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Note

High-performance liquid chromatography of biflavones from *Ginkgo biloba* L.

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The ability of high-performance liquid chromatography (HPLC) to resolve complex natural mixtures of flavonoids is well established[1-5]. However, the use of this technique for the analysis of biflavonoids has not been much investigated[6]. This work was carried out in order to develop a rapid and reliable method for the identification and determination of biflavones in complex plants extracts. Such a method may be useful for observing qualitative and quantitative changes in biflavonoid contents, particularly in biosynthetic studies.

The leaves of *Ginkgo biloba* L. contain a biflavone mixture of the amento-flavone type. They differ from each other in the number and position of the methoxyl groups (Table I). This paper describes the HPLC determination of the relative abundance of the main substances in a purified mixture of biflavones isolated from *Ginkgo biloba* L.[7].

TABLE I

STRUCTURES OF THE BIFLAVONES OF THE AMENTOFLAVONE TYPE PRESENT IN THE LEAVES OF *GINKGO BILOBA* L.
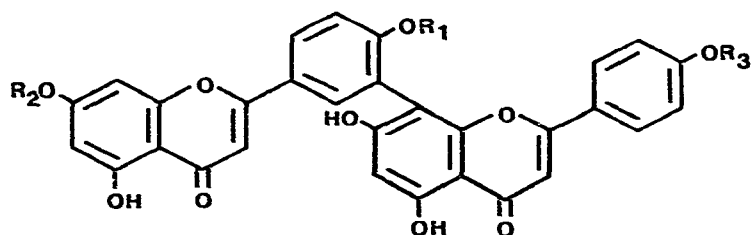
Peak No.	Compound	R_1	R_2	R_3
1	Amentoflavone	H	H	H
2	Bilobetin	CH$_3$	H	H
3	Isoginkgetin	CH$_3$	H	CH$_3$
4	Ginkgetin	CH$_3$	CH$_3$	H
5	Sciadopitysin	CH$_3$	CH$_3$	CH$_3$

EXPERIMENTAL

Solvents

Acetonitrile, chloroform and tetrahydrofuran (analytical-reagent grade) (Merck, Darmstadt. G.F.R.) were filtered with a 0.5-μm filter (Millipore, Bedford. MA, U.S.A.). Acetic acid (analytical-reagent grade) was obtained from Merck.

Deionized water was filtered with a 0.45-μm Millipore filter.

Standards

A crude mixture of biflavones from *Ginkgo biloba* L. and prepared authentic samples of bilobetin, ginkgetin, isoginkgetin and sciadopitysin, were extracted and purified according to the reported techniques[7,8]. Amentoflavone was prepared by demethylation of sciadopitysin[9]. α-Naphthoflavone was supplied by Carl Roth (Karlsruhe, G.F.R.).

Instrumentation

The separation of biflavones was carried out at room temperature with a 3B liquid chromatograph with a programmable gradient (Perkin-Elmer, Norwalk, CT, U.S.A.), equipped with a Rheodyne Model 7125 injection valve (Rheodyne, Berkeley, CA, U.S.A.) with a 20-μl loop. The detection of peaks was carried out at 330 nm with an LC-75 variable-wavelength spectrophotometric detector connected to an LC-75 autocontrol module (Perkin-Elmer). The baseline was corrected for gradient absorption. An SP-4100 integrator (Spectra-Physics, Santa Clara, CA, U.S.A.) was used for measuring retention times and peaks areas.

The columns tested for the resolution of the biflavones mixture were as follows: Perkin-Elmer columns:

Silica A (10 μm) (25 cm × 0.26 cm I.D.).

C_8 (10 μm) (25 cm × 0.46 cm I.D.).

C_{18} (10 μm) (25 cm × 0.46 cm I.D.).

Whatman column:

Partisil M9-ODS 2 (10 μm) (25 cm × 0.94 cm I.D.).

