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Short Communication

Separation of honey flavonoids by micellar electrokinetic capillary chromatography

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Abstract

The separation of thirteen flavonoids from honey by micellar electrokinetic capillary chromatography (MECC) is described. All the flavonoids were separated on a fused-silica column (75 cm × 75 μm I.D.) with 0.2 *M* sodium borate buffer (pH 8.0)–50 m*M* sodium dodecyl sulphate–10% methanol. These conditions were applied to the separation of flavonoids from lavender, rosemary, citrus and heather honey samples, to establish correlations between the flavonoid profiles and the botanical origin of honey. Citrus honey was characterized by the accumulation of hesperetin, lavender by luteolin, rosemary by 8-methoxykaempferol and heather by some unidentified flavonoids. The influence of the geographical origin on the honey flavonoid pattern was also studied by MECC. Honey samples from Spain, Mexico and Canada were analysed and no significant differences were found.

1. Introduction

Capillary electrophoresis (CE) was first applied to the separation of biological molecules such as proteins and nucleic acids. Terabe *et al.* [1] developed the technique of micellar electrokinetic chromatography (MEKC) or micellar electrokinetic capillary chromatography (MECC), which further widened the applications of CE to include separations of neutral substances.

In the last 3 years, plant flavonoids have been separated by CE. As they are charged molecules in alkaline media, they can be separated by capillary zone electrophoresis (CZE). Thus the flavonoids from sugar cane [2] and the flavonol glycosides from *Sambucus* [3] were separated by CZE. However, most flavonoid separations by CE have been achieved by MECC. Thus, the flavonoid glycosides from *Ginkgo biloba* [4] and *Tilia* [5] and other commercial flavonoid glycosides [6–9] were separated by MECC using sodium dodecyl sulphate micelles. In addition, a preliminary study on the separation of some flavonoids from honey by CE has recently been reported [9]. All these publications suggest that CE is a very promising technique for flavonoid separations.

The separation of the flavonoids present in honey is useful in the determination of their

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geographical and botanical origins [10–12], and for this reason the optimization of the separation of honey flavonoids by HPLC has recently been achieved [13]. In that HPLC study, it was demonstrated that some flavonoid markers eluted as a single peak under different separation conditions and it was necessary to run the same sample under three different solvent conditions to detect the presence of some flavonoids.

As part of our research programme to evaluate the use of flavonoids as markers for the botanical and geographical origins of honey, the aim of this work was the separation of honey flavonoids by CE, and the results were applied to the study of the flavonoids present in some selected honey samples with different geographical and botanical origins to establish correlations between their flavonoid composition and their origins.

2. Experimental

2.1. Materials

The following flavonoids were used to prepare a standard mixture containing the main flavonoids isolated from honey to date [11,13] (as the amounts available of some markers were very small, the different flavonoid markers were not weighed to prepare the standard mixture): the flavanones were eriodictyol, naringenin, hesperetin, pinobanksin and pinocembrin and the flavones were myricetin, quercetin, kaempferol, 8-methoxykaempferol, luteolin, apigenin, chrysin and galangin (Table 1). All these compounds had previously been isolated and identified from honey and propolis [11].

Honey samples were supplied and authenticated by the Centro Regional Apicola (Castilla-La Mancha) and by the SOIVRE (Valencia).

2.2. Sample preparation

The flavonoids for MECC analysis were extracted from honey as reported previously [11]. Honey (*ca.* 200 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, Buchs, Switzerland). The column was washed with acidic water (100 ml) and then with 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 (40°C), redissolved in methanol (0.5 ml) and analysed by MECC.

Table 1
Honey flavonoids separated by MECC

No.	Flavonoid	Structure	Migration time (min)
1	Pinobanksin	3,5,7-Trihydroxyflavanone	8.54
2	Naringenin	5,7,4′-Trihydroxyflavanone	9.12
3	Hesperetin	5,7,3′-Trihydroxy-4′-methoxyflavanone	9.50
4	8-Methoxykaempferol	3,5,7,4′-Tetrahydroxy-8-methoxyflavone	10.34
5	Myricetin	3,5,7,3′,4′,5′-Hexahydroxyflavone	10.73
6	Quercetin	3,5,7,3′,4′-Pentahydroxyflavone	11.10
7	Luteolin	5,7,3′,4′-Tetrahydroxyflavone	11.43
8	Eriodictyol	5,7,3′,4′-Tetrahydroxyflavanone	11.74
9	Pinocembrin	5,7-Dihydroxyflavanone	12.45
10	Kaempferol	3,5,7,4′-Tetrahydroxyflavone	13.08
11	Apigenin	5,7,4′-Trihydroxyflavone	13.51
12	Chrysin	5,7-Dihydroxyflavone	15.89
13	Galangin	3,5,7-Trihydroxyflavone	16.60

