Apidologie (1995) 26, 53-60
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Original article

Flavonoid patterns of French honeys with different floral origin

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(Received 9 August 1994; accepted 16 November 1994)

Summary — The flavonoid profiles of 12 different unifloral French honey samples were analysed by HPLC to evaluate if these substances could be used as markers of the floral origin of honey. In this analysis, the characteristic flavonoids from propolis and/or beeswax (chrysin, galangin, tectochrysin, pinocembrin and pinobanksin) were separated from those originating mainly from nectar and/or pollen (polyhydroxylated flavonoid aglycones), which would be related to their floral origin. All the analysed samples contained a common flavonoid profile consisting of polyhydroxylated flavonoid aglycones including 8-methoxykaempferol, kaempferol, quercetin, isorhamnetin, luteolin and apigenin, suggesting that flavonoid analysis does not generally prove differences between French monofloral honey samples. However, some individual honey samples showed potential floral markers. Thus, heather honey was characterized by the presence of myricetin, calluna honey by ellagic acid and citrus honey by the flavanone hesperetin. In other samples, the relative amount of 1 individual flavonoid could be related to the floral origin. Thus, sunflower honeys contained an important relative amount of quercetin, and in alder honey only 8-methoxykaempferol was detected. This preliminary study shows that flavonoid and phenolic compound analyses could be a very valuable complementary biochemical technique in the objective determination of the floral origin of some specific monofloral honey samples, but further studies with a larger number of samples is necessary to confirm the observed differences.

honey / flavonoid / botanical origin / HPLC

INTRODUCTION

At present, the principal objective means of determining the geographical and floral origin of honey is pollen analysis (Maurizio, 1951; Louveaux *et al*, 1978). However, the technique is tedious and very dependent on expert ability and judgement. Alternative methods that could be more widely and accurately used for characterizing honeys have been sought for many years. Bonaga and Giumanini (1986) have suggested that the next step in this type of research will be to correlate floral source with the pres-

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ence of certain compounds originating either in the nectar or in some biochemical modification of nectar compounds carried by the bee. In fact, aromatic compounds and degraded carotenoid-like substances (Tan *et al*, 1988, 1989), amino acids (Davis, 1975; Bosi and Battaglini, 1978), degradation products of phenylalanine (Speer and Montag, 1987), aromatic aldehydes and heterocycles (Häusler and Montag, 1990) and aromatic acids and their esters (Speer and Montag, 1984; Steeg and Montag, 1988) have all proved quite useful for this purpose.

In the same way, recent studies have revealed that the analysis of flavonoids and other phenolic compounds constitute a very promising technique to study the geographical and floral origin of honey (Amiot *et al,* 1989; Ferreres *et al,* 1992; Ferreres *et al,* 1993; Ferreres *et al,* 1994c). The flavonoids present in nectar and pollen are sometimes characteristic of the species, and can be used for the analysis of the floral origin of honey. The utility of these substances in the determination of the origin of citrus (Ferreres *et al,* 1993; Ferreres *et al,* 1994b), rosemary (Ferreres *et al,* 1992) and heather (Ferreres *et al,* 1994a) honeys have recently been reported.

In the present work, the flavonoid profiles of 12 different unifloral French honeys were analysed by HPLC to evaluate differences in their flavonoid patterns which could be related to their botanical origin. It has been recently shown that honey flavonoids come either from propolis and/or beeswax or from nectar and/or pollen and that they have different structural features depending on their origin (Tomás-Barberán *et al*, 1993; Ferreres *et al*, 1994c). Using Sephadex LH-20 chromatography, it is possible to fractionate honey phenolic compounds, since the flavanones pinobanksin and pinocembrin, the flavones chrysin and tectochrysin, characteristic of propolis/beeswax, and the phenolic acid derivatives elute essentially in a first fraction, while the polyhydroxylated flavonoids originating mainly from nectar/pollen (although some have also been detected in propolis) elute together in a second fraction (Ferreres *et al*, 1994a). For the purposes of this study, only the flavonoids present in the second fraction were collected and analysed by HPLC.

