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Direct analysis of alkylphenols in *Ginkgo biloba* leaves by thermochemolysis–gas chromatography/mass spectrometry in the presence of tetramethylammonium hydroxide

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ABSTRACT

Thermochemolysis–gas chromatography/mass spectrometry (GC/MS) in the presence of tetramethylammonium hydroxide (TMAH) was applied to the determination of alkylphenols in *Ginkgo biloba* leaves directly using a vertical microfurnace pyrolyzer. TMAH thermochemolysis–GC enabled the highly sensitive determination of alkylphenols including ginkgolic acids and ginkgols in *G. biloba* leaves as their methyl derivatives on the resulted pyrograms. On the basis of their peak areas, the contents of the alkylphenols in *G. biloba* leaf sample were rapidly and precisely determined without using any tedious and time-consuming pretreatment.

1. Introduction

Ginkgo biloba extracts (EGb) containing many kinds of bioactive constituents such as terpene trilactones and flavonol glycosides possess important pharmacological properties especially in the treatment of cardiovascular disease [1,2]. However, some alkylphenols including ginkgolic acids and ginkgols (Fig. 1) were proved to exist in ginkgo leaves, and occurred in different concentrations and proportions in commercial extracts [3,4] due to the different manufacturing processes. These compounds appear allergenic, mutagenic and slight neurotoxic, and should be well characterized and avoided in commercial extracts. Therefore, a maximum concentration of alkylphenols in EGb had been proposed in the draft monographs of US [5,6] and European pharmacopeias [7,8] by establishing a limit value of 5 ppm. Thus, it is requested to develop a practical method to determine the contents of alkylphenol components in *G. biloba* leaves or extracts rapidly and precisely.

So far, high-performance liquid chromatographic (HPLC) technique with preliminary treatments including enrichment by liquid–liquid extraction and concentration of the organic layer has been used for the determination of alkylphenols in *G. biloba* leaves and EGb. This procedure is time-consuming and lacks in reproducibility [9]. LC–ESI-MS in the negative mode has been successfully applied to sensitively analyze ginkgolic acids in *G. biloba* leaves [10,11]. However LC–MS/MS is a rather heavy method for routine quality control of medicinal plants and their extracts. A simple HPLC-UV method has been reported for allowing the quantification of ginkgolic acids in ginkgol extracts and not requiring enrichment procedures [12]. However, only 4 kinds of ginkgolic acids had been detected among alkylphenol components. Furthermore, it was difficult to separate the positional double bond isomers (Δ8 or Δ10) of C15:1 ginkgolic acid in *G. biloba* leaves by HPLC method described above, even though a dual column was used [13]. A method for the scale-up separation and qualitatively analysis of ginkgolic acids and ginkgols simultaneously by the supercritical fluid extraction (SFE) and gas chromatography/mass spectrometry (GC/MS) as trimethylsilyl derivatives was reported by Verotta and Peterlongo [3]. Furthermore, a separation of the positional double bond isomers (Δ8 or Δ10) of C15:1 was also achieved. However, no sensitivity and recovery data were given by this method.

Recently it was demonstrated that thermochemolysis–gas chromatography (GC) in the presence of TMAH was effective for the rapid and direct characterization of phenolic acids from some wood extractives [14,15] and wine tannin [16]. González-Vila et al. [14] applied this technique to the qualitative analysis of wood extractives including lipids, terpenoids and phenolic compounds from *Eucalyptus globules* in the presence of TMAH. Ishida et al. [15]

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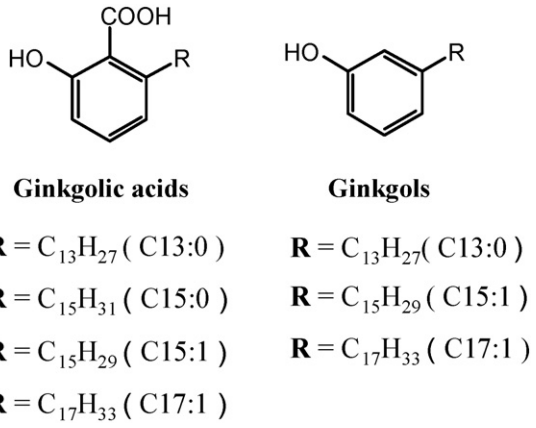

Fig. 1. Main components of *Ginkgo biloba* alkyphenols. *Double bonds have the *Z*-configuration.

quantitatively analyzed phenolic extractives in wood by this technique in the presence of tetrabutylammonium hydroxide. Galletti et al. [16] qualitatively analyzed the tannin fraction including flavonoids, phenolic acids and fatty acid components isolated from red wine by this technique in the presence of TMAH. This technique for quantitative determination of components including fatty acid, aliphatic alcohol and phenolic acid in carnauba wax [17] was also reported successfully. The results demonstrated that in addition to the carbonyl groups, almost all the hydroxyl groups can be alkylated simultaneously at about 300–400 °C in the presence of TMAH, resulting in highly sensitive determination of phenolic acids. Although thermochemolysis in the presence of TMAH is well developed as rapid repetitive analysis method, no reference to the use of it for determination of alkylphenols in *G. biloba* leaves and their extract, appears to exist.

