometric tools for distinguishing between comb waxes of different ages and the cuticular waxes from different castes and sexes of *A. m. carnica*. Previously there had been no studies on chemical composition of different age classes of comb waxes using quantitative classification tools.

When Frölich et al. (2000) analyzed their fractions by gas chromatography, 56–75 % of the total mass of the wax samples could be identified (Table 16.5). All comb waxes of different age classes were dominated by long-chain aliphatic compounds, with chain lengths ranging in length from C_{21} to C_{54} (Fig. 16.2). The chain lengths exhibited a bimodal distribution, and there were no differences in chain length distributions among wax scales, new, middle-aged, and old comb waxes respectively. The respective medians for the shorter and longer chain length distributions were also fairly close. Chain lengths were in the range of C_{42} to C_{44} for all comb wax classes (Fig. 16.2). These data are consistent with those of other studies on *A. mellifera* (Basson and Reynhardt 1988), as well as waxes of the Asian honeybee species (Narayana 1970; Phadke et al. 1971).

Table 16.4 Comparison of the compound composition of derivatised comb waxes of *A. mellifera, A. cerana*, *A. florea, A. andreniformis, A. dorsata* and *A. laboriosa* by GC–FID analysis on a SOP-50-PFD column (modified from Aichholz and Lorbeer 1999)

Structure	Peak	*Apis mellifera*	*Apis cerana*	*Apis florea*	*Apis andreniformis*	*Apis dorsata*	*Apis laboriosa*
Alkane C23	1	0.4	0	0	1.1	0.4	0.3
Alkane C25	3	1.5	0.9	1.5	7	4.3	3.8
Alkane C27	10	6.2	8.2	6.3	4.9	3.6	3.6
Alkane C29	17	2.6	2.3	3	2.8	1.2	1.7
Alkane C31	22	1.5	0	1.2	1.8	0.9	1
Alkane C33	26	0.3	0	0.5	0.5	0.4	0.4
Alkane C35	30	0.3	0	0	0.4	0	0
Alkene C27	8	0	0	0.6	0.5	0	0
Alkene C29	16	0	0.6	1	1	0	0
Alkene C31	21	0.8	0	2.3	0	0	0.3
Alkene C33	25	2.1	0.4	3	0	0.6	1.9
Alkene C35	29	0	5.4	0.6	1	0	1.7
Alkene C37	34	0	1	0	1.4	0	0.8
Alkene C39	38	0	0	0	1.3	0	0.6
Alkene C41	41	0	0	0	0.7	0	0
Fatty acid C20	13	1.1	0	0	0.8	0.8	0
Fatty acid C22	19	0.7	0	0	0	0.3	0.4
Fatty acid C24	24	6	0	0	0	1.4	0.7
Fatty acid C26	27	2.1	0.5	0	0	0	0
Fatty acid C28	31	2.6	1.2	0.4	0.5	0	0
Fatty acid C30	35	2.1	1.9	0.4	0.4	0	0
Fatty acid C32	39	1.6	0	0	0.2	0.3	0.6
Fatty acid C34	43	1.5	0	0	0.3	1.4	1.8
Fatty acid C36	46	0.3	0	0	0.4	0.7	0.8
Fatty alcohol C33	32	0.3	1.8	0.4	0	0	0
Fatty alcohol C35	36	0.3	0	0	0	0	0
Diene C35	28	0	0	0	0.4	0	0
Diene C37	33	0	0	0	0.9	0	0
Diene C39	37	0	0	0	1.1	0	0
Diene C41	40	0	0	0	1	0	0
Diester C54	67	0	0	0	0	1	0.6
Diester C54	68	1.2	0	0.7	0.7	5.6	4.1
Diester C56	69	0	0	0	0	1	0.9
Diester C56	70	1.2	0.6	1	1	2.4	2
Diester C58	72	0	0	0.8	0.6	0.5	0.3
Diester C58	73	1.4	2.3	5.2	4.2	1	0.9
Diester C60	75	0	1.1	1.1	0.9	0	0
Diester C60	76	2	5.3	4.2	3.4	0.4	0
Diester C62	78	0	0.7	0.7	0	0	0
Diester C62	79	1.2	1.6	1.7	1.6	0	0
Diester C64	81	0.4	0.6	0.3	0.5	0	0

(continued)

Table 16.4 (continued)

