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Chiral and achiral separation of ten flavanones using supercritical fluid chromatography. Application to bee pollen analysis

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ABSTRACT

The separation of ten flavanones (flavanone, 2′-hydroxyflavanone, 4'-hydroxyflavanone, 6-hydroxyflavanone, 7-hydroxyflavanone, naringenin, naringin, hesperetin, pinostrobin, and taxifolin) using supercritical fluid chromatography and considering achiral and chiral approaches has been studied in this work. For this purpose, different stationary phases and organic modifiers have been checked. Considering the achiral separation, the best results were obtained with the Lichrospher 100 Diol column at 35 °C, 3 mL/min, 150 bar and a gradient of 2-propanol from 5% to 50%. The baseline separation of the ten compounds was achieved in 18 min. Using the chiral column Chiralpak AD, the separation of the ten pairs of enantiomers was obtained in 32 min. In this case, the chromatographic conditions were 30 °C, 3 mL/min, 150 bar and the organic modifier was a mixture ethanol/methanol (80:20) containing 0.1% of trifluoroacetic acid applied in an elution gradient from 15% to 50%. The applicability of the proposed chiral method was assessed by analysing bee pollen samples and 2S-pinostrobin was determined in some of them.

1. Introduction

Flavanones are a sub-class of flavonoids produced by plants as compounds of the secondary metabolism. They are widely distributed in nature and have caught the researchers' interest due to their health benefits and important properties; in particular, they have been reported to have antioxidant, anticarcinogenic, cardioprotective or anti-inflammatory activities [1–5]. Flavanones have one chiral centre at the C2 position and 3-hydroxyflavanones possess two chiral centres at the C2 and C3 positions. In nature, they can exist both as free aglycones and as glycosidic conjugates, and the 2S configuration is the predominant one [6].

One of the principal human sources of flavanones are fruits, especially those of the *Citrus* genus [1]. However, they have also been found in other foods such as tomatoes [7,8], peanuts [9], or bee products [10–13].

Traditionally the analysis of flavanones has been performed using liquid chromatography (LC) coupled to UV-visible diode-array (DAD) or mass spectrometry (MS) detectors. The possibilities and applications of these methods have been widely discussed in several reviews [6,14,15]. Reverse phase mode on C_{18} columns with binary mobile phases, composed of an acidic aqueous solution and an organic solvent (methanol or acetonitrile), has been the choice for achiral separations. On the other hand, flavanones are chiral compounds and bioactive agents such as hormones, neurotransmitters etc. very often exhibit stereoselectivity, thus, one pair of enantiomers can show different pharmacokinetics or pharmacodynamics properties. Stereochemical differences have been proven to affect the bioavailability of flavonoids, as was shown for example for catechin [16]. Moreover, differences in bioactivity or pharmacodynamics processes have been also found between the enantiomers of hesperetin [17] and pinostrobin [18]. To further investigate the mechanisms of action of the flavanones enantiomers and their distribution in natural products, enantiomeric methods of analysis are necessary. It should be mentioned that chiral liquid chromatography had also been applied to perform this task, although the number of published works is much lower. In this case, polysaccharide derivatives or cyclodextrins based columns were mostly employed [19]. It should be noted that the number of enantiomeric pairs simultaneously resolved is usually small; in most cases, the enantiomeric separation of flavanones is studied individually [20–23]. Some papers have described the simultaneous enantiomeric determination of three [24] or six [25] flavanones. However, the baseline

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separation of all the enantiomers was not achieved and the determination was based on the use of MS detectors. Considering that in natural products these compounds often occur in the presence of each other, methods enabling the chiral separation of more than one flavanone are of great interest.

Although the determination of flavanones has been successfully achieved using LC, especially for non-chiral separations, in the last decade, the capabilities of supercritical fluid chromatography (SFC) for determining phenolic compounds in general and flavanones in particular, has also been explored [26,27]. Due to the singular properties of supercritical fluids, SFC offers several advantages over LC such as higher efficiencies and resolutions, shorter analysis times and lower consumption of organic solvents, which is one of the principles of the Green Analytical Chemistry [28]. Moreover, the introduction of a new generation of instruments, with improved robustness and performance, has contributed to renew the interest in this technique. SFC has been widely used in chiral separations with successful results [29–31], but also the number of papers related to achiral separations have been increased in the last years [32]. In this way, several papers have described the achiral separation of flavanones from other phenolic compounds [33–35]. Polar stationary phases like silica, diol, or 2-ethylpyridyne were predominantly selected, and elution gradients of organic modifiers containing acidic additives were required for eluting the most polar compounds; even wide elution gradients that reached a 100% of organic modifier have been used with great success [36]. Considering chiral separations, SFC has been scarcely employed in the chiral analysis of flavanones and, as in LC, limited to one compound [37].

Therefore, the main goal of this work was to study, for the first time, the separation of ten flavanones (flavanone, 2′-hydroxyflavanone, 4'-hydroxyflavanone, 6-hydroxyflavanone, 7-hydroxyflavanone, naringenin, naringin, hesperetin, pinostrobin, and taxifolin), which were present in nature and commercially available, by using SFC. Taking into account that neither achiral nor chiral separation of the ten compounds was previously described; both approaches were studied. Moreover, the results obtained in these studies would contribute to a better knowledge of the capabilities of SFC in the analysis of flavanones. In this regard, four achiral and seven chiral stationary phases were assayed and the effect of different organic modifiers were evaluated with the aim of achieving the best separation in the shortest time.

Moreover, a secondary goal of this work was to apply the proposed chiral method to the analysis of a complex real sample such as bee pollen, which is rich in bioactive compounds, including flavonoids [38].

2. Material and methods

2.1. Reagents and standards

All the organic solvents employed (methanol, ethanol, isopropanol, ethyl acetate) were HPLC grade and obtained from LAB-SCAN (Dublin, Ireland). Racemic solid standards of flavanone (FLV), 2′-hydroxyflavanone (2'-OHFLV), 4'-hydroxyflavanone (4'-OHFLV), 6-hydroxyflavanone (6-OHFLV), 7-hydroxyflavanone (7-OHFLV), naringenin (NGEN), naringin (NGIN), hesperetin (HESP), pinostrobin (PINO), and taxifolin (TAXI) were purchased from Sigma-Aldrich (Madrid, Spain). Their standard stock solutions were prepared in methanol at the 500 µg/mL level and were stored at 4 °C. The working solutions were prepared by appropriate dilution of the stock solutions with methanol. Trifluoroacetic acid (TFA), acetic acid, ammonium sulphate and phosphoric acid were of analytical grade and obtained from Sigma-Aldrich (Madrid, Spain). Carbon dioxide was SFC grade and obtained from Carburos Metálicos (Barcelona, Spain).

