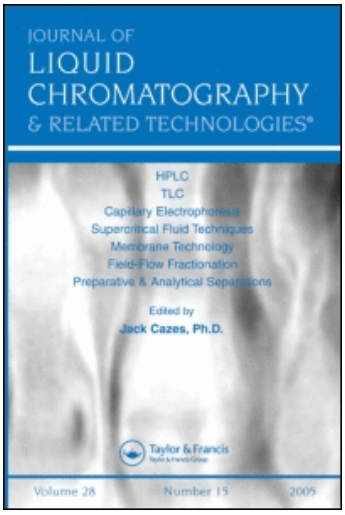

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND THIN-LAYER CHROMATOGRAPHY OF PHENOLIC ACIDS FROM *GINKGO BILOBA* L. LEAVES COLLECTED WITHIN VEGETATIVE PERIOD

M. Ellnain-Wojtaszek[a]; G. Zgórka[b]
[a] Dept. of Pharmacognosy, University of Medical Sciences, Poznań, Poland [b] Dept. of Pharmacognosy, University Medical Academy, Lublin, Poland

Online publication date: 13 January 2005

To cite this Article Ellnain-Wojtaszek, M. and Zgórka, G.(1999) 'HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND THIN-LAYER CHROMATOGRAPHY OF PHENOLIC ACIDS FROM *GINKGO BILOBA* L. LEAVES COLLECTED WITHIN VEGETATIVE PERIOD', Journal of Liquid Chromatography & Related Technologies, 22: 10, 1457 — 1471
To link to this Article: DOI: 10.1081/JLC-100101744
URL: http://dx.doi.org/10.1081/JLC-100101744

J. LIQ. CHROM. & REL. TECHNOL., 22(10), 1457–1471 (1999)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND THIN-LAYER CHROMATOGRAPHY OF PHENOLIC ACIDS FROM *GINKGO BILOBA* L. LEAVES COLLECTED WITHIN VEGETATIVE PERIOD

M. Ellnain-Wojtaszek,[1,]* G. Zgórka[2]

[1] Dept. of Pharmacognosy
University of Medical Sciences
10 Sieroca Street
61-771 Poznań, Poland

[2] Dept. of Pharmacognosy
University Medical Academy
12 Peowiaków Street
20-007 Lublin, Poland

ABSTRACT

In this paper, thin-layer chromatography and high-performance liquid chromatography for the qualitative and quantitative analysis of phenolic acids in *Ginkgo biloba* L. leaves are described. The amounts of both free and bound phenolic acids were determined within the whole vegetative period. For this purpose, a special extraction procedure, comprising acid and alkaline hydrolyses, was used. The described method allowed us to identify and estimate the concentration levels of eight phenolic acids: protocatechuic, *para*-hydroxybenzoic, vanillic, caffeic, isovanillic, *para*-coumaric, ferulic, and sinapic.

Depending on the part of the plant vegetation, significant differences in the content of the compounds examined were observed and discussed. The elaborated method could be used in studies on the seasonal distribution of phenolic acids in plant material.

INTRODUCTION

The leaves of *Ginkgo biloba* L. (*Ginkgoaceae*) have been known for a long time for their therapeutic properties. Extracts from the leaves are active components of pharmaceutical preparations used in the treatment of cardiovascular and central nervous system disorders. They also inhibit platelet-activating factor and the formation of free radicals.[1-3]

Flavonoids, i.e., flavonol glycosides, biflavones, proanthocyanidins,[1,4-11] and terpenes (ginkgolides, bilobalide),[1,6,12] are the main constituents responsible for the multidirectional pharmacological activity of *G. biloba* leaves. Phenolic acids (both derivatives of benzoic and cinnamic acid) are another interesting group of compounds found in the leaves of this plant.[1,13-15]

Table 1

R_f Values of Phenolic Acids Identified in *G. biloba* Leaves by 2D-TLC

Compound No.	Phenolic Acids	R_f Values in Solvent Systems S_1	S_2
1	Protocatechuic	0.03	0.61
2	*para*-Hydroxybenzoic	0.29	0.69
3	Vanillic	0.69	0.69
4	Caffeic	0.04	0.38 (0.70)*
5	Isovanillic	0.54	0.68
6	*para*-Coumaric	0.35	0.55 (0.80)*
7	Ferulic	0.71	0.42 (0.77)*
8	Sinapic	0.70	0.34 (0.73)*

* R_f values of the *cis*-isomers of the corresponding phenolic acids.

