

## ON NATURAL WAXES. XXIII.\*

HYDROXY ESTERS AND FREE ALCOHOLS IN THE WAX OF THE HONEYBEE (*Apis mellifera* L.)

K.STRÁNSKÝ, K.ÚBIK and M.STREIBL

*Institute of Organic Chemistry and Biochemistry,  
Czechoslovak Academy of Sciences, Prague 6*

Received January 21st, 1972

Unsaponified virgin beeswax was chromatographed on silica gel to isolate two different groups of hydroxy esters and a group of free primary alcohols. The individual types of substances obtained by saponification of the two groups of hydroxy esters were separated by column chromatography and analyzed by thin-layer chromatography, gas-liquid chromatography, and by IR and mass spectrometry. Homologous series of saturated monocarboxylic acids (predominantly  $C_{16}$ ), monoalcohols ( $C_{24}$ – $C_{34}$ ), monocarboxylic ( $\omega - 1$ ) – and ( $\omega - 2$ )-monohydroxy acids (mostly  $C_{16}$ ) and of (1, $\omega - 1$ )-diols ( $C_{22}$ – $C_{32}$ ) were identified. The free alcohols of beeswax form a homologous series from  $C_{20}$  to  $C_{34}$ . The chemical composition of some minor components is discussed.

The present communication describes the analysis of two further groups of beeswax compounds which were separated by gradient elution chromatography on silica gel<sup>1</sup>. The fraction VII contains hydroxy esters, fraction VIII other hydroxy esters and free primary alcohols. The presence of hydroxy acids or hydroxy esters in the beeswax has been recognized for a long time<sup>2–15</sup>. The most detailed and, due to use of advanced physical methods, the most convincing analysis of the hydroxy esters of beeswax was presented by Tulloch<sup>16,17</sup>. Free alcohols in beeswax are mentioned on the basis of functional group analysis by Findley and Brown<sup>8</sup> but Tulloch<sup>17</sup> did not describe free alcohols in his detailed study.

Fractions IV–VI were not yet analyzed here. According to Tulloch<sup>17</sup>, our fraction IV may contain a group of "triesters".

## EXPERIMENTAL

*Material and isolation of hydroxy esters.* Beeswax, silica gel and all solvents were as described before<sup>1</sup>. Fractions VII and VIII were rechromatographed on silica gel in chloroform–hexane–ether (40 : 10 : 1) and monitored by thin-layer chromatography. A total of 601 mg fraction VII and 945 mg fraction VIII were obtained, representing 6.0 and 9.5% beeswax, respectively.

\* Part XXII: Fette, Seifen, Anstrichmittel 74, 566 (1972).

*Saponification on hydroxy esters and separation.* 300 mg of fraction VII and VIII were saponified with ethanolic KOH as described before<sup>1,18</sup>. After isolation and esterification with diazomethane the mixtures were separated on a column (2 × 65 cm) containing 100 g silica gel (60–125 μm) in chloroform–hexane–ether (13 : 6 : 1). The chromatography was monitored by thin-layer chromatography. From each of the hydroxy ester fractions 9 groups of compounds were obtained and designated A-I (Table I).

TABLE I  
Overall Composition of Hydroxy Esters after Saponification

Group	Fraction VII		Fraction VIII	
	% (w/w)	mean m.w. <sup>a</sup>	% (w/w)	mean m.w. <sup>a</sup>
A	0.2	—	0.5	—
B	5.0	260	6.9	260
C	1.1	—	4.7	—
D	—	—	2.0	—
E	33.9	440	43.1	430
F	36.7	275	13.7	280
G	1.7	—	3.0	—
H	10.8	405	12.9	385
I	10.6	—	13.5	—
Total	100		100	

<sup>a</sup>From gas chromatography.

TABLE II  
Composition (%) of Hydroxy Esters

Number of C atoms	Fraction VIII series A	Number of C atoms	Fraction VII series B	Fraction VIII	
				series A	series B
20	+	32	0.1	5.0	0.3
22	+	34	+	0.9	—
23	+	36	0.2	—	—
24	0.1	38	0.7	—	1.0
25	+	40	6.9	—	15.7
26	0.2	42	10.7	—	11.7
27	0.2	44	20.0	—	16.4
28	0.6	46	36.0	—	27.1
29	0.2	48	22.1	—	15.0
30	3.5	50	3.3	—	1.9
31	0.2				
		<i>Total</i>	<i>100</i>	<i>10.9</i>	<i>89.1</i>

+ Stands for traces.

*Reduction with lithium aluminium hydride.* 4 and 2 mg of compounds of groups C and D of fraction VIII were dissolved in ether and refluxed for 30 min with 30 mg  $\text{LiAlH}_4$ . After acidification with sulfuric acid the reaction products were extracted with ether. Totals of 3.9 and 1.6 mg of mixtures of primary alcohols were obtained, respectively.