2.2. Sample procurement and treatment

Bee pollen samples were obtained (n=3) from a local market (Valladolid, Spain) or were kindly donated (n=4) by the Centre for Agroenvironmetal and Apicultural Investigation (CIAPA; Marchamalo, Guadalajara, Spain). They were ground and sieved through 40 mesh, then they were dried overnight at 30 °C and three subsamples were submitted to analysis. The extraction of flavanones was performed according to a previous published methodology [35]. Briefly, 5 g of sample was mixed with 25 mL of ethyl acetate; then 12.5 mL of 40% ammonium sulphate and 2.50 mL of 20% phosphoric acid were added. The flask was stirred for 20 min and centrifuged for 10 min (1000 rpm). The remaining solid residue was submitted to a second extraction process, and the supernatants were combined and transferred to a separation funnel. The organic phase was collected (top phase) and the aqueous phase was extracted again with 25 mL of ethyl acetate. All the organic phases were collected in a flask and concentrated to dryness in a vacuum rotary evaporator at 30 °C. Finally, the residue was dissolved in 2 mL of ethanol and filtered through 0.45 µm pore size nylon filter. During all the process, the extracts were protected from light using aluminium foil.

2.3. Instrumentation

The SFC system was manufactured by Jasco (Tokyo, Japan). It was equipped with two pumps, PU-2080-CO2 and PU-2080, for supplying the carbon dioxide and the modifier respectively. The autosampler was an AS-2059-SF model and the injection volume was set at 10 µL. The column was thermostated in a CO-2065 oven. The pressure was controlled by a BP-2080 pressure regulator and the detector employed was a MD-2015 photodiode-array detector (PDA). Circular dichroism (CD) data were obtained using another SFC Jasco system equipped with two PU-4180 pumps, an AS-4350 autosampler, a CO-4065 oven a BP-4340 pressure regulator and a CD-4095 circular dichroism detector. System control and data acquisition were performed by ChromNav 1.009.02 software from Jasco.

The columns employed in this work are listed in Table 1.

A 5810 R refrigerated bench-top centrifuge from Eppendorf (Hamburg, Germany), an R-3 rotary evaporator from Buchi (Flawil, Switzerland), and Nylon syringe filters (17 mm, 0.45 μm; Nalgene, Rochester, NY) were employed for sample treatment.

2.4. Method performance

Performance of the chiral chromatographic method was evaluated in terms of repeatability, intermediate precision, accuracy, limit of detection (LOD), limit of quantification (LOQ) and linearity.

Instrumental repeatability was evaluated by injecting a 10.0 µg/mL standard solution six times during the same day. Intermediate precision was determined at three different levels: 2.5, 10.0 and 50.0 µg/mL and each standard was injected three times during three consecutive days. In all cases the relative standard deviation of retention times and peak areas were calculated.

Accuracy was determined at three concentration levels (2.5, 10.0 and 50.0 µg/mL), by injecting three replicates of each solution and the ratio of the calculated concentration to the nominal concentration was evaluated. LOD and LOQ were calculated as 3 and 10 times the signal to noise ratio (S/N) respectively.

Finally, linearity was assessed using calibration standards prepared at six concentration levels (LOQ, 5.0, 10.0, 25.0, 50.0 and 100.0 µg/mL). Each calibration level was prepared by triplicate and from different stock solutions.

Table 1
Columns employed in the work.

Achiral columns	Column	Stationary phase	Dimensions	Supplier
	Hypersil silica	Bare silica	250 × 4.6 mm, 5 µm	Phenomenex (Madrid, Spain)
	Lichrospher CN	Cyanopropyl bonded to silica gel	250 × 4.6 mm, 5 µm	Phenomenex (Madrid, Spain)
	Lichrospher 100 diol	Propanediol bonded to silica gel	250 × 4.6 mm, 5 µm	Merck (Madrid, Spain)
	DCpak PBT	polybutylene terephthalate (PBT) coated on silica gel	250 × 4.6 mm, 5µm	Chiral Technology Europe (Illkirch, France)
Chiral columns	Chiralpak AD	Amylose-tris(3,5-dimethylphenylcarbamate) coated on silica gel	250 × 4.6 mm, 10µm	Chiral Technology Europe (Illkirch, France)
	Chiralcel OD	Cellulose tris(3,5-dimethylphenylcarbamate) coated on silica gel	250 × 4.6 mm, 10µm	Chiral Technology Europe (Illkirch, France)
	Lux i-Amylose-3	Amylose tris(3-chloro-5-methylphenylcarbamate) inmobilized on silica gel	250 × 4.6 mm 3 µm	Phenomenex (Madrid, Spain)
	Lux Cellulose-2	Cellulose tris(3-chloro-4-methylphenylcarbamate) coated on silica gel	250 × 4.6 mm, 5 µm	Phenomenex (Madrid, Spain)
	Lux Amylose-2	Amylose tris(5-chloro-2-methylphenylcarbamate) coated on silica gel	250 × 4.6 mm, 5 µm	Phenomenex (Madrid, Spain)
	Regis S,S-Whelk-O1	1-(3,5-Dinitrobenzamido)-1,2,3,4,-tetrahydrophenanthrene bonded to silica gel	150 × 4.6 mm, 3.5µm	Regis Technology (Chicago USA)
	Regis Reflect I-Cellulose C	Cellulose tris(3,5-dichlorophenylcarbamate) inmobilized on silica gel	150 × 4.6 mm, 3 µm	Regis Technology (Chicago USA)