In this work, thermochemolysis–gas chromatography technique in the presence of an organic alkali, tetramethylammonium hydroxide ($(CH_3)_4NOH$, TMAH) was applied to the quantitative analysis of alkylphenols including ginkgolic acids and ginkgols in *G. biloba* leaves or their extracts directly without using preliminary solvent extraction of the *G. biloba* leaf sample. The method is simple and fast to implement, requires no laborious manipulation.

2. Experimental

2.1. Materials

Ginkgolic acids GA15:1-Δ8 (>99%) was purchased from Kexiang Company (Kunmin, China), and a mixture standard of GA13:0, GA15:1-Δ8, GA15:0, GA17:1-Δ12 (relative percentage (%):18:6:42:34), was purchased from Tongtian Company (Shanghai, China). A methanol solution of TMAH (25%, w/w), supplied by Aldrich Chemical Co. Inc., was used as the organic alkali reagent.

G. biloba leaves harvested in Shandong province of China in autumn were examined in this work. The dried leaf samples were milled into fine powders in order to homogenize and improve the efficiency of TMAH thermochemolysis.

The ethanol extract of leaf sample for TMAH thermochemolysis–GC trial was prepared as follows: *G. biloba* leaves were dried and milled into powders. A 1.0-g amount of leaves was carefully weighed and extracted three times using 10 ml of ethanol with 30 min assisted with ultrasonic. Then, the ethanol was combined, filtered and dried under vacuum. The residue was dissolved with 10 ml ethanol in a volumetric flask at last.

2.2. Conditions for thermochemolysis–GC/MS

A vertical microfurnace pyrolyzer [Frontier Lab (Koriyama, Japan), PY 2020 iD] was directly attached to a gas chromatograph [Varian (Avondale, PA, USA) model CP-3800] equipped with a flame ionization detector (FID). About 500 μg of *G. biloba* dried leaf sample (or 2 μl of the ethanol extract of the leaf sample) and 4 μl of TMAH methanol solution taken into a small platinum cup were first mounted at the waiting position of a pyrolyzer at near room temperature, and then dropped into the heated center under a flow of nitrogen carrier gas. The optimum pyrolytic methylation temperature of 300 °C to obtain the highest yield of the methyl derivatives of alkylphenols was empirically determined after examining various temperatures between 200 and 500 °C. A fused-silica capillary column (30 m × 0.25 mm i.d.) coated with 50% diphenyl–50% dimethylpolysiloxane, 0.25 μm film thickness (CP-Sil 24 CB) was used. The flow rate of 30 ml/min of carrier gas at the pyrolyzer was reduced to 1.0 ml/min at the capillary column by means of a splitter. The column temperature was increased from an initial temperature of 50–280 °C at 10 °C min^{-1}, maintained at this temperature for 10 min. The FID detector temperature was kept at 300 °C.

Identification of the peaks on the resulting programs was carried out by use of a GC/MS system [Thermo Finnigan (Austin, TX, USA), trace DSQ] to which the same type of pyrolyzer was also attached. For the MS measurement, ionization was executed by electron impact (EI) at 70 eV. Samples were analyzed by full scan MS from 40 to 500 amu. The peaks on the resulting pyrograms were identified by both their mass spectra and retention times. Identification of all alkylphenols was carried out through direct comparison of commercially available mass spectra database using the dedicated library searching system together with the interpretation of their mass spectral fragmentation patterns.

3. Result and discussion

Fig. 2 shows typical chromatograms obtained from ginkgolic acid GA15:1-Δ8 standard solution (500 ppm, 2 μl) at 300 °C (a) without the addition of TMAH and (b) in the presence of TMAH. On the chromatogram of Fig. 2(a), no any peak of ginkgolic acid GA15:1-Δ8 but a weak peak of ginkgol C15:1-Δ8 was observed at 23.28 min, mainly due to the decarboxylation of ginkgolic acid at injector heated above 200 °C [18,19]. This result suggests ginkgolic acids cannot be analyzed by direct GC because ginkgols are main components of alkylphenols originality present in *G. biloba* leaves. On the other hand, on the chromatogram obtained in the presence

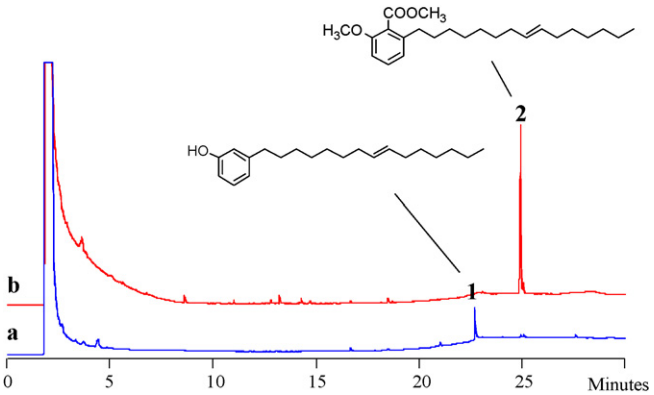

Fig. 2. Typical chromatograms of ginkgolic acid C15:1-Δ8 by thermochemolysis–GC at 300 °C: (a) without the addition of TMAH and (b) in the presence of TMAH. Chromatographic peaks: (1) ginkgol C15:1-Δ8 and (2) methyl derivatives of ginkgolic acid C15:1-Δ8.