MATERIALS AND METHODS

Honey samples

Honey samples and data on their floral and geographical origin were provided by Dr Morlot (Bernard Michaud, SA, Jurançon) *via* Dr Delgado, from INP-ENSC, Toulouse (France) (Delgado, 1993).

Flavonoid extraction from honey

Flavonoids were extracted from honey as reported previously (Ferreres *et al,* 1994c). Honey (*ca* 200 g) was diluted with 5 parts acid water (pH 2–3, adjusted with HCl) until completely fluid and then filtered through cotton. The filtrate was passed through an Amberlite XAD-2 column (Sigma) and washed with acid water (100 ml) and 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 (Pharmacia). The elution was followed under UV light at 360 nm to separate polyhydroxylated flavonoids, which were more retained in the column, from the flavanones and flavones with an unsubstituted ring B, characteristic of propolis and/or beeswax, which eluted first (Tomás-Barberán *et al*, 1993). The polyhydroxylated flavonoid fraction had a dark-purple colour and was evaporated to dryness under reduced pressure (40°C), redissolved in methanol (0.5 ml), and 20 ml were injected for HPLC analysis (Ferreres *et al,* 1994a).

HPLC analysis of honey flavonoids

HPLC analysis was carried out on a reversed-phase column LiChrochart RP-18 (Merck, Darmstadt) (12.5 x 0.4 cm, 5 μm particle size) using water-formic acid (19:1) (solvent A) and methanol (solvent B) as solvents (Ferreres *et al*, 1994c). Detection was performed with a diode array detector, and chromatograms were recorded at 290 and 340 nm. For the purposes of the present work quantification of the individual flavonoids was not necessary, and the presence of the different flavonoids was evaluated as the percentage of the total absorbance of the whole chromatogram for each individual flavonoid.

Flavonoid identification

The various flavonoids were identified in the chromatograms by cochromatographic comparisons with authentic markers previously isolated from honey, and by their UV spectra recorded with an on-line diode array detector (Ferreres *et al*, 1993; Ferreres *et al*, 1994c).

RESULTS

The flavonoids present in the different honey samples available were extracted and analysed by HPLC and the results are summarized in table I. It is remarkable that 8-methoxykaempferol (P) (3,5,7,4'-tetrahydroxy-8-methoxyflavone) was present in 100% of the samples analysed, and the flavonoids, kaempferol (K) (3,5,7,4'-tetrahydroxyflavone), apigenin (A) (5,7,4'-trihydroxyflavone), isorhamnetin (I) (3,5,7,4'-tetrahydroxy-3'-methoxyflavone), quercetin (Q) (3,5,7,3',4'-pentahydroxyflavone) and luteolin (L) (5,7,3',4'-tetrahydroxyflavone) were detected in the majority of the samples analysed. In spite of the very different floral origin of the honey samples, they show flavonoid patterns composed of only a reduced number of common compounds. On the other hand, some compounds were detected in only 1 unifloral honey type, and could be considered as potential floral mark-

Table I. Flavonoids from the various honey samples analysed.

Honey	*EA*	*M*	*H*	*Q*	*L*	*P*	*K*	*A*	*I*
Heather *(Erica)*	–	32.21	–	7.15	6.18	14.35	7.50	24.01	8.60
Rape *(Brassica)*	–	–	–	11.51	2.82	25.08	42.10	11.76	6.73
Chesnut *(Aesculus)*	–	–	–	–	–	18.59	12.22	43.56	25.63
Calluna *(Calluna)*	21.29	–	–	5.60	8.58	5.92	12.72	31.07	14.83
Sunflower *(Helianthus)*	–	–	–	28.69	7.97	7.88	25.46	16.63	13.37
Rosemary *(Rosmarinus)*	–	–	–	–	–	22.17	27.85	29.17	20.81
Fir *(Abies)*	–	–	–	–	–	50.48	12.91	26.42	10.19
Alder *(Frangula)*	–	–	–	–	–	100.00	–	–	–
Lavender *(Lavandula)*	–	–	–	4.69	12.21	13.05	18.54	31.25	20.25
Orange *(Citrus)*	–	–	6.96	19.84	–	10.22	36.37	12.71	13.90
Rhododendron *(Rhododendron)*	–	–	–	–	13.90	16.15	69.94	–	–
Lime tree *(Tilia)*	–	–	–	2.84	2.94	45.73	19.49	19.53	9.46