2.3. Micellar electrokinetic capillary chromatography

MECC separations were carried out using a Beckman P/ACE System 2200 apparatus equipped with a 75 cm × 75 μm I.D. fused-silica capillary. The running buffer was 0.2 *M* sodium borate (pH 8.0)–50 m*M* sodium dodecyl sulphate (SDS) (Sigma)–5, 10 or 15% methanol. The voltage was 20 kV with an average current of 58.4 μA and the samples were injected by hydrodynamic injection for 2 s. All electropherograms were recorded on a Merck–Hitachi (Darmstadt, Germany) integrator. The on-column detector was operated at 280 nm.

The capillaries were conditioned daily by washing sequentially with 1 *M* hydrochloric acid (5 min), 1 *M* sodium hydroxide (5 min), 0.1 *M* sodium hydroxide (3 min) and the buffer (3 min). All washings and runs were carried out at 30°C. Between two analyses, the capillary tubes were flushed with 1 *M* HCl (2 min), distilled water (2 min), 1 *M* NaOH (2 min) and buffer (3 min) in order to improve the reproducibility of the migration times.

3. Results and discussion

3.1. Separation of honey flavonoids by MECC

To establish the analytical conditions for the separation of honey flavonoids by CE, a mixture of the different honey flavonoid standards available was prepared. In previous reports, flavonoids were successfully separated by CE using buffers at pH > 10 (CZE) [2,3] and by using borate buffers (30–200 m*M*) at pH 7.0–8.5 with addition of SDS (30–50 m*M*) (MECC) [4–9]. Some of the flavonoids present in honey, namely