Structure	Peak	*Apis mellifera*	*Apis cerana*	*Apis florea*	*Apis andreniformis*	*Apis dorsata*	*Apis laboriosa*
Hydroxydiester C50	71	0	0.7	0	0.4	1	0.7
Hydroxydiester C52	74	0	0	0	0.6	0.4	0.4
Hydroxydiester C54	77	0	1	1.1	1.6	0	0
Hydroxydiester C56	80	0	1	0.6	0.9	0	0
Hydroxydiester C58	82	0	0.3	0.6	0.4	0	0
Hydroxymonoester C40	48	0	0	0	0.4	3.3	2.3
Hydroxymonoester C40	49	0.9	0	0	0.4	9.6	8.4
Hydroxymonoester C42	51	0	0	0	0	4	4.5
Hydroxymonoester C42	52	0.8	0.4	0.4	0.8	2.5	2.6
Hydroxymonoester C44	54	0	2.8	0	0	1.3	1.3
Hydroxymonoester C44	55	1.8	0	3.3	4.3	0.5	0.6
Hydroxymonoester C46	57	0.9	9.2	0	0	0.4	0.4
Hydroxymonoester C46	58	2.3	0	2.9	4.7	0.3	0.4
Hydroxymonoester C48	61	0.6	4.4	0	0	0.3	0.5
Hydroxymonoester C48	62	1.6	0	1.5	1.9	0.5	0.9
Hydroxymonoester C50	64	0	0.5	0	0.8	0.3	0.7
Hydroxymonoester C50	65	0.3	0.8	0.7	0	0.3	0.5
Hydroxymonoester C52	66	0	0	0.3	0.3	0	0.5
Monoester C38	42	0	0	0	0	0.5	0.7
Monoester C40	44	6.6	0.7	1.5	1.3	26.8	24.9
Monoester C42	47	4.6	0.9	3.4	1.5	4.7	4.5
Monoester C44	50	5.7	4.8	9.7	7.7	0.7	1
Monoester C46	53	11.9	23.7	17	10.7	0.9	1.6
Monoester C48	56	9	2.2	7.3	4.7	1.7	2.7
Monoester C50	60	2.6	0.6	1.8	1.3	1.2	1.6
Monoester C54	63	0.4	0.5	0.4	0.3	0.4	0.5

Table 16.5 Analytical yields derived from gas chromatographic analyses of *A. m. carnica* scale and comb waxes (Frölich et al. 2000)

Sample type	Relative amounts of masses (%), Means ± 95 % confidence intervals[a]		
	Identified in GC	Unidentified in GC	Polar fraction
Comb waxes			
Wax scales	71 ± 2.2	4.2 ± 2.24	25 ± 1.8
New wax	68 ± 2.1	3.0 ± 2.13	29 ± 6.6
Middle-aged wax	70 ± 1.9	4.6 ± 1.93	26 ± 2.6
Old wax	69 ± 1.5	5.4 ± 1.51	26 ± 4.0
Cuticular wax			
Workers	67 ± 0.7	1.6 ± 0.76	31 ± 5.1
Drones	54 ± 0.7	2.2 ± 0.72	43 ± 6.2
Queens	57 ± 2.8	7.7 ± 2.79	36 ± 9.7

[a] Fractions 1–3 were subjected to gas-chromatographic (GC) analysis. The values given are related to the total mass of fractions 1–4. The limit of detection was 0.01 % and the decimals were set accordingly