Merck Hibar Columns:

LiChrosorb CN (10 μm) (25 cm × 0.4 cm I.D.).

LiChrosorb Diol (10 μm) (25 cm × 0.4 cm I.D.).

LiChrosorb RP-18 (5 μm) (25 cm × 0.4 cm I.D.).

Chromatographic conditions

It was found that the best separation of biflavones was obtained with the following two systems.

System 1. The mobile phase used with the Perkin-Elmer C_{18} column was a mixture of acetonitrile (stronger solvent = solvent A) and water (weaker solvent = solvent B), each containing 5 % of acetic acid. The initial composition of A–B (60:40) was kept constant for 4 min, then the percentage of A was increased linearly during 10 min to reach 75 %. This composition (75:25) was allowed to remain constant for 10 min and then the system was allowed to reach equilibrium at least 10 min before the next injection. The flow-rate was 1.5 ml/min, with a column head pressure of 8 MPa.

System 2. The mobile phase used with the LiChrosorb Diol column was a

mixture of tetrahydrofuran (solvent A) and chloroform (solvent B). The initial composition of A–B was 0.1:99.9. The percentage of A was increased linearly to reach 5% in 4 min, then 50% in 10 min. This composition (50:50) was kept constant for 8 min, then the system was allowed to reach equilibrium after 10 min under the initial conditions. The flow-rate was 1 ml/min, with a column head pressure of 1.3 MPa.

Identification of peaks

The identification of the resolved peaks was carried out using the co-injection technique with authentic samples.

Quantitative determination

The internal standard technique was chosen for quantitative determinations, using α-naphthoflavone as standard.

Linearity of response. The linearity of the detector response was checked for each of the biflavones and the internal standard by injecting known amounts of pure samples, integral measurement of the peaks areas and plotting these areas against mass injected.

Limit of detection. This was determined as the smallest amount of biflavone that gave a peak height of ten times the peak threshold with the detector and integrator set at the highest sensitivity.

Response factors. The response factor of a constituent (i) was determined by the integrator according to the equation

$$RF_i = \frac{C_i}{C_{IS}} \cdot \frac{A_{IS}}{A_i}$$

where

RF_i = response factor of constituent i;
C_i = concentration of constituent i;
C_{IS} = concentration of internal standard;
A_i = peak area of constituent i;
A_{IS} = peak area of internal standard.

Quantitative determination of biflavones in the crude mixture extracted from the leaves of Ginkgo biloba L. A certain concentration of both the internal standard and the crude mixture in tetrahydrofuran–chloroform (10:90) was chromatographed in order to determine the percentage of the four main biflavones in the mixture.

RESULTS AND DISCUSSION

Selection of the system

It was found that the best separation was achieved by the following two systems.

Reversed-phase chromatography with the C_8 and C_{18} bonded columns. The chromatogram illustrated in Fig. 1 shows the resolution obtained with the Perkin-Elmer C_{18} column and the gradient system 1. The peaks are strongly tailing and the two isomers, ginkgetin and isoginkgetin, were not separated at all. Further attempts were made to improve these results, using other elution conditions with the same column
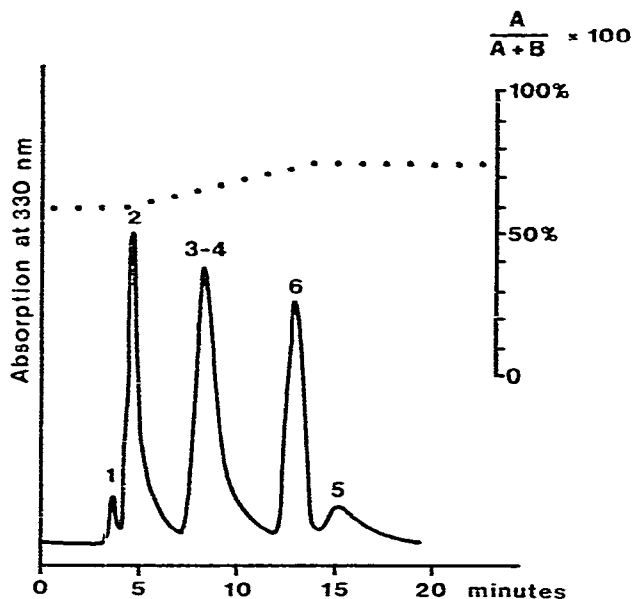
$$\frac{A}{A+B} \times 100$$

