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Short Communication

Supercritical fluid chromatographic analysis of polyprenols in *Ginkgo biloba* L

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ABSTRACT

A supercritical fluid chromatographic (SFC) procedure for the quantitation of three major polyprenols present in the leaves of *Ginkgo biloba* was developed. In contrast to previously reported high-performance liquid chromatographic (HPLC) methods, the SFC method does not require extensive pre-purification for polyprenol analysis. The SFC analytical procedure described shows a very broad range of linearity and detects many known polyprenol isoprenologs with baseline separation. Dodecaprenol was used as the internal standard. The coefficient of variation of the method was 5.8% for the quantitation of C_{85}, C_{90} and C_{95} polyprenols. The SFC assay results showed that the content of polyprenols in ginkgo leaves were higher than the previously published values. In addition, the chromatogram of the highly concentrated leaf extract revealed the presence of an isoprenolog (C_{120}) not previously detected by HPLC methods.

INTRODUCTION

Several types of polyprenols have been found in the plant kingdom. Most of the polyprenols isolated from the angiosperms have the ω-t_3-c_n-OH structure. However, polyprenols from many gymnosperms are known to have the ω-t_2-c_n-OH structure (see Fig. 1) [1] and are therefore similar to the structure of the animal dolichols. Dolichols function in the transfer of the sugar moieties during the process of protein glycosylation [2]. Numerous recent studies have shown that dilochols are elevated in the urine of alcoholics suggesting that urinary dilochols could serve as an important laboratory marker for alcoholism [3].

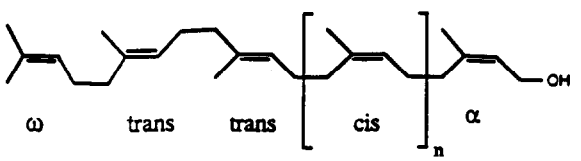

Fig. 1. Structure of Ginkgo polyprenols (di-*trans*,poly-*cis*-prenols), $n = 10$–18. Mixtures of polyprenols have different numbers of isoprene units. For example, if $n = 11$, it represents pentadecaprenol and if $n = 12$, it represents hexadecaprenol.

The precise physiological role of polyprenols in plants is not fully understood and it has been suggested that they may have physiological roles similar to that of dilochols in animals [4]. The ginkgo

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leaves have a very high content of polyprenols that have a chemical structure slightly different from that of animal dolichols. Therefore, they could provide an abundant source of an intermediate that could be used in the synthesis of dolichols [5,6].

In 1983, Ibata *et al.* [7] isolated and characterized polyprenols from the leaves of *Ginkgo biloba.* Using high-performance liquid chromatographic (HPLC) separation, field-desorption mass spectroscopy (FD-MS), ^{1}H NMR spectroscopy and/or ^{13}C NMR spectroscopy [1,7], they were able to show that the ginkgo polyprenol mixture contained compounds which had 14 to 22 isoprene units with 17, 18 and 19 isoprene-containing compounds being predominant. Numerous other studies using very laborious and elaborate purification schemes with HPLC separation have characterized and quantitated polyprenols in plant, and dolichols in animal samples [3,8–11].

Our studies describe the use of a very simple purification protocol followed by chromatographic separation using supercritical fluid chromatography (SFC) to quantitate the various polyprenols present in the leaves of *G. biloba.*

EXPERIMENTAL

Instrumentation and chromatographic conditions

A Series 600 capillary supercritical fluid chromatograph (Lee Scientific, Salt Lake City, UT, USA) was used for all analyses. The SFC system was equipped with a flame ionization detector and Valco injection system with a 200-nl injection loop operated in a time-split mode. All analyses were performed with the supercritical fluid carbon dioxide as the mobile phase (Scott Specialty Gases, PA, USA). For the separation of polyprenols, a chemically bonded SB-Phenyl-50 capillary column (10 m × 50 mm I.D., film thickness 0.25 mm) purchased from Lee Scientific was used. The injection time used was 0.2 s and the detector temperature was maintained at 325°C. Separation of the polyprenols was achieved by pressure gradient in which the initial pressure of 200 atm was increased at a rate of 20 atm/min to a final pressure of 400 atm. The oven temperature was maintained at 100°C. All of the data were processed by a Dionex AC1600 SFC–gas chromatographic system controller in an IBM PS/2 Model 60-071 computer.

