Flavonoids from *Apis mellifera* Beeswax

F. A. Tomás-Barberán, F. Ferreres, F. Tomás-Lorente
C.E.B.A.S. (CSIC) P.O. Box 4195, Murcia 30080, Spain

and

A. Ortiz
Laboratorio de Mieles, Centro Regional Apícola, SIA, JJ-CC Castilla-La Mancha, Marchamalo 19180, Spain

Z. Naturforsch. **48c**, 68–72 (1993); received November 19, 1992/January 1, 1993

Beeswax, *Apis mellifera*, Flavonoids, Honey, Propolis, Botanical Origin, Characterization

The flavonoids present in beeswax produced in "La Alcarria" region were analyzed by HPLC. Pinocembrin, pinobanksin, pinobanksin 3-acetate, chrysin, galangin and techtochrysin were detected as the main flavonoid constituents. This is the first detailed report on the flavonoids of beeswax. These substances are already present when wax scales are secreted by bees. The same flavonoid compounds were generally present in honey, propolis and *Populus nigra* bud exudates collected in the same geographical region. These results indicate that beeswax flavonoids originate from those of honey and/or propolis, and suggest that analysis of beeswax flavonoids could be used as an adjunct in the detection of beeswax adulterations.

Introduction

Beeswax is obtained by melting and purifying the honeycomb of *Apis mellifera*. This is a true wax, consisting of about 80 per cent of myricyl palmitate (myricin) and a little myricyl stearate. It also contains about 15 per cent of free cerotic acid, cerolein, hydrocarbons, lactones, cholesteryl esters and pollen pigments [1]. To the best of our knowledge no report on the presence of flavonoids in beeswax has been published.

As part of our research programme to study flavonoids from honey and other bee-products in order to use flavonoid analysis in the determination of the geographical and botanical origin of honey [2, 3], we have studied in this work the flavonoids present in beeswax, their natural origin and their relationship with honey flavonoids. To achieve these objectives the flavonoids present in beeswax, virgin wax scales, honey, propolis and poplar bud exudates, collected or produced in the same geographical area were studied.

Reprint requests to Dr. F. A. Tomás-Barberán.

Verlag der Zeitschrift für Naturforschung,
D-W-7400 Tübingen
0939–5075/93/0100–0068 $ 01.30/0

Materials and Methods

Materials

All materials were collected from hives of La Alcarria región (Spain). Beeswax was obtained by normal apicultural procedures, melting the honeycomb with water at 100 °C for 20 min. *Populus nigra* buds were collected during early spring 1991 in La Alcarria. Virgin wax scales were carefully collected by hand from the bees just after their secretion.

Flavonoid extraction

For beeswax and virgin wax scales, 2.57 g of beeswax and 0.36 g of wax scales were dissolved in 50 and 10 ml of *n*-hexane respectively. The hexane solutions were then filtered and extracted with the same volume of 2 N NaOH in a separation funnel. The aqueous layer was then taken to pH 2 with 10 N HCl, and the flavonoids extracted with ethylic ether. This was removed under reduced pressure, and the remaining flavonoids dissolved in 0.5 ml of methanol. 20 µl of these solutions were HPLC analyzed. 50 g of honey were processed for flavonoid extraction as described previously [2]. The flavonoid fraction was dissolved in 0.5 ml methanol and 20 µl of this solution HPLC analyzed. 1 g of propolis was dissolved with 5 ml methanol for 30 min. The extract was filtered and HPLC analyzed. For *Populus nigra* buds, the fresh poplar buds were rinsed in methanol for 5 min to

remove the exudated resins, and this rinse was filtered and HPLC analyzed.

Flavonoid identification

The different flavonoids were identified by their UV spectra recorded with a diode-array detector coupled to the HPLC, and by co-chromatography with authentic markers. Pinocembrin, pinobanksin and pinobanksin 3-acetate, isolated from poplar bud exudates [4], were kindly provided by Prof. Dr. E. Wollenweber. Chrysin (Roth) tectochrysin (Fluka) and galangin (Fluka), were commercial markers.

