Journal of Chromatography, 257 (1983) 431–438
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 15,972

Note

Analysis of terpenes from *Ginkgo biloba* L. by high-performance liquid chromatography

ANNELISE LOBSTEIN-GUTH*, FRANÇOISE BRIANÇON-SCHEID and ROBERT ANTON
Laboratoire de Pharmacognosie, Faculté de Pharmacie, B.P. 10, 67048 Strasbourg Cédex (France)
(Received May 6th, 1983)

Ginkgo biloba L., used in the treatment of vascular diseases, occupies a unique position in botany and chemistry: as this "living fossil" has remained unchanged for 200 million years and is the sole surviving species of the Ginkgoales group, it shows many botanical originalities[1,2]. From the chemical point of view, it synthesizes terpenoid compounds called the "bitter principles of the ginkgo tree"[3], whose structures are very complex and unusual. Three diterpenes and one sesquiterpene are reported to be present. The ginkgolides (diterpenes) were first isolated from the root bark[4], and later from the leaves of the Ginkgo[5]. They contain three γ-lactone rings and a *tert.*-butyl group. They differ from each other in the number and the position of the hydroxyl groups (Fig. 1). Bilobalide (a sesquiterpene) was isolated only from the leaves[6,7]. Its structure is very similar to those of the ginkgolides, as shown in Fig. 1.

Several workers have achieved, with much difficulty the separation of these terpenes by liquid (LC) and thin-layer chromatography (TLC). These techniques are still limited and unsatisfactory, especially the latter, which suffers from a lack of sensitivity and specificity because of detection problems.

This paper describes the high-performance liquid chromatographic (HPLC) separation of a mixture of these terpenes and the detection of small amounts of three of them in a purified extract prepared from *Ginkgo biloba* L. leaves.

EXPERIMENTAL

Chemicals

The solvents used for the HPLC analysis (acetonitrile, tetrahydrofuran and methanol) were of analytical-reagent grade (Merck, Darmstadt, F.R.G.) and filtered with a 0.5-μm filter (Millipore, Bedford, MA, U.S.A.). Fresh deionized, glass-distilled water, filtered with a 0.45 μm Millipore filter, was used.

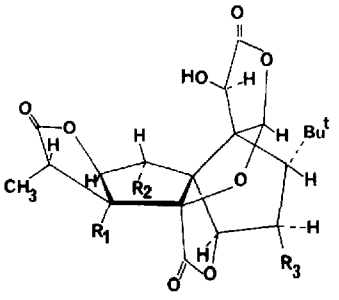
Other solvents (cyclohexane, chloroform, ethyl acetate, diethyl ether and ethanol) and chemicals (hydrochloric acid, acetic anhydride, anhydrous sodium sulphate) used for the standard mixture and the Ginkgo leaf extract preparations were of analytical-reagent grade.

Apparatus

Analytical liquid chromatographic separations were conducted with a Perkin-

0021-9673/83/$03.00

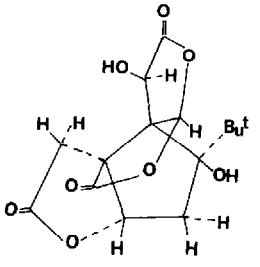
Diterpenes:



GINKGOLIDES

	R_1	R_2	R_3
Ginkgolide A	OH	H	H
Ginkgolide B	OH	OH	H
Ginkgolide C	OH	OH	OH

Sesquiterpene:



BILOBALIDE

Fig. 1. Structures of terpenes from *Ginkgo biloba* L.

Elmer Model 3B liquid chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.) equipped with a Rheodyne Model 7125 injection valve and a 20-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.). Detection was carried out with an LC-75 variable-wavelength spectrophotometric detector connected to an LC-75 Autocontrol module (Perkin-Elmer). A Hibar column (25 × 0.4 cm I.D.) containing LiChrosorb RP-18 (10 μm) (Merck) was used for separations.

Preparative LC purifications were conducted with a Chromatospac Prep 10 (Jobin-Yvon, Longjumeau, France) with a silica stationary phase [250 g of silica H 60, 0.063–0.2 mm (Merck) in a 40 mm I.D. column].

