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Identification and determination of the flavonoids from *Ginkgo biloba* by high-performance liquid chromatography

A. Hasler and O. Sticher

Department of Pharmacy, Swiss Federal Institute of Technology (ETH) Zurich, CH-8092 Zurich (Switzerland)

B. Meier

Zeller AG, CH-8590 Romanshorn (Switzerland)

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ABSTRACT

Qualitative and quantitative reversed-phase high-performance liquid chromatographic methods have been developed for the separation and determination of the flavonoids found in the leaves and therapeutically used extracts of *Ginkgo biloba*. The first method includes hydrolysis of the flavonoids and subsequent quantitative chromatographic assay of the obtained aglycones and qualitative analysis of the biflavones. The second method is a "fingerprint" procedure to identify unambiguously 33 flavonoids of *Ginkgo biloba*.

INTRODUCTION

Extracts of the leaves of *Ginkgo biloba* L. are used as phytomedicines to increase peripheral and cerebral blood flow. The Ginkgo extracts contain as active compounds flavonoids and terpene lactones (ginkgolides and bilobalide). They show effects on vascular and cerebral metabolic processes and they inhibit platelet-activating factor [1–3].

There have been only a few investigations on the separation and determination of flavonoids in *Ginkgo biloba*. Briançon-Scheid and co-workers [4,5] and with superior results Pietta *et al.* [6] reported high-performance liquid chromatographic (HPLC) separations of biflavones. According to present knowledge, the biflavones represent characteristic markers for the identification of Ginkgo leaves, but they do not show the desired activity and are therefore not suitable for standardization.

The great variety of genuine flavonoid glycosides can be reduced by hydrolysis to the three major aglycones isorhamnetin, kaempferol and quercetin. The minor flavonoids in Ginkgo, apigenin, luteolin and myricetin, can also be identified, if necessary, by our previously described method [7]. The efficient chromatographic procedure is advantageous compared with a similar method described by Wagner *et al.* [8], especially for serial analyses in quality control and stability tests of herbal remedies. The reduction of the genuine compounds by hydrolysis has already been established in the quality control of phytopharmaceuticals, *e.g.*, for the standardization of willow preparations [9].

To identify extracted plant material as a Ginkgo preparation, the erstwhile proposed quantification [7] is, owing to the omnipresent flavonoid aglycones, not sufficient. Therefore, we have developed a chromatographic procedure to determine the

Correspondence to: Dr. O. Sticher, Department of Pharmacy, Swiss Federal Institute of Technology (ETH) Zürich, CH-8092 Zürich, Switzerland.

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aglycones and to qualify the characteristic biflavones of *Ginkgo biloba* in one run. To obtain more information about the genuine flavonoid pattern of *Ginkgo biloba* we have also elaborated a "fingerprint" analysis. Fingerprint chromatography was introduced, and subsequently accepted by WHO [10], several years ago for the quality control of herbal medicines. The possibility of on-line diode-array detection offered real progress in fingerprint chromatography [11]. Wagner *et al.* [8] described a fingerprint HPLC separation of Ginkgo leaves. Twenty peaks were detected within 55 min but only two flavonoids (rutin and astragalin) and the four biflavones (bilobetin, ginkgetin/isoginkgetin and sciadopitysin), ginkgol, shikimic acid and 6-hydroxykynurenic acid could be assigned. Recently, Pietta *et al.* [12] reported an HPLC method for the separation of fifteen known Ginkgo flavonoids within 50 min. The assignment was done with reference compounds for six flavonoids. The other flavonoid glycosides were assigned by their UV–VIS spectra using a diode-array detector, although their absorptions were less than 10 milliabsorption units (mAU). Such assignments, without any other investigations, are very speculative. In both methods the separation and identification of the flavonoids are not very developed and not complete. Lobstein *et al.* [13] used gradient elution with acetonitrile and 0.1 *N* phosphoric acid to separate flavonoids and biflavones within 50 min. No diode-array detector was coupled to the HPLC system. Additionally, the lack of reference compounds prevented complete peak assignment. Kaempferol and quercetin 3-O-coumaroyl glucorhamnoside and the biflavones in leaves were determined. A small study of seasonal variations was carried out.