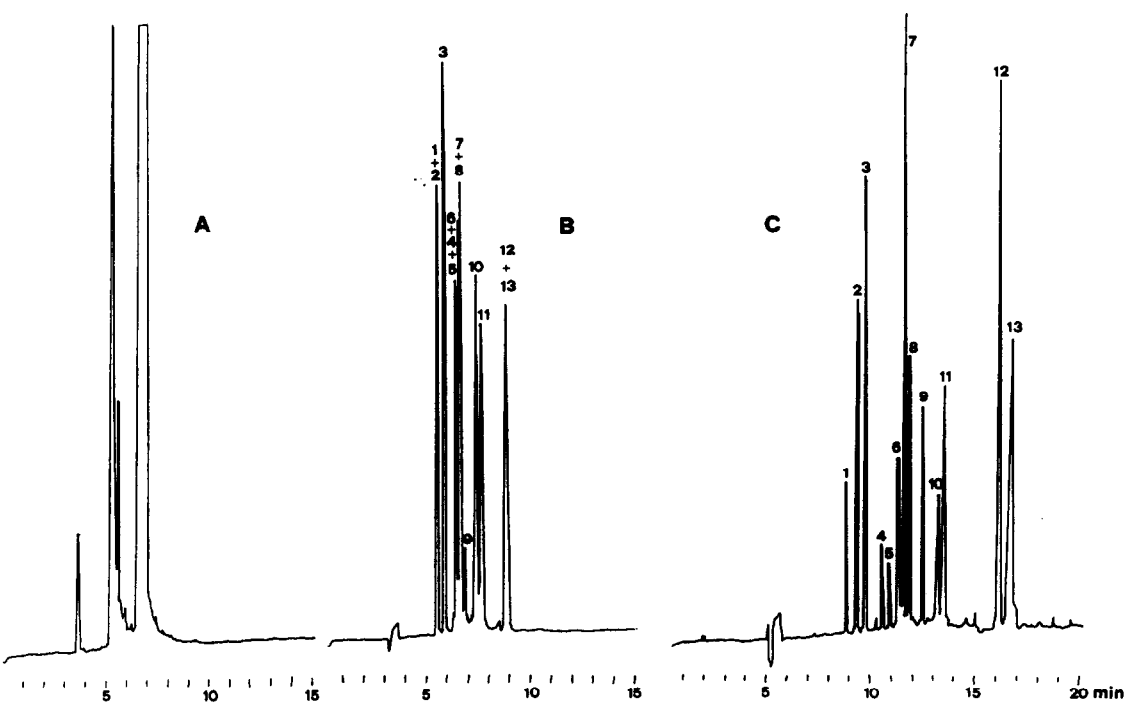

Fig. 1. Electropherograms of the standard mixture of honey flavonoids. For peak identification, see Table 1. Detection at 280 nm. (A) 200 m*M* Borate buffer (pH 8.0); (B) 200 m*M* borate buffer (pH 8.0)–50 m*M* SDS; (C) 200 m*M* borate buffer (pH 8.0)–50 m*M* SDS–10% methanol.

myricetin, quercetin, kaempferol, etc., decompose in alkaline media, and for this reason CZE of honey flavonoids was not attempted. In order to separate the flavonoids present in the standard mixture by MECC, different concentrations of borate buffer were tried (0.05, 0.1 and 0.2 *M*) and also different pH values (7.5, 8.0 and 8.5) and SDS concentrations (30, 50 and 100 m*M*). Capillary temperatures of 25, 30 and 40°C were also studied but no significant differences were found and 30°C was selected for subsequent analyses. The best separations were obtained with 0.2 *M* sodium borate (pH 8.0)–50 m*M* SDS (Fig. 1). However, under these conditions, some flavonoids eluted together. For final optimization of the separation of flavonoids, methanol (5, 10 or 15%) was added to the buffer as a modifier and a concentration of 10% led to the best separation (Fig. 1). Fig. 1 shows that the flavonoids were not at all separated with borate buffer at pH 8, that the addition of SDS (to achieve MECC) increases the separation considerably and that with the addition of 10% of methanol all the flavonoids present in the standards mixture were well separated. This separation is an improvement over previously reported CE separations of honey flavonoids [9], as it clearly resolves luteolin (**7**) from quercetin (**6**) and chrysin (**12**) from galangin (**13**). This separation also improves on previous analysis by HPLC [13], in which some flavonoid pairs such as naringenin, pinobanksin and quercetin, or eriodictyol and myricetin, were not resolved in a single analysis.

3.2. Separation of the flavonoid extracts obtained from the selected honey samples by MECC

The flavonoids present in several honey samples of different geographical and botanical origins were subjected to MECC under the conditions described. Honey samples from lavender (*Lavandula*), rosemary (*Rosmarinus*), orange