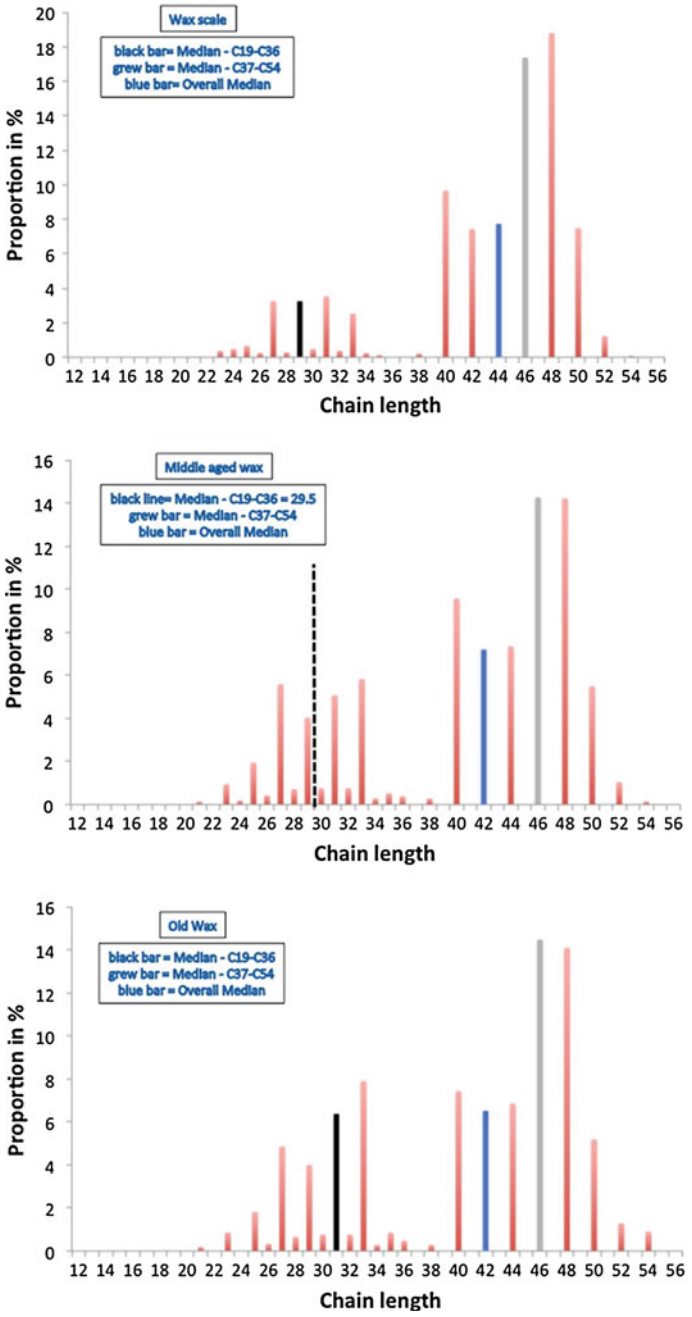

Fig. 16.2 Distribution of chain lengths of *A. m. carnica* comb waxes. $Median_1$ refers to the chains ranging from C_{19} to C_{36}; $Median_2$ refers to the chains ranging from C_{37} to C_{54}; and $Median_{all}$ characterizes the whole range of chain lengths (Frölich et al. 2000)

Table 16.6 Relative chemical composition of *A. m. carnica* comb waxes of different ages (Frölich et al. 2000)

Substance classes	Relative amounts of masses (%), Means ± 95 % confidence intervals[a]			
	Wax scales ($N = 6$)	New wax ($N = 6$)	Middle-age wax ($N = 6$)	Old wax ($N = 6$)
Alkanes	11 ± 4.9	13 ± 1.7	15 ± 1.7	14 ± 1.1
Alkenes	3.4 ± 1.43	6.0 ± 1.04	8.8 ± 0.98	12 ± 1.3
Alkadienes	0.06 ± 0.044	0.24 ± 0.041	0.72 ± 0.077	2 ± 0.21
Branched alkanes	0.00 ± 0.008	0.19 ± 0.117	0.46 ± 0.053	0.95 ± 0.12
Esters	57 ± 6.9	57 ± 3.6	47 ± 4	48 ± 4.3
Unsaturated alkyl esters	13 ± 3.3	11 ± 0.7	12 ± 1.4	9.5 ± 1.54
Hydroxzalkyl esters	8.0 ± 3.08	7.9 ± 5.72	8.1 ± 1.57	6.4 ± 0.98
Acids	1.3 ± 2.00	0.14 ± 0.158	0.51 ± 0.338	0.08 ± 0.10
Alcohols	0.41 ± 0.239	0.53 ± 0.317	0.74 ± 0.128	0.48 ± 0.20
Unidentified	5.6 ± 2.97	4.2 ± 2.99	6.2 ± 2.59	7.3 ± 2.03

[a] The values given related to the total mass of fractions 1–3; limit of detection at 0.01 %, decimals were set accordingly

Table 16.7 Relative chemical composition of *A. m. carnica* hydrocarbon fractions of comb waxes of different ages (Frölich et al. 2000)

Substance classes	Relative amounts of masses (%), Means ± 95 % confidence intervals[a]			
	Wax scales (N = 6)	New Wax (N = 6)	Middle-age wax (N = 6)	Old wax (N = 6)
Alkanes	75 ± 1.2	67 ± 0.9	60 ± 0.3	50 ± 0.6
Alkenes	24 ± 1.1	31 ± 0.7	35 ± 0.2	40 ± 0.5
Alkadienes	0.38 ± 0.071	1.2 ± 0.03	2.9 ± 0.05	7.0 ± 0.09
Branched alkanes	0.05 ± 0.048	1.0 ± 0.31	1.8 ± 0.05	3.3 ± 0.08

[a] The values given related to the total mass of fraction 1; the limit of detection was 0.01 % and the decimals were set accordingly

The chemical compositions of all waxes were dominated by long-chain alkyl esters contributing 47 % ± 4.0 to 57 % ± 6.9 of the total of fractions 1–3 (Table 16.6).

