Identification and the Retention Index of Bilobalide and Ginkgolides Using Capillary Gas Chromatography

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Abstract

***Ginkgo biloba* L. is known to contain the unique terpene trilactone compounds bilobalide and ginkgolides. Capillary gas chromatography is used for the quantitative identification of bilobalide and the main ginkgolides (ginkgolide A, B, and C). The retention indices of these compounds are also determined. Retention indices of bilobalide and ginkgolide A, B, and C substitute the use of their standards at their routine identification. Our procedure does not require temperature-programmed operation.**

Introduction

The *Ginkgo biloba* L. plant contains several compounds of pharmacological importance that can be divided into two distinct chemical groups. One of these groups contains the flavonoids (which are widespread in plants), and the second group is formed by unique terpene trilactone structures, the characteristic compounds of *Ginkgo biloba* L. (1,2). The sesquiterpene or pentanorditerpene (3) bilobalide (Figure 1) and the diterpene ginkgolides A, B, and C (Figure 2) are the terpene constituents of *Ginkgo biloba* L. Bilobalide shows neuroprotective properties (4) and ginkgolides are known as platelet-activating-factor antagonists (5,6). Fast, simple, and reliable analytical methods are necessary to identify the terpene trilactones because they are the compounds (and also the flavonoids) that are considered to be responsible for the pharmacological activity of the standardized and therapeutically used leaf extract of *Ginkgo biloba* L. (7,8).

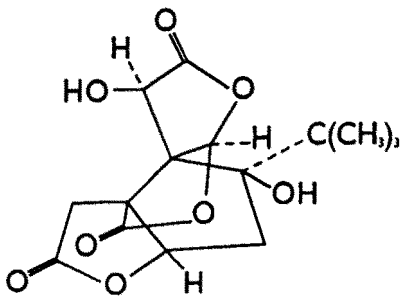

Figure 1. Structure of bilobalide.

Diverse methods have been described for the separation and quantitative determination of bilobalide and the ginkgolides in the extracts, leaves, and cell cultures. Pietta et al. (9) published a reversed-phase high-performance liquid chromatographic (HPLC) method for the separation of the various components of *Ginkgo biloba* L. extract. The quantitation of bilobalide and the ginkgolides was published by van Beek (10); the samples were the leaves of *Ginkgo biloba* L., and the phytopharmaceuticals also originated from *Ginkgo biloba* L.

Neither bilobalide nor the ginkgolides can be analyzed directly by gas chromatography (GC). For the GC analysis, the trimethylsilyl ether derivatives were formed from the terpene trilactones on the basis of their active hydrogen functions. There are two active hydrogen functions in the structures of the sesquiterpene bilobalide and the diterpene ginkgolide A. Ginkgolide B and ginkgolide C contain three and four active hydrogen functions, respectively.

Carrier et al. (11), Hasler et al. (12), Huh and Staba (13), and Jeon et al. (14) applied GC to the trimethylsilyl derivatives. Carrier et al. (11) reported the use of GC combined with online mass spectrometry (MS) for the identification of the trimethyl-

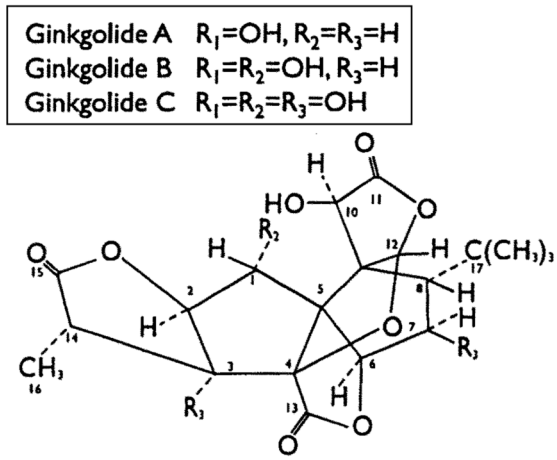

Figure 2. Structures of the ginkgolides.