Preparation of standard mixture

A purified mixture of ginkgolides and bilobalide was prepared by extraction from an already purified *Ginkgo biloba* L. leaf extract, prepared according to reported techniques[8]. Dried, powdered, purified *Ginkgo biloba* L. leaf extract (120 g) was treated with boiling ethyl acetate (7 × 600 ml). The absence of terpenes in the last ethyl acetate extract was controlled by TLC examinations. It was chromatographed over aluminium sheets coated with silica gel 60F 254 (20 × 20 cm, layer thickness 0.2 mm; Merck) using cyclohexane–ethyl acetate (50:50) as the solvent. The developed chromatograms were air-dried, sprayed with acetic anhydride reagent and heated at

150°C for 20–30 min. Under these conditions, ginkgolides and bilobalide show a faint orange fluorescence at 365 nm.

The combined ethyl acetate extracts were concentrated to 300 ml, treated with the same volume of chloroform, cooled overnight, decanted and finally filtered. The filtrate was evaporated to dryness under vacuum and the residue was extracted with several successive portions of diethyl ether until free from terpenes (TLC examination). The combined diethyl ether extracts were dried over anhydrous sodium sulphate, then distilled under reduced pressure to a dark syrupy liquid (21.5 g). The latter was chromatographed on a silica gel column (250 g) with a preparative apparatus (Chromatospac Prep-10) using cyclohexane–ethyl acetate (40:60) as eluting solvent. Fractions of 100 ml were collected, monitored by TLC as mentioned above, then the terpene-rich fractions were concentrated. It was found that further purification of ginkgolides and bilobalide can be obtained by successive precipitations with chloroform, diethyl ether and methanol.

Separation and identification of terpenes

The identity of each terpene of this pure mixture was established by TLC and HPLC examinations, in comparison with pure authentic reference compounds:

TLC examinations of the standard mixture were carried out as described above and revealed the presence of four spots (R_F = 0.46, 0.34, 0.30 and 0.15), corresponding to the reference compounds (bilobalide and ginkgolides A, B and C, respectively).

HPLC analyses were carried out at room temperature with the following isocratic systems:

System A: water–methanol (70:30), flow-rate 1 ml/min;

System B: water–acetonitrile (80:20), flow-rate 1 ml/min;

System C: water–tetrahydrofuran (80:20), flow-rate 1 ml/min;

System D: water–methanol–tetrahydrofuran (75:5:15), flow-rate 1.5 ml/min.

Detection was effected at a wavelength of 220 nm (range 0.02 a.u.). A volume of 15 µl of the standard methanolic solutions was injected with a Hamilton syringe.

Preparation of the Ginkgo biloba L. leaf extract for HPLC

Leaves of *Ginkgo biloba* L. were collected in October 1980 from the botanical

TABLE I

HPLC CHARACTERISTICS OF THE TERPENES FROM *GINKGO BILOBA* L.

Compound	*Peak*	*Elution system**							
		A		*B*		*C*		*D*	
		t_R**	*S****	t_R	*S*	t_R	*S*	t_R	*S*
Ginkgolide A	2	21.4	1.08	27.0	1.80	23.2	1.50	11.9	1.00
Ginkgolide B	3	24.2	1.12	27.0	1.80	37.4	1.25	19.3	1.20
Ginkgolide C	1	10.6	1.00	13.6	1.00	17.2	1.50	9.2	1.00
Bilobalide	4	8.8	1.17	10.0	1.00	24.8	1.06	13.6	1.14

* See *Separation and identification of terpenes.*

** t_R = retention time (min).

*** S = peak symmetry.

garden at Strasbourg. Air-dried, powdered leaves (30 g) were extracted with ethanol in a Soxhlet apparatus. The ethanol extract was filtered and then evaporated under vacuum to a thick residue, which was suspended in water (100 ml) and then extracted with cyclohexane (10 × 150 ml). The partially purified aqueous phase was acidified to pH 2 with hydrochloric acid (1 *N*) and extracted with ethyl acetate (10 × 150 ml). The organic phase was dried over anhydrous sodium sulphate and distilled under reduced pressure. The residue was dissolved in water (50 ml) and extracted with diethyl ether (15 × 50 ml). The combined ether extracts were dried over anhydrous