In summary, there are strategies, but no satisfactory method, for the determination of flavonoids in Ginkgo leaves and Ginkgo preparations for research teams and pharmaceutical companies. These are necessary to fulfil the demands of new guidelines for the assessment of herbal medicines, as proposed by the WHO in Munich in 1991, for example [10]. The isolation of 22 flavonoids, five of which have not been described previously and three others which have not been previously detected in Ginkgo leaves, during our research with *Ginkgo biloba* [14,15] stimulated us to develop effective HPLC methods. In this paper we describe the HPLC determination of flavonols after hydrolysis together with the separation of biflavones in one run and the fingerprint HPLC separation of 33 flavonoids in leaves and in therapeutically used extracts of *Ginkgo biloba.*

EXPERIMENTAL

Plant materials and plant extracts

Ginkgo leaves were purchased from several different dealers (Dixa, St. Gallen, Switzerland; Hänseler, Herisau, Switzerland; Siegfried, Zofingen, Switzerland) and collected from a female tree in Zurich (Mythenquai) from mid-May to mid-November, 1988. Plant extracts were either bought from Flachsmann (Zurich, Switzerland) or provided by Zeller (Romanshorn, Switzerland).

Drying

After collection, the leaves were immediately dried, for 72 h at 35°C in a Salvis TSK2 HL dryer (Salvis, Emmenbrücke, Switzerland) with forced ventilation.

Standards and solvents

The isolation and structure elucidation of all the flavonoid glycosides were carried out in our laboratory [14,15]. The aglycones (Rotichrom HPLC grade) were purchased from Roth (Karlsruhe, Germany) and the biflavones were provided by Dr. Willmar Schwabe (Karlsruhe, Germany). All the organic solvents used were of HPLC grade (Romil Chemicals, Shepshed, UK). Orthophosphoric acid (analytical-reagent grade) was obtained from Fluka (Buchs, Switzerland). Pure water was delivered by a NANOpure Cartridge System (Skan, Basle-Allschwil, Switzerland). Bond Elut C_{18} (500 mg) disposable extraction columns (Analytichem International, Harbor City, CA, USA) were used for sample clean-up.

Instrumentation and columns

All separations were carried out with a Hewlett-Packard system (Model 79994A Analytical Workstation, Model 1090 liquid chromatograph, Model 1040 diode-array detector). A Knauer (Berlin, Germany) prepacked column cartridge (100 × 4 mm I.D.) filled with Nucleosil 100-C_{18}, 3 μm (Machery–Nagel, Düren, Germany) was used.

TABLE I

MOBILE PHASE GRADIENT IN METHOD A

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
0.01	22.5	22.5	55.0
9.50	22.5	22.5	55.0
9.51	25.0	25.0	50.0
17.00	25.0	25.0	50.0
17.01	30.0	30.0	40.0
20.00	30.0	30.0	40.0

Chromatographic procedures

Method A. The mobile phase consisted of solvent A (methanol), solvent B [tetrahydrofuran (THF)] and solvent C (0.5% orthophosphoric acid) with the gradient shown in Table I. The flow-rate was 1 ml/min, the column temperature 30.0°C, the injection volume 10 μl and detection was effected at 370 nm.

Method B. The mobile phase consisted of solvent A [isopropanol–THF (25:65)], solvent B (acetonitrile) and solvent C (0.5% orthophosphoric acid) with the gradient shown in Table II. The flow-rate was 1 ml/min, the column temperature 30°C, the injection volume 10 μl and detection was effected at 350 nm.

Sample preparation

Method A. A 4-g amount of dried and pulverized plant material or a 2-g amount of dried plant extract was refluxed with 70 ml of methanol and 10 ml of 25% hydrochloric acid for 60 min. After cooling, the solution was filtered through a glass filter (G3, pore size 16–40 μm; Schott, Jena, Germany) covered with a filter-paper (LS 14, pore size 4.4 μm; Schleicher & Schüll, Feldbach, Switzerland). The supernatant was washed with 100 ml of methanol. The solution was evaporated under vacuum to about 80 ml and then diluted to 100 ml with methanol in a volumetric flask. A 5-ml volume of this solution was filtered through a Bond Elut C_{18} cartridge that was equilibrated with methanol. The cartridge was washed with 4 ml of methanol and the solution was diluted to 10 ml with methanol in a volumetric flask. A 10-μl volume of this solution was injected into the HPLC system.

