Journal of Chromatography, 634 (1993) *41-46*
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 SO6

High-performance liquid chromatography of honey flavonoids

Francisco A. Tomás-Barberán*, Federico Ferreres, M. Amparo Blázquez☆, Cristina Garcia-Viguera and Francisco Tom&Lorente

Laboratorio de Fitoquimica, CEBAS (CSIC), P.O. Box 4195, Murcia 30080 (Spain)

(First received October 20th, 1992; revised manuscript received December 7th, 1992)

ABSTRACT

Twenty flavonoid aglycones from honey were analysed by HPLC on reversed-phase columns. Different solvents were used in order to optimize the detection of those flavonoids which could be considered as markers for the floral origin of honey. None of the solvent systems used allowed the resolution of all the flavonoids from honey included in this analysis. The different solvent systems were then applied to the analysis of tlavonoids from citrus and rosemary honeys. The methanol-water system permitted the separation of hesperetin, the marker of citrus honey, whereas the acetonitrile-water system was the best for the separation of all the flavanones and the detection of apigenin, the marker of rosemary honey. The presence of the flavone techtochrysin was also demonstrated in both honeys. The use of a diode-array detector proved very useful for studies of the floral origin of honey by HPLC tlavonoid analysis.

INTRODUCTION

Reversed-phase HPLC is considered as the method of choice in flavonoid analysis [1–3]. It is a very useful technique in chemosystematic studies of plant species [4,5] and in the characterization of plant-derived foods [6]. In the last few years the possibilities of the application of flavonoid analysis in the determination of the geographical [7], and botanical origins [8,9] of honey have been suggested. However, HPLC of honey flavonoids has rarely been reported. Thus, Bogdanov [10] detected pinocembrin, chrysin and galangin in floral and honeydew honeys and Amiot *et al.* [8] analysed flavonoids and phenolic acids from honeys of different floral origin and detected rhamnetin, kaempferol, naringenin, quercetin and apigenin. In a recent paper the occurrence of sixteen flavonoids in La Alcarria honey was reported [7], but under the HPLC conditions reported in this paper these compounds were poorly resolved [9].

As the HPLC of honey flavonoids is a very promising technique in studies of the botanical and geographical origin of honies, the aim of this work was the optimization of the chromatographic conditions for the analysis of flavonoids from honey, with emphasis on those flavonoids which are markers of the botanical and geographical origins.

EXPERIMENTAL

Materials

The following flavonoids were used to make a standard mixture containing the different flavonoids isolated from honey to date (as the amounts available of some markers were very

* Corresponding author.
☆ Permanent address: Facultad de Farmacia, Universidad de Valencia, Valencia 46010, Spain.

0021-9673/93/$06.00

small, the different flavonoid markers were not weighed to prepare the standard mixture; to provide an idea of the amounts of flavonoids dissolved in the standard mixture we can say that 2 mg of chrysin were dissolved in 10 ml of methanol): the flavanones were eriodictyol (Extrasynthèse, Genay, France), naringenin (Koch-Light, Colnbrook, UK), hesperetin (Schuchart , Munich, Germany), pinocembrin (supplied by Professor Wollenweber, Darmstadt, Germany), pinobanksin (supplied by Professor Wollenweber); the flavones were myricetin (Fluka, Buchs, Switzerland), quercetin (Roth, Karlsruhe, Germany), kaempferol (Fluka), luteolin from *Thymus membranaceus* [11], apigenin from *Thymus membranaceus* [11], chrysin (Roth), galangin (Fluka), techtochrysin (Fluka), 8-methoxykaempferol (obtained after acid hydrolysis of the 3-sophoroside, a pigment present in almond bee pollen) [12], genkwanin from *Satureia obovatu* [13], quercetin 3-methyl ether from propolis and honey [7], kaempferol 3-methyl ether from *Ballota hirsuta* [14], isorhamnetin from *Heliunthemum lavundulaefolium* [15] and quercetin 3,3′-dimethyl ether and quercetin 3,7-dimethyl ether from honey and propolis [7].

Honey samples were supplied by the Centro Regional Apicola de Castilla-La Mancha (rosemary honey, Guadalajara) and Laboratorio Agrario Regional (citrus honey, Murcia) with known floral origin.