Values are percentage of total absorbance of flavonoids at 340 nm. (EA) Ellagic acid; (M) myricetin = 3,5,7,3',4',5'-hexahydroxyflavone; (H) hesperetin = 5,7,3'-trihydroxy-4'-methoxyflavanone; (Q) quercetin = 3,5,7,3',4'-pentahydroxyflavone; (L) luteolin = 5,7,3',4'-tetrahydroxyflavone; (P) 8-methoxykaempferol = 3,5,7,4'-tetrahydroxy-8-methoxyflavone; (K) kaempferol = 3,5,7,4'-tetrahydroxyflavone; (A) apigenin = 5,7,4'-trihydroxyflavone; (I) isorhamnetin = 3,5,7,4'-tetrahydroxy-3'-methoxyflavone.

ers. Thus, ellagic acid (EA) (a dimer of gallic acid) seems to be characteristic of calluna honey, myricetin (M) of heather honey and the flavanone hesperetin (H) of citrus honey. In figure 1, the HPLC chromatograms of the flavonoids present in these honey samples are shown, and the differences are clearly observed. The flavonoids coming mainly from pollen and/or nectar (although they are also present in propolis as minor constituents), which are related to the floral origin of honey, were

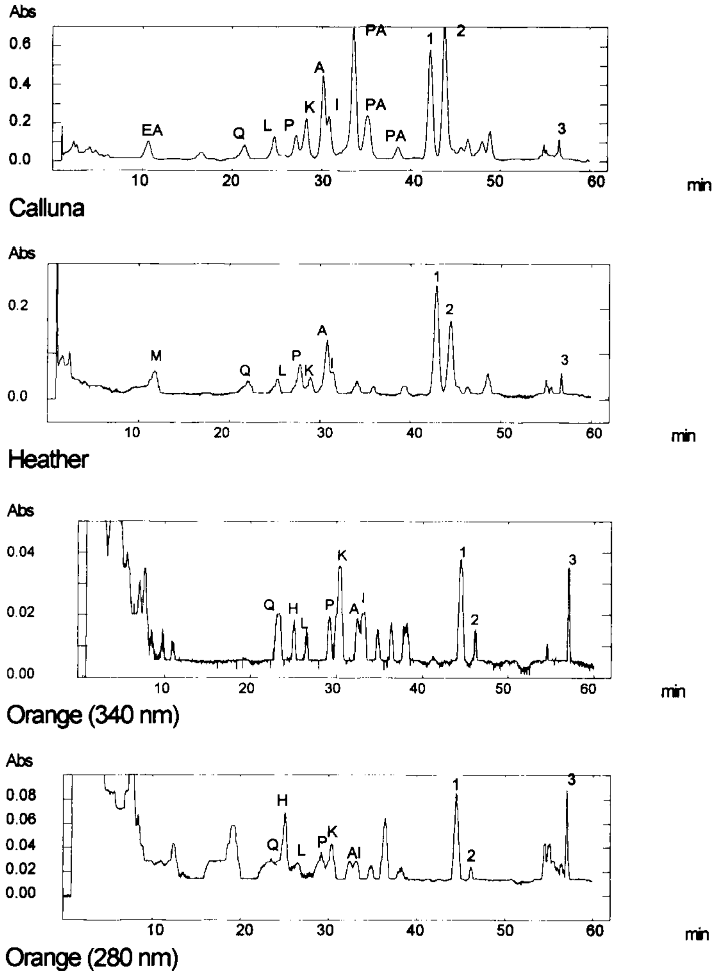

Fig 1. HPLC flavonoid profiles of calluna, heather and orange honey samples. Detection at 340 nm (and 280 nm for orange honey). (**EA**) Ellagic acid; (**Q**) quercetin; (**L**) luteolin; (**P**) 8-methoxykaempferol; (**K**) kaempferol; (**A**) apigenin; (**I**) isorhamnetin; (**PA**) caffeic acid esters; (**M**) myricetin; (**H**) hesperetin; (**1**) chrysin = 5,7-dihydroxyflavone; (**2**) galangin = 3,5,7-trihydroxyflavone; (**3**) tectochrysin = 5-hydroxy-7-methoxyflavone.