TABLE II

MOBILE PHASE GRADIENT IN METHOD B

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
0.01	15.0	1.5	83.5
7.00	15.0	1.5	83.5
7.01	12.0	5.0	83.0
12.00	12.0	5.0	83.0
16.00	15.0	13.0	72.0
20.00	5.0	25.0	70.0
20.01	5.0	30.0	65.0
24.00	8.0	42.0	50.0
24.01	0.0	48.0	52.0
30.00	0.0	78.0	22.0

Method B. A 4-g amount of dried and pulverized plant material or a 2-g amount of dried plant extract was extracted using a Polytron PT-MR 3000 high-speed mixer (20 000 rpm) (Kinematica, Littau, Switzerland) with 50 ml of 80% ethanol for 2 min. The solution was filtered through a glass filter (G3, pore size 16–40 μm; Schott) covered with a filter-paper (LS 14, pore size 4.4 μm; Schleicher & Schüll). The supernatant was extracted with the Polytron a second time with 30 ml of 80% ethanol for 1 min and then washed with 20 ml of 80% ethanol. The solution was evaporated under vacuum to about 40 ml and then diluted to 50 ml with 80% ethanol in a volumetric flask. A 5-ml volume of this solution was filtered through a Bond Elut C_{18} cartridge that was equilibrated with 80% ethanol. The cartridge was washed with 4 ml of methanol and the solution was diluted to 10 ml with 80% ethanol in a volumetric flask. A 10 μl-volume of this solution was injected into the HPLC system.

Identification and purity of peaks

The identification of the peaks was carried out with automated library search software (Hewlett-Packard, operating software, Rev. 5.03, 1988). In the library the standards were saved with their retention times (Table III) and their UV spectra.

Determination of the aglycones

The determination of the aglycones was established with external standards. Each sample was injected three times for HPLC. The linearity of the determination of the threee flavonols was verified by regression analysis. The correlation coefficients

TABLE III

THE GRADIENT ELUTION PROFILE OF THE NATURALLY OCCURRING FLAVONOIDS OF *GINKGO BILOBA* L.

Compound No.	t_R (min)	Flavonoid
1	3.21	3-O-{2-O-[6-O-(*p*-Hydroxy-*trans*-cinnamoyl)-β-D-glucosyl]-α-L-rhamnosyl}-7-O-(β-D-glucosyl)quercetin
2	3.47	3-O-[2-O, 6-O-Bis(α-L-rhamnosyl)-β-D-glucosyl]quercetin
3	4.09	3-O-[2-O, 6-O-Bis(α-L-rhamnosyl)-β-D-glucosyl]isorhamnetin
4	4.71	3-O-[2-O, 6-O-Bis(α-L-rhamnosyl)-β-D-glucosyl]kaempferol
5	4.97	3-O-[6-O-(α-L-Rhamnosyl)-β-D-glucosyl]myricetin
6	5.96	3-O-[6-O-(α-L-Rhamnosyl)-β-D-glucosyl]-3'-methylmyricetin
7	6.53	3-O-(2-O-{6-O-[*p*-(β-D-Glucosyl)oxy-*trans*-cinnamoyl]-β-D-glucosyl}-α-L-rhamnosyl)quercetin
8	7.19	3-O-[6-O-(α-L-Rhamnosyl)-β-D-glucosyl]quercetin
9	8.73	3-O-(2-O-{6-O-[*p*-(β-D-Glucosyl)oxy-*trans*-cinnamoyl]-β-D-glucosyl}-α-L-rhamnosyl)kaempferol
10	8.73	3-O-[6-O-(α-L-Rhamnosyl)-β-D-glucosyl]isorhamnetin
11	9.68	3-O-(β-D-Glucosyl)quercetin
12	10.37	3-O-[6-O-(α-L-Rhamnosyl)-β-D-glucosyl]kaempferol
13	10.74	3-O-[2-O-(β-D-Glucosyl)-α-L-rhamnosyl]quercetin
14	11.39	3-O-(β-D-Glucosyl)isorhamnetin
15	13.00	3-O-(β-D-Glucosyl)kaempferol
16	13.58	7-O-(β-D-Glucosyl)apigenin
17	14.58	3-O-[2-O-(β-D-Glucosyl)-α-L-rhamnosyl]kaempferol
18	15.22	3-O-(α-L-Rhamnosyl)quercetin
19	16.65	3'-O-(β-D-glucosyl)luteolin
20	17.05	3-O-(α-L-Rhamnosyl)kaempferol
21	17.60	3-O-{2-O-[6-O-(*p*-Hydroxy-*trans*-cinnamoyl)-β-D-glucosyl]-α-L-rhamnosyl}quercetin
22	18.51	3-O-{2-O-[6-O-(*p*-Hydroxy-*trans*-cinnamoyl)-β-D-glucosyl]-α-L-rhamnosyl}kaempferol
23	18.76	Myricetin
24	20.60	Luteolin
25	21.50	Quercetin
26	22.22	Apigenin
27	22.76	Isorhamnetin
28	22.91	Kaempferol
29	24.02	Amentoflavon
30	25.06	Bilobetin
31	27.00	Ginkgetin
32	27.17	Isoginkgetin
33	29.35	Sciadopitysin