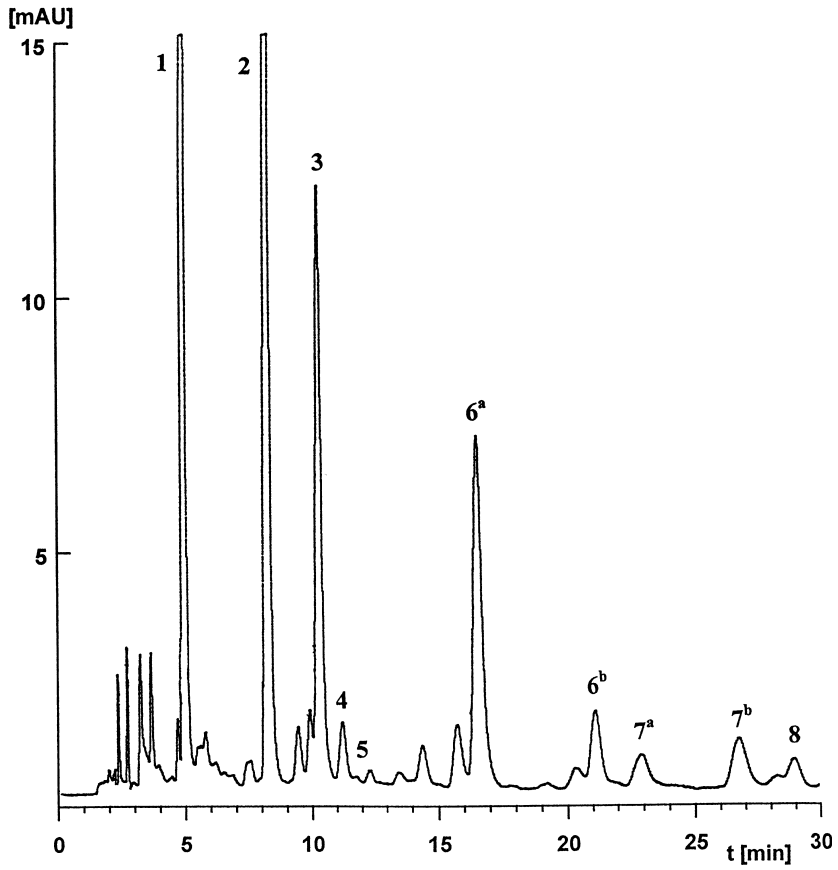

Figure 1. Separation of phenolic acids from *G. biloba* leaves, carried out on ODS Hypersil (200 x 4.6 mm I.D., 5μm) column under isocratic conditions. Mobile phase: methanol-water (25:75). Flow-rate, 1 mL min^{-1}; detection, 254 nm. Compounds numbered according to Table 1; [a] – *trans*, [b] – *cis* isomers.

Naturally occurring phenolic acids show various pharmacological properties and act as cholagogues, stomach stimulants, tranquilizers, and immuno-stimulants, as well as anti-inflammatory, antibacterial, and antifungal agents.[16-21] The antibacterial activity was also determined for five phenolic acids from *G. biloba* leaves.[22]

Chromatographic methods—thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas chromatography (GC)—are often used for quick qualitative and quantitative analysis of natural

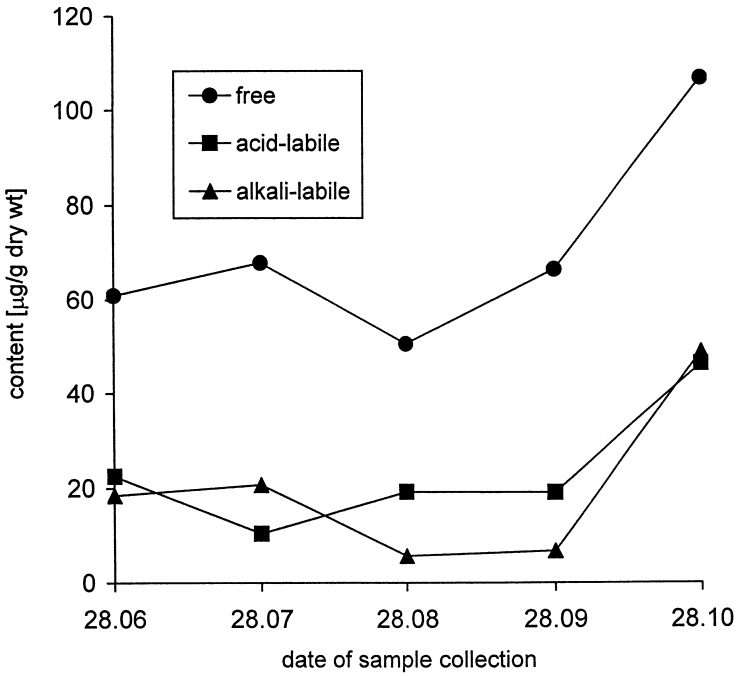

Figure 2. Protocatechuic acid content in *G. biloba* leaves within vegetative period.