Sample preparation

The flavonoids for HPLC analysis were extracted from honey as reported previously [7]. Honey (*ca.* 50 g) was diluted with five parts of water (pH 2-3, adjusted with HCl) until completely fluid and then filtered. The filtrate was passed through a column of Amberlite XAD-2 (Fluka). The column was washed with acidic water (100 ml) and then with neutral distilled water (300 ml). The phenolic fraction was then eluted with methanol (300 ml). This fraction was concentrated under reduced pressure and the flavonoids were further purified by dissolving them in methanol and passing the solution through a Sephadex LH-20 column. The flavonoids were concentrated under reduced pressure, redissolved in methanol (1 ml) and analysed by HPLC.

Chromutographic conditions

The liquid chromatograph consisted of an L-6000 pump (Merck-Hitachi, Darmstadt, Germany), a high-pressure mixing chamber, a sampling valve (Rheodyne), a 20-μl sample loop and an L-3000 (Merck-Hitachi) diode-array UV detector coupled to a computer. A Spherisorb ODS-2 column (Tecnokroma, Barcelona, Spain) (25 x 0.4 cm I.D., 3 μm particle size) and a LiChroCART RP-18 column (Merck, Darmstadt, Germany) (12.5 × 0.5 cm I.D., 5 μm particle size) were used (the solvent conditions are given in the figure captions).

RESULTS AND DISCUSSION

To optimize the HPLC conditions for the analysis of flavonoids from honey, an artificial mixture was prepared containing the sixteen flavonoids previously reported from honey plus four other flavonoids (myricetin, hesperetin, eriodictyol and techtochrysin), that had been detected in propolis and pollen and therefore they could be present in honey. The structures of these flavonoids and their common names and plant origin (propolis, nectar or pollen) are given in Table I.

The study in honey of those flavonoids originating from pollen or nectar gives information on the botanical origin of honey [9]. This is important as the quality of honey generally depends on its floral origin. Thus, some monofloral honeys, such as citrus, rosemary, *Calluna* and white clover honeys are generally more appreciated than the multifloral types. On the other hand, the study of honey flavonoids originating from propolis seems to be more useful in geographical origin determinations [7,9]. For these reasons it is important to discriminate in the chromatograms between the flavonoids originating from pollen-nectar and those from propolis .

In a previous report on honey flavonoid analysis in which we used reversed-phase columns with the solvents methanol and water, the flavanone pinobanksin (4) (from propolis) and

TABLE I

HONEY FLAVONOIDS

Flavonoids	Structure	Origin"	No.
Flavanones			
Eriodictyol	5,7,3′,4′-Tetrahydroxyflavanone	Pollen-nectar	1
Naringenin	5,7,4′-Trihydroxyflavanone	Pollen-nectar	2
Hesperetin	5,7,3′-Trihydroxy-4′-methoxyflavanone	Pollen-nectar	3
Pinobanksin	3,5,7-Trihydroxyflavanone	Propolis	4
Pinocembrin	5,7-Dihydroxyflavanone	Propolis	5
Flavones			
Myricetin	3,5,7,3′,4′,5′-Hexahydroxyflavone	Pollen-nectar	6
Quercetin	3,5,7,3′,4′-Pentahydroxyflavone	Pollen-nectar	7
Kaempferol	3,5,7,4′-Tetrahydroxyflavone	Pollen-nectar	8
8-Methoxykaempferol	3,5,7,4′-Tetrahydroxy-8-methoxyflavone	Pollen-nectar	9
Luteolin	5,7,3′,4′-Tetrahydroxyflavone	Pollen-nectar	10
Apigenin	5,7,4′-Trihydroxyflavone	Pollen-nectar	11
Chrysin	5,7-Dihydroxyflavone	Propolis	12
Galangin	3,5,7-Trihydroxyflavone	Propolis	13
Techtochrysin	5-Hydroxy-7-methoxyflavone	Propolis	14
Genkwanin	5,4′-Dihydroxy-7-methoxyflavone	Propolis	15
Quercetin 3-methyl ether	5,7,3′,4′-Tetrahydroxy-3-methoxyflavone	Propolis	16
Kaempferol 3-methyl ether	5,7,4′-Trihydroxy-3-methoxyflavone	Propolis	17
Isorhamnetin	3,5,7,4′-Tetrahydroxy-3′-methoxyflavone	Pollen-nectar	18
Quercetin 3,3′-dimethyl ether	5,7,4′-Trihydroxy-3,3′-dimethoxyflavone	Propolis	19
Quercetin 3,7-dimethyl ether	5,3′,4′-Trihydroxy-3,7-dimethoxyflavone	Propolis	20