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silyl derivative of ginkgolide A from the cell culture of *Ginkgo biloba* L.; however, no data were published on bilobalide and ginkgolides B and C.

Hasler et al. (12) used capillary GC (CGC) instead of HPLC to determine the characteristic terpene compounds of *Ginkgo biloba* L. Also, CGC was proposed for the routine quantitation of the terpene constituents in the leaves and the extract of *Ginkgo biloba* L. Huh and Staba (13) proposed to use GC for the identification of ginkgolide A, B, and C after extraction and purification.

Jeon et al. (14) described GC–MS for the identification and quantitation of ginkgolide B in *Ginkgo biloba* cell cultures using the slightly modified method of Huh and Staba (13).

The retention index (I) introduced in 1958 by E.Sz. Kováts (15) is the most widely accepted way for compound identification. It is conveniently calculated according to the following equation (16,17):

$$I_i^{st.ph.}(T) = 100\,[((\log t_{R'i} - \log t_{R'z}) / (\log t_{R'(z+1)} - \log t_{R'z})) + z] \quad \text{Eq. 1}$$

where i is the compound of interest, st. ph. is the stationary phase, z is the *n*-alkane with a z carbon number, z+1 is the *n*-alkane with a z+1 carbon number, and t_R' is the adjusted retention time.

$$t_R' = t_R - t_M \quad \text{Eq. 2}$$

where t_R is the retention time and t_M the gas holdup time. In this study, we described the quantitative identification of bilobalide and the ginkgolides using isothermal GC after the formation of silyl ethers, and we also determined the Kováts retention indices.

Experimental

Materials

Standard compounds, such as bilobalide and ginkgolide A, B, and C, were kindly supplied by Dr. Teris van Beek (Phytochemical Section, Laboratory of Organic Chemistry, Agricultural University, Dreijenplein, The Netherlands). Cholesterol and *n*-alkanes were purchased from Fluka (Buchs, Switzerland). The derivatization reagents, *N*,*O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS), were also purchased from Fluka.

An HP-1 column (OV-1 methylsilicone) (25-m × 0.2-mm i.d.) with a 0.5-µm stationary phase film thickness was used for the analysis. The carrier gas was helium (Gumpoldskirchen, Austria) with a 125-kPa inlet pressure.

Table I. Retention Times, Adjusted Retention Times, and Holdup Times of the Trimethylsilyl Ethers of the Compounds of Interest at 310°C

Trimethylsilyl Ethers of Compounds	t_R (min)	$t_{R'}$ (min)	t_M (min)
Cholesterol	16.283	14.219	2.064
Bilobalide	5.730	3.666	2.064
Ginkgolide A	14.886	12.822	2.064
Ginkgolide B	17.125	15.061	2.064
Ginkgolide C	17.901	15.837	2.064

Chromatographic conditions

An HP 5890 Series II GC (Agilent, Palo Alto, CA) was used equipped with a flame ionization detector (FID) and connected to an IBM AT 386 computer. A volume of 1 µL of the sample was injected using the split ratio of 1:100. The column was used for