*Isolation and identification of monoalcohols.* 100 mg of a mixture of compounds from fraction VIII was heated for 1 h with 1 ml acetyl chloride<sup>19</sup>. After evaporation of the acetyl chloride, the mixture was chromatographed on a column (1 × 35 cm) containing 15 g silica gel (60–125  $\mu\text{m}$ ) in a mixture of hexane and ether (24 : 1). A total of 7.4 mg (6.7%) acetates of the original free alcohols was obtained (the  $R_F$  value on a thin layer of silica gel in 23 : 2 hexane-ether, corresponding to the acetates of synthetic primary alcohols was 0.68). Hydroxy esters which in the free state give a single spot, were separated after acetylation into 3 groups of substances: 79.9 mg (72.3%), 14.2 mg (12.8%) and 9.1 mg (8.2%). The corresponding  $R_F$  values in the above system were 0.47, 0.32 and 0.20. The hydroxy esters of fraction VII were separated after acetylation into groups of the above  $R_F$  values but the group of monoalcohols was completely missing.

The  $R_F$  of free alcohols on a thin layer of silica gel (identical with the  $R_F$  value of synthetic standards) in chloroform-methanol (19 : 1) was 0.76, the  $R_F$  of free hydroxy esters was 0.88. The retention times of free alcohols of beeswax as well as of their acetates obtained from gas chromatography were identical with the retention times of the synthetic standards.

All *IR spectra* were recorded in a UR-10 spectrophotometer in 0.01 cm cuvettes. The concentration of the unsaponified esters (fractions VII and VIII) and of the primary alcohols (group E) in chloroform was 7%, the concentration of the other groups of compounds in tetrachloromethane was 5%.

*Gas chromatography* was done in a Pye series 104 Chromatograph, Model 24, with flame ionization detectors and a dual system of glass columns (0.4 × 150 cm). The original unsaponified esters were chromatographed on 2.5% SE-30 G.C. Grade (General Electric, USA) placed on Gas-Chrom P (100–120 mesh), all the other groups of compounds<sup>20,21</sup> on 3% SE-30 placed on Gas-Chrom Z (100–120 mesh). The quantitative evaluation of the chromatograms was done in such a way as to compare the products of the retention times and of the corresponding wave heights (without using correction factors).

*Mass spectrometry* was done in a AEI 902 apparatus connected with a gas chromatograph (Pye Series 104 Chromatograph, Model 64). The liquid phase was 3% OV-17 on Chromosorb G (100–120 mesh), the flow of helium was 60 ml/min, ion source temperature 250°C, 70 eV. The free alcohols of beeswax were identified in the form of acetates on the basis of the peak at  $m/e$  61 (protonated acetic acid) and of the M-60 peak (formed after elimination of acetic acid from the molecule of acetate).

## RESULTS AND DISCUSSION

While the less polar fractions (I–IV) eluted by gradient elution chromatography of beeswax on silica gel<sup>1</sup> could be distinguished relatively sharply, the mutual separation of compounds of fractions VII and VIII was not so perfect. To be able to analyze each of these separately a new chromatographic separation had to be carried out. In contrast with the hydrocarbons (fraction I), esters 1 (fraction II) and esters 2 (fraction III) analytical chromatography on a thin layer of silica gel (impregnated with silver nitrate) of the individual pure fractions VII and VIII revealed only traces of unsaturated hydroxy esters. For further work, these unsaturated hydroxy esters

TABLE III

Composition (%) of Monocarboxylic Acids (Group B) and Monocarboxylic Monohydroxy Acids (Group F) of Hydroxy Esters

Number of C atoms	Fraction VII		Fraction VIII	
	Group B	Group F	Group B	Group F
12	0.1	—	+	—
14	0.3	+	0.2	0.1
16	87.7	87.6 <sup>b</sup>	91.1	82.6
18	3.4 7.5 <sup>a</sup>	10.0 <sup>c</sup>	3.2 4.9 <sup>a</sup>	12.9
20	0.3	2.4	0.3	4.0
22	0.3	—	0.1	0.4
24	0.4	—	0.2	—
<i>Total</i>	<i>100</i>	<i>100</i>	<i>100</i>	<i>100</i>

+ Stands for traces. <sup>a</sup>Oleic acid. <sup>b</sup>Mixture of the ( $\omega - 1$ )- and ( $\omega - 2$ )-isomers (approximately 1 : 1). <sup>c</sup>Mixture of the ( $\omega - 1$ )- and ( $\omega - 2$ )-isomers.