marked with letters in the chromatograms, and those coming exclusively from propolis and/or beeswax, and which do not have any relationship with the floral origin of honey, were marked with numbers. Both flavonoid types were readily distinguished in the chromatograms.

In other samples, it seems that the relative amount of 1 individual flavonoid could be related to the floral origin of honey. Thus, sunflower honey contains a considerable amount (around 30%) of quercetin, while the sample of alder honey contained 8-methoxykaempferol as the only flavonoid and other unidentified phenolic acid derivatives which were not present in the rest of the samples.

To evaluate if these possible markers were present in the same proportion in different honey samples with the same floral origin, but with different geographical origin, 3 sunflower honey samples produced in 3 French regions (Ariège, Aude and Loir-et-Cher) were extracted and their flavonoids

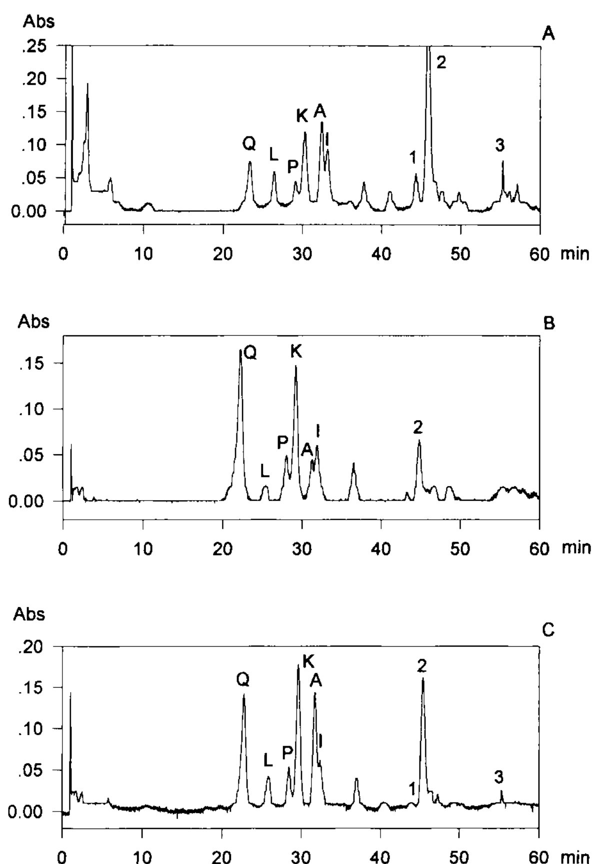

Fig 2. HPLC flavonoid profiles from 3 sunflower honey samples produced in 3 different geographic regions in France. Detection at 340 nm (**Q**) quercetin; (**L**) luteolin; (**P**) 8-methoxykaempferol; (**K**) kaempferol; (**A**) apigenin; (**I**) isorhamnetin; (**1**) chrysin = 5,7-dihydroxyflavone; (**2**) galangin = 3,5,7-trihydroxyflavone; (**3**) tectochrysin = 5-hydroxy-7-methoxyflavone. (**A**) Aude; (**B**) Loir-et Cher; (**C**) Ariège.

analysed. The results are shown in figure 2. The 3 samples have an identical flavonoid profile, with slight differences in the relative amounts of some components, and they are characterized by the relative importance of the flavonoid quercetin.