phenolics.[23-25] These methods were employed for the evaluation of the content of flavonoids,[5,6,26] terpenoids,[6] and phenolic acids[14-15] in *G. biloba* leaves. Some assays dealt with the examination of seasonal variations of flavonoids and biflavones,[5-7,27,28] as well as ginkgolides and bilobalide concentrations[28-30] in *G. biloba* leaves.

In this paper, the identification and determination of free and covalently-bound phenolic acids by TLC and RP-HPLC methods in *G. biloba* leaves, harvested from June to October, are described.

EXPERIMENTAL

Plant Material

G. biloba leaves were collected every month, between June 28 and October 28, 1997, from an approximately 70-year-old male tree, in the Botanical Garden

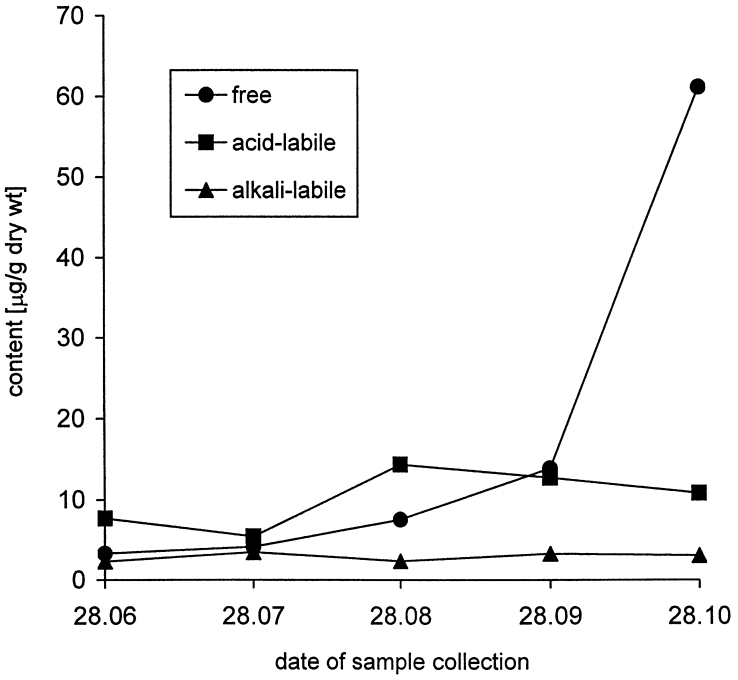

Figure 3. *Para*-Hydroxybenzoic acid content in *G. biloba* leaves within vegetative period.

of A. Mickiewicz University (Poznań, Poland) and immediately dried at room temperature. The leaves collected between June 28 and August 28 were green, those from September 28 – green-yellow, and from October 28 – yellow. The samples were marked: 1-5 – from June to October, respectively.

Extraction Procedure

Dried, powdered samples (1-5) of *G. biloba* leaves (25 g) were extracted twice with boiling 85^{o} methanol for 1h. Methanolic extracts were concentrated under vacuum, below 60^{o}C, diluted with hot, distilled water (100 mL), and filtered. Water extracts were partitioned into several fractions, according to a liquid-liquid extraction procedure elaborated for phenolic acids.[18,31,32]

For this purpose, each of the water extracts was purified by shaking with petroleum ether (2 x 30 mL) – organic layer was rejected after extraction - then extracted (15 x 50 mL) with diethyl ether (Et_2O), finally providing water "A"