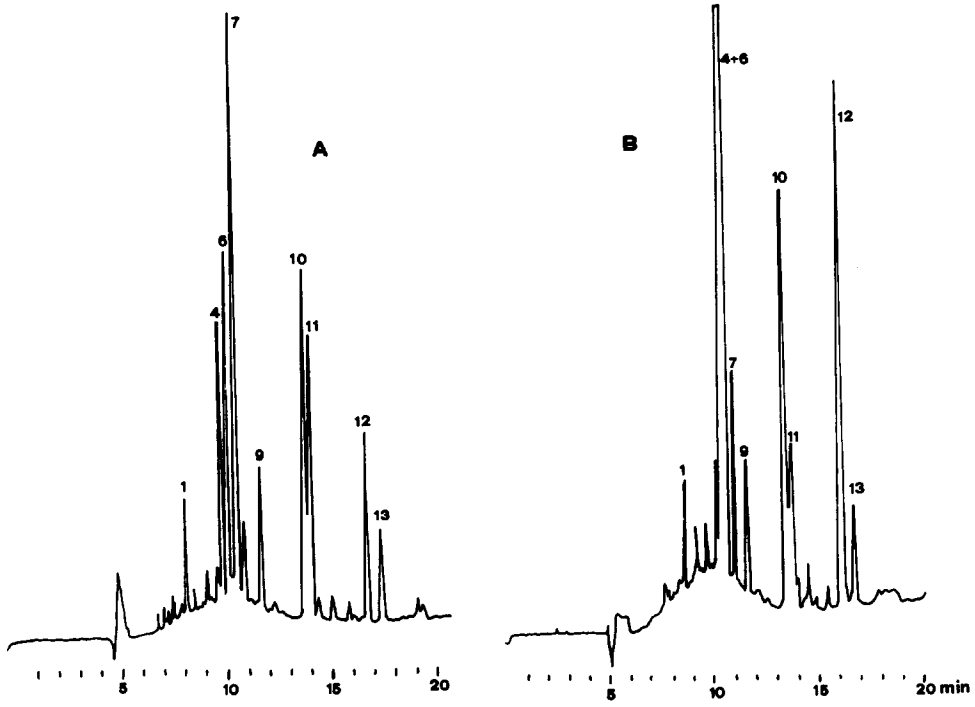

Fig. 2. Electropherograms of honey flavonoids. (A) Lavender honey; (B) rosemary honey. For flavonoid identification, see Table 1. Detection at 280 nm.

tree (*Citrus*) and heather (*Erica*) were analysed. In previous studies we have shown that some flavonoids are useful markers for the floral origin of honey [14]. In Fig. 2, the MECC separations of the flavonoids from lavender and rosemary honeys are shown. Both samples show the same flavonoid pattern (the same flavonoids are detected in both electropherograms), but their relative amounts are different. Thus, lavender honey contains luteolin (**7**) as the main flavonoid whereas rosemary honey contains 8-methoxykaempferol (**4**) as the main constituent. In the latter case, the huge amount of 8-methoxykaempferol present in the extract leads to compound **6** being masked within the peak of the former and the migration times of **4**, **6**, **7** and **9** appear slightly modified. This common flavonoid profile could be expected as both lavender and rosemary belong to the same plant family, the Labiatae.

In Fig. 3, the MECC separations of the flavonoids from citrus and heather honeys are presented. The differences in the flavonoid profiles shown in the electropherograms are clear. Citrus honey is characterized by the presence of hesperetin (**3**), which was not detected in any other honey sample, and agrees with previous reports in which this flavonoid was suggested as a marker for the floral origin of citrus honey [14]. It is interesting that the flavanones naringenin (**2**) and eriodictyol (**8**) were not detected in the citrus honey analysed, in spite of the fact that these flavanones are common constituents of citrus fruits and pollens, but they were not detected in citrus nectar [14]. On the other hand, heather honey is characterized by several unidentified flavonoids and by the presence of myricetin (**5**), 8-methoxykamepferol (**4**) and quercetin (**6**).

All four samples analysed so far had different floral origins but they all were produced in Spain (same geographical origin). All samples showed significant amounts of pinobanksin (**1**), pinocembrin (**9**), chrysin (**12**) and galangin (**13**) which