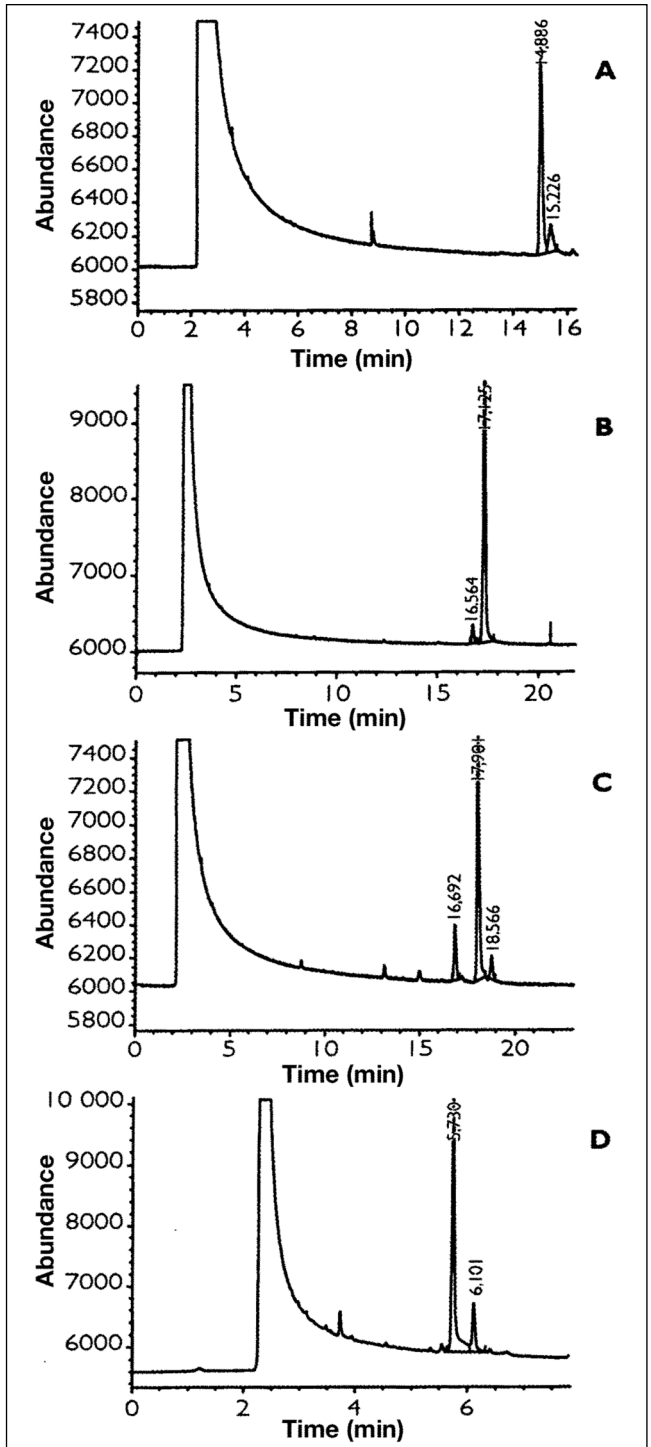

Figure 3. Chromatograms of the trimethylsilyl ethers of (A) ginkgolide A, (B) ginkogolide B, (C) ginkogolide C, and (D) bilobalide at 310°C.

the isotherm temperatures of 290°C, 300°C, or 310°C. Both the detector and the injector temperatures were 300°C. The diluted gas was 30-mL/min nitrogen, and the auxiliary gas was hydrogen with 140 kPa and air with 250 kPa inlet pressure.

Derivatization

A volume of 500 µL of the derivatization reagent (BSTFA also containing 1% TMCS) was added to the vials containing either 3 mg internal standard (cholesterol) or one of the terpenes. The vial was placed into a 120°C thermostat for 45 min.

Results

The retention times of the trimethylsilyl ether derivatives of bilobalide and ginkgolide A, B, and C were determined at 310°C and are listed in Table I. The chromatograms of the constituents are shown in Figure 3.

The retention indices were determined at three temperatures (290°C, 300°C, and 310°C) using isothermal conditions. The difference between the retention indices of the compound of interest measured at 290°C and 300°C (ΔI_1/10°C) and at 300°C and 310°C (ΔI_2/10°C) was calculated. The data are shown in Table II.

Using the retention indices determined at three different temperatures and equation 3 (18,part II of 19), the constants *a* and *b* can be calculated for each compound. The values of the constants are listed in Table III.

The retention index of the compound of interest can be calculated according to the following equation:

$$I = at + b \qquad \text{Eq. 3}$$

where *t* is the column temperature (°C).

The retention indices of the compounds of interest can be calculated at any extended range of temperature on the OV-1 stationary column using *a* and *b* constants. As an example, the calculated values of the retention indices (I_{280}) at 280°C are listed in Table IV.

The retention indices determined at three different temperatures were suitable for the identification of the compounds of interest. When the dependence of the retention index on the temperature is known, values of the retention indices of the compounds can be calculated at any extended range of temperature.