"The main botanical origins of the different flavonoids are given.

the flavone quercetin (7) (from pollen-nectar) eluted in a single peak [9]. The same happened with the flavones luteolin (10) (from pollen–nectar) and quercetin 3-methyl ether (16) (from propolis), and apigenin (11) (from pollen-nectar) and kaempferol 3-methyl ether (17) (from propolis). The first approach to improve the resolution was attempted by using a longer column (25 cm) with a smaller particle size (3 μm). In Fig. 1A, the separation of the flavonoid standards with such a column is shown. Generally, there is a better resolution in the chromatogram obtained with the longer column than that obtained with the shorter column (12.5 cm length and particle size 5 μm) (Fig. 1B). However, some of the unresolved flavonoid pairs still eluted together using this column, although the resolution of several compounds such as 3 and 18 was much better with the longer column. The advantages observed with the longer column with the optimum conditions (new column) disappeared after a few analyses, and then no significant differences were found between the columns. However, in the longer column, naringenin (2) was clearly separated from pinobanksin (4), which co-eluted with quercetin (7), whereas in the shorter column naringenin and pinobanksin eluted together (Fig. 1).

In a second attempt to optimize the conditions, water-acetonitrile mixtures were used. After trying different solvent gradients, the best resolution was obtained with the conditions shown in Fig. 2A for the mixture of standards. Under these conditions, the five flavanones were well separated from flavones, but luteolin (10) and quercetin (7) eluted together, as did kaempferol 3-methyl ether (17) and isorhamnetin (18). However, as 17 and 18 showed clear differences in their UV spectra, the presence of these two compounds can be detected in honey by means of a diode-array detector.

A third attempt to improve the conditions was made by using the Prisma system for isocratic separations involving mixtures of four solvents

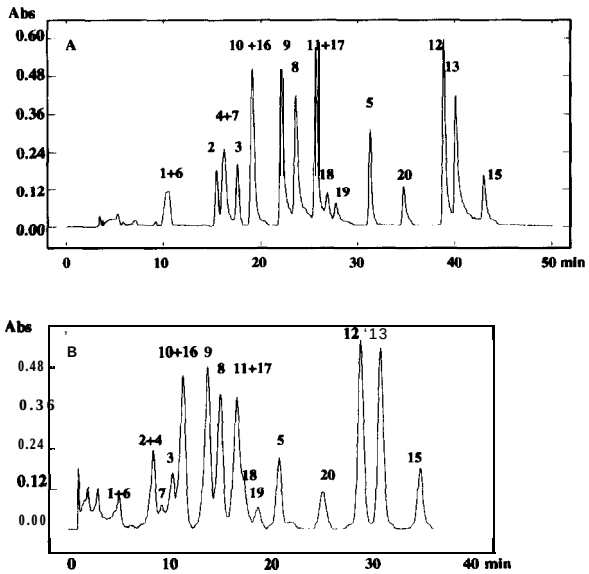

Fig. 1. HPLC of the mixture of flavonoid standards. Solvent system, methanol-water. Detection at 340 nm. The following solvent gradient was used for both columns: solvent a = water-formic acid (95 : 5); solvent b = methanol; the gradient started with 40% b to reach 45% b at 10 min and 60% b at 35 min, then the system remained isocratic until 50 min. (A) Column, Spherisorb ODS-2 (25 × 0.4 cm I.D., 3 μm particle size); solvent flow-rate, 0.7 ml min^{-1}. (B) Column, LiChroCART RP-18 (12.5 × 0.5 cm I.D., 5 μm particle size); solvent flow-rate, 1 ml min^{-1}. For flavonoid identification, see Table I.