were 0.994 for isorhamnetin, 0.998 for kaempferol and 0.999 for quercetin. Standard solutions for calibration showed a very high stability with a loss of <4% within 12 months and storage at 8°C in a refrigerator. Seven different weight ratios from 0.2 to 2.0 μg (injected, resulting peak heights 250–2200 mAU) of the three flavonols were used. Known amounts of the aglycones dissolved in methanol were submitted three times to the sample preparation process and the recovery was determined: isorhamnetin = 99.8, kaempferol = 101.5 and quercetin = 100.6%.

Reproducibility of the fingerprint method

The reproducibility of the fingerprint method was demonstrated with columns from several batches and with the successful application in the laboratories of Zeller, where the analyses were carried out on an HP 1090 liquid chromatograph with a PV5 system (ternary solvent-delivery system controlled by a proportioning valve) instead of a DR5 system (three low-pressure pumps) on which the separation was developed in the laboratories of the ETH.

RESULTS AND DISCUSSION

Hydrolysis of the glycosides followed by a spectrophotometric determination of the aglycones as an aluminium chelate complex is the current method in several pharmacopeias to determine the concentration of flavonoids in herbal drugs. In the case of Ginkgo leaves this method was not reproducible [14], propably owing to the large amount of disturbing proanthocyanidines. Further, a detailed determination of the qualitative and quantitative composition of the obtained aglycones is not possible.

For more than 10 years, HPLC has been the method of choice for qualitative and especially quantiative analyses of flavonoids. It is logical to combine the well-established hydrolysis in the pharmacopeias with the modern technique of HPLC. The kinetics of hydrolysis were tested with rutin. It decomposed completely to the calculated amount of quercetin within 60 min. The separation of the aglycones on a reversed-phase (RP) column can be realized with complete resolution and different elution orders with two different solvents (method A and ref. 7). The work-up procedure consists of two steps: extraction and hydrolysis of the glycosides, and sample clean-up. Extraction and hydrolysis are performed by refluxing the pulverized plant material or a plant extract with 10 ml of hydrochloric acid (25%) in 70 ml of methanol for 60 min, while sample clean-up is carried out using C_{18} solid-phase extraction cartridges. An aliquot of the resulting final solution is then injected into the HPLC system. Isorhamnetin, kaempferol and quercetin can easily be determined by RP-HPLC using a gradient with 0.5% (v/v) orthophosphoric acid in water and methanol [7] or a methanol–THF mixture and UV detection at 370 nm. The elution profile of isorhamnetin and kaempferol is inverted on using THF as organic solvent.

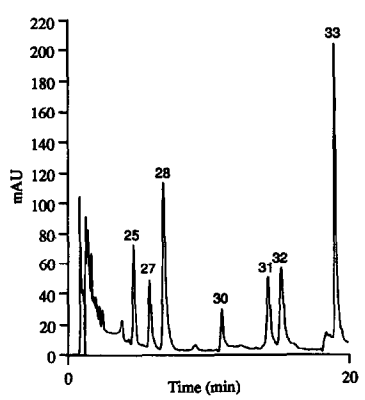

Fig. 1. Chromatogram of a leaf extract from *Ginkgo biloba* after hydrolysis, using Nucleosil 100-C_{18} (3 μm) with method A. Peak numbering as in Table III. Aglycones for quantitative assay and biflavones for identification are detected.