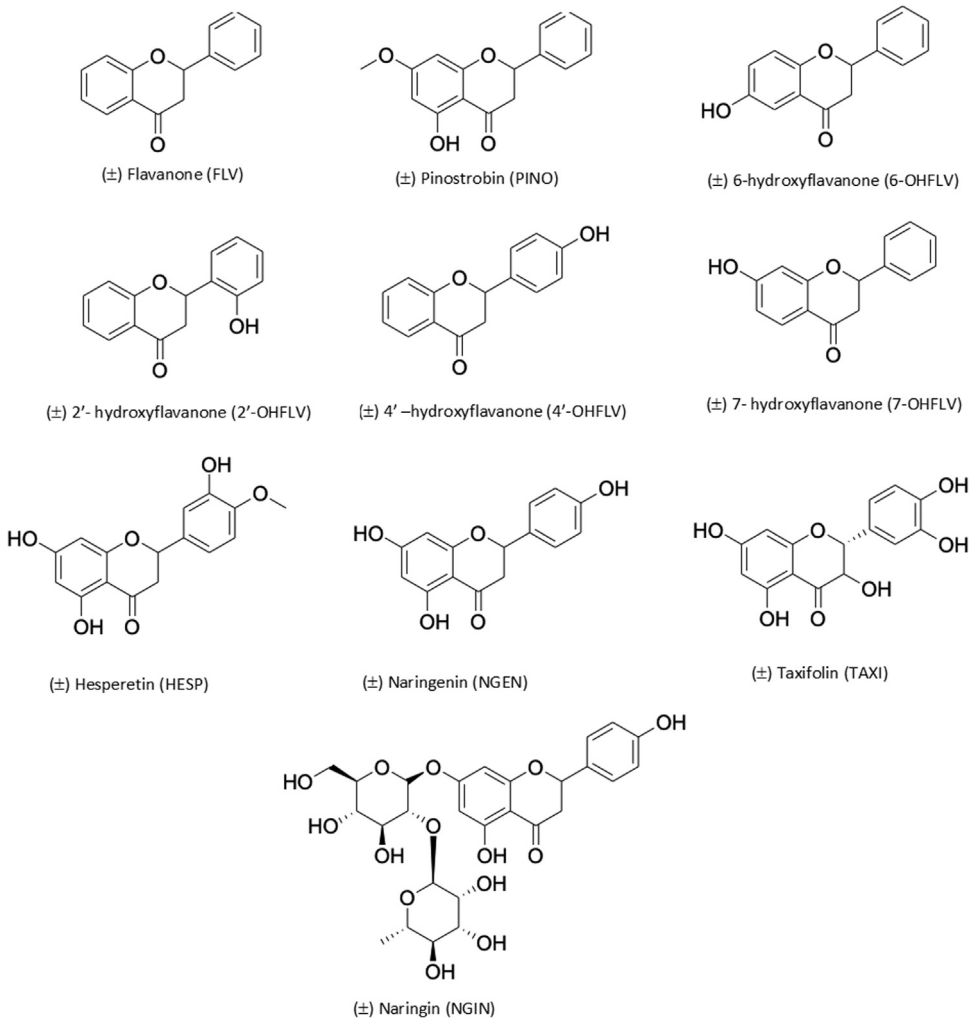

Fig. 1. Names and structures of the compounds studied.

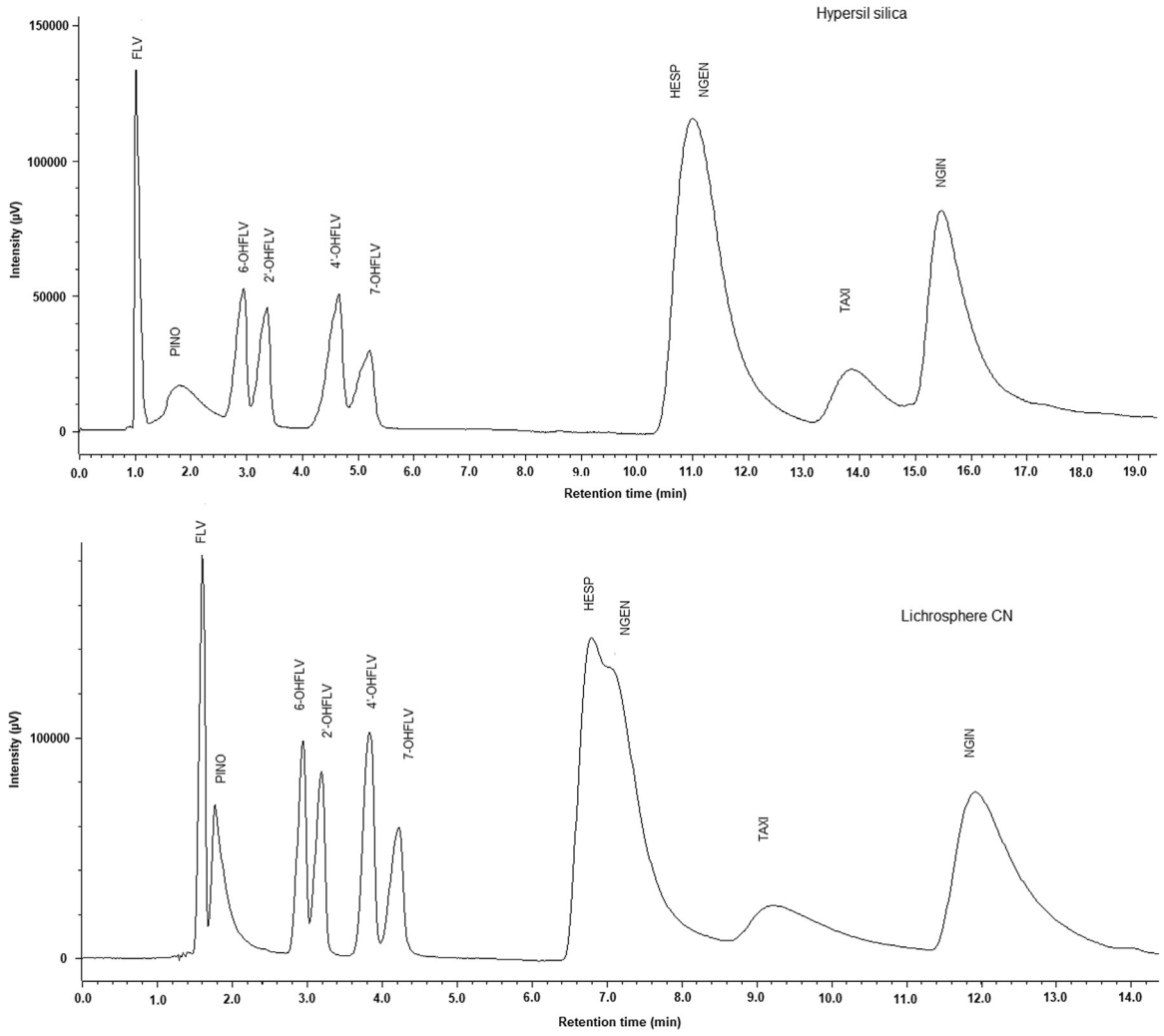

Fig. 2. Chromatograms obtained with the Hypersil silica and Lichrospher CN columns. The Chromatographic conditions were 35 °C, 150 bar, 3mL/min, gradient of methanol: from 0.0 to 5.0 min it was held at 3%, from 5.0 to 10.0 min it was increased to 20 %, from 10.0 to 15.0 min it was increased to 50% which was held for 5 min. Detection at 220 nm.

3. Results and discussion

3.1. Achiral separation

The separation of the flavanones was studied using four different types of stationary phases: silica, cyano, diol, and poly(butylene terephthalate). The selection of the stationary phases was based on the published papers related to the achiral SFC separation of polyphenols, including flavanones [35,39–41]. The use of an organic modifier was necessary to obtain reasonable retention times, as the analytes have several functional groups (see Fig. 1) that can interact with the stationary phases through hydrogen bonding and/or π-π interaction. Three organic modifiers were checked in this work: methanol, ethanol and 2-propanol. In all the cases, the compounds with the higher number of hydroxyl groups (naringenin, hesperetin, taxifolin and naringin) showed the highest retention and their elution was achieved increasing the percentage of organic modifier, thus working in gradient elution mode was mandatory. Retention increased in the order methanol<ethanol<2-propanol as the polarity of the organic modifier decreased.

Silica and cyano stationary phases did not provide satisfactory results. The peaks obtained were broad and several compounds coeluted (see Fig. 2). The best results were obtained with the diol and poly(butylene terephthalate) based columns.

On the Lichrospher 100 diol column the retention was lower than using the DCpak PBT one. This could be probably because on the last column the π-π interactions are favoured causing an increase on the retention. Generally, on both columns, the retention increased as the number of hydroxyl groups incremented, but the elution order in each column was different (see Table 2). Pinostrobin showed a much higher retention on the DCpak PBT column and the elution order of the pairs 6-hydroxyflavanone/2'-Hydroxyflavanone, 7-hydroxyflavanone/4'-hydroxyflavanone and hesperetin/naringenin was reversed with respect to that observed on the Lichrospher 100 diol. Different organic modifiers and gradients were checked to improve the resolution between 6-hydroxyflavanone, 2'-hydroxyflavanone, 4'-hydroxyflavanone, 7-hydroxyflavanone and pinostrobin. In the case of the Lichrospher 100 diol column, the best results were obtained when working at 35 °C, 3mL/min, 150 bar and using 2-propanol as modifier delivered according with the following gradient: from 0.0 to 2.0 min it was held at 5%, from 2.0 to 3.0 min it was increased to 15%, from 3.0 to 8.0 min it was increased to 20%, from 8.0 to 13.0 min it was increased to 50% which was held for 7 min. Under these conditions the compounds were separated in 18 min with resolutions higher than 1.5 (see Fig. 3a).