HPLC analysis of flavonoids

These were analyzed dissolved in methanol and chromatographed on a reversed phase column Licrochart RP-18 (12.5 × 0.5 cm, 5 μm particle size) (Merck, Darmstadt) using a solvents water-5% formic acid (solvent A) and methanol (solvent B), with a flow rate of 1 ml min^{-1} and detection with a diode array detector. Chromatograms were recorded at 280 nm. Elution of flavonoids was performed first with an isocratic elution pumping 30% B until 15 min, then installing a gradient to reach 40% B at 20 min, 45% B at 30 min, 60% B at 50 min, 80% B at 52 min and then became isocratic again until 60 min. Flavonoid quantitation was achieved by the absorbance recorded in the chromograms relative to external standards of chrysin and pinocembrin. Reproducibility of the analyses was ± 5%.

Results

Flavonoids from beeswax

The flavonoids present in beeswax produced in "La Alcarria" hives, were extracted as described in Materials and Methods, and identified by UV spectrophotometry and co-chromatographic comparisons with authentic markers (HPLC). The HPLC flavonoid profile (Fig. 1) showed that seven main flavonoids were present (the other peaks present in the chromatogram were not flavonoids as revealed their UV spectra recorded with a diode array detector). The flavones chrysin (**4**), galangin (**5**) and techtochrysin (**7**), and the flavanones pinobanksin (**1**), pinocembrin (**2**), pinobanksin 3-ace-

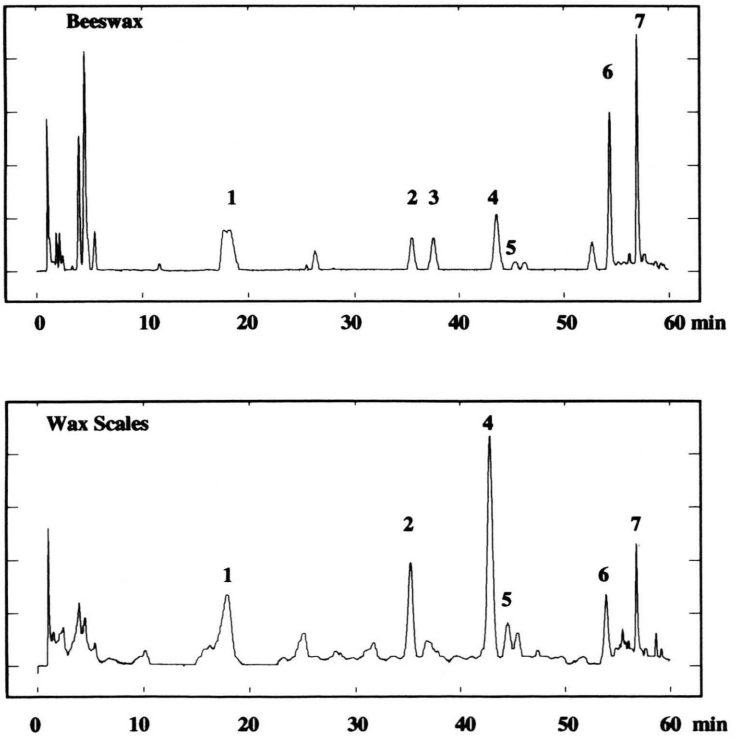

Fig. 1. HPLC chromatograms of beeswax and virgin wax scales flavonoids. Detection at 280 nm. For flavonoid identification see Table I.

Table I. Flavonoids from beeswax, virgin wax scales and honey from "La Alcarria".