DISCUSSION

These results do not demonstrate that differences occur between the flavonoid profiles of different monofloral honeys, since they have a common flavonoid profile, as shown by a previous work on the flavonoids of La Alcarria honey (Fererres *et al*, 1992). However, in some cases, 1 individual flavonoid could be a potential marker for the floral origin of honey. Therefore, the presence of hesperetin in the French citrus honey agrees with previous reports on Spanish citrus honey (Ferreres *et al*, 1994b) and supports its potential use as a marker of its origin (Ferreres *et al*, 1993). The same applies to myricetin, since it has recently been reported to be a possible marker of the botanical origin in Portuguese heather honey (Fererres *et al*, 1994a) and was found in our French sample.

In addition, the flavonoids that are common to honeys with different floral origin could be useful as an adjunct in the objective determination of the plant origin of honey when they are present as major components in the flavonoid profile. Therefore, the importance of the relative amount of quercetin observed in the 3 French samples of sunflower honey, agrees with previous work on Spanish (Ferreres *et al*, 1992) and French sunflower samples (Sabatier *et al*, 1992). This observation supports the possible use of the determination of the relative amount of quercetin in honey as a complementary analysis in determining the origin of sunflower honey. It is not surprising that quercetin is one of the main flavonoids in the chromatograms of sunflower honey, since rutin (quercetin 3-rutinoside) has been detected as the main flavonoid in sunflower pollen (Ferreres *et al*, 1992) and nectar (Tomás-Barberán, unpublished results). Quercetin was also present in heather, rape, calluna, lavender, citrus and lime tree honey, but with smaller relative amounts than in the case of sunflower honey.

Although the profiles of flavonoid glycosides present in pollen can be used to differentiate pollens with different botanical origin (Tomás-Barberán *et al*, 1989), they reflect glycosidic combinations of a relatively limited number of polyhydroxylated aglycones (quercetin, luteolin, 8-methoxykaempferol, kaempferol, apigenin and isorhamnetin). These flavonoid aglycones also constitute the main flower- derived flavonoids in honey.

We conclude that the flavonoid profiles detected in the different French honey samples analysed are very similar, and it is not easy to differentiate all honey samples by their flavonoid profiles. However, some individual compounds seem to be useful biochemical markers of the floral origin of specific honey samples (calluna, citrus and heather), and the relative amount of other common flavonoids could help in the determination of the floral origin of other honey samples (*eg*, quercetin in sunflower honey). These results are quite encouraging, but measurements in more monofloral honeys of the same type should be conducted to confirm which flavonoids could be important for the characterization of a particular monofloral honey.

ACKNOWLEDGMENTS

The authors are grateful to the Spanish CICYT (Grants ALI91-0486 and ALI92-0151) for financial support of this work, and to Drs Morlot and Delgado for providing honey samples.

Résumé — Les flavonoïdes de miels français de diverses origines florales.

Ces dernières années on s'est efforcé de mettre au point de nouvelles techniques analytiques objectives pour déterminer l'origine des miels. Les flavonoïdes, les acides phénoliques, les dérivés des caroténoïdes, les acides aminés et les composés aromatiques ont été utilisés dans ce but. Dans ce travail les flavonoïdes de 12 miels monofloraux provenant de France ont été analysés par chromatographie liquide haute pression (HPLC) afin de voir s'il était possible de corréler le spectre des flavonoïdes et l'origine botanique des miels. Les flavonoïdes ont été extraits et purifiés sur une colonne Amberlite XAD-2, puis sur une colonne Sephadex LH-20 et analysés en HPLC. Les flavonoïdes provenant principalement du pollen et/ou du nectar (marqués d'une lettre) et ceux caractéristiques de la propolis et/ou de la cire d'abeille (marqués d'un chiffre) se différenciaient nettement sur les chromatogrammes (fig 1). Les premiers étaient principalement des composés polyhydroxylés, les seconds des flavonoïdes lipophyles avec un cycle B non substitué. Tous les miels monofloraux analysés avaient en commun un spectre constitué d'un petit nombre de flavonoïdes (tableau I). Néanmoins certains miels contenaient un composé phénolique spécifique, susceptible d'être utilisé pour déterminer l'origine des miels. Ainsi le miel de bruyère renfermait de la myricétine (M) celui de callune ; de l'acide ellagique (EA) celui d'oranger ; la flavanone hespérétine (H). D'autres miels se caractérisaient par l'accumulation d'un flavonoïde assez commun. Ainsi, le miel de tournesol contenait des quantités de quercetine nettement supérieures à celles des autres échantillons. Des quantités voisines ont été trouvées dans 3 miels de tournesol produits dans différentes régions de France (fig 2). Cette étude montre que l'analyse des flavonoïdes et des composés phénoliques peut être une technique biochimique complémentaire de valeur pour déterminer l'origine botanique de miels monofloraux spécifiques, mais que des mesures complémentaires sur des échantillons d'origine géographique variée sont nécessaires pour confirmer l'utilisation de certains flavonoïdes comme marqueurs de l'origine botanique des miels.