Standards and identification

Dodecaprenol (C_{60}) from *Rhus typhina* was used as the internal standard and it was provided by Drs. E. Swiezewska and T. Chojnacki, Polish Institute of Physics and Chemistry (Warsaw, Poland). A polyprenol mixture from *G. biloba* was provided by Dr. Takigawa, Kuraray (Okayama, Japan). Polyprenol standards (C_{85}, C_{90}, C_{95}) were purchased from Sigma (St. Louis, MO, USA) and all reagents used were of analytical-reagent grade. Individual standards curves for C_{85}, C_{90} and C_{95} polyprenols were prepared in hexane at concentrations of 0.5, 1.0, 1.5 or 2.0 mg/ml for each compound with 0.5 mg of dodecaprenol (C_{60}) as internal standard. The ratio of the area under each polyprenol peak and the dodecaprenol peak was plotted against the individual polyprenol concentration. Standard curves were generated each day that samples were analyzed.

Plant materials

Ginkgo leaves were collected and pooled from three trees located on the campus of the University of Minnesota (Minneapolis, MN, USA) during the following seasons: spring (May), summer (August), fall (early October) and the late fall (late October) of 1990. The leaves were oven-dried at 60°C and pulverized. Ginkgo leaves were also collected from 3-month-old seedlings germinated and grown in a green house (College of Pharmacy, University of Minnesota).

Extraction and purification

Pulverized leaves (about 200 mg) spiked with 500 μg of the internal standard dodecaprenol were extracted for 1 h in *n*-hexane with occasional stirring. The resulting *n*-hexane extract was washed three times with 90% aqueous methanol and then vigorously stirred with potassium carbonate and methanol for 12 h at room temperature. After a further washing once with water and twice with saturated sodium chloride solution, the resulting *n*-hexane solution was passed through sodium sulfate. The eluted *n*-hexane solution was evaporated under nitrogen. The residue was reconstituted with 1 ml of *n*-hexane prior to separation of the polyprenols by SFC.

Quantitation and reproducibility of the method

Two ginkgo leaf samples collected for each sea-

son were extracted, purified and subjected to separation by SFC. The concentration of each polyprenol (C_{85}, C_{90} and C_{95}, respectively) was calculated from the standard curves generated, and the content of each polyprenol (mg/g of dried leaves) was calculated. The reproducibility of the SFC analysis method was validated by analyzing ten different samples of a batch of leaves that were harvested on October 25th, 1990. Polyprenol contents were calculated as described and the relative standard deviation (R.S.D.) for each polyprenol was determined.

RESULTS AND DISCUSSION

The use of SFC enabled us to rapidly separate the different polyprenols that were present in the leaves of the ginkgo (see Fig. 2). The extraction and saponification techniques we used were similar to those were described by Takigawa *et al.* [1] with the exception that we omitted the purification steps with adsorbent carbon treatment and column chromatography. Even though we were using a semi-purified mixture, the use of SFC enabled us to obtain baseline separation between different poly-