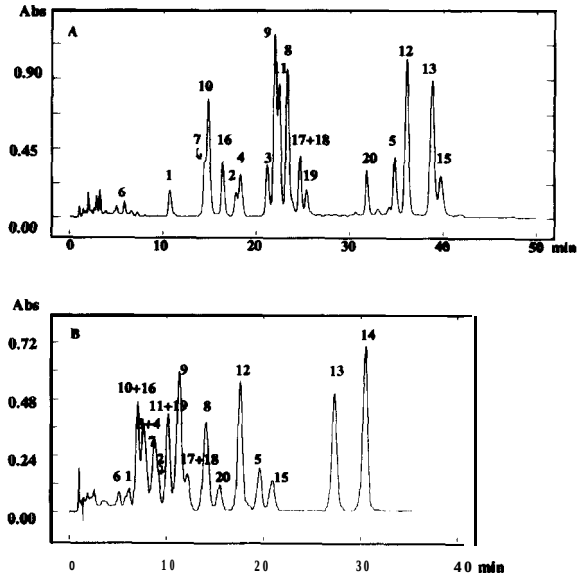

Fig. 2. HPLC of the mixture of flavonoid standards. Solvent systems, acetonitrile-water and Prisma conditions. Column, LiChroCART RP-18 (12.5 x 0.5 cm I.D., 5 μm particle size); detection, 340 nm; solvent flow-rate, 1 ml min^{-1}. (A) Solvent a = water + 5% formic acid; solvent b = acetonitrile. The system was isocratic until 5 min with 20% b. A gradient was then applied to reach 25% b at 15 min and 35% b at 30 min. The system then remained isocratic until 50 min. (B) Prisma conditions: solvent a = methanol-tetrahydrofuran-water containing 5% formic acid (25 : 15 : 60); solvent b = methanol. The system was isocratic with 0% b until 5 min, and a gradient was then applied to reach 10% b at 20 min and 25% b at 30 min. The system then remained isocratic until 40 min.

(water, methanol, acetonitrile and tetrahydrofuran) [16]. This system was applied to the isocratic resolution of the flavonoids in the first half of the chromatogram, and a gradient was then applied to elute the other compounds. The best conditions found were those shown in Fig. 2B, in which the flavonols quercetin (7) and kaempferol (8) were well resolved, but the flavanones pinobanksin (4) (from propolis) and hesperetin (3) (from pollen-nectar) eluted together, in addition to three other flavonoid pairs (Fig. 2B). Under these conditions, the flavone techtochrysin (14) was detected. This is a good system for the detection of naringenin (2) as this flavanone, which eluted as a shoulder on the peak of quercetin (7) at 340 nm, can readily be detected with a diode-array detector. A significant change in the elution order of flavonoids when compared with the elution order with methanol-water or acetonitrile-water mixtures was also observed with the Prisma conditions (Figs. 1 and 2). This means that tetrahydrofuran induces changes in the elution order when compared with methanol or acetonitrile.

HPLC analysis of flavonoids from citrus and rosemary honeys

After optimization of the HPLC solvent conditions for the separation of the standard mixture, this was applied to the flavonoid analysis of two honey samples of different floral origin. Thus, the flavonoids from citrus and rosemary honeys were analysed under different solvent conditions.

Fig. 3 shows the HPLC of the flavonoids from both honey samples chromatographed using methanol-water mixtures. Some significant differences were observed. The flavanones eriodictyol (1) and hesperetin (3) are only detected in

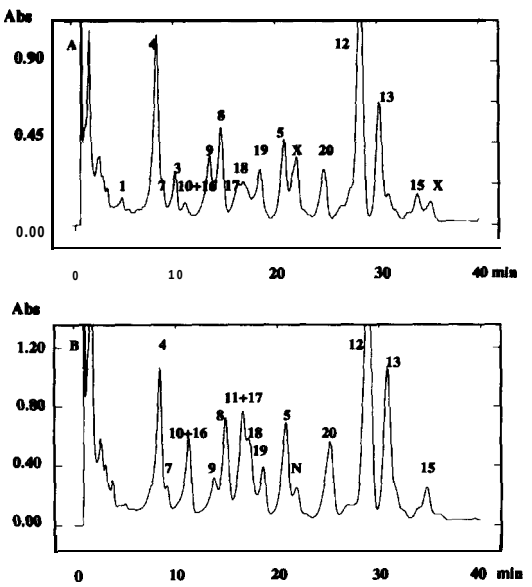

Fig. 3. HPLC of honey flavonoids. Solvent system methanol-water (see Fig. 1). (A) Citrus honey; (B) rosemary honey. X = not flavonoid; N = unidentified flavonoid. Detection at 340 nm.

