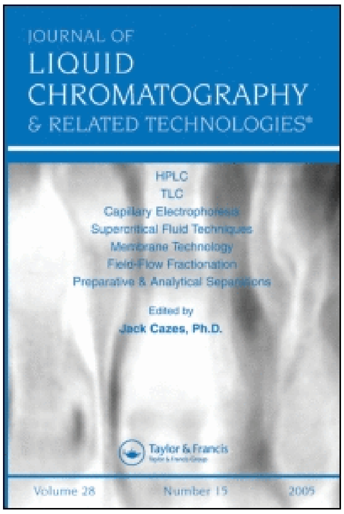

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ABSTRACT

An RP-HPLC method for the simultaneous separation of the 16 main flavonoid aglycones and 3 important glycosides present in natural samples was developed. The separation was performed on a Supelcosil LC-318 column with eluants containing water, acetonitrile, and trifluoroacetic acid. The method was successfully applied to the analysis of propolis extract, a complex natural product containing flavonoid aglycones.

Hydrolysis conditions able to convert all the flavonoid glycosides into the corresponding aglycones were optimised on different plant extracts. The chromatographic analysis supplied an accurate description of the qualitative and quantitative composition of the aglycones in these samples.

INTRODUCTION

Flavonoids are polyphenolic compounds containing multiple substituents on the 2-phenyl-benzo[α]pyran. The family includes monomeric flavanols, flavanones, anthocyanidins, flavones, and flavonols. It is well known[1,2] that most of these compounds exhibit two major absorption bands in the ultraviolet/visible region: Band I, which is found in the 320-385 nm range, and Band II in the 250-285 nm range.

They have mainly been described for their antiinflammatory,[3] antiallergic,[4] and vasoprotective[5] properties. For many flavonoids a direct free radical-scavenging activity has also been reported,[6-8] as has the ability to inhibit lipid peroxidation[9] and several important enzymes in cellular systems.[10] In recent studies, these compounds have even been evaluated as inhibitors of HIV replication.[11] Given their wide range of biological functions, there is a growing interest in isolating and separating the flavonoids from natural sources. They occur in high concentrations in natural products such as honey and propolis, and in plant extracts where they are normally found as glycosides. Although they present, from one plant to another, different spectra of glycosidation that include a large number of sugars, the flavonoid glycosides derive from only a few aglycones. In the literature, the conversion of glycosides into aglycones obtained by acid hydrolysis has been described to quantify the aglycones in plant extracts by their spectrophotometric detection as aluminium chloride chelate complexes.[12]

An HPLC method for the simultaneous separation of the most important flavonoid aglycones and 3 main glycosides of biological interest was developed. Our aim was to achieve a successful separation and identification of aglycones from different kinds of natural samples, including very complex ones such as propolis extract.

Specific hydrolysis conditions for the total conversion of the glycosides into the corresponding aglycones were tested and optimised in seven plant extracts. HPLC analysis provides a more detailed description of the qualitative and quantitative composition of the aglycones in these samples than that obtained by spectrophotometric detection.

EXPERIMENTAL

Chemicals and Reagents

Flavonoid standards of the highest grade available were purchased from Sigma (St.Louis, MO, USA) and Fluka (Buchs, Switzerland). Anhydrous ethyl alcohol for HPLC, acetonitrile, and hydrochloric acid (analytical reagent-grade) were obtained from Carlo Erba, Italy. *N,N*-dimethylformamid and trifluoracetic acid (TFA) were purchased from Fluka and HPLC-grade methanol was from

PROLABO (Fontenay S/Bois, France). The water used in the mobile phase was double-distilled and filtered through a 0.22 μm filter (Millipore, Bedford, USA) in our laboratory. Stock solutions of flavonoids were prepared by dissolving a weighed mass of dried material in ethyl alcohol, which was then stored at 4°C. The only exception, hesperidin, was dissolved in *N,N*-dimethylformamid.