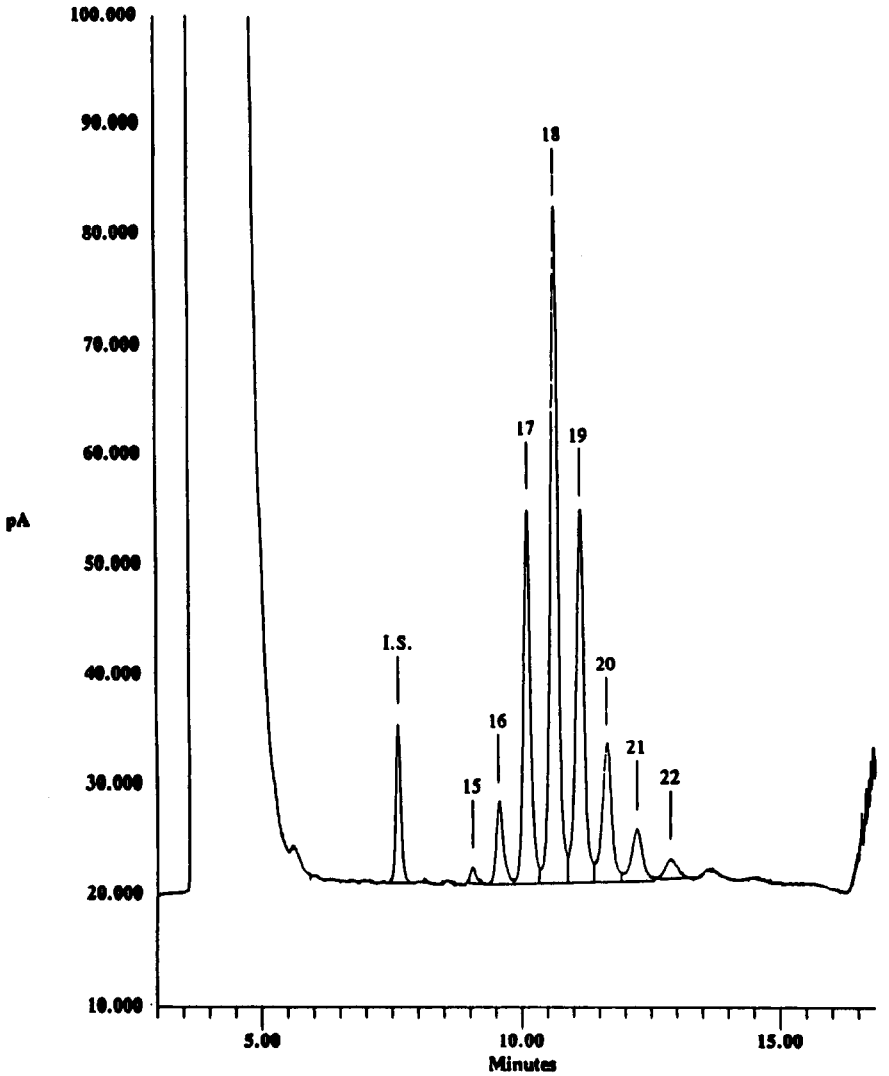

Fig. 2. SFC separation of polyprenol extract from ginkgo leaves. Numbers above the peaks represent isoprene units. Sample: leaf collected late October; 263 mg of dried leaves were extracted, saponified and reconstituted with 1 ml of *n*-hexane. Injection volume, 0.1 μl. I.S. = Internal standard.

prenols (see Fig. 2). Dodecaprenol (C_{60}, containing 12 isoprene units) from *R. typhina* was selected as the internal standard because this compound is not present in ginkgo but it has similar physicochemical properties to the other polyprenols that are present in the ginkgo. The use of this internal standard enabled the correction of recovery of polyprenols during the extraction process.

This present study shows for the first time the application of SFC to the separation and quantitation of polyprenols in plant tissues. A number of other investigators have used HPLC techniques to quantitate polyprenols in plant tissues [1,7,10]. Their techniques involve the use of very elaborate and laborious purification steps prior to HPLC. The use of SFC eliminates the need for extreme prepurification steps.

Fig. 2 shows the presence of polyprenols containing 15 isoprene units to 22 isoprene units to be present in the ginkgo leaf extract. As previously reported [7] and confirmed by our SFC techniques, polyprenols containing 17, 18 and 19 isoprene units