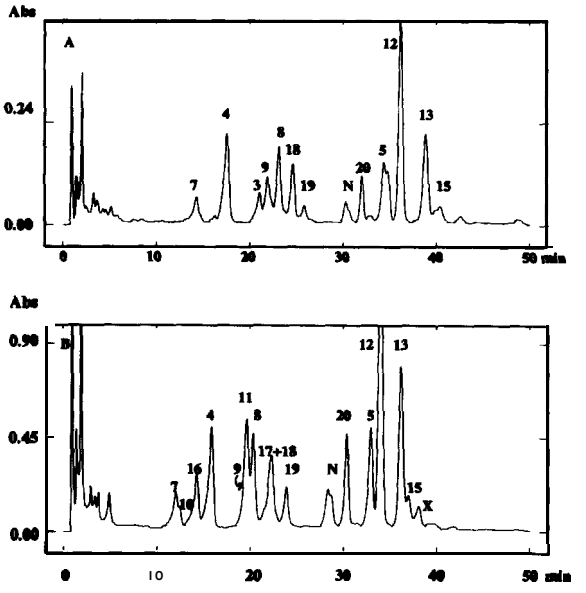

Fig. 4. HPLC of honey flavonoids. Solvent system, acetoni-Me-water (see Fig. 2). (A) Citrus honey; (B) rosemary honey. X = not flavonoid; N = unidentified flavonoid. Detection at 340 nm.

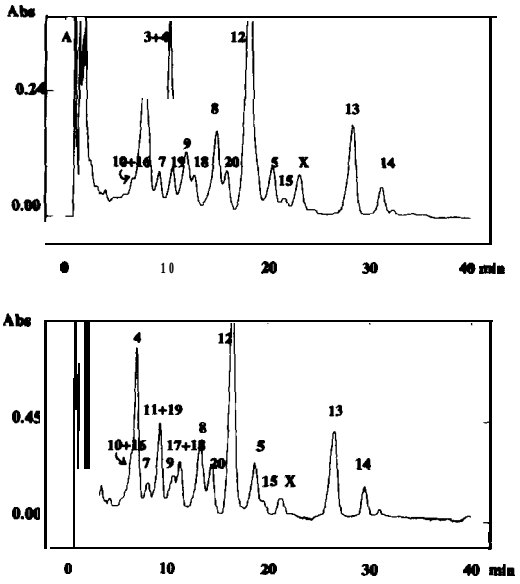

Fig. 5. HPLC of honey flavonoids. Prisma conditions (see Fig. 2). (A) Citrus honey; (B) rosemary honey. X= not flavonoid. Detection at 340 nm.

citrus honey (Fig. 3A), whereas the peaks for luteolin (10) plus quercetin 3-methyl ether (16) and apigenin (11) plus kaempferol 3-methyl ether (17) seem to be much more abundant in rosemary honey (Fig. 3B). The flavonoids coming from propolis are present in similar percentages in both honey chromatograms. As luteolin and apigenin originate from pollen and/or nectar and the 3-methyl ethers of quercetin and kaempferol come from propolis, the discrimination between both compound pairs would be essential for floral origin analysis.

When the flavonoids from both honey samples were analysed using acetonitrile-water (Fig. 4), these two flavonoid pairs (10–16 and 11-17) were resolved, indicating that the amount of luteolin (10) in the rosemary honey is not significant, whereas that of apigenin (11) is important and could be related to its floral origin. In addition, hesperetin (3) is still well separated under these conditions in the citrus honey (Fig. 4A). The presence of naringenin was not confirmed in any honey sample. The diode-array detector showed that the peak 17 + 18 only contained isorhamnetin (18) in the citrus honey.