Apparatus

A liquid chromatographic system from Beckman, the Gold System, (Beckman, Berkely, CA, USA) was used throughout this work. The HPLC apparatus consisted of two model 125 pumps, a Model 7010 sample injection valve (Rheodyne, CA, USA), a 5 μL injection loop and a 168 diode-array detector. The system was interfaced with a personal computer utilising System Gold software for control and data collection. A 5 μm Supelcosil LC-318 analytical column (25 cm x0 4.6 mm I.D.) (Supelco, Bellafonte, PA, USA), protected with a guard column (2 cm x0 2.1 mm I.D.), 40 μm Pelliguard LC-18 pellicular packing resin, was used.

HPLC Conditions

The mobile phase used for the separation of flavonoids consisted of 0.1% (v/v) TFA (Buffer A), and 0.1% (v/v) TFA in acetonitrile (Buffer B).

The following gradient of Buffer B was used: 10-50% in 35 min, 50-100% in 1 min, and 5 min at 100%. The initial conditions were then restored in 7 min. The flow rate was 1.2 mL/min. Detection was performed at 254 nm and 340 nm and the diode-array detector was set from 190 nm to 400 nm.

Hydrolysis Conditions and Sample Preparation

For each of the flavonoid aglycones, six micro tubes with screw caps, containig 50 μL of a 1 M standard solution, 250 μL of methanol and 100 μL of 1M HCl, were prepared. At one-hour intervals during the hydrolysis, one micro tube was removed and neutralised with 100 mL of 1.5 mM Tris, pH 8.8, centrifuged and injected to control the stability of the aglycones over time.

Three grams each of dried *Matricaria* c*hamomilla, Betula alba, Ginkgo biloba, Achillea millefolium, Passiflora incarnata, Malva sylvestris* and *Calendula officinalis* were extracted with 30 mL of 60% ethanol, in the dark and at room temperature for 5 days. The best conditions for the acid hydrolysis of the seven plant extracts were determined utilising the same procedure and amounts used for the standards. Dewaxed ethanolic extract of propolis (66,8%) was a commercially available product.