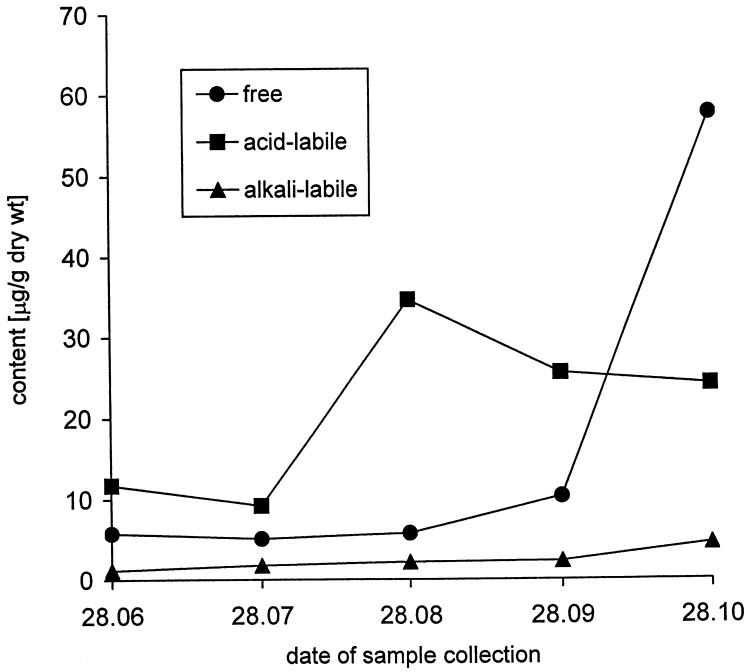

Figure 4. Vanillic acid content in *G. biloba* leaves within vegetative period.

and Et_2O layers. Phenolic acids contained in the Et_2O layers were further treated with 5% $NaHCO_3$ (10 x 10 ml). Bicarbonate layers were acidified with 36% HCl until pH 3 and thereafter extracted with 10 portions of 25 mL of Et_2O. This procedure led to water layers "B" and purified Et_2O layers. Each of Et_2O extracts 1-5 was evaporated to dryness, and the residues were dissolved in methanol (25 mL), providing fractions of free phenolic acids, marked: 1/L-1 to 5/L-1.

The remaining water layers "A" and "B" were combined, divided in two equal parts "C" and "D" and subjected to acid and alkaline hydrolyses in accordance with the standard protocol.[18,31,32]

Hydrolysis Procedure

Acid hydrolysis was carried out by refluxing "C" solutions, previously treated with 36% HCl until 2 M, on a boiling water bath for 1 h. The liberated phenolic acids were further separated by extraction with Et_2O (10 x 50 mL).

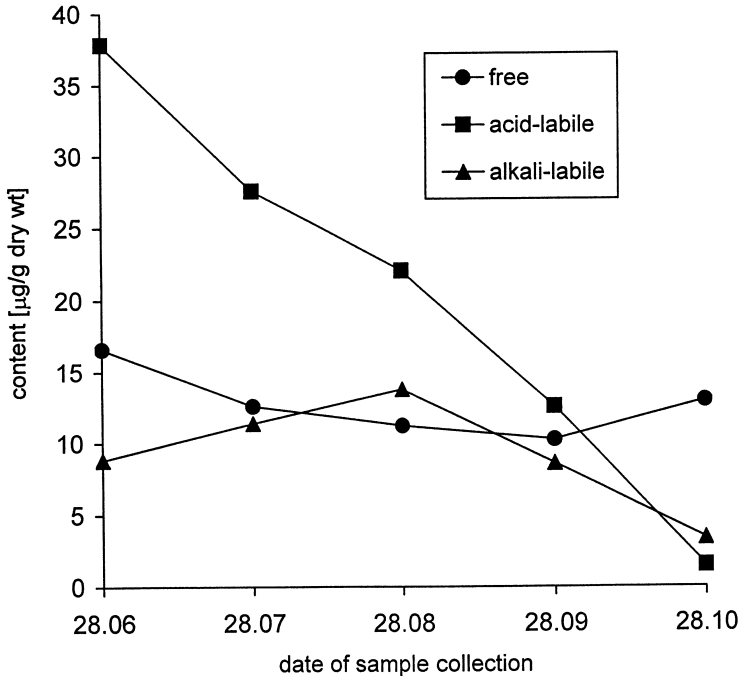

Figure 5. Caffeic acid content in *G. biloba* leaves within vegetative period.

The residues, after evaporation of the solvent, were dissolved in methanol (25 mL), giving fractions of acid-labile phenolic acids, marked from 1/L-2 to 5/L-2. Alkaline hydrolysis was performed by refluxing "D" solutions on a boiling water bath, after alkalization with 1% $Ba(OH)_2$ to pH 12-13 and addition of 1g of $NaBH_4$, for 15 min. After cooling, the hydrolyzate was acidified with 96% H_2SO_4. The precipitates of $BaSO_4$ were filtered off, and the acidic filtrates were further extracted with Et_2O (10 x 100 mL). After evaporation of the solvent, the residues were dissolved in methanol (25 mL), providing fractions of alkali-labile phenolic acids, labelled: 1/L-3 to 5/L-3.