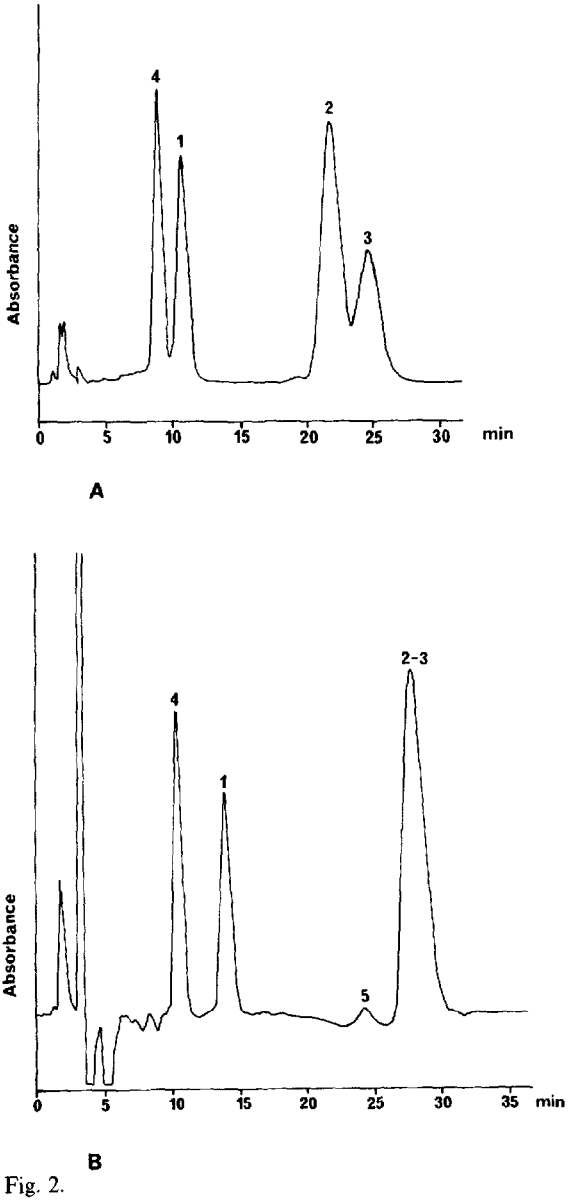


Fig. 2.