Meanwhile, the separation of the compounds on the DCpak PBT column was achieved at 40 °C and using a gradient of methanol

Table 2
Comparison between retention on Lichrosphere 100 diol and DCpak PBT columns. Chromatographic conditions: 35 °C, 150 bar, 3 mL/min. Gradient of methanol: 0.0 min–5.0 min (10%), 15.0–25.0 min (30%).

Lichrosphere 100 diol Compound	t_r (min)	DCpak PBT Compound	t_r (min)
Flavanone (FLV)	1.32	Flavanone (FLV)	2.68
Pinostrobin (PINO)	1.45	2'- hydroxyflavanone (2'-OHFLV)	3.77
6-hydroxyflavanone (6-OHFLV)	2.78	6-hydroxyflavanone (6-OHFLV)	3.95
2'- hydroxyflavanone (2'-OHFLV)	2.82	7- hydroxyflavanone (7-OHFLV)	4.09
4' –hydroxyflavanone (4'-OHFLV)	2.95	4' –hydroxyflavanone (4'-OHFLV)	4.40
7- hydroxyflavanone (7-OHFLV)	3.31	Pinostrobin (PINO)	4.25
Hesperetin (HESP)	8.75	Naringenin (NGEN)	10.09
Naringenin (NGEN)	9.23	Hesperetin (HESP)	10.45
Taxifolin (TAXI)	10.51	Taxifolin (TAXI)	11.12
Naringin (NGIN)	11.73	Naringin (NGIN)	12.48

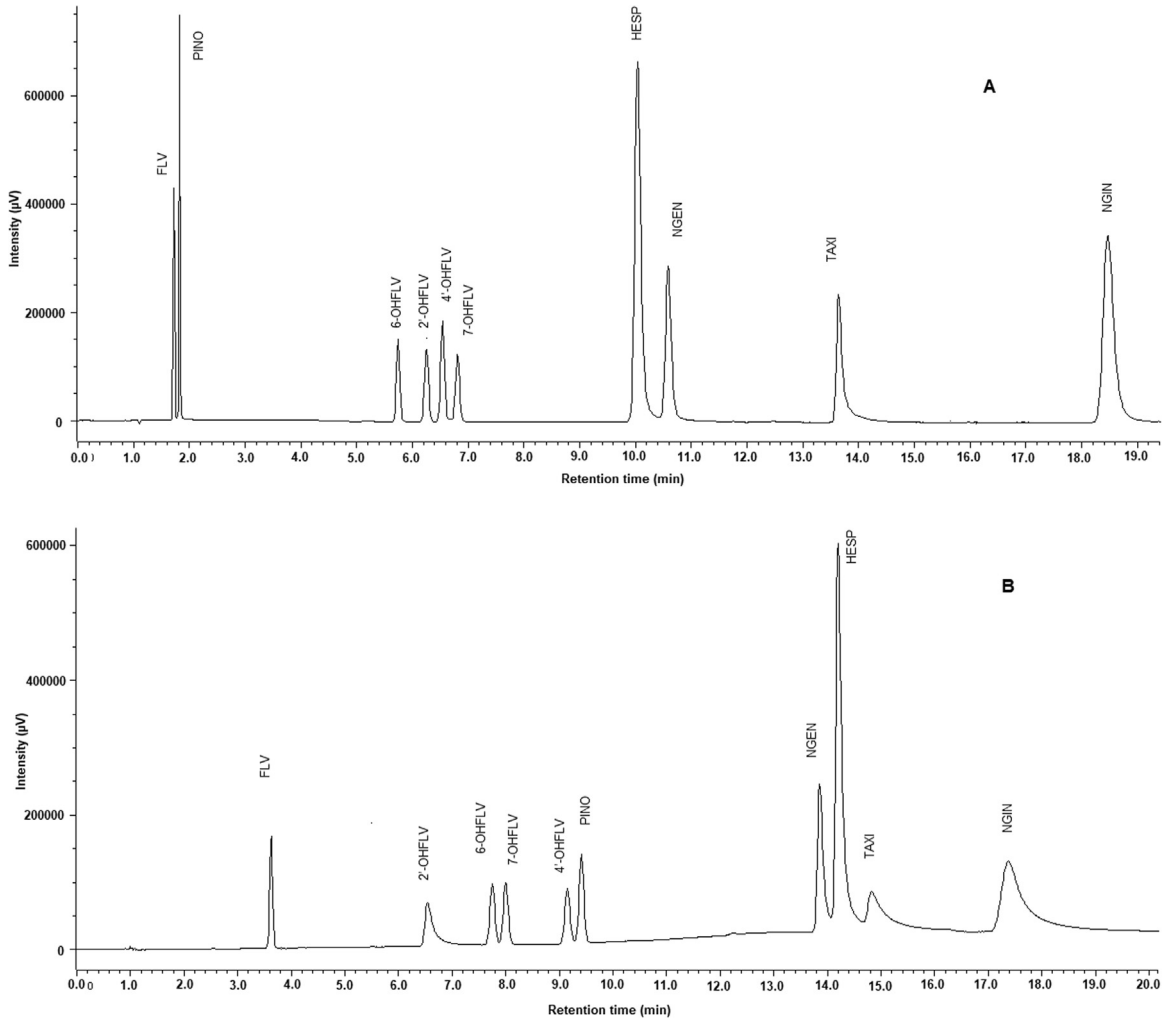

Fig. 3. Chromatogram obtained with the achiral columns. A- Lichrospher 100 diol column. Chromatographic conditions: 35 °C, 3mL/min, 150 bar. Gradient of 2-propanol: from 0.0 to 2.0 min it was held at 5%, from 2.0 to 3.0 min it was increased to 15%, from 3.0 to 8.0 min it was increased to 20%, from 8.0 to 13.0 min it was increased to 50% which was held for 7 min. B- DCpak PBT column. Chromatographic conditions: 40 °C, 3 mL/min, 150 bar. Gradient of methanol: it started at 5%, at 7.0 min it increased to 10%, from 7.0 to 11.0 min it was increased to 20%, which was held for 9.0 min. Detection at 220 nm.