Thin-Layer Chromatography

Methanolic aliquots, containing phenolic acid fractions, were subjected to qualitative TLC analysis on cellulose plates (20 x 20 cm, Merck, Darmstadt, Germany). Samples (100 μL) of 0.1%-0.2% (w/v) solutions of the solutes in methanol were spotted 2.5 cm from the edge of the plate and developed over a

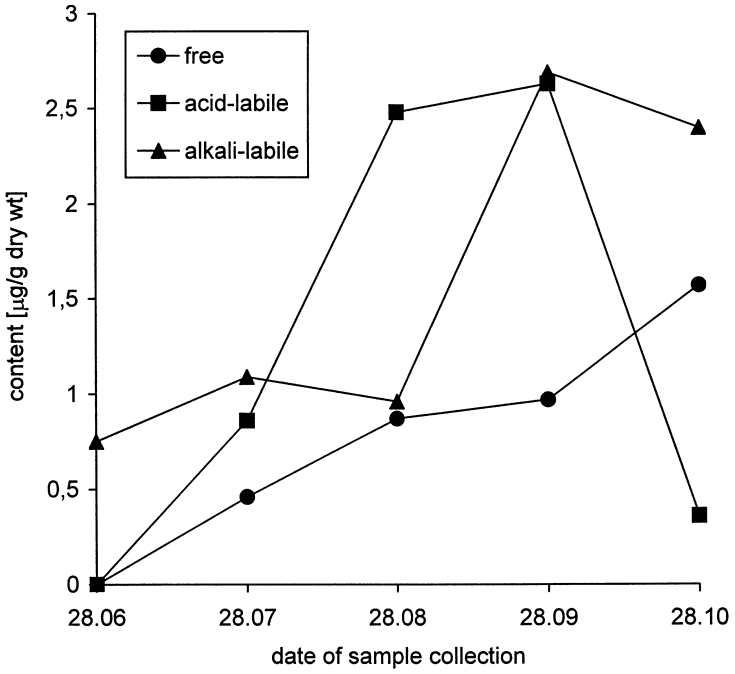

Figure 6. Isovanillic acid content in *G. biloba* leaves within vegetative period.

distance of 15 cm by the two-dimensional technique (2D-TLC) in horizontal DS chambers (CHROMDES, Lublin, Poland), using the following mobile phases - S_1: benzene – glacial acetic acid – water (6 : 7 : 3) in the first direction, and S_2: glacial acetic acid – water (15 : 85) in the second. Run time in solvent S_1 was approximately 1h and in solvent S_2 – 2h. After each development, the plates were dried for 1h at room temperature.

Chromatograms were examined under UV_{254nm} and UV_{365nm} light. The spots were further visualized with general sprays for detecting phenols, such as: a 1:1 mixture of diazotized sulfanilic acid and 20% aqueous Na_2CO_3, 1% methanolic KOH, or 2% aqueous $FeCl_3$.[14,33] Co-chromatography of the investigated samples was run against 23 reference phenolic acids. Table 1 shows R_f values of phenolic acids identified in *G. biloba* leaves.

High-Performance Liquid Chromatography

The HPLC analysis was performed using a Hewlett-Packard (Palo Alto, CA, USA) Model 1050 liquid chromatograph, equipped with a 20-µL sample

Table 2

Free, Acid- and Alkali-Labile Phenolic Acids of *G. biloba* Leaves Identified by 2D-TLC

Fractions	Phenolic Acids (Numbered according to Table 1)							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1/L-1	+	+	+	+	-	+	+	-
2/L-1	+	+	+	+	-	+	+	-
3/L-1	+	+	+	+	-	+	+	-
4/L-1	+	+	+	+	-	+	+	-
5/L-1	+	+	+	+	+	+	+	+
1/L-2	+	+	+	+	-	+	+	-
2/L-2	+	+	+	+	-	+	+	-
3/L-2	+	+	+	+	+	+	+	+
4/L-2	+	+	+	+	+	+	+	+
5/L-2	+	+	+	+	-	+	+	+
1/L-3	+	+	-	+	-	+	+	-
2/L-3	+	+	-	+	-	+	+	-
3/L-3	+	+	-	+	-	+	+	+
4/L-3	+	+	-	+	+	+	+	-
5/L-3	+	+	+	+	+	+	+	-

injector (Rheodyne, Cotati, CA, USA) and a variable wavelength UV-VIS detector. The chromatograms were recorded with a 3396A reporting integrator (Hewlett-Packard). A stainless-steel column (200 x 4.6 mm I.D.) packed with 5 μm ODS Hypersil (Shandon, Cheshire, UK) was used.