No.	Flavonoid	Structure	Beeswax	Wax scales	Honey
1	Pinobanksin	3,5,7-OH-flavanone	n.d.	24.0	4.7
2	Pinocembrin	5,7-OH-flavanone	2.3	14.7	2.9
3	Pinobanksin 3-acetate	3,5,7-OH-flavanone 3-acetate	2.5	–	–
4	Chrysin	5,7-OH-flavone	10.0	88.7	2.3
5	Galangin	3,5,7-OH-flavone	1.2	13.0	1.1
6	Flavanone?	unknown	7.0	9.2	n.d.
7	Techtochrysin	5-OH-7-OMe-flavone	19.6	22.2	0.6
–	Other		n.d.	n.d.	9.1
	Total		42.6	171.8	20.7

Reproducibility was ±5% (determined only for honey and beeswax). **1–3** and **6** quantified as pinocembrin; **4, 5** and **7** quantified as chrysin. (n.d.) not determined; (–) not detected. Values are μg per gram of material.

tate (**3**) and an unknown flavanone (**6**) were detected. A total amount of 42.3 μg of flavonoids per gram of beeswax were found (Table I), the main flavonoids being chrysin (**4**), techtochrysin (**7**) and the unknown flavanone (**6**). It is interesting that all these flavonoids have an unsubstituted ring B, and that they have previously been detected as constituents of propolis and/or honey [2, 5]. This is the first detailed study of flavonoids from beeswax. A question arises after these results. Are these flavonoids natural constituents of the wax secreted by the bee, or otherwise, do these substances contaminate the wax once it has been secreted? To answer to these questions virgin wax scales were collected and analyzed.

Analysis of flavonoids from virgin wax scales

Wax is secreted by worker bees in cells on the ventral surface of the last four segments of their abdomen. Collection of wax scales is a difficult task, since 1 g of beeswax is produced with approximately 1.250 wax scales. The wax scales produced by the bees were carefully collected before contamination with any other material from the hive, and their flavonoids were extracted and HPLC-analyzed (Fig. 1). In this case six out of the seven flavonoids found in beeswax were present. In the scales we failed to detect any pinobanksin 3-acetate (**3**), and, on the contrary, pinobanksin (**1**) was present as a major constituent. Another significant difference between wax scales flavonoids and those from beeswax was that the relative amount of the more lipophilic flavonoids (**6** and **7**) was smaller in the wax scales. The amount of flavonoids is four times larger in wax scales than in the beeswax (Fig. 1 and Table I). These results indicate that flavonoids are already present when the wax scales are secreted by the bees. It is remarkable the absence of pinobanksin 3-acetate in the virgin wax scales, while this is present in beeswax. Pinobanksin, chrysin and galangin decrease in beeswax after secretion whereas pinobanksin 3-acetate increases.

The flavonoids present in beeswax and virgin wax scales have been found to be constituents of the flavonoid fraction of honey and propolis as well [2–6]. This suggests that the flavonoids present in the original wax scales secreted by the bee, could originate either from propolis and/or honey, and that they should be digested by the bee before incorporating them into wax, as indicates the absence of pinobanksin 3-acetate and the significant amount of pinobanksin detected.

Analysis of flavonoids from honey, propolis and poplar bud exudate

Since the flavonoids detected in beeswax had previously been described as constituents of honey and/or propolis [2, 6], we considered essential the analysis of these substances from propolis, honey and the natural source for propolis (*Populus nigra* bud exudates), under the same HPLC conditions, to understand the origin of beeswax flavonoids and their fate in the hive.