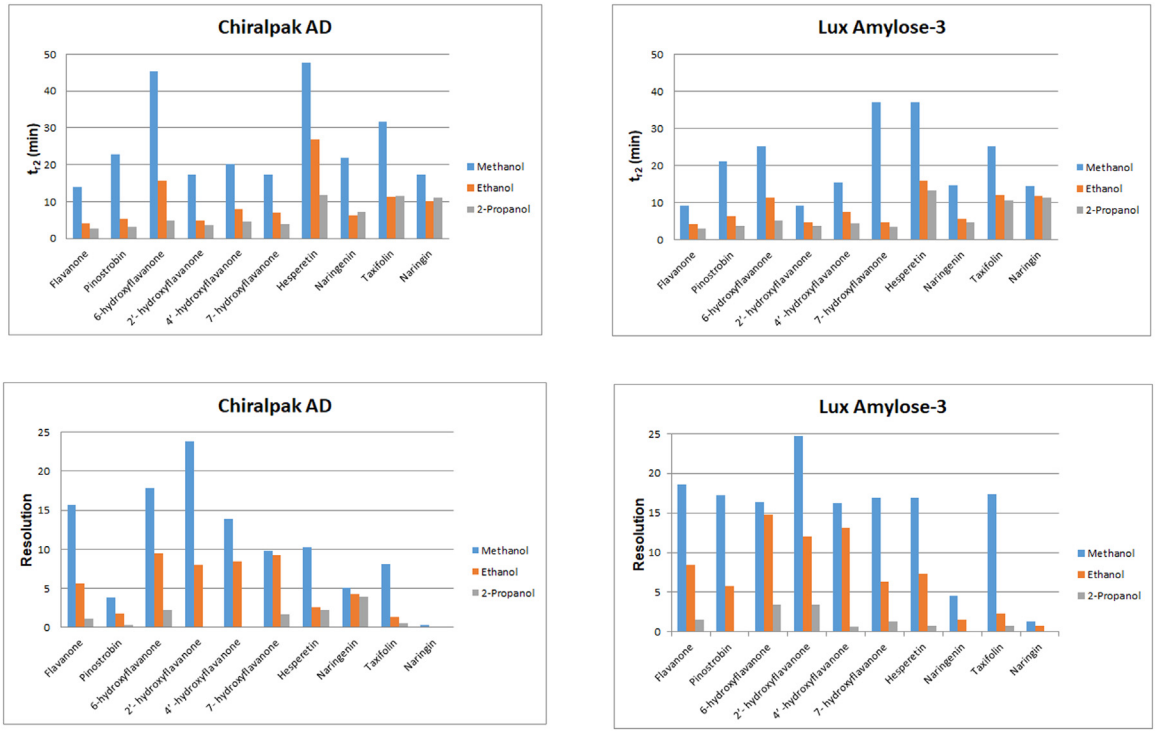

Fig. 4. Effect of the organic modifier on enantioresolution and retention time of the second eluted enantiomer, using the Chiralpak AD and Lux Amylose-3 columns. Chromatographic conditions: 35 °C, 2mL/min and 150 bar. Gradient of methanol: from 0.0 to 2.0 min it was held at 20%, from 2.0 to 20.0 min it was increased to 40%, which was held for 30.0 min. Detection at 220 nm.

(it started at 5%, at 7.0 min it increased to 10%, from 7.0 to 11.0 min it was increased to 20%, which was held for 9.0 min). As it can be seen in Fig. 3b, the analysis time was similar to that obtained on the Lichrospher 100 diol column; but the column efficiency was lower especially for 2'-hydroxyflavanone, taxifolin and naringin.

3.2. Chiral separation

3.2.1. Column selection and mobile phase optimization

The enantiomeric separation of the flavanones was studied using seven different chiral columns. The chiral selectors employed included Pirkle-type as well as cellulose and amylose carbamate derivatives (Table 1). Based on the previous experiments (data not shown), the initial conditions were 35 °C, 2mL/min and 150 bar. The use of a gradient of organic modifier was necessary in order to decrease the retention time of the compounds with a high number of hydroxyl groups. Initially, methanol was the organic modifier selected and it was delivered according to the following gradient: from 0.0 to 2.0 min, it was held at 20%, from 2.0 to 20.0 min it was increased to 40%, which was held for 30.0 min. The results obtained are presented in Table 3. As can be seen, the best chiral separations were obtained with the amylose derived columns, especially with Chiralpak AD and Lux Amylose-3, which provided the highest enantioresolutions for all the compounds studied. In general, the retention was also higher on the amylose columns, obtaining the longest retention times on the Chiralpak AD column. Taking into account these results, Chiralpak AD and Lux Amylose-3 were the columns selected to continue the work.

In order to achieve the simultaneous resolution of the ten pairs of enantiomers, different organic modifiers (methanol, ethanol and 2-propanol) and gradients were checked on both columns. Considering the effect of the type of organic modifier, in all cases, retention and resolution increased in the order 2-propanol<ethanol<methanol (Fig. 4). It should be noted that 2-propanol has a lower polarity than the other two modifiers, thus the opposite retention behaviour could have been expected. Nevertheless, this behaviour has also been observed for other compounds using amylose based columns [35,42]. It could be explained in terms of the hydrogen bond accepting ability. The studied compounds have several functional groups with hydrogen bond accepting ability and their retention is lower when the hydrogen bond-accepting ability of the modifier increases; on the contrary, the enantioresolution increases when the hydrogen bond accepting ability of the modifier decreases. The hydrogen bond accepting ability of the modifiers assayed increases in the order methanol<ethanol<2-propanol. The latter provided the lower retentions especially for the second eluted enantiomers, which in some cases caused the loss of the enantioresolution. Therefore, it can be concluded that the highest enantioresolutions were achieved with methanol and ethanol.

The best results, using the Lux Amylose-3 column, were obtained using methanol as organic modifier and the following gradient: from 0.0 to 2.0 min, it was held at 25%, from 2.0 to 15.0 min it was increased to 40%, which was held for 25 min. As it can be observed in Fig. 5, using this column, the enantiomers from different compounds coeluted, and the simultaneous chiral separation of the ten pairs of enantiomers was not possible. Further changes in temperature and pressure did not improve the separation. In relation to the Chiralpak AD column, the best performance was obtained at 30 °C, 3mL/min, 150 bar and using as modifier a mixture ethanol/methanol (80:20; v/v) delivered according to the following gradient: from 0.0 to 10.0 min it was held at 15%, from 10.0 to 23.0 min it was increased to 28%, and from 23.0 to 40.0 min it was increased to 50%. Under these conditions, good results were obtained for the simultaneous chiral separation of the ten pairs of enantiomers, and except for naringin, all the enantiomers were resolved. Taxifolin presented severely tailed peaks (Fig. 6a), but this issue was circumvented by using 0.1% of trifluoroacetic acid (TFA) as additive (Fig. 6b), moreover the separation between 2'- hydrox-

Table 3
Resolutions and retention times (minutes) obtained with the chiral columns studied and for the individual injection of each flavanone . Chromatographic conditions: 35 °C, 2 mL/min, 150 bar and methanol as organic modifier delivered according with the following gradient: 0.0–2.0 min (20%), 20.0–50.0 min (40%).