TABLE IV

CONVERSION OF THE FLAVONOID AGLYCONE CONTENT INTO A "GINKGO FLAVONE" GLYCOSIDE CONTENT

The conversion factors for the aglycones isorhamnetin (MW 316.27), kaempferol (MW 286.24) and quercetin (MW 302.2) are determined with the molecular weight of flavonol coumaroyl ester glycosides, *e.g.*, 3-O-{2-O-[6-O-(*p*-hydroxy-*trans*-cinnamoyl)-β-D-glucosyl]-α-L-rhamnosyl}quercetin (MW 756.7).

Compound	Conversion factor[a]
Isorhamnetin	2.39
Kaempferol	2.64
Quercetin	2.51
Average	2.51

[a] Calculation: $\sum$(aglycones) · 2.51 = concentration of "Ginkgo flavone" glycosides.

The analytical validation of methods used for the determination of phytopharmaceuticals is part of the modern documentation ordered by the national regulatory authorities. The specification, precision and accuracy have to be documented. To determine the accuracy of the determination of flavonoids, method A was developed in addition to the earlier described method [7]. Identical values resulted for both methods (Table V). In addition, method A can be used for the identification of the crude drug: the three main aglycones (**25–28**) and the characteristic and therefore specific biflavones (**30–33**) were separated in the same run (Fig. 1). The ubiquitous aglycones alone are not specific enough to identify Ginkgo. This method cannot be applied to special extracts like EGb 761 (Dr. Willmar Schwabe), where the biflavones have been removed.

In the standardization of phytomedicines, direct determination of the naturally occurring active principles would be desirable. The complex flavonoid profile of *Ginkgo biloba* demands a reduction

TABLE V

DETERMINATION OF THE FLAVONOID AGLYCONES IN GINKGO LEAVES AND EXTRACTS

Values in parentheses are relative standard deviations (%) (n = 3)

Harvest month, 1988	Isorhamnetin (%)		Kaempferol (%)		Quercetin (%)		∑(Aglycones) (%)	
	A	C[a]	A	C[a]	A	C[a]	A	C[a]
May	0.13 (2.5)	0.14 (2.5)	0.38 (2.8)	0.38 (1.8)	0.18 (2.2)	0.18 (1.2)	0.70	0.70
June	0.06 (3.5)	0.06 (3.2)	0.17 (3.0)	0.18 (2.5)	0.09 (3.3)	0.09 (2.0)	0.32	0.33
July	0.05 (3.1)	0.06 (2.1)	0.16 (3.6)	0.17 (2.6)	0.08 (3.0)	0.08 (1.7)	0.29	0.31
August	0.06 (1.9)	0.07 (2.1)	0.17 (1.6)	0.17 (2.0)	0.09 (1.9)	0.09 (0.9)	0.32	0.33
September	0.06 (2.5)	0.06 (2.5)	0.16 (2.8)	0.17 (2.8)	0.08 (1.2)	0.09 (1.2)	0.30	0.32
October	0.06 (2.0)	0.06 (3.0)	0.16 (2.0)	0.16 (3.0)	0.07 (2.2)	0.07 (2.2)	0.29	0.29
November[b]	0.07 (2.8)	0.07 (2.8)	0.17 (3.2)	0.17 (3.2)	0.08 (2.8)	0.08 (2.8)	0.32	0.32
Commercial sample								
Hänseler 912050	0.04 (3.0)	0.05 (0.8)	0.10 (2.8)	0.10 (3.1)	0.09 (3.1)	0.10 (3.2)	0.25	0.25
Dixa 38747	0.04 (2.8)	0.04 (3.1)	0.13 (2.7)	0.13 (2.7)	0.10 (3.0)	0.10 (2.7)	0.27	0.27
Siegfried 147974-02	0.04 (1.8)	0.05 (3.8)	0.11 (2.2)	0.11 (2.8)	0.09 (2.0)	0.09 (3.6)	0.24	0.25
Extract								
Flachsmann 6 L 035	0.10 (2.2)	0.10 (2.5)	0.39 (2.5)	0.39 (2.8)	0.50 (2.3)	0.51 (3.0)	0.99	1.00
Zeller 32/89	0.14 (3.0)	0.14 (2.9)	0.42 (2.8)	0.42 (1.9)	0.55 (2.8)	0.55 (2.8)	1.11	1.11