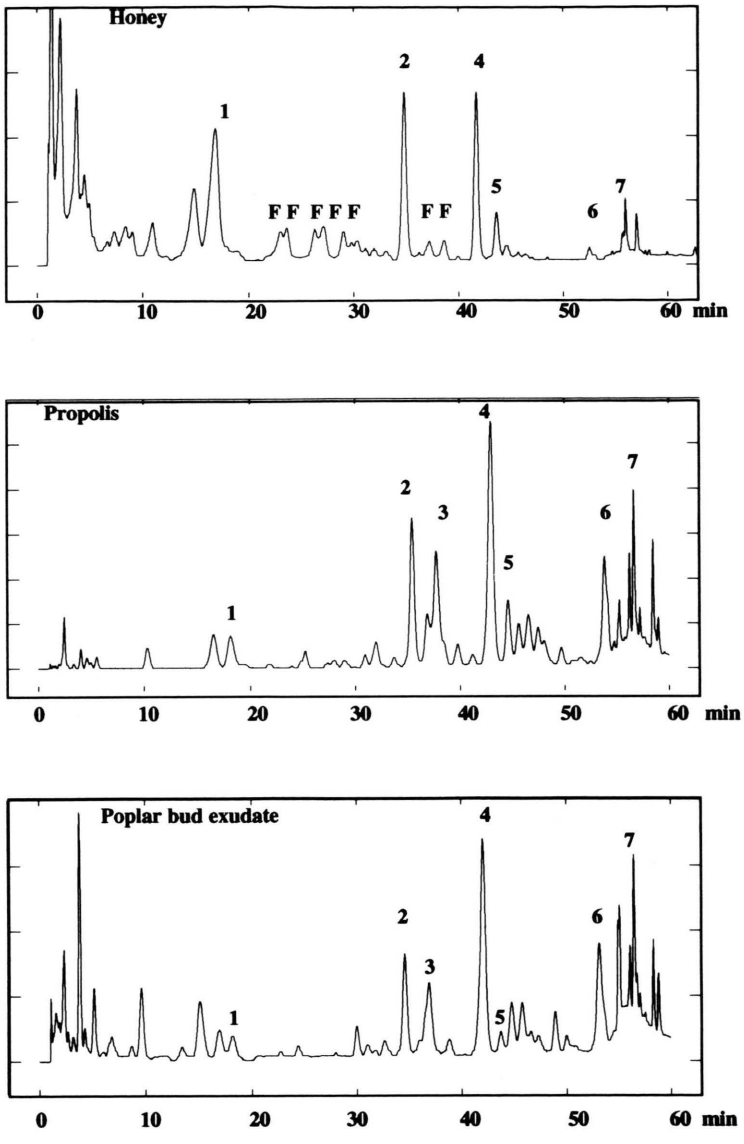

Fig. 2. HPLC chromatograms of honey, propolis and *Populus nigra* bud exudate flavonoids. Detection at 280 nm. For flavonoid identification see Table I.

When honey flavonoids were analyzed six out of the seven flavonoids detected in beeswax were present. Only pinobanksin 3-acetate (**3**) was not found in honey. In addition a number of flavonols and flavones (marked with an F in the chromatogram) which have previously been reported [2] were detected (Fig. 2). The amount of flavonoids present in honey was smaller than that of beeswax (Table I). Propolis has been reported as the main source for honey flavonoids, in geographical areas where propolis is produced from poplars [7–9]. The HPLC profile of the flavonoids present in the propolis produced in "La Alcarria", clearly indicates that the seven flavonoids detected in beeswax and the six of honey were present (Fig. 2). Moreover, when the flavonoids present in the natural source for propolis in this region, *Populus nigra* bud exudates, were HPLC-analyzed, the same profile was found confirming that this was the natural source for propolis and honey flavonoids.

The only difference found between honey and propolis flavonoids was that pinobanksin 3-ace-

tate (**3**), one important constituent of propolis, was not detected in honey.