TABLE IV

Composition (%) of Alcohols (Group E) and Diols (Group H) of Hydroxy Esters

Number of C atoms	Fraction VII			Fractions VIII			
	alcohols B <sub>E</sub>	alcohols C <sub>E</sub>	diols	alcohols A <sub>E</sub>	alcohols B <sub>E</sub>	alcohols C <sub>E</sub>	diols
22	—	—	—	—	—	—	0.7
24	+	2.5	30.8	—	0.2	10.3	65.6
25	—	—	—	—	0.1	—	—
26	0.1	5.5	20.0	0.3	0.3	9.7	17.1
27	—	—	—	—	0.2	—	—
28	0.1	11.7	41.8	0.3	0.3	12.7	15.6
29	—	—	—	—	0.2	—	—
30	0.5	41.4	7.4	0.4	0.7	33.8	1.0
31	—	—	—	—	0.4	—	—
32	0.7	34.9	+	0.7	0.8	25.8	+
33	—	—	—	—	0.4	—	—
34	—	2.6	—	+	—	2.4	—
<i>Total</i>	<i>1.4</i>	<i>98.6</i>	<i>100</i>	<i>1.7</i>	<i>3.6</i>	<i>94.7</i>	<i>100</i>

+ Stands for traces.

were not separated. The IR spectra of the two fractions showed absorption maxima for the free hydroxyl group ( $3610\text{ cm}^{-1}$ ) and for the ester group ( $1782$  and  $1725\text{ cm}^{-1}$ ). High-temperature gas chromatography showed that both fractions contain hydroxy esters (homologous series B) from  $C_{32}$  to  $C_{50}$  with a maximum at  $C_{46}$  (Table II). The chromatogram of fraction VIII, moreover, contained another homologous series of compound from  $C_{20}$  to  $C_{34}$  (series A). Using the  $R_F$  values, elution data of gas chromatography and mass spectra, the compounds were identified as free primary alcohols. According to gas chromatography, fraction VIII contains some 11% of them which corresponds to 1% of beeswax; isolation in the form of acetates yielded only 6.7%, i.e. 0.63% of beeswax. These amounts are in agreement with the assessment of Findley and Brown<sup>8</sup>.

For a detailed study of the individual types of components the two groups of the original hydroxy esters (fractions VII and VIII) were saponified and, after esterification with diazomethane, the mixture was separated on a column of silica gel into 9 groups of compounds (A-I). The principal of these (B, C, D, E, F and H) were identified by IR spectroscopy, mass spectrometry and gas chromatography using synthetic standards. Fraction VII thus yielded a homologous series of methyl esters of normal monocarboxylic acids (B), a series of primary alcohols (E), a series of methyl esters of  $(\omega - 1)$ - and  $(\omega - 2)$ -hydroxy acids (F) and a series of  $(1, \omega - 1)$ -diols (H) (Tables III and IV). All the types of compounds were identified before<sup>18</sup> in saturated as well as unsaturated esters 2 (fraction III), only the series of methyl esters of  $(\omega - 2)$ -hydroxy acids being novel. The position of the OH group was defined by mass spectrometry of the trimethylsilyl derivative on the basis of the base peak at  $m/e$  131

$[\text{CH}_3\text{---CH}_2\text{=}\overset{+}{\text{O}}\text{---Si}(\text{CH}_3)_3]$ . Fraction VIII contained the same groups of compounds, with the exception of the series of  $(\omega - 2)$ -hydroxy acids in group F. The amount of primary alcohols (E) formed by saponification is increased by the free alcohols present in the unsaponified fraction VIII. In contrast with fraction VII it was possible to isolate from fraction VIII minute amounts of compounds of groups C and D. In the IR spectrum they absorb at  $1740\text{ cm}^{-1}$  and heavily at  $1120\text{ cm}^{-1}$ . Reduction with lithium aluminium hydride of the two groups of substances (C and D) yielded practically always a single homologous series of primary alcohols. The gas chromatograms of the original compounds contained always three homologous series of compounds  $A_C, B_C, C_C$  and  $A_D, B_D, C_D$  (Table V). The mass spectra of the corresponding homologues from the two groups of compounds were identical. Their analysis allowed to identify only the homologues of series  $B_C$  and  $B_D$  as acetates of higher alcohols.

The composition of the individual components of hydroxy esters of the present sample of virgin beeswax is in fine agreement with the recently published results<sup>17</sup> and supports also the suggested structural patterns for the naturally occurring hydroxy esters of beeswax.