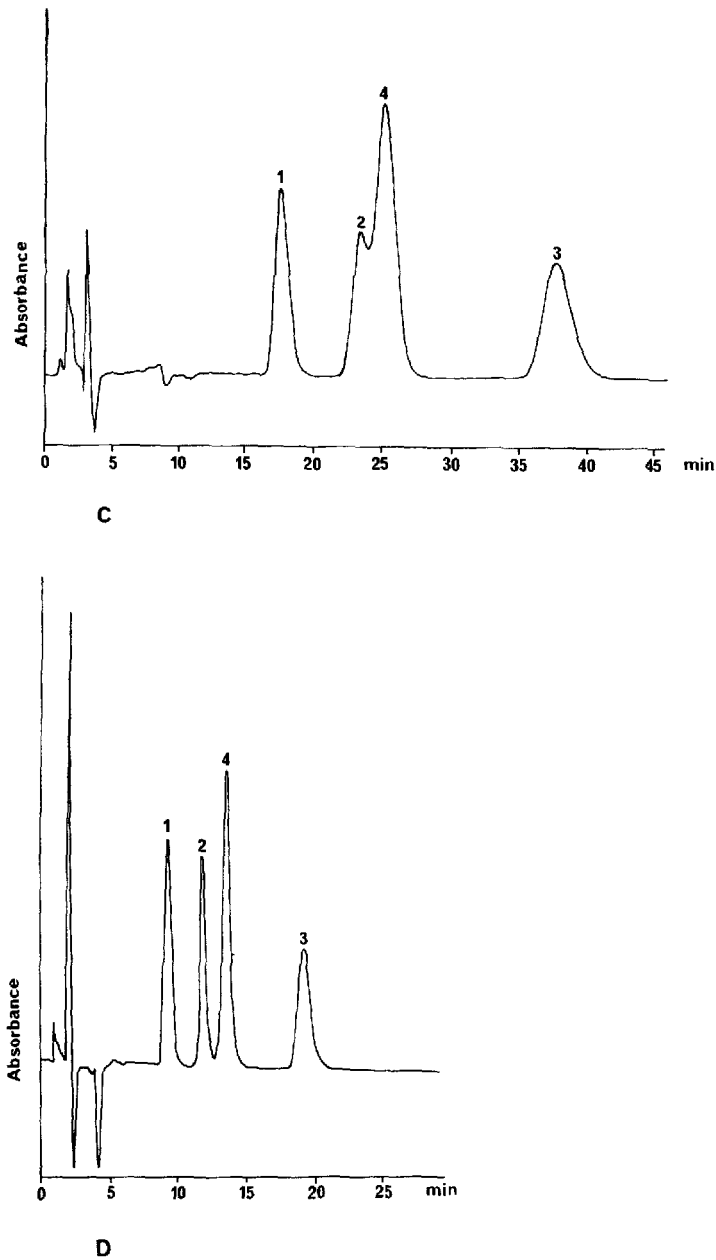


Fig. 2. HPLC traces of terpene mixture from *Ginkgo biloba* L. Conditions: (A) solvent system A; (B) system B; (C) system C; (D) system D. Peaks: 1 = ginkgolide C; 2 = ginkgolide A; 3 = ginkgolide B; 4 = bilobalide.

sodium sulphate and the solvent was evaporated to dryness under vacuum. The residue was dissolved in methanol (1 ml) and kept for HPLC analysis. Detection was effected at a wavelength of 220 nm (range 0.08 a.u.).

RESULTS AND DISCUSSION

Selectivity of the elution systems

Fig. 2 shows the HPLC resolution of a pure mixture of standards, in four isocratic sytems.

Table I lists the different HPLC characteristics of the Ginkgo terpenes.

System A. With these first elution conditions, all four peaks are symmetrical (Table I). The resolution factor of peaks 2 and 3 (0.82) is less satisfactory than those of peaks 1 and 4 (0.97). Several attempts made to improve the resolution limit of the ginkgolides A and B, using gradient elution or flow programmes, were unsuccessful.

Other isocratic systems were chosen according to Schoenmakers *et al.*[9]. The procedure permits one to establish, for the elution power of a particular water–methanol mixture, the equivalent compositions of water–acetonitrile or water–tetrahydrofuran mixtures. In our case, the theoretical optimal isocratic conditions were water–acetonitrile (80:20) = system B and water–tetrahydrofuran (80:20) = system C.

System B. With these second elution conditions, the peaks are also symmetrical (Table I). Peaks 1 and 4 are totally separated (resolution factor = 4), but peaks 2 and 3 overlap. Any gradient elution or flow programme ameliorated their separation. Therefore, this chromatographic system was eliminated.

Nevertheless, it is of interest to note that under these conditions, a minor peak was appeared (peak 5, retention time, t_R = 23.4 min). This peak could be either a Ginkgo terpene such as the ginkgolide M present in very small amounts in the Ginkgo leaves and previously described by Nakanishi[4], or a new di- or sesquiterpene. Its identification needs further investigation.

System C. All the peaks still show good symmetry (Table I). The resolution factors of peaks 2 and 3 (4.08) and peaks 1 and 4 (2.81) are much greater than in the two last elution systems, but those of peaks 2 and 4 (0.56) are now unsatisfactory. For this reason, this chromatographic system was also eliminated.

Nevertheless, these conditions could be useful for the separation of a mixture of ginkgolides A and B; the separation of these two diterpenes usually needs a 10–15-step fractional crystallization procedure and is complicated by a strong tendency of ginkgolide A to exhibit polymorphism[4]. Therefore, this new chromatographic system could provide more satisfactory results for the resolution of this particular problem.

System D. According to Roggendorf and Spatz's results on the selectivity benefits of a ternary mobile phase containing tetrahydrofuran in reversed-phase liquid chromatography[10], a fourth elution system was tried with a ternary mixture: water–methanol–tetrahydrofuran. The best results were obtained with the proportions 75:5:15 (system D).

These chromatographic conditions allowed satisfactory separations, as the four constituents of the standard mixture gave perfectly symmetrical and well resolved peaks (Table I). The resolution factors of peaks 1 and 2, 2 and 4, and 4 and 3 are 1.60, 1.06 and 2.32, respectively. Moreover, the analysis time is reduced (20 min instead of 25, 27 or 38 min for the systems A, B and C, respectively). For these reasons, this elution system was the system of choice for the analysis of a purified extract of *Ginkgo biloba* L. leaves.

HPLC of a purified extract of Ginkgo biloba L. leaves

The ginkgolides and bilobalide are present in very small amounts in *Ginkgo biloba* L. At the wavelength of analysis, they have low UV absorption coefficients; their minimum detectable amounts are approximately 30 μg. Therefore, it is impossible to detect them in a crude extract of Ginkgo leaves.