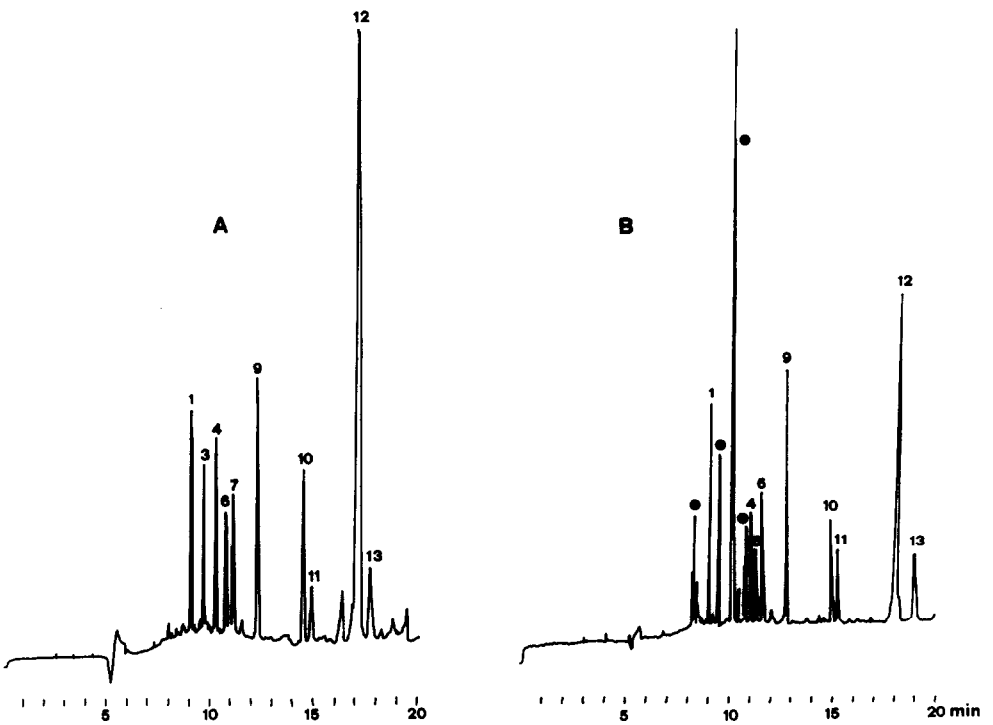

Fig. 3. Electropherograms of honey flavonoids. (A) Citrus honey; (B) heather honey. For flavonoid identification, see Table 1. Detection at 280 nm. ● unidentified flavonoid.

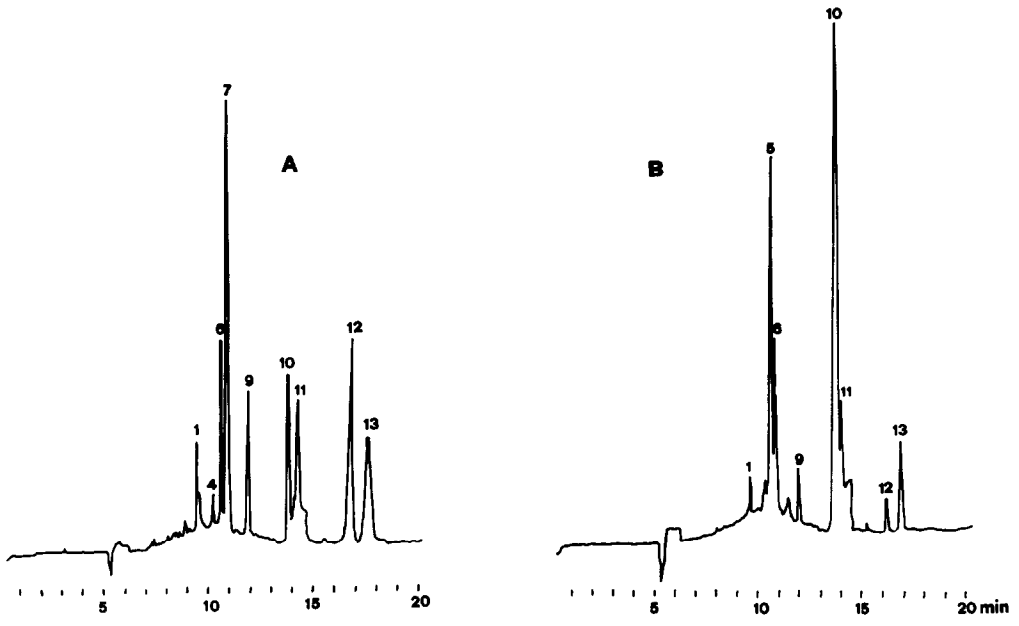

Fig. 4. Electropherograms of honey flavonoids from different locations: (A) Mexico; (B) Canada. For flavonoid identification, see Table 1. Detection at 280 nm.

have been considered as possible markers for the geographical origin of honey, as they are incorporated into honey from the poplar bud exudates [15,16].

Two samples from other geographical origins (Mexico and Canada) were also analysed by MECC. In Fig. 4, the electropherograms of the flavonoids from honey samples collected in Mexico and Canada are shown. It is remarkable that the amount of galangin (**13**) in the sample from Canada is higher than that of chrysin (**12**), this being a difference from the Spanish samples, and agrees with previous studies carried out by HPLC [15]. However, both samples contain pinocembrin, pinbanksin, chrysin and galangin, and suggest that the role of these substances as markers for the geographical origin of honey needs to be re-examined. The sample from Mexico accumulated luteolin (**7**) as the main flavonoid, while that from Canada accumulated myricetin (**5**) and kaempferol (**10**) instead.

To conclude, this study suggests that MECC is a very promising technique in the separation and identification of honey flavonoids, as it allows the separation of the main honey flavonoids with a single analysis.

4. Acknowledgements

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