All reagents (methanol, acetic acid) were of chromatographic grade (J. T. Baker B. V., Deventer, Holland) and, in all experiments, bidistilled water was used. Phenolic acids were purchased from Sigma (St. Louis, MO, USA). The amount of 1 mg of each standard was weighed, dissolved in 10 mL of methanol, and applied to the column. Successive dilutions of the phenolic acid solutions were also prepared for the estimation of calibration curves. Samples of 10 μL were injected into the HPLC system.

For qualitative and quantitative analysis of phenolic acids, methanol - water (25 : 75) with 1% (v/v) addition of acetic acid, was used as the mobile phase. Compounds were detected at 254 nm. All measurements were done at a flow-rate of 1 mL/min at ambient temperature.

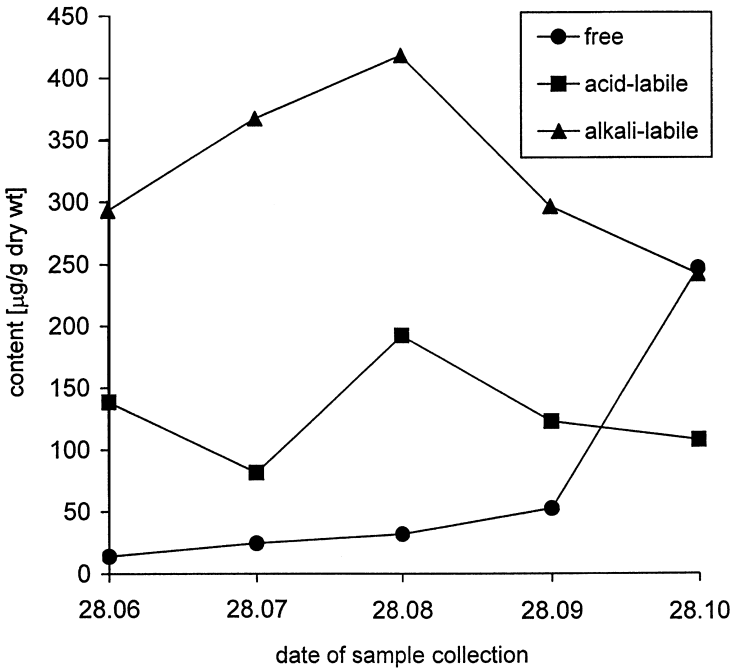

Figure 7. *Para*-Coumaric acid content (*cis* + *trans* form) in *G. biloba* leaves within vegetative period.

Dried fractions of phenolic acids, obtained by liquid-liquid extraction, were dissolved in 50% methanol prior to HPLC analysis. The identification of phenolic acids was accomplished by the comparison of their retention times with standards.

Quantitative determination was carried out using the external standard method. The amounts of phenolic acids were calculated from calibration curves, comparing the mean (n = 3) peak areas with standard concentrations.

RESULTS AND DISCUSSION

This paper presents the results of qualitative and quantitative analysis of phenolic acids in *G. biloba* leaves, both free and liberated by acid and alkaline hydrolysis. At the first step, qualitative analysis of phenolic acids was carried out by TLC. Two-dimensional chromatography on cellulose plates revealed the presence of the following compounds: protocatechuic, *para*-hydroxybenzoic,

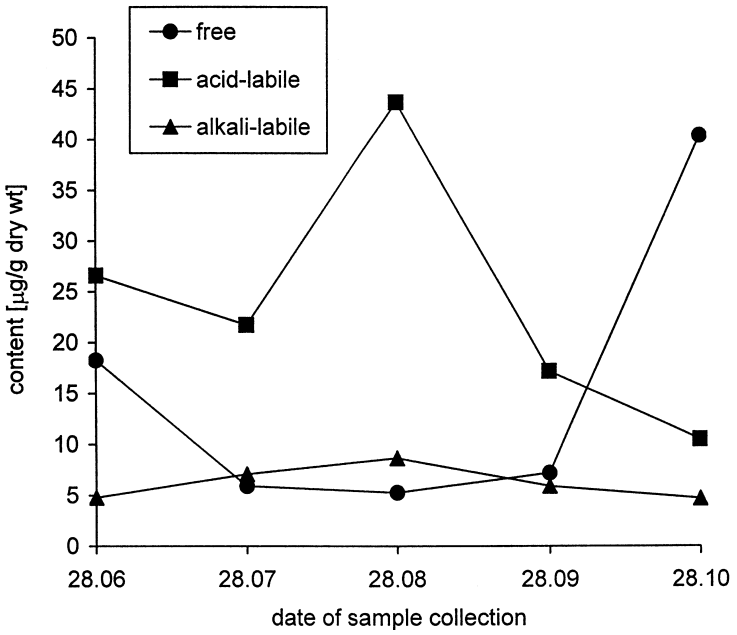

Figure 8. Ferulic acid content (*cis* + *trans* form) in *G. biloba* leaves within vegetative period.