Accordingly, several attempts were made to purify this crude extract prior to HPLC examination. The major impurities were found to be waxes, chlorophylls and polyphenolic compounds. The elimination of waxes and chlorophylls was first tried with different chromatographic procedures using several minicolumns (alumina, Florisil, silica gel, silica gel bounded C_{18}, Sephadex), without satisfactory results. Treatment of the crude ethanol extract suspended in water with sodium hydroxide or lead acetate[11] can remove major polyphenolic impurities but results in a partial loss of terpenes.

Finally, good purification of the crude ethanolic extract suspended in water was obtained by extraction with cyclohexane, followed by ethyl acetate after acidification, and finally with diethyl ether in neutral medium. The diethyl ether extract was then evaporated to dryness and the residue dissolved in 1 ml of methanol prior to injection.

The HPLC trace of this partially purified *Ginkgo biloba* L. leaf extract is shown in Fig. 3. It shows the three main terpenes of the Ginkgo leaves previously recorded in the literature, *i.e.*, ginkgolides A and B and bilobalide. Ginkgolide C is not detectable under these chromatographic conditions.

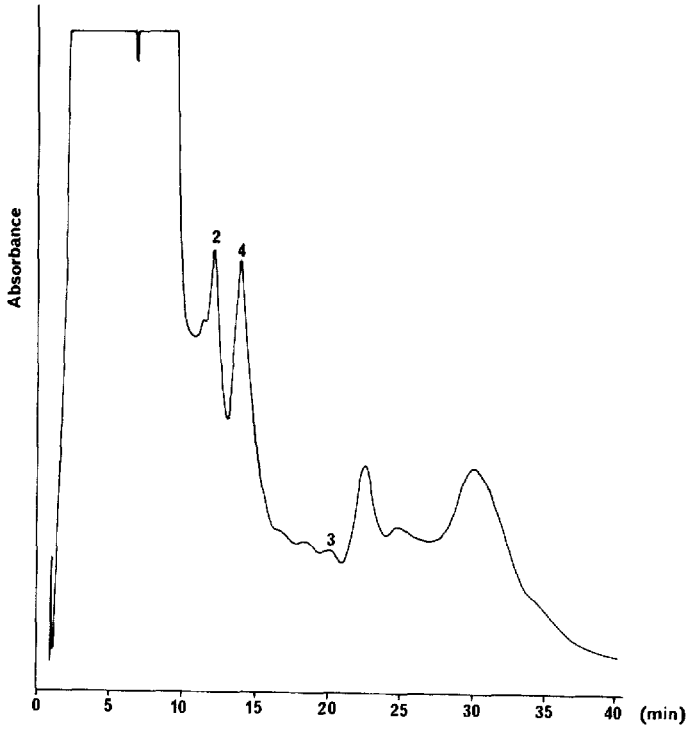


Fig. 3. HPLC trace of a partially purified *Ginkgo biloba* L. leaf extract. Conditions: solvent system D.

CONCLUSION

HPLC is a useful technique for the separation of terpenes from *Ginkgo biloba* L. Their detection was found to be impossible in a crude extract, but can be achieved with a partially purified extract. The purification process needs further amelioration in order to make possible their qualitative and quantitative determination in *Ginkgo biloba* L. extracts.

REFERENCES

1 A. H. Franklin, *Virginia J. Sci. N.S.*, 10 (1959) 131.
2 L. Emberger, *Les Plantes Fossiles dans leurs Rapports avec les Végétaux Vivants*, Masson, Paris, 1968, p. 490.
3 S. Furukawa, *Sci. Pap. Inst. Phys. Chem. Res.*, 19 (1932) 27.
4 K. Nakanishi, *Pure Appl. Chem.*, 14 (1967) 89.
5 K. Okabe, K. Yamada, S. Yamamura and S. Takada, *J. Chem. Soc.*, (1967) 2201.
6 R. T. Major, *Science*, 157 (1967) 1270.
7 K. Weinges and W. Bähr, *Justus Liebigs Ann. Chem.*, 724 (1969) 214.
8 R. Anton, *Plant. Méd. Phytother.*, 11, No. Spécial (1977) 189.
9 P. J. Schoenmakers, H. A. H. Billiet and L. de Galan, *J. Chromatogr.*, 205 (1981) 13.
10 E. Roggendorf and R. F. Spatz, *J. Chromatogr.*, 204 (1981) 263.
11 P. Ribereau-Gayon, *Les Composés Phénoliques des Végétaux*, Dunod, Paris, 1968, p. 35.