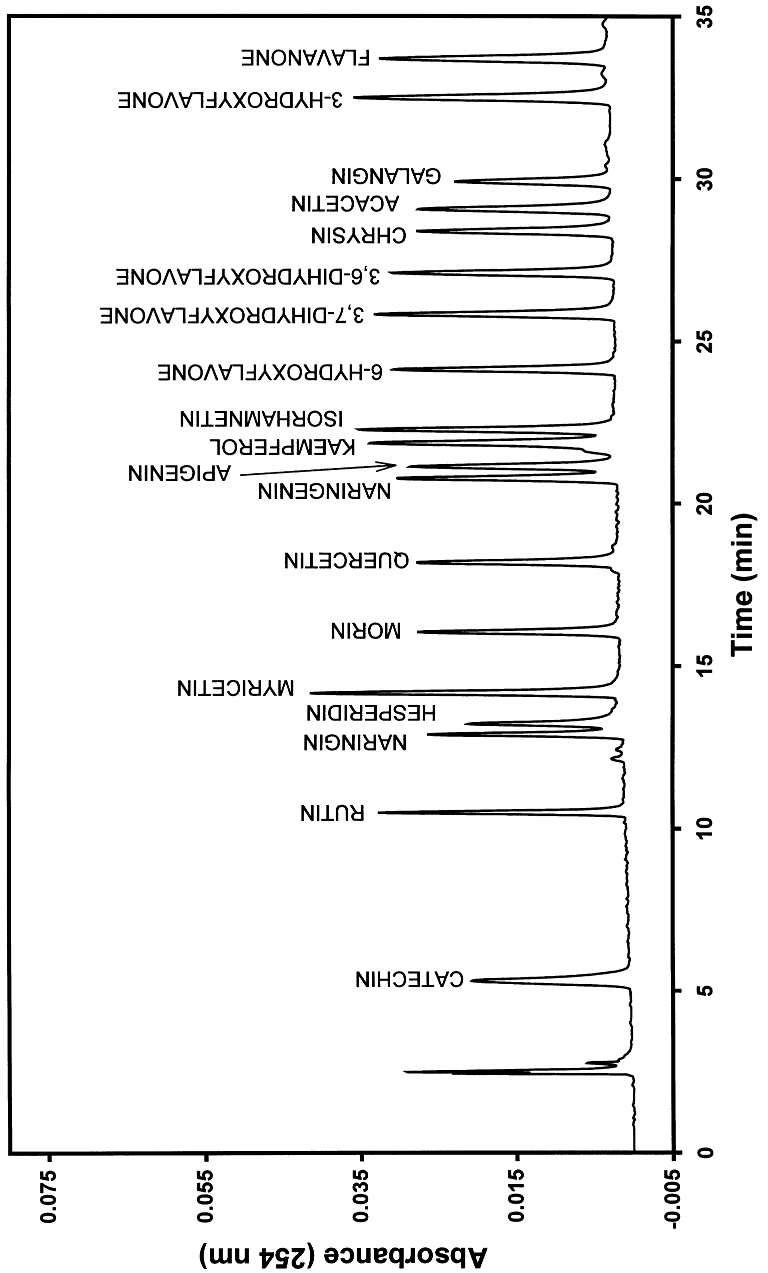

Figure 1. Reversed-phase HPLC on a 5 µm (25 cm × 4.6 mm i. d.) Supelcosil LC-318 column of a standard mixture of 16 flavonoid aglycones and 3 glycosides, using the chromatographic conditions reported under "Materials and Methods". The amount injected was 5 µL and the compounds were monitored at 254 nm.

Calibration Curves

Calibration curves were constructed using a linear regression method. Solutions of various concentrations were prepared and five determinations were carried out for each sample. By plotting the area responses versus the amount of each reference substance, a linear response was observed in the concentration range examined (0.02-0.5 mM).

RESULTS AND DISCUSSION

HPLC Separation of Flavonoids

The aim of this work was to develop a HPLC method allowing the simultaneous separation of the flavonoid aglycones usually present in natural products.

Although various methods including paper chromatography[13] and thin-layer chromatography (TLC)[14,15] have been reported in the literature, RP-HPLC is generally considered the method of choice for the separation of flavonoid compounds.

As shown in Figure 1, the RP-HPLC method used allowed the complete separation of a standard mixture of 16 different flavonoid aglycones and 3 glycosides, all of wide biological interest, in 35 min. As can be seen, the gradient used does not cause an excessive baseline drift. This factor, along with the high efficiency of the column, enhances the sensitivity of the method and as a consequence also permits the easy determination of the compounds present in low concentrations. The sensitivity of the method was tested by determining the amount of sample that must be injected into the column to produce a peak approximately three times the noise level. The detection limit was found to range from 0.135 mg/L for quercetin (2 pmol injected) to 0.580 mg/L for catechin (10 pmol injected).

The reproducibility of the method was tested by injecting 5 µL of a standard mixture ten times. As reported in Table 1, good reproducibility can be observed in the peak area and in retention time.

Given the fact that flavonoids exhibit absorption bands in the 320-385 nm range and also in the 250-285 nm range, we examined, at the same time, the UV-detection of samples at 340 nm and at 254 nm. Utilising the diode-array detection of peaks we obtained the on-line spectrum analysis, able to confirm the identification of the aglycones and even to distinguish flavonoids from the phenolic compounds since both of them present characteristic UV-absorbance.

Table 1

Reproducibility of Retention Times and Peak Areas of Flavonoids Obtained by Injecting 5 μL of a Standard Mixture Ten Times

Flavonoids	Retention Time (min)	SD	CV (%)	Peak Area	SD	CV (%)
Catechin	5.38	0.126	2.35	10.89	0.527	4.83
Rutin	11.05	0.113	1.03	2.66	1.139	5.24
Naringin	13.43	0.099	0.74	2.66	0.148	5.57
Hesperidin	13.88	0.079	0.57	3.26	0.179	5.49
Myricetin	14.31	0.181	1.26	1.93	0.117	6.07
Morin	16.02	0.189	1.18	6.64	0.348	5.24
Quercetin	18.17	0.189	1.04	3.35	0.175	5.23
Naringenin	20.54	0.220	1.07	4.08	0.202	4.94
Apigenin	21.16	0.183	0.86	4.77	0.233	4.88
Kaempferol	21.72	0.215	0.99	3.17	0.169	5.34
Isorhamnetin	22.53	0.149	0.66	4.32	0.212	4.91
6-Hydroxyflavone	24.43	0.125	0.51	3.47	0.177	5.08
3,7-Dihydroxyflavone	25.89	0.161	0.62	3.57	0.192	5.38
3,6-Dihydroxyflavone	27.19	0.152	0.56	3.96	0.194	4.91
Chrysin	28.26	0.186	0.66	3.18	0.168	5.29
Acacetin	28.93	0.187	0.65	2.72	0.136	5.01
Galangin	29.65	0.194	0.66	2.64	0.147	5.56
3-Hydroxyflavone	32.36	0.149	0.46	5.57	0.270	4.85
Flavanone	33.35	0.172	0.52	5.92	0.281	4.74