Compounds	Columns																			
	Regis S,S- Welk-O1			Regis Reflect I- Cellulose C			Chiralcel OD			Lux Cellulose-2			Chiralpak AD			Lux Amylose-2			Lux Amylose-3		
	Rs	t_1	t_2	Rs	t_1	t_2	Rs	t_1	t_2	Rs	t_1	t_2	Rs	t_1	t_2	Rs	t_1	t_2	Rs	t_1	t_2
FLV	0	3.0	3.0	1.9	3.2	3.4	3.0	3.9	4.4	0.9	3.7	3.8	15.7	6.8	13.9	7.9	6.8	9.1	18.6	4.9	9.1
PINO	0.2	3.8	3.9	1.5	4.5	4.7	5.0	5.0	6.1	1.0	5.7	5.9	3.8	19.4	22.8	4.1	9.3	11.5	17.2	11.7	21.1
6-OHFLV	0	4.0	4.0	0.9	8.1	8.2	2.2	5.2	5.6	1.1	6.4	6.6	17.9	20.6	45.3	11.9	12.4	17.9	16.4	14.6	25.2
2'-OHFLV	3.0	3.4	3.8	0	7.0	7.0	3.7	4.3	4.9	1.5	4.6	4.9	23.8	6.2	17.2	12.2	10.9	15.1	24.7	3.7	9.2
4'-OHFLV	1.2	4.7	4.9	0	9.5	9.5	0	5.6	5.6	1.8	7.6	8.1	13.9	11.7	20.2	5.5	12.9	14.6	16.3	9.7	15.4
7-OHFLV	0	5.3	5.3	1.3	14.9	15.2	0	11.2	11.2	2.9	12.1	13.9	9.8	11.7	17.2	0	19.6	19.6	16.9	20.5	37.0
HESP	0	8.5	8.5	1.3	14.9	15.2	0	11.2	11.2	2.9	12.1	13.9	10.3	30.3	47.6	0	19.6	19.6	16.9	20.5	37.0
NGEN	1.1	9.5	9.7	0.5	12.0	12.1	1.1	8.7	9.2	2.7	8.6	9.9	5.1	18.3	21.8	1.3	15.2	15.6	4.6	12.6	14.7
TAXI	1.5	9.3	10.0	1.3	16.6	17.6	0	16.8	16.8	0	24.2	24.2.	8.1	18.6	31.6	–			17.4	11.1	25.2
NGIN	1.1	9.5	9.7	2.0	24.8	26.1	––			1.5	32.0	34.9	0.3	17.2	17.3	0	26.1	26.1	1.3	12.9	14.3

– Not eluted in 50 min.

Fig. 5. Chiral separation using the Lux Amylose-3 column. Chromatographic conditions: 35 °C, 2mL/min and 150 bar. Gradient of methanol: from 0.0 to 2.0 min it was held at 25%, from 2.0 to 15.0 min it was increased to 40%, which was held for 25 min. Detection at 220 nm. A) mixture 1: 2'-OHFLV, 7-OHFLV, 6-OHFLV and HESP. B) mixture 2: FLV, 4'-OHFLV, PINO and NGEN. C) mixture 3: TAXI and NGIN.

yflavanone(1)/flavanone(2) and 4'–hydroxyflavanone(1)/7- hydroxyflavanone(2) improved.

Therefore, it can be concluded that the best overall performance was obtained with the Chiralpak AD column under the above-mentioned conditions.

3.2.2. Enantiomers elution order

The elution order of the enantiomers was determined using the Chiralpak AD column and a CD detector coupled to the SFC system. As can be seen in Table 4, in all the cases, except for naringenin, the first eluted enantiomer presented a positive CD signal, while the opposite behaviour was observed for the second

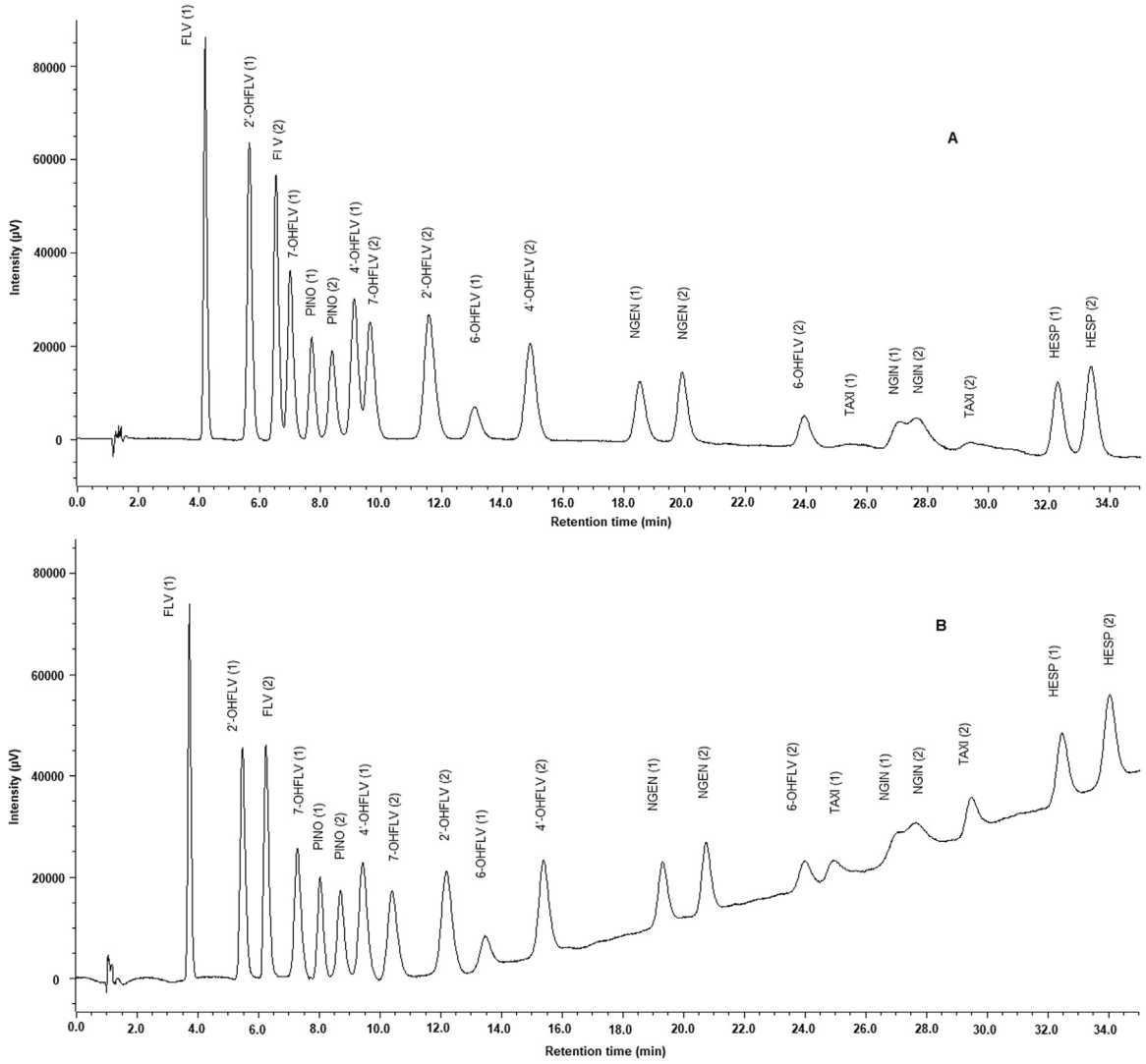

Fig. 6. Chiral separation using the Chiralpak AD column. Chromatographic conditions 30 °C, 3mL/min, 150 bar. Gradient of ethanol/methanol (80:20): from 0.0 to 10.0 min it was held at 15%, from 10.0 to 23.0 min it was increased to 28% and from 23.0 to 40.0 min it was increased to 50%. A- without using TFA. B- Using 0.1% TFA as additive. Detection at 220 nm.