With the increasing age of comb wax, the overall median of the different age classes decreases, but the relative contributions by alkenes, alkadienes and branched alkanes increased from 3.4 % ± 1.43 (alkenes), 0.06 % ± 0.044 (alkadienes) and 0.00 % ± 0.008 (branched alkanes) in wax scales, to 12 % ± 1.3, 2.0 % ± 0.21 and 0.95 % ± 0.129 in old comb wax respectively. These systematic changes of alkene, alkadiene, and branched alkane contents were even more pronounced when the hydrocarbon fraction (fraction 1) alone was analysed. In this case, the contributions of the three substance classes to the total of hydrocarbons increased from 24 % ± 1.1, 0.38 % ± 0.071 and 0.05 % ± 0.048 in wax scales, to 40 % ± 0.5, 7.0 % ± 0.09 and 3.3 % ± 0.08 in old comb wax respectively (Table 16.7).

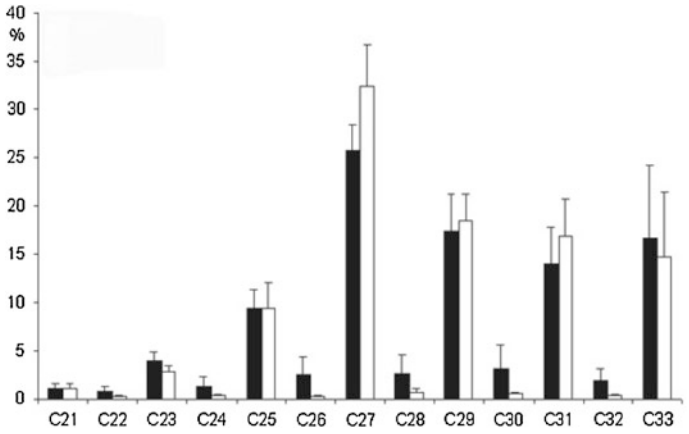

Fig. 16.3 Histogram of the averaged peak areas of the alkanes extracted from *light coloured* (*white columns*) and *dark coloured* (*black columns*) *A. m. ligustica* beeswax samples. The relative peak areas are normalized to the most abundant alkane. Cx refers to *n*-alkane with x carbons in its chain. Y axis = % (from Namdar et al. 2007)

More recently, Namdar et al. (2007) published GC and GC/MS analyses of light and dark coloured *A. m. ligustica* and *A. m. syriaca* combs (Fig. 16.3). They found that, as beeswax ages and darkens, its *n*-alkane composition changes. The amount of even numbered *n*-alkanes (C_{22}–C_{32}), is significantly higher in darker coloured beeswax compared to light beeswax. They attributed these differences, at least in part, to the accumulation of cuticular residues known to contain C_{23} to C_{32} odd and even numbered *n*-alkanes. They determined the presence of odd and even numbered *n*-alkanes, and showed that there was a clear predominance of the C_{27} alkane, with only very small amounts of even numbered *n*-alkanes in the range of C_{22}–C_{32}. Also, darker beeswax contains on average about 3 times more even numbered *n*-alkanes than lighter coloured beeswax.

16.3.2 Discrimination and Classification of Beeswaxes

Before introducing this topic, it is often important to identify and separate pure beeswax from contaminant resins, such as slumgum, which occur in beeswax samples (Grout 1946; Morales-Corts et al. 2010). It was recently reported that waxes and contaminating resins can readily be identified by differential scanning calorimetry (Zhang et al. 2012). Quantitative criteria for the distinction between comb age classes, castes are possible based on chemical features of the respective waxes are both desirable and possible Frölich et al. (2000) subjected their data to a discriminant function analysis which allows the predictive classification of cases (wax samples) by computation of classification functions. These functions are not

to be confused with discriminant functions. Only substance classes that could be positively identified by gas chromatography-mass spectrometry, were included. The results of their analysis functions achieved 99.3 % unambiguous discrimination into the classes: wax scales, new wax, middle aged wax and old wax.