Analysis of Propolis Extract

The method described allows the simultaneous determination of many flavonoid aglycones and thus can be utilised to analyse samples with different flavonoid composition.

In order to test the efficacy of the method, we applied it to the analysis of propolis extract, a natural complex sample. Since ancient times, this extract has been used as a natural medicine because of its remarkable pharmacological properties, which include anti-inflammatory, antibacterial, antifungal, antiviral, and antioxidant activities.[16-19] These effects have been associated with the main organic constituents of propolis, which are flavonoid compounds and caffeic acid esters.

As shown in the chromatogram in Figure 2, the peaks are well resolved under the conditions employed. The absorption spectra resulting from diode-array detection were analysed to distinguish between the peaks due to the flavonoids and those due to other UV-absorbing components, and it was found that many peaks present the characteristic spectrum of caffeic acid and, thus, they all can be

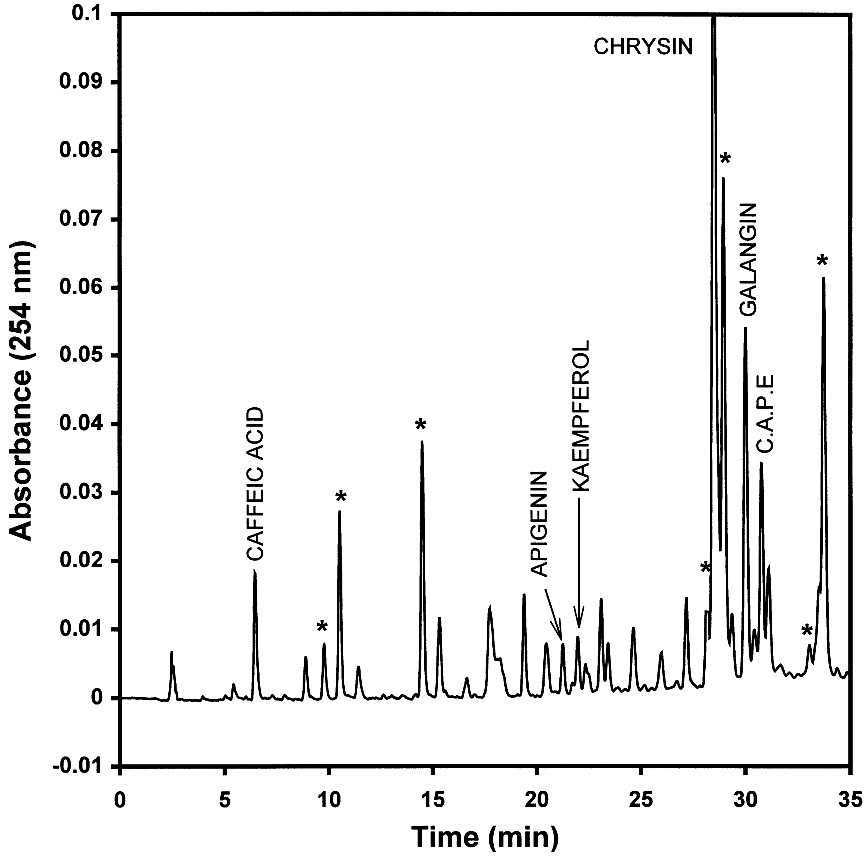

Figure 2. Reversed-phase determination of flavonoids in a hydroalcoholic propolis extract (66.8%). 5 μL of the extract, diluted 1:250, were injected under the same chromatographic conditions used for the separation shown in Figure 1. *Caffeic acid derivatives.