[a] The values in column C were obtained with the earlier described method [7].
[b] Yellow and fallen leaves.

to the basic principles, because most reference compounds are not commercially available and a complete analysis for all compounds is tedious. However, the aglycone content obtained can be correlated with the total flavonoid glycoside content (Table IV). This is done in accordance with the practice in the pharmaceutical industry. The producers of Ginkgo preparations convert the obtained aglycone content into a "Ginkgo flavone" glycoside content. As "Ginkgo flavone" glycosides, the flavonol coumaroyl ester glycosides **21** and **22** (Table III) with an average molecular weight of *ca.* 760 are documented (the described interglycosidic linkage [16,17] has to be revised from 1→4 to 1→2 owing to new NMR investigations [14,15]).

Typical HPLC results for a hydrolysed Ginkgo extract have been presented previously [7]. Generally, kaempferol and quercetin are the main peaks and the concentration of isorhamnetin is approximately five times lower. The very small minor peaks represent further aglycones, *e.g.*, apigenin and luteolin. They could also be determined if they occurred at higher concentration levels. Our investigations have shown that dried Ginkgo leaves obtained commercially contain an aglycone content of 0.2–0.4% (w/w), corresponding to a calculated "Ginkgo flavone" glycoside content of 0.5–1% (w/w) (Table V). Green leaves are considered to be of better quality. However, concerning the total flavonoid content we could not see any significant differences in our ontogenetic studies between June and November 1988. The self-collected and self-dried leaves show a higher concentration of flavonoids than the commercially available leaves and the ratio of kaempferol to quercetin is different (Table V). Ginkgo full extracts contain about 2–4% (w/w) and enriched extracts (*e.g.*, EGb 761) about 24–27% (w/w) of "Ginkgo flavone" glycosides [3].

Further characterization of Ginkgo extracts is possible with the fingerprint analysis of the flavonoids. A chromatographic system (method B) was developed to separate the isolated flavonoids of *Ginkgo biloba*. Owing to the complex mixture of very polar (triglycosides), polar (mono- and diglycosides) and apolar (biflavones) flavonoids, the proposed separation within 30 min requires a sophisticated HPLC procedure including a three-pump system and a diode-array detector. It is possible to identify unambiguously 22 flavonoid glycosides, six flavonoid aglycones and five biflavones in leaves