The chemical changes recorded by Frölich et al. (2000) during the ageing process of comb wax, seem to consist of two parallel processes. They proposed that the decrease in chain length with age (process 1), may be due to lipolytic enzymes (Kurstjens et al. 1985; Davidson and Hepburn 1986; Hepburn 1986), which bees add to the wax scales during their conversion into comb wax. These enzymes might be esterases, and this could result in a decrease in long-chain esters and subsequently an increase in shorter chains. The second process (2), may be due to spontaneous physical and chemical processes rather than the direct influence of the bees. The olfactory system of the honeybee is very sensitive to hydrocarbon compounds (Page et al. 1991), the clearly distinguishable wax compositions may be cues for the honeybees to distinguish different regions of the nest for allocating tasks, or to identify nestmate bees they meet in the darkness of the nest (Tautz 2009) (cf. Chap. 5). Phiancharoen et al. (2011) calculated the weighted frequency distributions of the compounds in Table 16.4 to determine the average chain length of each type of wax as shown in Table 16.8. There were no significant differences among the waxes, although there is a trend suggesting that the waxes of the dwarf honeybees have the longest chain lengths. This is surprising because, as a general rule, stiffness, strength, yield stress and other properties increase with increasing carbon chain length in polymers (Salamone 1996), but this relationship does not hold for beeswaxes.

In a further study on wax discrimination Phiancharoen et al. (2011) performed a cluster analysis of beeswax composition, based on the data of Aichholz and Lorbeer (1999) (Table 16.4) to assess their relative affinities, as measured by the Euclidean distances using the unweighted pair-group centroid amalgamation rule. A parsimonious unweighted pair-group analysis based on the distribution of the chemical constituents for 82 elution peaks of the derivatized comb waxes of *A. andreniformis*, *A. cerana*, *A. dorsata, A. florea*, *A. laboriosa* and *A. mellifera* is shown in Fig. 16.4. The giant honeybee group (*A. dorsata* and *A. laboriosa*) is clearly separated from the other species, as are the dwarf species (*A. andreniformis* and *A. florea*), while *A. mellifera* is placed close to its sister-group, *A. cerana*.

The Euclidean distances of beeswaxes presented a very similar picture, which is consistent with the recent analyses of *Apis* species, in which three distinct clusters of sister-groups result from morphometric (Alexander 1991), behavioural (Raffiudin and Crozier 2007) and DNA sequence analyses (Arias and Sheppard 2005): (1) dwarf bees (*A. andreniformis* and *A. florea*); (2) giant honeybees (*A. dorsata* and *A. laboriosa*); and (3) a cluster consisting of the medium-sized bees (*A. cerana*, *A. koschevnikovi*, *A. mellifera*, *A. nigrocincta* and *A. nuluensis*). In any event, the close proximity of the beeswax unweighted pair-groups to those based on DNA and morphometrics, suggests that the wax glands and the products of secretions were highly conserved features during honeybee evolution (Fig. 16.4).

Table 16.8 Weighted frequency distributions for carbon chain length variation in *A. mellifera, A. cerana, A. florea, A. andreniformis*, *A. dorsata* and *A. laboriosa* waxes calculated from the data in Table 16.4 (Phiancharoen et al. 2011)