Fig. 1. HPLC trace of the biflavones from *Ginkgo biloba* L. using the C_{18} (10 μm) column. Mobile phase: A = acetonitrile–acetic acid (95:5); B = water–acetic acid (95:5). Flow-rate: 1.5 ml/min.

or other reversed-phase columns, but they were not successful. Peak 1 could be amentoflavone (the retention time and UV spectrum are identical with those of an authentic sample).

Normal-phase chromatography with the LiChrosorb Diol bonded column. This allowed a satisfactory separation as shown in Fig. 2. The four main constituants of the mixture and the internal standard gave symmetrical and well resolved peaks, with the retention times listed in Table II. The high reproductibility of the separation is shown by the relative standard deviations of the retention times.

TABLE II

RETENTION TIMES OF THE BIFLAVONES FROM *GINKGO BILOBA* L.

For conditions, see Fig. 2.

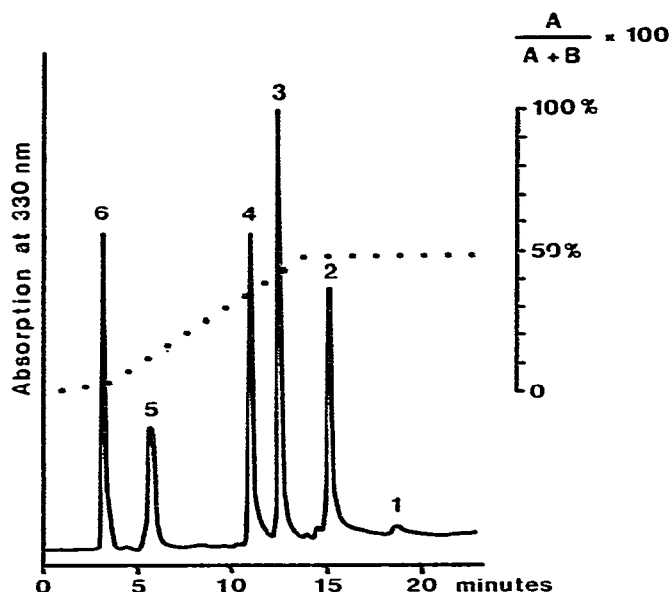| Compound	Peak No.	Retention time* (min)	Standard deviation	Relative standard deviation of the retention time (%)
α-Naphthoflavone	6	3.06	0.010	0.34
Sciadopitysin	5	5.44	0.021	0.38
Ginkgetin	4	10.62	0.051	0.48
Isoginkgetin	3	12.11	0.049	0.41
Bilobetin	2	14.90	0.081	0.54

* Average values of 21 measurements.

Fig. 2. HPLC trace of the biflavones from *Ginkgo biloba* L. using the LiChrosorb Diol (10 μm) column. Mobile phase: A = tetrahydrofuran; B = chloroform. Flow-rate: 1 ml/min.

It was also found that neither the silica nor the LiChrosorb CN bonded columns gave any separation under any elution conditions tried.

Quantitative determination

According to the above study, the quantitative determination was performed using the LiChrosorb Diol bonded column and elution system 2.

Calibration graphs. In order to check the linear relationship between UV absorption intensity and the amount of biflavone, calibration graphs were established for each of the biflavones and also for α-naphthoflavone. The results obtained are shown in Fig. 3. For all least-squares analyses, the correlation coefficients are approximatively unity.