Table 4
Sign of circular dichroism signal obtained for the first (E1) and second (E2) eluted enantiomers of the flavanones studied, at the wavelength selected. Chromatographic conditions: Chiralpak AD column, 30 °C, 3 mL/min, 150 bar, organic modifier ethanol/methanol (80:20) containing 0.1% TFA delivered according to the following gradient from 0.0 to 10.0 min it was held at 15%, from 10.0 to 23.0 min it was increased to 28% and from 23.0 to 40.0 min it was increased to 50%.

Compound	(E1)	(E2)	Wavelength (nm)
FLV	(+)	(-)	280
2'-OHFLV	(+)	(-)	250
7-OHFLV	(+)	(-)	280
PINO	(+)	(-)	280
4'-OHFLV	(+)	(-)	280
6-OHFLV	(+)	(-)	280
NGEN	(-)	(+)	280
TAXI	(+)	(-)	290
NGIN	(+)	(-)	280
HESP	(+)	(-)	280

enantiomer. According to Gaffield [43], flavanones with 2S configuration (having the 2 position substituted equatorially to the heterocyclic ring) and 3-hydroxyflavanones with 2R3R configuration (having the 2 and 3 position substituted diequatorially to the heterocyclic ring) present a negative Cotton effect in the region of 280-290 nm corresponding to the transition $\pi \rightarrow \pi^*$. In addition, 2S flavanones present a positive Cotton effect at 245–270 nm. Based on this work, we can tentatively conclude that, on the conditions selected, the first eluted enantiomer of flavanone, 7-hydroxyflavanone, pinostrobin, 4-hydroxyflavanone, 6-hydroxyflavanone, naringin and hesperetin, had the 2R configuration and the second had the 2S one. In the case of naringenin and 2-hydroxyflavanone, the situation was the opposite, the first eluted enantiomer had the 2S configuration and the second had the 2R one. In the case of taxifolin, which is a 3-hydroxyflavanone, the first eluted enantiomer had the 2R3S configuration, while the second eluted enantiomer the 2R3R.

It should be noted that 2-hydroxyflavanone presented a weak CD signal at 280-290 nm and subsequently the measurement was performed at 250 nm.

Table 5
Limits of detection (LOD) and quantification (LOQ) obtained with the PDA detector at 220 nm. Chromatographic conditions: Chiralpak AD column, 30 °C, 3 mL/min, 150 bar, organic modifier ethanol/ethanol (80:20) containing 0.1% TFA delivered according with the following gradient from 0.0 to 10.0 min it was held at 15%, from 10.0 to 23.0 min it was increased to 28% and from 23.0 to 40.0 min it was increased to 50%.

Compound	LOD (µg/mL)	LOQ (µg/mL)
FLV (1)	0.21	0.68
FLV (2)	0.27	0.92
2'-OHFLV (1)	0.24	0.79
2'-OHFLV (2)	0.32	1.07
7-OHFLV (1)	0.38	1.86
7-OHFLV (2)	0.56	1.49
PINO (1)	0.26	0.85
PINO (2)	0.51	1.70
4'-OHFLV (1)	0.45	1.49
4'-OHFLV (2)	0.51	1.70
6-OHFLV (1)	0.33	1.10
6-OHFLV (2)	0.51	1.70
TAXI (1)	0.33	1.12
TAXI (2)	0.47	1.57
NGEN (1)	0.40	1.33
NGEN (2)	0.48	1.60
NGIN	0.89	2.63
HESP (1)	0.32	1.07
HESP (2)	0.43	1.43

3.2.3. Analytical performance of the chiral method

Taking into account that chiral separation provides a comprehensive information about the occurrence of the selected compounds in samples, the performance of the chromatographic method was evaluated using the Chiralpak AD column and the chromatographic conditions selected in Section 3.2.1. Considering that the sensitivity of the CD detector was lower than that provided by the PDA detector, the latter was selected for the study of the method performance. It should be noted that this is just an evaluation of the performance of the chiral chromatographic method to show its potential; the evaluation of the chiral method for the analysis of a specific sample/matrix has not been performed. In this case, evaluation of the method performance should have to be repeated once the sample preparation has been optimized.

Limits of detection (LOD) and quantification (LOQ) were calculated as 3 and 10 times the signal to noise ratio (S/N) respectively. They were determined at 220 nm except for naringin where 280 nm was used. Considering that the baseline separation of naringin enantiomers was not achieved, both enantiomers were determined together. As can be seen in Table 5, the LOD values ranged from 0.21 to 0.89 µg/mL; meanwhile, LOQ values varied between 0.68 to 2.63 µg/mL. The relative standard deviation (%RSD) of peak areas and retention times, evaluated for the instrumental repeatability, were in all the cases below 4% and 1.5% respectively (Table 6). Meanwhile, in the case of the intermediate precision, the %RSD values were close to 10% and 5% for the peak areas and retention times, respectively; and the accuracy values were between 84.1% and 115.7%. Finally, the determination coefficients obtained for the standard calibration curves were above 0.99 in all cases and the absence of bias was confirmed by a *t* test and by studying the distribution of residuals (data not shown).

3.3. Application to the analysis of bee pollen samples

In order to examine the feasibility of the proposed chiral SFC method in the analysis of real samples; it was applied to the enantiomeric determination, of the selected flavanones, in bee pollen

Table 6
. Repeatability, intermediate precision and accuracy of the chiral analytical method (see Table 5 for chromatographic conditions).

Compound	Repeatability (%RSD)		Intermediate precision (% RSD peak area)			Intermediate precision (% RSD retention time)			Accuracy (%)		
	Area	Retention time	2.5 µg/mL	10.0 µg/mL	50.0 µg/mL	2.5 µg/mL	50.0 µg/mL	100.0 µg/mL	2.5 µg/mL	50.0 µg/mL	100.0 µg/mL
FLV (1)	1	0.5	6.6	2.4	1.6	2.2	2.3	2.6	86.2	96.2	98.5
FLV (2)	1.6	0.7	7.2	2.6	1.7	2.4	2.5	2.4	84.1	97.2	105.3
2'-OHFLV (1)	1.3	0.6	6.9	2.3	1.5	2.3	2.3	2.2	85.2	102.1	97.6
2'-OHFLV (2)	3.1	1.1	8	4.3	1.9	3.5	3.1	3.3	112.3	98.1	103
7-OHFLV (1)	2.1	0.7	7.4	4.5	2.5	2.2	2.3	2.5	88.2	96.7	98.4
7-OHFLV (2)	2.3	1	7.8	3.8	2.6	2.2	2.4	2.3	85.8	103.2	99.1
PINO (1)	2.3	0.7	7.9	3.1	2.8	2.4	2.2	2.1	106.8	97.9	108.1
PINO (2)	2.1	0.8	8.1	4	2.6	2.6	2.5	2.6	108.9	98.2	102.6
4'-OHFLV (1)	2.9	0.8	8	4.8	2.7	2.4	2.3	2.6	86.4	102.4	98.3
4'-OHFLV (2)	3.1	1.1	8.2	4.5	2.9	3.6	3.2	3.4	85.8	94.8	98
6-OHFLV (1)	3.6	1.3	9.1	4.8	2.6	3.8	3.6	3.5	111.6	91.5	105.8
6-OHFLV (2)	3.7	1.4	9.2	5	3.6	4.1	3.8	4	114.1	93.7	96.8
TAXI (1)	3.9	1.3	9.4	5.3	4.1	4.3	4	4.2	85.2	110.3	94.6
TAXI (2)	3.2	1.2	8.7	5	4.1	4.4	4.1	4.3	86.4	92.8	95.1
NGEN (1)	2.7	1.3	7.3	4.1	2.5	4.2	4	4.2	87.3	96.1	103.5
NGEN (2)	2.6	1.2	7.5	4	2.3	4	4.1	4	88.1	98.2	98.7
NGIN	3.8	1.4	9.4	4.8	2.7	4.8	4.6	4.7	115.7	92.1	95.1
HESP (1)	2.3	1.3	7.8	3.8	2.6	4.1	3.9	4.2	87.5	96.8	108.3
HESP (2)	2.5	1.3	8	3.7	2.2	4.4	4.5	4.3	86.9	96.5	94.8