considered substituents of it. These peaks are labelled in the chromatogram with a star symbol; and it is evident that they make up a large part of the propolis extract. Furthermore, we were able to identify the caffeic acid and the caffeic acid phenethylester (C.A.P.E.) by co-chromatography with internal standards. Their presence in propolis extracts may explain its activity as lipooxygenase inhibitor.[20] In addition, it has been reported that C.A.P.E. induces differential growth inhibition of cancer cells compared to normal cells[21] and modulates oxidative stress in several systems.[22,23]

Table 2

Flavonoid Aglycone Content in Propolis Extract

Flavonoids	(mg/mL)
Kaempferol	0.537
Apigenin	0.597
Chrysin	11.813
Galangin	5.513

The propolis extract contained only flavonoid aglycones and those identified are: apigenin, chrysin, galangin and kaempferol. The identification was made by comparing the retention times and by co-chromatography with standards; moreover, the correlation coefficients, assigned to each peak by comparing the on-line UV spectra with those of the pure standards, reached 0.99. The concentrations of flavonoids in propolis extract are reported in Table 2.

Analysis of Different Plant Extracts

The advantages of using HPLC analysis to determine the qualitative and quantitative composition of the aglycones in plant extracts instead of the spectrophotometric detection method described in several Pharmacopoeias have already been underlined by Hasler. For this purpose, optimal hydrolysis conditions for the complete conversion of glycosides into the corresponding aglycones are necessary. At the same time, during the hydrolysis all the phenolic acids and their derivatives are broken down, co-elution problems during the HPLC separation of the sample become unusual and the chromatogram can be easily examined. For all these reasons, we tested and optimised specific hydrolysis conditions for some plant extracts.

Betula alba, Ginkgo biloba, Passiflora incarnata, *Calendula officinalis*, *Matricaria chamomilla, Achillea millefolium, Malva sylvestris* extracts were prepared with 60% ethanol with the aim of analysing the hydroalcoholic solution usually utilised in herbal remedies.

The stability of the flavonoids under hydrolysis was verified as described in "Materials and Methods." After six hours, all of the aglycones included in the standard mixture were found to be stable, with the exception of flavanone and catechin, which were found to have already degraded after only one hour.

Figure 3 (right). Reversed-phase determination of flavonoid aglycones in the *Betula alba*, *Calendula officinalis* and *Ginkgo biloba* extracts after the hydrolysis conditions reported under "Materials and Methods". The amount injected was 5 μL.

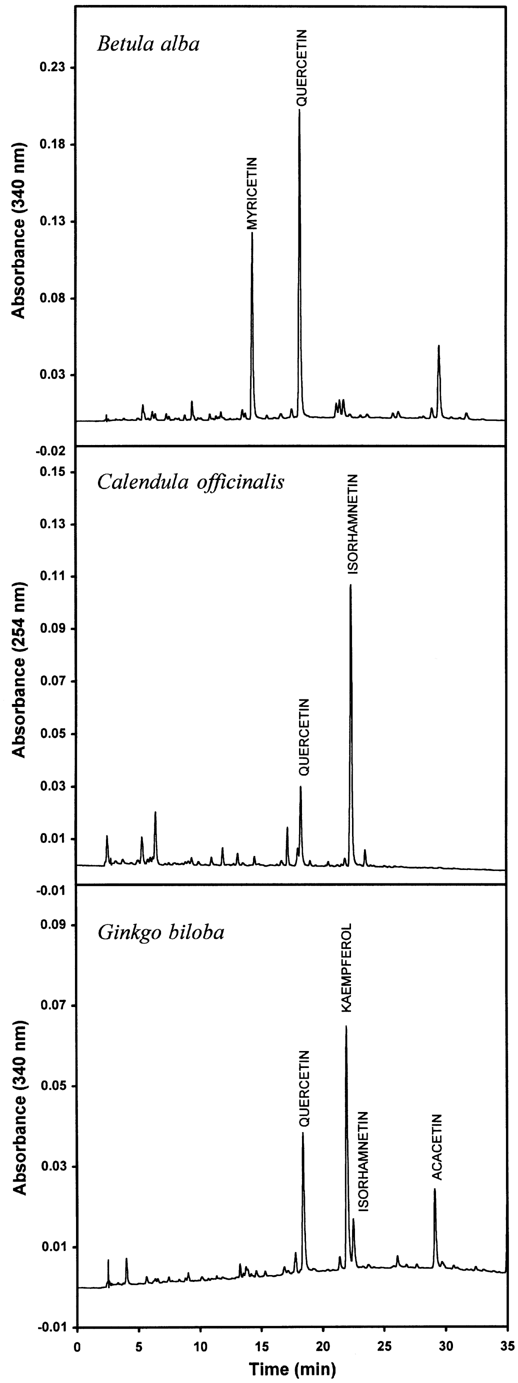
Betula alba
Absorbance (340 nm)
0.23
0.18
0.13
0.08
0.03
-0.02
MYRICETIN
QUERCETIN
Calendula officinalis
Absorbance (254 nm)
0.15
0.13
0.11
0.09
0.07
0.05
0.03
0.01
-0.01
QUERCETIN
ISORHAMNETIN
Ginkgo biloba
Absorbance (340 nm)
0.09
0.07
0.05
0.03
0.01
-0.01
QUERCETIN
KAEMPFEROL
ISORHAMNETIN
ACACETIN
0 5 10 15 20 25 30 35
Time (min)