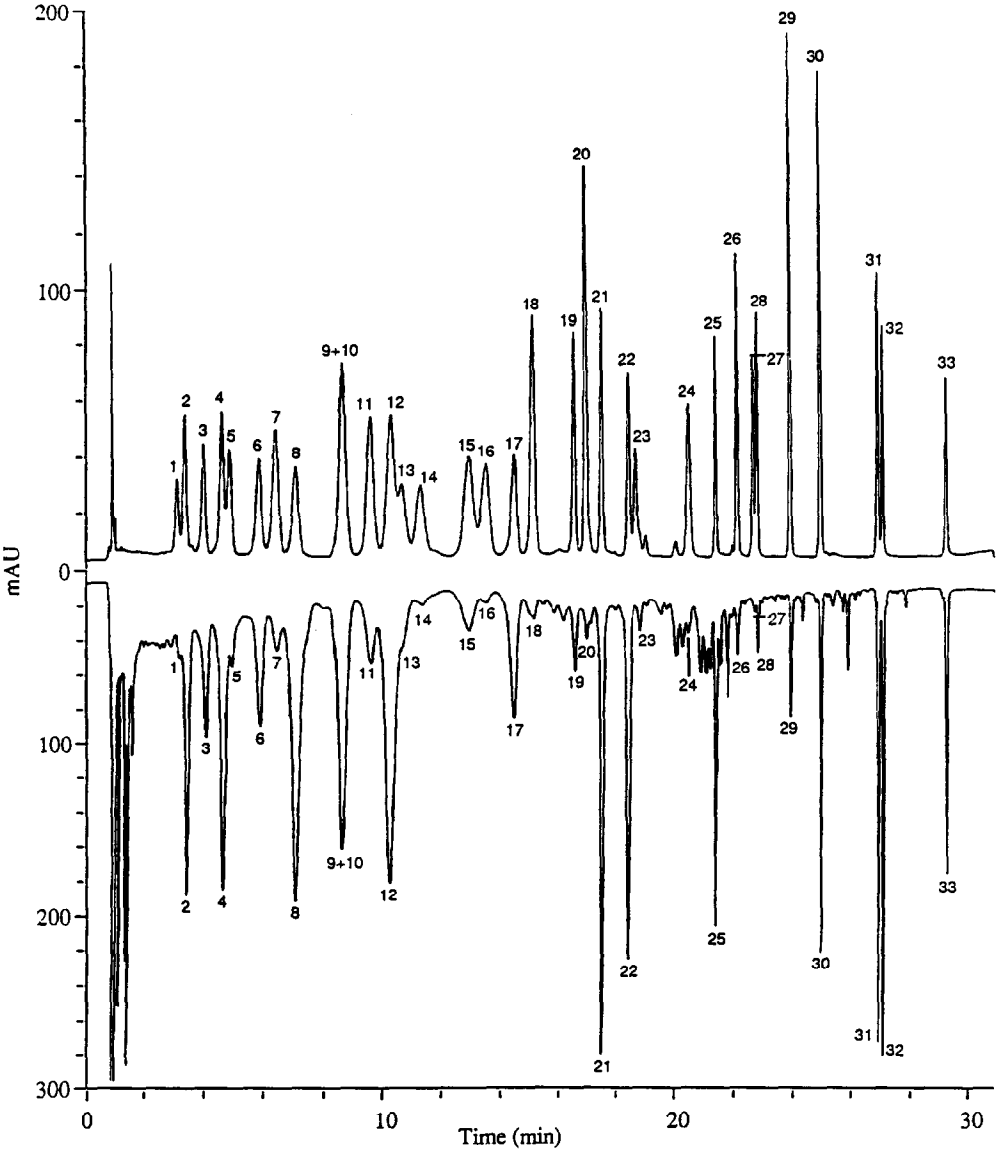

Fig. 2. (Top) chromatogram of the pure reference flavonoids from *Ginkgo biloba* and (bottom) fingerprint chromatogram of a therapeutically used dry extract of the leaves from *Ginkgo biloba* (Zeller, Lot-No. 32/89), using Nucleosil 100-C_{18} (3 μm). Peak numbering as in Table III.

and extracts from the elution order and UV spectra. An elution profile and the chromatographic results of the separation of reference compounds and of an extract used for herbal remedies produced by Zeller are shown in Table III and Fig. 2. The reference run has been stored together with UV spectra in the data system and can be used for peak assignment.

The fingerprint analysis is especially useful in stability tests. It can be shown that the flavonoid glycosides and the biflavones are stable and that the ratio of the compounds does not change. This is a demand of the EEC guideline 75/318 "Quality of Herbal Drugs" [18]. An increase in the aglycones and a decrease in the glycosides would indicate an

undesirable degradation process in the extract. The fingerprint analysis especially allows us to identify the very typical flavonol coumaroyl ester glycosides **21** and **22**. Both are well separated from each other and from other compouds. A more selective system is required in order to separate **9** and **10**. However, the UV spectra of the tested extracts showed a dominance of **10**.

CONCLUSION

New guidelines for herbal medicines demand ever better developed analytical methods to describe the quality of phytopharmaceuticals. *Ginkgo biloba* produces a large number of flavonoids in leaves, mainly derivatives of isorhamnetin, kaempferol and quercetin. The flavonoid assay can be controlled by the determination of the aglycones. The flavonols are commercially available as standards. The standardization of extracts is normally based on this assay. The presented HPLC fingerprint separation for checking the identity and the stability of products is applicable. The assignment can be done from the on-line UV spectra and elution profile. The analysis of a second group of compounds with therapeutic value, the assay of ginkgolides and bilobalide, has been described recently [19]. Analytical methods to guarantee a constant quality of Ginkgo products are now available.

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