The analyses under the Prisma optimized conditions of both honey samples showed that this system was less useful than the previous two solvent systems for the differentiation of these

two honeys by flavonoid analysis (Fig. 5). However, quercetin (7) is well separated from other compounds, and the flavone techtochrysin (14) is detected in both honey samples, this being the first time that this flavonoid has been reported in honey. In addition, the flavanone naringenin (2), which had been previously reported from honey samples [8], was not detected in any of these samples. These conditions are not useful for the determination of the floral origin of these honeys by flavonoid analysis, as the flavonoids which for these two samples are markers of the floral origin [hesperetin (3) for citrus honey and apigenin (11) for rosemary honey] are not resolved from the flavonoids originating from propolis [pinobanksin (4) and quercetin 3,3′-dimethyl ether (19)].

CONCLUSIONS

In studies of the floral origin of citrus and rosemary honeys, the flavonoid markers of the botanical origin, hesperetin and apigenin, can be readily analysed by HPLC using as solvents methanol-water or acetonitrile-water mixtures. The Prisma optimized conditions were only useful for the separation of quercetin and kaempferol and for the detection of techtochrysin. Techtochrysin could also be eluted with methanol-water and acetonitrile-water mixtures, increasing the percentage of organic solvent at the end of the elution programmes. The use of diode-array detectors is essential in studies of the floral origin of honey by HPLC analysis of its flavonoids.

ACKNOWLEDGEMENTS

The authors are grateful to the Spanish CICYT (Grant ALI91-0486) and to the Consejeria de Agricultura de Castilla-La Mancha for financial support of this work. The authors are also grateful to Professor E. Wollenweber (Darmstadt , Germany) for providing authentic samples of pinocembrin and pinobanksin. M.A.B is indebted to the Spanish MEC for a fellowship.

REFERENCES

1 D.J. Daigle and E.J. Conkerton, *J. Liq. Chromatogr.*, 11 (1988) 309.
2 F.A.T. Barberán, F. Tomás, L. Hernández and F. Ferreres, *J. Chromatogr.*, *347 (1985) 443.*
3 K. Vande Casteele and C.F. Van Sumere, *J. Chromatogr.*, *240 (1982) 81.*
4 J.B. Harborne and B.L. Turner, *Plant Chemosystematics*, Academic Press, London, 1984, pp. 128-179.
5 A. Lenherr, B. Meier and 0. Sticher, *Planta Med., 50 (1984) 403.*
6 C. Garcia-Viguera, *Thesis,* University of Murcia, Murcia, 1991.
7 F. Ferreres, F.A. Tomás-Barberán, M.I. Gil and F. Tomás-Lorente, *J. Sci. Food Agric.*, 56 (1991) 49.
8 M.J. Amiot, S. Aubert, M. Gonnet and M. Tacchini, *Apidologie, 20 (1989) 115.*
9 F. Ferreres, A. Ortiz, C. Silva, C. Garcia-Viguera, F.A. Tomás-Barberán and F. Tomás-Lorente, Z. *Lebensm.-Unters.-Forsch.*, *194 (1992) 139.*
10 S. Bogdanov, *J. Apic. Res.*, 28 (1989) 55.
11 F. Ferreres, F. Tomás, F.A.T. Barberán and L. Hernández, *Plant Med. Phytother.*, *19 (1985) 89.*
12 F. Ferreres, F.A. Tomás-Barberán, F. Tomás-Lorente, J.L. Nieto, A. Rumbero and J.M. Olías, *Phytochemistry*, *28 (1989)* 1901.
13 F. Ferreres, F.A.T. Barberán and F. Tomás, *Int. J. Crude Drug Res.*, 2.5 (1987) 246.
14 F. Ferreres, F.A. Tomás-Barberán and F. Tomás-Lorente, *J. Nat. Prod., 49 (1986) 554.*
15 F. Ferreres and F. Tomás-Lorente, *Afinidad*, *39 (1982) 152.*
16 S. Nyiredy, B. Meier, C.A.J. Erdelmeier and 0. Sticher, *J. High Resolut. Chromatogr. Chromatogr. Commun., 8 (1985) 186.*