Table 3

Flavonoid Aglycone Content (mg/mL) in Different Plant Extracts

Plant	Isorhamnetin	Quercetin	Myricetin	Kaempferol	Apigenin	Acacetin
Achillea Millefolium (flowers)	----	3.151	----	----	0.0504	----
Betula alba (leaves)	----	0.429	0.067	----	----	----
Calendula officinalis (flowers)	0.686	0.142	----	----	----	----
Matricaria chamomilla (flowers)	----	0.192			0.147	----
Ginko biloba (leaves)	0.0349	0.0714	----	0.122	----	0.0355

Under these conditions the *Betula alba, Ginkgo biloba, Passiflora incarnata,* and *Calendula officinalis* extracts were completely hydrolysed after one hour, while for the *Matricaria chamomilla, Achillea millefolium,* and *Malva sylvestris* extracts more drastic conditions were needed (5 hours). In Figure 3, three representative chromatograms of the plant extracts after hydrolysis are reported.

The identification of peaks was performed by comparison of the retention times and co-chromatography with standards. Moreover, the identity was confirmed by comparing their on-line UV spectra with those of the corresponding standards. The correlation coefficients assigned to each peak reached a value higher than 0.90.

Table 3 reports the flavonoid aglycones present in each extract and their amounts. *Passiflora incarnata* (leaves) and *Malva sylvestris* (leaves and flowers) extracts are not reported in the table because none of the peaks analysed corresponded to the flavonoid standards considered in this paper. Under the same analytical conditions, flavonoid glycosides are also sharply separated and can be identified after collecting and hydrolysing the peaks. The corresponding aglycones can be detected by HPLC, while the sugars can be identified by gas chromatography as acetyl derivatives.[24]

CONCLUSIONS

Recently, much attention has been given to the beneficial effects of the flavonoids present in natural products and to their possible role in disease prevention.

The RP-HPLC method described in this study allows the simultaneous determination of the most important aglycones of biological and pharmacological interest present in natural samples. We successfully utilised it to evaluate the aglycone content in propolis extract. By analysing the absorption spectra of the peaks, it was also possible to note the great amount of caffeic acid and its derivatives present in the sample. Caffeic acid and caffeic acid phenethylester were identified. The high sensitivity of the method also allowed an accurate description of the qualitative and quantitative composition of the aglycones in plant extracts. To reach this goal, we needed to optimise specific hydrolysis conditions able to quantitatively convert all the flavonoid glycosides to the corresponding aglycones in the different plant samples. The hydrolysis was prolonged until the total degradation of the phenolic compounds was obtained, to assure the purity of the peaks used for the quantitative determination of the aglycones.

In conclusion, the data obtained in this study demonstrate that this method can be utilised to obtain a very detailed description of the aglycone content in natural products and is of even greater value for the analysis of samples or mixtures with a complex and unknown content of flavonoids.

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