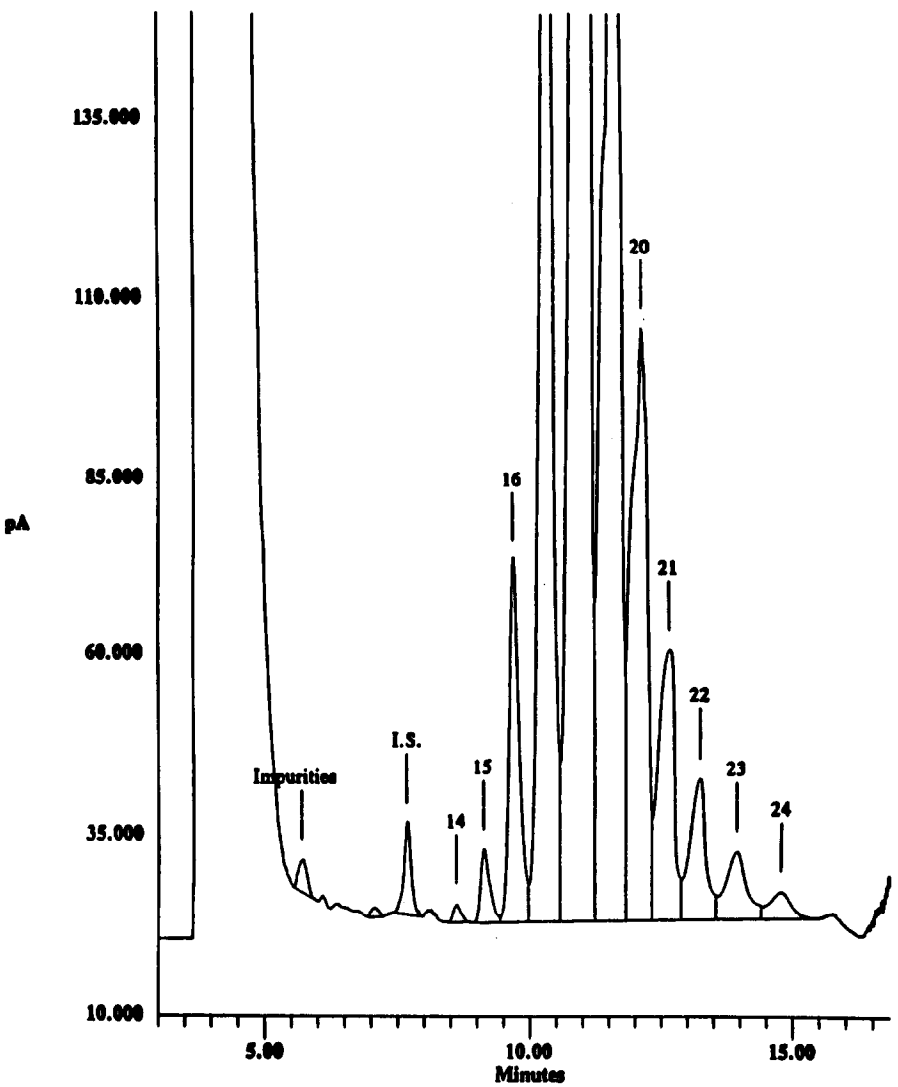

Fig. 3. SFC separation of concentrated ginkgo polyprenol extract. Numbers above the peaks represent isoprene units. Sample: leaf collected late October; 2.99 g of dried leaves were extracted, saponified and reconstituted with 1 ml of *n*-hexane. Injection volume, 0.1 μl.

were the predominant species present and they represented about 80% of the total ginkgo polyprenols.

In Fig. 3 shows that when we injected a highly concentrated extract from the leaves, we were able to show the presence of an additional peak with retention time of 14.8 min that appears to have 24 isoprene units (C_{120}). Such a polyprenol has not been previously described to be present in the ginkgo. Further characterization of this compound is currently being carried out to verify that it is indeed a C_{120}-containing polyprenol.

The calibration curves obtained for the three major polyprenols C_{85}, C_{90} and C_{95} show a wide range of linearity (see Fig. 4), up to 2 mg/ml for each compound. The reproducibility in quantitating ten different samples from the same batch of pulverized leaves was excellent (see Table I). The R.S.D. for the amounts of each of three compounds was less than 10%, and the value of the sum of total polyprenol content of these three species in the ten samples extracted had an R.S.D. of less than 6%. The recovery of the ginkgo polyprenols based on the recovery of our internal standard (500 μg) was 85.5 $\pm$ 13.7% ($n=9$).

The content of the three major polyprenols in ginkgo leaves analyzed by SFC and expressed in mg/g dry weight of the leaves was approximately

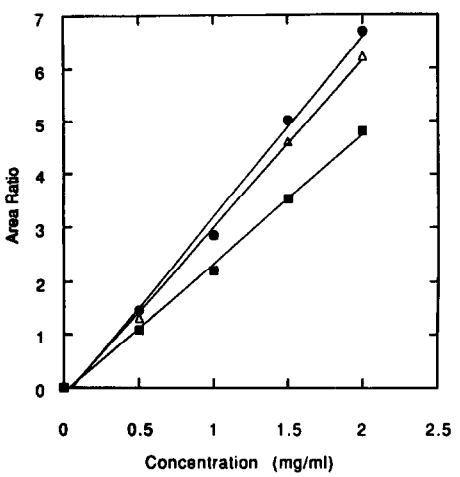

Fig. 4. Standard curves for three major polyprenols. The concentration coefficients of the regression lines of C_{85} polyprenol (△), C_{90} polyprenol (■) and C_{95} polyprenol (●) were 0.9989, 0.9990 and 0.9969, respectively.

TABLE I

REPRODUCIBILITY OF POLYPRENOL ANALYSIS BY SFC

Ten different samples from the same batch of pulverized leaves were subjected to the extraction and saponification procedure. Each of the polyprenols C_{85}, C_{90} and C_{95} was quantitated.

Polyprenol	Content (mean ± S.D.) (mg/g dry weight)	R.S.D. (%)
C_{85}	4.37 ± 0.296	6.78
C_{90}	10.58 ± 0.742	7.01
C_{95}	4.85 ± 0.405	8.35
Sum	19.57 ± 1.146	5.86

TABLE II

SEASONAL VARIATION OF POLYPRENOLS IN GINKGO LEAF

Season	Age of leaf (weeks)	Content (mg/g dry weight)	
		Present study	Ibata *et al.* [7][a]
Spring	6	1.5	1.0
Summer	20	10.2	8.0
Fall	26	16.9	14.0
Late fall	28	19.6	16.0

[a] Values calculated from Fig. 5 of ref. 7.

20% higher than the previously reported values [7]. This yield discrepancy might be resulted from either the quantitation methods used or geographical origin and/or environmental difference of the leaf samples. Our data also show seasonal variation in ginkgo polyprenol content (see Table II) and is in agreement with the findings of Ibata *et al.* [7].

The present study shows that SFC is an excellent alternative to HPLC for the analysis and quantitation of plant polyprenols and possibly of dolichols in animal tissues.

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