TABLE V  
Composition (%) of Unidentified Compounds (Groups C and D) and of Primary Alcohols after their Reduction with  $\text{LiAlH}_4$  (Fractions VIII)

Number of C atoms	Group C				Group D			
	series A <sub>C</sub>	series B <sub>C</sub>	series C <sub>C</sub>	alcohols	series A <sub>D</sub>	series B <sub>D</sub>	series C <sub>D</sub>	alcohols
21	0.6	—	0.1	—	0.4	—	—	—
22	—	0.2	—	0.1	—	0.1	—	—
23	0.1	—	0.2	—	0.3	—	0.2	—
24	—	0.3	—	12.7	—	0.3	—	3.8
25	2.4	—	3.7	—	0.5	—	1.6	—
26	—	4.8	—	12.2	—	1.6	0.1	8.1
27	2.3	—	3.8	—	1.4	—	3.5	0.3
28	—	5.2	—	15.1	—	3.5	0.4	14.9
29	2.9	—	4.6	0.4	1.8	—	6.0	0.5
30	—	6.3	—	36.8	—	5.7	0.8	41.1
31	6.6	—	11.3	—	3.5	—	17.0	0.5
32	—	15.5	—	21.9	—	15.7	0.6	28.8
33	4.7	—	8.7	—	3.1	—	13.9	—
34	—	12.6	—	0.8	—	13.7	—	2.0
35	0.6	—	1.0	—	0.6	—	1.6	—
36	—	1.5	—	—	—	2.1	—	—
<i>Total</i>	<i>20.2</i>	<i>46.4</i>	<i>33.4</i>	<i>100</i>	<i>11.6</i>	<i>42.7</i>	<i>45.7</i>	<i>100</i>

The above work has in principle elucidated the chemical composition of the principal components of beeswax but very little is known about the origin of these compounds in the bee body<sup>22-25</sup>. Since the separation and identification methods for analyzing beeswax have now been verified and since the feeding experiments on bees using labelled compounds appear to be relatively simple the way for a thorough analysis of the biosynthesis of beeswax is now open. It is known that for the production of beeswax over a certain limited period, a bee requires the supply of only oxygen, water and monosaccharide<sup>26</sup>.

*We are indebted to Mrs K. Matoušková and Mr P. Formánek for the recording of IR spectra, and to Dr J. Smolíková for their interpretation. The competent technical assistance of Mrs I. Dusová is acknowledged.*

## REFERENCES

1. Stránský K., Streibl M.: This Journal 36, 2267 (1971).
2. Lipp A., Kovács E.: J. Prakt. Chem. 99, 243 (1919).
3. Lipp A., Casimer E.: J. Prakt. Chem. 99, 256 (1919).
4. Ikuta H.: J. Soc. Chem. Ind., Japan 36, Suppl. Binding, 444 (1933); Analyst 59, 161 (1934).
5. Toyama Y., Hirai H.: J. Chem. Soc. Japan, Ind. Chem. Sect. 54, 293 (1951).
6. Toyama Y., Hirai H.: Fette u. Seifen 53, 556 (1951).
7. Toyama Y., Toyama Y.: Research Rept. Nagoya Ind. Sci. Research Inst. No 6, 28 (1953).
8. Findley T. W., Brown J. B.: J. Am. Oil Chem. Soc. 30, 291 (1953).
9. Fuchs W., de Jong A.: Fette, Seifen, Anstrichmittel 56, 218 (1954).
10. Warth A. H.: *The Chemistry and Technology of Waxes*, 2nd Ed., p. 88. Reinhold, New York 1956.
11. Downing D. T., Kranz Z. H., Lamberton J. A., Murray K. E., Redcliffe A. H.: Australian J. Chem. 14, 253 (1961).
12. Mehta T. N., Murthy B. N.: Indian J. Appl. Chem. 24, 158 (1961).
13. Callow R. K.: Bee World 44, 95 (1963).
14. Horn D. H. S., Kranz Z. H., Lamberton J. A.: Australian J. Chem. 17, 464 (1964).
15. Carlier A., Ghaigneau M., Giry L., Puisieux F., Le Hir A.: Compt. Rend. Acad. Sci., Paris, Ser. C. 265, 1240 (1967).
16. Tulloch A. P.: Lipids 5, 247 (1970).
17. Tulloch A. P.: Chem. Phys. Lipids 6, 235 (1971).
18. Stránský K., Streibl M., Kubelka V.: This Journal 36, 2281 (1971).
19. Purdy S. J., Truter E. V.: Proc. Roy. Soc. (London) Ser. B 158, 536 (1963).
20. Streibl M., Konečný K., Stránský K.: Fette, Seifen, Anstrichmittel 72, 777 (1970).
21. Streibl M., Stránský K.: Fette, Seifen, Anstrichmittel 72, 856 (1970).
22. Piek T.: Koninkl. Ned. Akad. Wetenschap., Proc., Ser. C, 64, 648 (1961).
23. Piek T.: *Thesis*. University of Utrecht, Netherlands 1962.
24. Young R. G.: Life Sci. 2, 676 (1963).
25. Piek T.: J. Ins. Physiol. 10, 563 (1964).
26. Svoboda J.: Private communication.

Translated by A. Kotyk.