Sensitivity. Under the experimental conditions used, it was found that the least detectable amount for each of the biflavones was about 10 ng.

TABLE III

RESULTS OF THE DETERMINATION BY HPLC OF THE MAIN BIFLAVONES IN THE MIXTURE EXTRACTED FROM *GINKGO BILOBA* L.

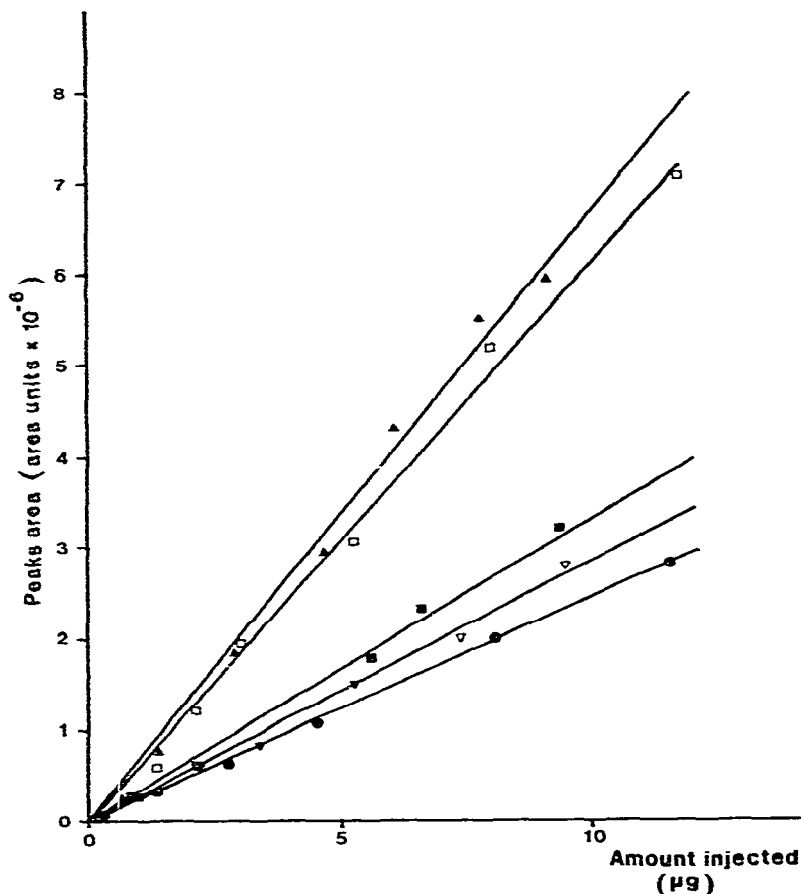| Compound	Response factor	Proportion in the mixture (%)	Standard deviation (%)	Relative standard deviation (%)
Sciadopitysin	0.373	8.5	0.394	4.6
Ginkgetin	0.893	40.5	0.88	2.2
Isoginkgetin	0.757	39	0.6	1.5
Bilobetin	0.409	12	1.6	13

Fig. 3. Calibration graphs of the reference substances (each data point represents a single determination). Correlation coefficients: sciadopitysin (▲), 0.996; ginkgetin (▽), 0.994; isoginkgetin (▉), 0.997; bilobetin (□), 0.998; α-naphthoflavone (●), 0.999.

Determination of the relative percentage of the biflavones in the crude mixture. The response factors for each of the biflavones were separately determined using α-naphthoflavone as a standard, from which the percentage of each was determined in the mixture. The values obtained are given in Table III.

CONCLUSION

It is clear that HPLC is a useful method for the separation of biflavones. It would be effective in the analysis of complex plant extracts.

Using HPLC, amentoflavone was detected for the first time in *Ginkgo biloba* L. The isolation and the spectral analysis will be investigated further in order to confirm the full identification of this peak.

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