and vanillic,[1,13] as well as caffeic, *para*-coumaric, and ferulic acids, which had been reported by us previously.[14,15] These acids were dominant in every sample containing free phenolic acids, collected from June to October. Moreover, sinapic and isovanillic acid were found among free phenolic acids in sample 5/L-1, as well as in some samples containing acid- and alkali-labile phenolic acids (Table 2). It was the first time that these two compounds were identified in *G. biloba* leaves.

Qualitative HPLC analysis confirmed the results of 2D-TLC (Fig. 1) and showed the presence of eight phenolic acids, both free and bound, in fractions examined, even if the concentration levels of some compounds (sinapic, isovanillic) in plant material were very low (~ 1.0-5.0 μg/g dry wt). Based on the quantitative results of HPLC analysis, the growing tendency, as regards to the concentrations of free phenolic acids, was observed during the whole vegetative period (Figs. 2-9). It was also found that covalently-bound, i.e., acid- and alkali-labile phenolic acids, were present in considerably greater amounts than the free ones in *G. biloba* leaves collected in several months of plant vegetation.

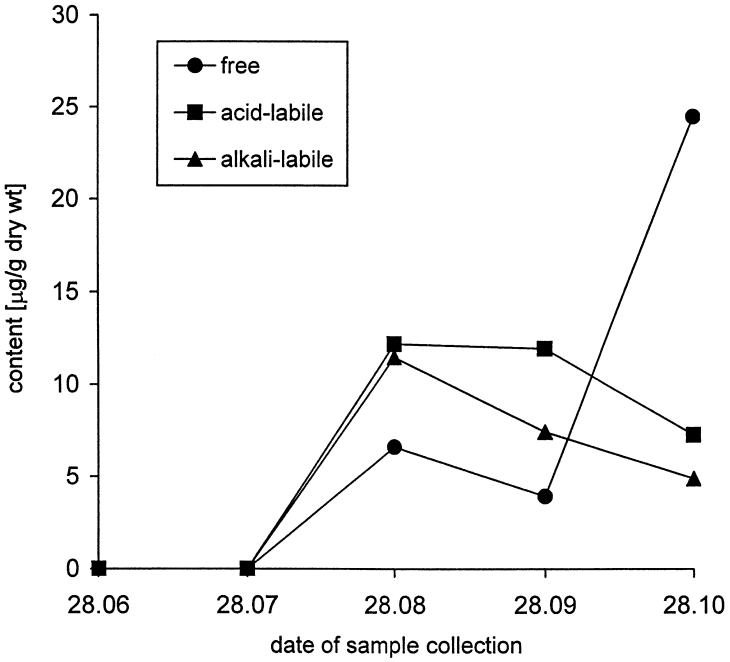

Figure 9. Sinapic acid content in *G. biloba* leaves within vegetative period.

Only the free form of protocatechuic acid in every case predominated (Fig. 2). Similarly, the bound, alkali-labile *para*-coumaric acid was predominant in all samples (Fig. 7).

These results seem to prove that this compound mainly occurs in the ester form in *G. biloba* leaves. On the other hand, acid-labile vanillic, caffeic and ferulic acids were present in the highest concentrations in samples collected from June to August (Figs. 4, 5, 8). It proved that these phenolics predominated as glycoside forms in *G. biloba* leaves. The highest amounts of all free phenolic acids in the last month (October) of plant vegetation were observed when the collected leaves were yellow. As regards to acid- and alkali-labile forms of phenolic acids, the biggest accumulation was in the middle of the vegetative period (August) for almost all compounds examined (Figs. 2-9).

Summing up, the application of TLC and HPLC methods, and a special extraction procedure, enabled the study of the distribution of both free and covalently-bound phenolic acids.

Such investigations broaden the scientific knowledge on the biochemistry of plant phenolics. On the other hand, they enable the estimation the proper time when *G. biloba* leaves should be collected to possess the best pharmacological properties.