The advantage of the method is that it is reliably applicable in the case of less than 1% concentration.

If the compounds of interest can be suspected in the sample of a plant or a phytopharmaceutical product, after suitable preparation (silylation) the compounds can be determined without standards.

Table II. Retention Indices of the Trimethylsilyl Derivatives of the Compounds of Interest at 290°C, 300°C, and 310°C and the Differences Between the Retention Indices Measured at 290°C and 300°C (ΔI_1/10°C) and 300°C and 310°C (ΔI_2/10°C)

Trimethylsilyl ethers of compounds	Retention indices 290°C	300°C	310°C	ΔI_1/10°C	ΔI_2/10°C
Cholesterol	3174.8	3201.4	3229.2	26.6	27.8
Bilobalide	2481.9	2504.2	2526.7	22.3	22.5
Ginkgolide A	3130.7	3153.1	3175.6	22.4	22.5
Ginkgolide B	3211.0	3235.0	3259.0	24.0	24.0
Ginkgolide C	3241.9	3263.4	3285.0	21.5	21.6

Table III. Values of *a* and *b* Constants and the Correlation Coefficients (*r*) of the Trimethylsilyl Ethers of the Compounds of Interest

Trimethylsilyl ethers of compounds	*a*	*b*	*r*
Cholesterol	2.720	2386.11	0.999919
Bilobalide	2.240	1832.27	0.999997
Ginkgolide A	2.245	2479.60	0.999999
Ginkgolide B	2.400	2515.00	1.000000
Ginkgolide C	2.155	2616.93	0.999999

Table IV. Calculated Values of the Retention Indices (I_{280}) of the Trimethylsilyl Derivatives of the Compounds of Interest at 280°C

Trimethylsilyl ethers of compounds	I_{280} (min)
Cholesterol	3149.3
Bilobalide	2459.8
Ginkgolide A	3108.4
Ginkgolide B	3187.0
Ginkgolide C	3221.0

Discussion

Standardized extracts of *Ginkgo biloba* leaves are mainly used in the treatment of peripheral and cerebral circulation disorders. The leaf extracts contain biologically active terpene lactones. These unique terpene trilactones are the diterpene ginkgolides and the sesquiterpene or pentanor-diterpene bilobalide. Diverse methods have been described for the separation and quantitative determination of the terpene constituents.

The neuroprotective effects (4) of *Ginkgo biloba* extract have been known for a long time. Recent clinical studies have demonstrated that a standard extract of *Ginkgo biloba* (EGb 761) was effective for mild-to-moderate dementia of Alzheimer's disease patients (20). Certain studies (21,22) enlist several other beneficial effects of the extract of *Ginkgo biloba* (EGb 761) (21) and also of the bilobalide, such as direct effects against necrosis and the apoptosis of neurons, improvement of neural plasticity, regulating ion balance, and facilitating behavioral adaption to stress. (20–22). Bilobalide has had protective effects on amyloid beta-peptide 25-35 induced PC12 cell cytotoxicity (22). In order to declare the exact mechanism of the physiological and pharmacological activity, an increasing attention has been paid to the anal-

ysis of ginkgo leaf extract in order to evaluate its active components. Recent analyses have been performed using liquid chromatography (LC)–atmospheric pressure chemical ionization (APCI)–MS (23,24), the sophisticated method for the detection and identification of bilobalide and the ginkgolides of multicomponent mixtures. LC–APCI–MS has been widely used to analyze ginkgo leaf extracts and phytopharmaceuticals (23) and also the plasma of volunteers dosed with *Ginkgo biloba* L. extracts (24).

The hereby described CGC method uses numerical calculations for the reliable identification of bilobalide and the ginkgolides in an inexpensive and simple way. The determination of bilobalide and the ginkgolides of the standardized extracts of ginkgo leaves or phytopharmaceuticals requires a fast and simple method. The described analytical method is fast, simple, and reliable to identify the terpenes in the standardized and therapeutically used leaf extract of *Ginkgo biloba* L.

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