miel / flavonoïde / origine botanique / chromatographie liquide haute pression

Zusammenfassung — Das Flavonoidmuster unterschiedlicher unifloraler Blütenhonige französischer Herkunft. In den letzten Jahren wurden einige Anstrengungen unternommen, um die Herkunft von Honigen mit neuen, objektiven, analytischen Methoden beurteilen zu können. Hierzu wurden bisher Flavonoide, Phenolsäuren, Karotinoid-Abkömmlinge, Aminosäuren und aromatische Inhaltsstoffe benutzt. In dieser Arbeit wurden die Flavonoidprofile von 12 verschiedenen unifloralen Honigen französischer Abstammung durch HPLC analysiert. Mögliche Unterschiede des Flavonoidmusters sollten zu der botanischen Herkunft des Honigs in Beziehung gesetzt werden. Die Honigflavonoide wurden extrahiert und mit einer Kombination von Amberlite XAD-2 und Sephadex LH-20 Chromatographie gereinigt, und durch Hochdruckflüssigchromatographie mit Phasenumkehr analysiert. Die hauptsächlich aus Pollen und/oder Nektar stammenden Flavonoide (mit einem Buchstaben gezeichnet) waren in den HPLC-Chromatogrammen klar von den für Propolis und/oder Bienenwachs charakteristischen Flavonoiden (mit einer Zahl gezeichnet) getrennt (Abb 1). Die Flavonoide aus Pollen und/oder Nektar bestanden hauptsächlich aus polyhydroxilierten Anteilen. Die aus Propolis und/oder Bienenwachs stammenden Flavonoiden waren lipophil mit einem unsubstituierten Ring B. Die verschiedenen unifloralen Honige hatten alle ein übereinstimmendes Flavonoidmuster (Tabelle I). Einige der Honige enthielten allerdings spezifische phenolische Komponenten, die

zur Herkunftsbestimmung genutzt werden könnten. So enthielt der Heidehonig Myricetin (M), Honig der Besenheide *(Calluna)* Ellagsäure (EA) und Zitrushonig das Flavanon Hesperetin (H) (Abb 1). Andere Honige wurden durch das gehäufte Vorkommen von eher gewöhnlichen Flavonoiden charakterisiert. Beispielsweise enthielt Sonnenblumenhonig anteilsmäßig mehr Quercetin als der Rest der analysierten Proben. Die Höhe dieses Anteils war bei 3 verschiedenen Honigen aus unterschiedlichen Regionen Frankreichs ähnlich (Abb 2). Die Studie ergab damit, daß die Analyse der Flavonoide und der phenolischen Komponenten eine sehr nützliche zusätzliche biochemische Technik zur objektiven Bestimmungen einiger bestimmter monofloraler Honige darstellt. Allerdings sind weitere Messungen an Proben aus verschiedenen geographischen Regionen erforderlich, um die Verwendbarkeit einiger Flavonoide als Marker für die Blütenherkunft von Honigen zu bestätigen.

Honig / Flavonoide / Blütenherkunft / Hochdruckflüssigchromatographie

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