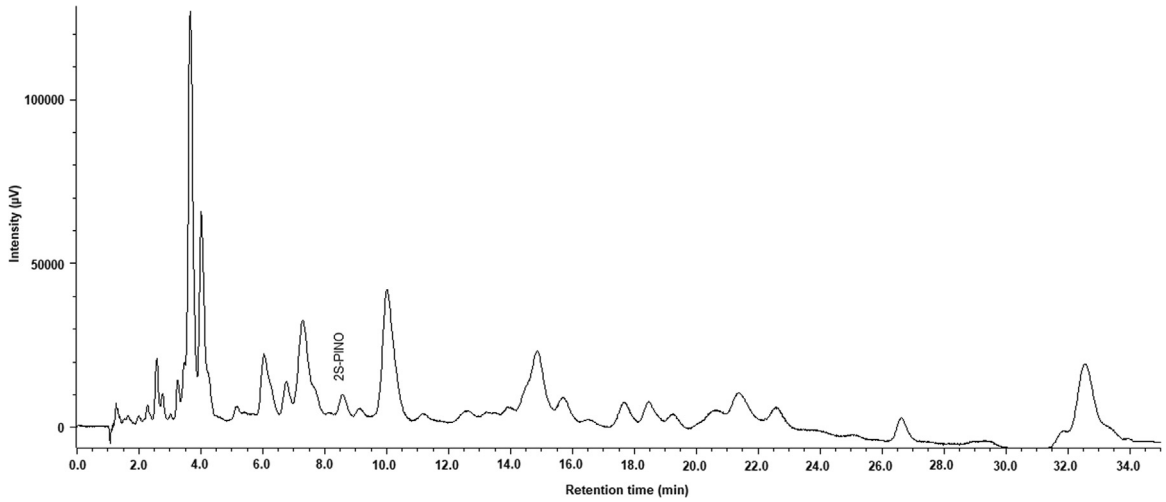

Fig. 7. Chromatogram of a bee pollen sample using the Chiralpak AD column. Detection at 280nm. See Fig. 6 for other chromatographic conditions.

samples. Peak identification was based on retention time, peak spectra and peak purity. Peak purity was determined by the software, and it was based on the comparison of the spectra recorded during the peak elution. The software calculated a match factor. Values higher than 990 indicate that the spectra are similar and the peak can be considered pure.

In Fig. 7, the chromatogram obtained for one sample is shown. As it can be seen, although several peaks appeared at similar retention times to those of the flavanones studied, only the second eluted enantiomer of pinostrobin (2S configuration) could be identified in some of the samples, more specifically in two of the non-commercial samples, M1 (80 mg/kg) and M2 (8.3 mg/kg). This is a first attempt to apply the proposed chiral SFC method to the analysis of flavanones in bee pollen. The results obtained are the preliminary ones and show that to enhance the determination of these compounds in a complex matrix, sample treatment should be improved and/or a more selective and sensitive detector (MS) should be employed. In addition, it should be remarked that the absence of these compounds in the specific samples analyzed does not imply that they could not be present in samples from different geographical and botanical origins. Indeed, the proposed SFC method, after performing the above-mentioned improvements, could be used to evaluate the potential presence of these compounds in bee pollen samples with the aim of investigating their role as markers of origin, as it seems that their presence is dependent on the geographical and botanical origin [44].

4. Conclusions

The separation of ten flavanones was successfully achieved using SFC in both chiral and achiral approaches. Considering the retention, in both cases the retention increased with the number of hydroxyl groups. It should be noted that on the Chiralpak AD column the retention was higher than on the achiral stationary phases, which made necessary the use of a higher percentage of organic modifier. This could be because the chiral stationary phase is based on a polymer (amylose) which has different types of binding sites and the chiral selectors are also inside the cavities of the polysaccharide structure, thus the number of interaction sites is higher and so is the retention. As far as elution order is concerned, on the Chiralpak AD column hesperetin enantiomers eluted later than the naringin and taxifolin ones, although the number of hydroxyl groups of hesperetin is lower. This could be due to a higher steric hindrance especially for naringin, which could decrease the interaction with the amylose structure.

Using the chiral method, a comprehensive information about the occurrence of these compounds could be obtained. Nevertheless, in some instances may not be necessary to know the enantiomeric distribution. In this case, the achiral method would be the choice. Achiral columns are widespread in the laboratories and they are cheaper than the chiral ones. The results obtained in this work showed that diol or poly(butylene terephthalate) based stationary phases provided the best results for the achiral separation, rendering DCpak PBT column the highest retentions but the lowest efficiencies, especially for 2-hydroxyflavanone, taxifolin and naringin. Concerning the chiral separation, the best results were obtained with the amylose based columns, providing Chiralpak AD the simultaneous separation of the ten pairs of enantiomers. On this column, the use of TFA as additive was necessary in order to improve the peak symmetry of taxifolin enantiomers and in all the cases, the first eluted enantiomer had the 2R configuration except for naringenin, 2-hydroxyflavanone and taxifolin for which the first eluted enantiomer was the 2S one. The application of the proposed method to the analysis of bee pollen showed that, as it was described in the literature, the predominant enantiomeric form was 2S but only pinostrobin could be determined. For a better identification of the compounds, sample treatment should be improved and/or a more selective and sensitive detector, such as MS, should be used.

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Data availability

The datasets generated during the current study are included in this published article and the Supplementary Information, or they are available from the corresponding author on reasonable request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ana M. Ares: Conceptualization, Methodology, Supervision, Writing – review & editing. **José Bernal:** Funding acquisition, Conceptualization, Supervision, Project administration, Resources, Visualization, Writing – original draft, Writing – review & editing. **Andrea Janvier:** Investigation, Visualization. **Laura Toribio:** Conceptualization, Investigation, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Data availability

The datasets generated during the current study are included in this published article and the Supplementary Information, or they are available from the corresponding author on reasonable request.

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