ACKNOWLEDGMENTS

We wish to thank Alina Babula, M.Sc., from the Botanical Garden of A. Mickiewicz University (Poznań, Poland), for her permission to collect *G. biloba* leaves.

REFERENCES

1. E. W. Fünfgeld, **Rökan, Ginkgo biloba**, Springer-Verlag, Berlin, Heidelberg, New York, London, Paris, Tokyo, 1988.

2. Z. Janeczko, D. Sobolewska, T. Marszewska-Karmazyn, Farm. Pol., **50**, 1037-1041 (1994).

3. H. Schilcher, Z. Phytother., **9**, 119-127 (1988).

4. H. Huk, E. J. Staba, J. of Herbs, Species, Med. Plants, **1**, 91-124 (1992).

5. A. Hasler, O. Sticher, B. Meier, J. Chromatogr., **605**, 41-48 (1992).

6. O. Sticher, Planta Med., **59**, 2-11 (1993).

7. A. Lobstein, L. Rietsch-Jako, M. Haag-Berrurier, R. Anton, Planta Med. **57**, 430-433 (1991).

8. Ch. Nasr, M. Haag-Berrurier, A. Lobstein-Guth, R. Anton, Phytochemistry, **25**, 770-771 (1986).

9. Ch. Nasr, A. Lobstein-Guth, M. Haag-Berrurier, R. Anton, Phytochemistry, **26**, 2869-2870 (1987).

10. L. Verotta, E. Lolla, A. Moggi, Fitoterapia, **62**, 339-341 (1991).

11. F. Briancon-Scheid, A. Guth., R. Anton, J. Chromatogr., **245**, 261-267 (1982).

12. T. A. Van Beek, H. A. Scheeren, T. Rantino, W. H. Melger, G. P. Lelyveld, J. Chromatogr., **543**, 375-378 (1991).

13. A. Codignola, L. Verotta, P. Spanu, M. Maffei, S. Scannerini, P. Bonfante-Fasolo, New Phytol., **112**, 221-228 (1989).

14. M. Ellnain-Wojtaszek, Acta Pol. Pharm., **54**, 225-228 (1997).

15. M. Ellnain-Wojtaszek, Acta Pol. Pharm., **54**, 229-232 (1997).

16. B. Borkowski, Herba Pol., **39**, 139-144 (1993).

17. E. Dombrowicz, L. Swiątek, Farm. Pol., **43**, 639-642 (1987).

18. L. Swiątek, E. Dombrowicz, Farm. Pol., **40**, 729-732 (1984).

19. E. Dombrowicz, L. Swiątek, R. Zadernowski, Farm. Pol., **44**, 657-660 (1988).

20. T. Sawicka, J. Drozd, J. Prosińska, B. Borkowski, Herba Pol., **40**, 31-32 (1994).

21. T. Krzaczek, Farm. Pol., **40**, 475-477 (1984).

22. M. Ellnain-Wojtaszek, I. Mirksa, Acta Pol. Pharm., **55**, 81-84 (1998).

23. L. W. Wulf, W. C. Nagel, J. Chromatogr., **116**, 271-279 (1976).

24. L. Nagels, W. Van Dongen, J. De Brucker, H. De Pooter, J. Chromatogr., **187**, 181-187 (1980).

25. M. Vanhaelen, R. Vanhaelen-Fastre, J. Chromatogr., **187**, 255-260 (1980).

26. B. Meier, A. Hasler, O. Sticher, Planta Med., **58(Suppl. A)**, 688-689 (1992).

27. J. B. Harborne, **Methods in Plant Biochemistry. Vol. 1**, Academic Press, London, San Diego, New York, Boston, Sydney, Tokyo, Toronto, 1989.

28. A. Hasler, B. Meier, Planta Med., **59(Suppl. A)**, 632 (1993).

29. T. A. Van Beek, G. P. Lelyveld, Planta Med., **58**, 413-416 (1992).

30. W. Flesch, M. Jacques, L. Cosson, B. P. Teng, V. Petiard, J. P. Balz, Phytochemistry, **31**, 1941-1945 (1992).

31. L. Wiątek, Herba Pol., **23**, 201-209 (1977).

32. H. Wysokińska, L. Swiątek, Acta Pol. Pharm., **43**, 623-629 (1986).

33. A. Gawron-Gzella, Z. Kowalewski, Acta Pol. Pharm., **52**, 301-304 (1995).

Received May 13, 1998
Accepted July 24, 1998
Manuscript 4901-TLC

Request Permission or Order Reprints Instantly!