Structure	*A. mellifera*		*A. cerana*		*A. florea*		*A. andreniformis*		*A. dorsata*		*A. laboriosa*	
	Comp. %	Wt. freq.	Comp. %	Wt. freq.	Comp. %	Wt. freq.	Comp. %	Wt. freq.	Comp. %	Wt. freq.	Comp. %	Wt. freq.
C20	1.1	7.9	0.0	0.0	0.0	0.0	0.8	6.0	0.8	5.9	0.0	0.0
C22	0.7	5.5	0.0	0.0	0.0	0.0	0.0	0.0	0.3	2.4	0.4	3.2
C23	0.4	3.3	0.0	0.0	0.0	0.0	1.1	9.5	0.4	3.4	0.3	2.5
C24	6.0	51.8	0.0	0.0	0.0	0.0	0.0	0.0	1.4	12.3	0.7	6.1
C25	1.5	13.5	0.9	8.2	1.5	13.8	7.0	65.4	4.3	39.5	3.8	34.3
C26	2.1	19.6	0.5	4.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C27	6.2	60.2	8.2	80.4	6.9	68.8	5.4	54.5	3.6	35.7	3.6	35.1
C28	2.6	26.2	1.2	12.2	0.4	4.1	0.5	5.2	0.0	0.0	0.0	0.0
C29	2.6	27.1	2.9	30.5	4.0	42.8	3.8	41.2	1.2	12.8	1.7	17.8
C30	2.1	22.7	1.9	20.7	0.4	4.4	0.4	4.5	0.0	0.0	0.0	0.0
C31	2.3	25.7	0.0	0.0	3.5	40.1	1.8	20.9	0.9	10.3	1.3	14.6
C32	1.6	18.4	0.0	0.0	0.0	0.0	0.2	2.4	0.3	3.5	0.6	6.9
C33	2.7	32.1	2.2	26.4	3.9	47.5	0.5	6.2	1.0	12.1	2.3	27.4
C34	1.5	18.4	0.0	0.0	0.0	0.0	0.3	3.8	1.4	17.5	1.8	22.1
C35	0.6	7.6	5.4	68.6	0.6	7.8	1.8	23.5	0.0	0.0	1.7	21.5
C36	0.3	3.9	0.0	0.0	0.0	0.0	0.4	5.4	0.7	9.3	0.8	10.4
C37	0.0	0.0	1.0	13.4	0.0	0.0	2.3	31.8	0.0	0.0	0.8	10.7
C38	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	7.0	0.7	9.6
C39	0.0	0.0	0.0	0.0	0.0	0.0	2.4	35.0	0.0	0.0	0.6	8.4
C40	7.5	108.0	0.7	10.2	1.5	22.1	2.1	31.4	39.7	583.6	35.6	514.1
C41	0.0	0.0	0.0	0.0	0.0	0.0	1.7	26.0	0.0	0.0	0.0	0.0
C42	5.4	81.6	1.3	19.8	3.8	58.9	2.3	36.1	11.2	172.9	11.6	175.9
C44	7.5	118.8	7.6	121.4	13.0	211.1	12.0	197.3	2.5	40.4	2.9	46.1
C46	15.1	250.0	32.9	549.4	19.9	337.9	15.4	264.7	1.6	27.0	2.4	39.9

(continued)

Table 16.8 (continued)

Structure	*A. mellifera*		*A. cerana*		*A. florea*		*A. andreniformis*		*A. dorsata*		*A. laboriosa*	
	Comp. %	Wt. freq.	Comp. %	Wt. freq.	Comp. %	Wt. freq.	Comp. %	Wt. freq.	Comp. %	Wt. freq.	Comp. %	Wt. freq.
C48	11.2	193.5	6.6	115.0	8.8	155.9	6.6	118.4	2.5	44.1	4.1	71.1
C50	2.9	52.2	2.6	47.2	2.5	46.1	2.5	46.7	2.8	51.4	3.5	63.2
C52	0.0	0.0	0.0	0.0	0.3	5.8	0.9	17.5	0.4	7.6	0.9	16.9
C54	1.6	31.1	1.5	29.4	2.2	43.9	2.6	52.5	7.0	138.9	5.2	101.4
C56	1.2	24.2	1.6	32.5	1.6	33.1	1.9	39.8	3.4	70.0	2.9	58.6
C58	1.4	29.2	2.6	54.7	6.6	141.3	5.2	112.7	1.5	32.0	1.2	25.1
C60	2.0	43.2	6.4	139.4	5.3	117.4	4.3	96.4	0.4	8.8	0.0	0.0
C62	1.2	26.8	2.3	51.8	2.4	54.9	1.6	37.1	0.0	0.0	0.0	0.0
C64	0.4	9.2	0.6	13.9	0.3	7.1	0.5	12.0	0.0	0.0	0.0	0.0
Total	91.7		90.9		89.4		88.3		89.8		91.4	
Mean		39.7		43.9		44.4		42.5		40.9		40.7
SD		55.7		98.4		73.9		58.4		104.8		92.4

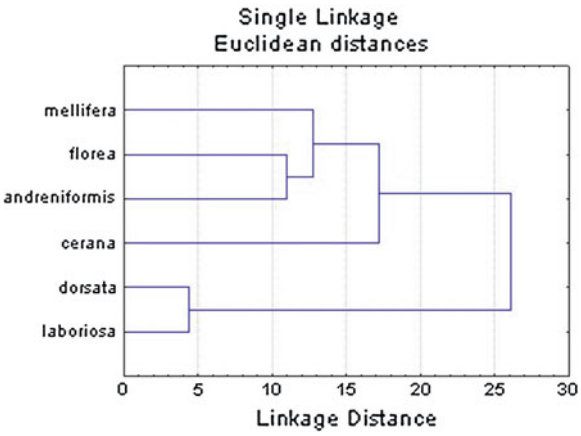

Fig. 16.4 Hierarchical clustering diagram derived from single linkage clustering compound composition of derivatized comb wax (Phiancharoen et al. 2011)