Discussion

The analysis of the flavonoids present in beeswax, honey and propolis, revealed that all these bee products have very similar flavonoid composition. It has already been demonstrated that the majority of honey flavonoids, and the flavonoids present in propolis, originate from poplar bud exudates collected by bees. In the case of honey, other flavonoid constituents coming from nectar and/or pollen are also present and they constitute nearly 50% of the flavonoid content of the honey sample analyzed in the present study (Table I). These results also show that when wax is secreted by bees it already contains the flavonoids originating from propolis, and therefore these compounds should be ingested by bees either by eating propolis or honey. Honey could easily be the source for these flavonoids since *ca.* 10 kg of honey are consumed by bees to produce 1 kg of wax. In honey some flavonoids are transformed by the bee enzymes. The lack of pinobanksin 3-acetate in honey could be explained by the fact that the bee enzymes (esterases) present in honey, should hydrolyze pinobanksin 3-acetate to render pinobanksin (**1**). This is clear from the chromatograms, since in propolis pinobanksin 3-acetate is a major constituent and pinobanksin is only present in smaller amounts, whereas in honey, pinobanksin 3-acetate is not detected and pinobanksin has become one of the major flavonoid constituents.

Propolis must also be a source for beeswax flavonoids, since the presence of compounds **6** and **7**, which are nearly absent from honey, and present in propolis, are relatively important constituents of beeswax. Once the wax is used to produce the honeycomb, there should be an additional contamination with propolis since pinobanksin 3-acetate is again present in the beeswax obtained from the honeycomb. There must occur an exchange of flavonoids between beeswax and honey, and the more polar flavonoids tend to accumulate in honey while those more lipophilic are mainly located in the beeswax. This is clear from the HPLC chromatogram of honey flavonoids (Fig. 2), were flavonoids **6** and **7** are only present as traces, and from the HPLC analysis of propolis flavonoids, where **6** and **7** are present as significant constituents, but they are not the main components as it happens in beeswax.

The differences in the flavonoids present in wax scales and beeswax, together with the amount of flavonoids which is four times larger in wax scales than in beeswax, indicate that beeswax incorporates some flavonoids after its secretion, whereas some part of the flavonoids are lost and transferred to other bee product (honey) or degraded (oxidized?).

If beeswax flavonoids originate from propolis, and propolis originates from poplars, which only grow native in temperate regions of the northern hemisphere, beeswax produced in hives placed in tropical regions should not contain these flavonoids. It would be very interesting to look at the phenolic compounds present in beeswax from tropical hives to confirm this hypothesis.

The occurrence of these flavonoids in beeswax opens a new possibility to detect adulterations of this bee product.

Acknowledgements

The authors are grateful to the Spanish C.I.C.Y.T. (grants ALI91-0486 and ALI92-0151) and to the Consejería de Agricultura de Castilla-La Mancha for financial support of this work. The authors are indebted to Prof. E. Wollenweber (Darmstadt, F.R.G.) for providing authentic samples of several flavonoids.

[1] G. E. Trease and W. C. Evans, Pharmacognosy, Baillière Tindall, London 1983.
[2] F. Ferreres, F. A. Tomás-Barberán, M. I. Gil, and F. Tomás-Lorente, J. Sci. Food Agric. **56,** 49–56 (1991).
[3] F. Ferreres, A. Ortiz, C. Silva, C. García-Viguera, F. A. Tomás-Barberán, and F. Tomás-Lorente, Z. Lebensm. Unters. Forsch. **194,** 139–143 (1992).
[4] E. Wollenweber, Biochem. Syst. Ecol. **3,** 35–45 (1975).
[5] W. Greenaway, S. English, E. Wollenweber, and F. R. Whatley, J. Chromatogr. **481,** 352–357 (1989).
[6] C. García-Viguera, W. Greenaway, and F. R. Whatley, Z. Naturforsch. **47c,** 634–637 (1992).
[7] W. Greenaway, T. Scaysbrook, and F. R. Whatley, Z. Naturforsch. **43c,** 301–305 (1988).
[8] W. Greenaway, S. English, and F. R. Whatley, Z. Naturforsch. **45c,** 931–936 (1990).
[9] V. Bankova, A. Dyulgerov, S. Popov, L. Evstatieva, L. Kuleva, O. Pureb, and Z. Zamjansan, Apidologie **23,** 79–85 (1992).