16.4 The Proteins of Beeswax

That beeswax might contain non-lipoidal material has been a very real possibility since Huber (1814) showed that beeswax scales and comb wax have different solubility characteristics. A century later Lineburg (1924) described in detail how worker bees chew and maul wax scales, adding a frothy substance to them. Kurstjens et al. (1985) pursued this probability as a by-product of their studies on the physical changes that occur in the conversion of wax scales into fashioned comb. They found that scale wax did not exhibit a detectable monoglyceride fraction, but had a relatively large pool of diglycerides. In comb wax there was a pronounced monoglyceride fraction, and the diglyceride fraction was considerably less than that in scale wax.

These gross chemical differences between wax scales and finished combs led directly to a search for proteinaceous material that could be added to the wax during chewing, and which might have the expected lytic properties, as had been noted decades earlier by Lineburg (1924). In the search for bee-derived proteins in beeswax, it was essential to preclude any contamination of the scale and comb waxes used in the analyses. Such wax was obtained by keeping small colonies of bees made from newly enclosed brood, confined in a laboratory with no opportunity to forage, nor access to pollen or honey. The bees were only fed a syrupy solution of sucrose. Kurstjens et al. (1985) were able to confirm that scale wax obtained under these conditions contained about 2 μg of protein /mg of wax, and that comb wax contained about 6 μg of protein/mg of wax.

Because beeswax is hydrophobic, it was surmised that it is transported through the pore canals to the exterior surface of the wax mirror by lipophorins. This appears to be the major transport mechanism of hydrophobic natural products in insects (Gilbert and Chino 1974; Haruhito and Chino 1982). Because the lipid composition changes in the conversion of scales into comb wax (Kurstjens et al. 1985), it is also likely that some lipolytic protein is introduced into the scale wax when the bees chew it (Lineburg 1924; Kurstjens et al. 1985). In a series of

Table 16.9 Volatile components of beeswax characterized by gas chromatography-mass spectrometry (Ferber and Nursten 1977)

Hydrocarbons	Alcohols	Carbonyls
p-cymene	*cis*-linalol oxide (5-membered)	Octanal
Durene	*trans*-linalol oxide (5-membered)	Nonanal
Isodurene	*cis*-linalol oxide (6-membered)	Decanal
Decane	*trans*-linalol oxide (6-membered)	
Dodecane	Hotrienol	
Tridecane	ά-terpineol	
Tetradecane	Guaiacol	
Pentadecane	Benzyl alcohol	
Hexadecane	2-phenethyl alcohol	
Naphthalene	Phenol	
ά-methylnaphthalene		
β-methylnaphthalene		

electrophoretic studies on the beeswax proteins of *A. m. capensis* and *A. m. scutellata*, Kurstjens et al. (1990) showed that the substructures of the wax scale and comb protein fractions contained 11 and 13 bands respectively. Seven of these bands were common to both scale and comb waxes for both subspecies.

The proteins ranged between 19 and 100 kD. Bands 1, 2, 6 and 17 (about 97, 89, 66, and 19 kD respectively), were unique to scale wax, while bands 3, 4, 10, 11 and 15 (87, 82, 54, 47 and 43 kD respectively), were unique to comb wax. The waxes shared bands 5, 7–9, 12, 14 and 16 (70, 60, 57, 55, 51, 44 and 29 kD respectively). The densitometric scans showed the relative molecular weight distributions of the bands, and that band 17 is dominant in scale wax, while bands 7–12 are collectively dominant in comb wax. Although wax scales and comb wax contain both unique and shared proteins, their functions are unknown. However, two kinds of lipophorins occur in honeybees (Ryan et al. 1984), and it was surmised that apolipophorin II of honeybees at 78 kD is very close to the 82-kD fraction of comb wax, and to the 70-kD fractions shared by both comb and scale waxes. Although workers chew wax during comb-building, sometimes almost intact scales can be seen in cell walls (Casteel 1912; Zhang et al. 2010), this too points to the addition of a salivary secretion because when incorporated in scale wax, the diacylglycerol component of scales is reduced, and the monoacylglycerol fraction of comb wax increases (Davidson and Hepburn 1986).

16.5 Plant-Derived Aromatic Volatiles and Colourants in Beeswax

Although beeswax has long been a very valuable commodity and its aroma one of its particularly favoured qualities, no analyses of these volatiles were undertaken until the work of Ferber and Nursten (1977). They used a combined GC-MS

Table 16.10 Components of propolis recovered from beeswax (Puleo 1991, and references therein)

1	Citronellol
2	Cinnamic acid
3	Cinnamyl alcohol
4	Coumaric acid, *p*-hydroxycinnamic acid
5	Coumaric acid, *p*-methoxycinnamic acid
6	Cinnamyl-*p*-coumate
7	Vanillin, 4-hydroxy-3-methoxybenzaldehyde
8	Isovanillin, 3-hydroxy-3-methoxybenzaldehyde
9	Caffeic acid, 3,4dihydroxycinnamic acid
10	Ferulic acid, 4-hydroxy-3-methoxycinnamic acid
11	Ferulic acid, 2-hydroxy-4-methoxyacetophenone
12	Ferulic acid, 2-hydroxy-4,6-methoxyacetophenone
13	Pterostilbene, 4-hydroxy-2-4-dimethoxystilbene
14	Pterostilbene, 2′-hydroxy-4′,6′-dimethoxychalcone
15	Pterostilbene, 2′-hydroxy-4-acetyl-5-hydroxy-2-methyl-2H-3H-naptho (1,8-b,c)pyran
16	Pterostilbene, 2′-hydroxy-4,4′6′-trimethoxychalcone
17	Pterostilbene, 2′-hydroxy-3,4,4′-trimethoxychalcone
18	Xanthorrhoeol, 4-acetyl-5-hydroxy-2-methyl-2H-3H-naptho(1,8-b,c)pyran
19	Xanthorrhoeol, 3,5-dimthoxybenzyl alcohol
20	Benzoic acid
21	Benzyl alcohol
22	Sorbic acid, hexa-2,4-dienoic acid
23	Eugenol, 4-aliyl-2-methoxyphenol
24	Lanosterol
25	Squalene
26	Cholesterol
27	Chrysin, 5,7-dihydroxyflavone
28	Techochrysin, 5-hydroxy-7-methoxyflavone
29	Acacetin, 5,7-dihydroxy-4′- methoxyflavone
30	Acacetin, 5-hydroxy-4′,7′- dimethoxyflavone
31	Quercetin, 3,3′4′,5,7-pentahydroxyflavone
32	Kaempferide, 3,5,7-trihydroxy-4′-methoxyflavone
33	Rhamnocitrin, 3,4′,5- trihydroxy-4′,7-methoxyflavone
34	Rhamnocitrin, 3,5-dihydroxy-4′,7-methoxyflavone
35	Galangin, 3,5,7-trihydroxyflavone
36	Isalpinin, 3,5-dihydroxy-7-methoxyflavone
37	Pectolinarigenin, 5,7-dihydroxy-4′,6-methoxyflavone
38	Apigenin, 4′5,7-trihydroxyflavone
39	Kaempferide, 3,4′5,7-tetrahydroxyflavone
40	Flavone, 5-hydroxy-4′7-methoxyflavenone
41	Pinostrobin, 5-hydroxy-7-methoxyflavenone
42	Pinocembrin, 5,7-dihydroxyflavenone
43	Sakuranetin, 4′,5-dihydroxy-7-methoxyflavenone
44	Quercetin-3,3′dimethyl ether, 4′,5′7-trihydroxy-33′-dimethoxyflavone
45	Pinobanksin, 3,5,7-trihydroxyflavenone
46	3-Acetylpinobanksin, 5,7-hihydroxy-3-acetylflavenone

approach, and for positive identification, they used retention indices of ±0.10 for unknowns and standards on each of two columns of differing polarity, as well as acceptable mass spectral data (Table 16.9). In view of the now well-established interactions between pheromones and comb and/or cuticular waxes (Breed et al. 1995a, b, 1998), it is essential to know the chemical composition of the waxes involved, and to be able to classify same. The aromatic volatiles detected in *A. mellifera* wax and listed by Ferber and Nursten (1977) could lead to unimagined possibilities for studies on nestmate recognition.

Subsequently Puleo (1991) performed an exhaustive analysis of the minor constituents of beeswax. Table 16.10 demonstrates the extraordinary diversity of plant-derived compounds (collectively, propolis). Among them is a large percentage of chromophoric (C = C, C = O, N = N, C–NO_2) and auxochromic (C–OH, CNH_2, COOH) groups, which contribute to the strong colour of beeswax. This results from the fact that the auxochromes enhance the colouring capacity of the chromophores (Puleo 1991).

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