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Short communication

Reversed-phase argentation high-performance liquid chromatography in phytochemical analysis of ginkgolic acids in leaves from *Ginkgo biloba* L.

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Abstract

A reversed-phase argentation high-performance liquid chromatographic method has been achieved for the determination of ginkgolic acids. Liquid chromatography coupled with electrospray ionization (ESI) mass spectrometry in the negative ion mode is applied to identify ginkgolic acids from ginkgo leaves. The leaves are extracted with ethanol and then cleaned-up by extraction of analytes with hexane after addition of an acidified saturated solution of sodium sulfate and siliceous earth to the matrix solution. Ginkgolic acids are determined within 30 min on a C_{18} column with methanol–5% aqueous acetic acid (90:10) containing 0.03 mol l^{-1} silver nitrate as eluent and with ultraviolet detection at 310 nm. Addition of silver ions as complexation agent into the mobile phase decreases retention time of ginkgolic acids with an unsaturated side chain. Four ginkgolic acids are successfully separated from each other and from other interfering components by the high selectivity of reversed-phase argentation HPLC, which is confirmed by the spectra identification. The average recovery of the method is around 97%. Good reproducibility is obtained with relative standard deviations varying from 2 to 5%. © 2002 Published by Elsevier Science B.V.

Keywords: Ginkgolic acids

1. Introduction

Extract of *Ginkgo biloba* leaves (EGb) and phytopharmaceutical preparations containing EGb, which contain many kinds of bioactive constitutions, possess important pharmacological properties especially in the treatment of cardiovascular disease. Ginkgolic acids (6-alkyl salicylic acids substituted with a long alkyl side chain) were proved to exist in ginkgo leaves and EGb [1–5]. These compounds are claimed to be allergenic and should be absent from phytopreparations in Germany [5–7]. Due to the different manufacturing processes, they occur in different concentrations and proportions in commercial extracts. Nevertheless several producers limit their presence to a certain maximum in commercial extracts. In the standardized extract of *Ginkgo biloba* (EGb761) the concentration of ginkgolic acids was lower than 10 mg kg^{-1}.

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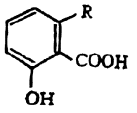

$GA_{15:1}$ R=$(CH_2)_7$-CH=CH-$(CH_2)_5CH_3$
$GA_{13:0}$ R=$(CH_2)_{12}CH_3$
$GA_{17:1}$ R=$(CH_2)_9$-CH=CH-$(CH_2)_5CH_3$
$GA_{15:0}$ R=$(CH_2)_{14}CH_3$

The structures of ginkgolic acids (GA)

At present, most papers on phytochemical analysis and quality control for ginkgo leaves and EGb focus on the main active substances such as flavonoids and terpene lactones, but seldom on ginkgolic acids. Thin-layer chromatography (TLC) and gas–liquid chromatography (GLC) were initially used for qualitative analysis [2,8]. Verotta and Peterlongo [1] reported the scale-up separation and identification by supercritical fluid extraction (SFE) and capillary gas chromatography–mass spectrometry (GC–MS) as trimethylsilyl derivatives, but no recovery data or extraction efficiencies were given. A closely related study using SFE was also reported and was applied to determination of ginkgolic acids from EGb [9]. Recently, liquid chromatography–electrospray ionization (ESI) MS has been successfully applied to analyze ginkgolic acids [4,5]. In this area there is scope for developing simple and less laborious analytical methods. Furthermore, no satisfactory high-performance liquid chromatography (HPLC) with UV detection method for determining four ginkgolic acids in the sample matrix has been published. In this paper, we describe a new reproducible method for the separation and determination of ginkgolic acids from ginkgo leaves by reversed-phase argentation HPLC, which contains a simple, rapid and quantitative sample preparation procedure and baseline separation HPLC conditions.

2. Experimental

2.1. Materials and chemicals

Hubei *Ginkgo biloba* L. leaves were collected from a 20-year-old male tree in our campus. The year and month of harvest are mentioned with the individual samples. The producers of phytopharmaceutical preparations provided the other leaves. Four ginkgolic acids standards were generously supplied by Professor L. Verotta (Italy). Their identities were checked by means of reversed-phase (RP) HPLC, UV spectroscopy, LC–photodiode array detection (PDA) MS and nuclear magnetic resonance (NMR) spectroscopy. Methanol, acetonitrile and tetrahydrofuran (THF) were of HPLC grade. Ethanol, hexane, chloroform and ether were of analytical-reagent grade. Water was doubly distilled. All solvents used for HPLC were degassed before use.

2.2. Chromatographic conditions

LC–MS analysis was performed on a SSQ7000 mass spectrometer (Finnigan, USA) coupled with a Model Waters996 liquid chromatograph (Waters, Milford, MA, USA), which was equipped with a photodiode array detector (Waters PDA996) set at 310 nm and connected to a Millennium 2010 data processing system. Chromatographic conditions were as follows: Waters symmetry shield RP18 (5 μm) column (150×2.1 mm) with sentry guard column; isocratic elution with methanol–0.5% aqueous acetic acid (90:10); flow-rate was 0.25 ml min^{-1}; temperature was 30°C. EM Volts 4200. The mass spectra were acquired in the negative ion mode and recorded by repeatedly scanning over the mass range m/z 200–400. The HPLC system was connected directly to the MS system without stream splitting.

LC in the SIM (selected ion monitoring) mode was performed under the same conditions used for LC–MS. The base peak ions of $[M-H]^-$ at m/z 319, m/z 345, m/z 347 and m/z 373 for $GA_{13:0}$, $GA_{15:1}$, $GA_{15:0}$ and $GA_{17:1}$, respectively, were monitored.

Reversed-phase argentation HPLC separation and determination were carried out on a Model LC-6A liquid chromatograph (Shimadzu, Japan) equipped with an SCL-6A injector with a 20-μl loop, a CTO-6A column oven and a Model SPD-6AV UV detector connected to a CR-3A data processor. Chromatographic conditions were as follows: KF-C_{18} (7 μm) analytical column (250×4.6 mm) (Dalian Elite, China), mobile phase was methanol–5% aqueous acetic acid (90:10) with 0.03 mol l^{-1} silver nitrate, column temperature 30°C, UV detection wavelength was 310 nm and the UV measuring range was set at $2\cdot10^{-2}$ AUFS, flow-rate 1.0 ml min^{-1}.

2.3. Sample preparation

The leaves were dried at 70°C in forced ventilation for 6 h and then pulverized mechanically. A 1.0-g amount of leaves was carefully weighed and refluxed with 40 ml of ethanol and a boiling stone for 90 min. After cooling, the solution was filtered and the flask and the leaves were washed three times with 3 ml of ethanol. The combined filtrates were diluted to 50 ml with ethanol in a volumetric flask. Then a 1.0-ml volume of this solution was transferred to a stopper tube followed by addition of 3.0 ml hexane. Then 10 ml of saturated sodium sulfate–sulfuric acid (pH 3) and a little adsorbent siliceous earth were also added to the matrix solution. Analytes were extracted with hexane by shaking the whole solutions for a few seconds. Then a 1.5-ml volume of the upper organic solvent taken with volumetric pipette was transferred to another volumetric flask. After evaporation to dryness by nitrogen gas, the residue was dissolved in 0.5 ml of chloroform and was ready for injection into the HPLC system. The total content of the four ginkgolic acids was calculated on the basis of ginkgolic acid $GA_{17:1}$.

3. Results and discussion

3.1. Identification of ginkgolic acids by HPLC–ESI-MS analysis

Electrospray is a low-energy, soft ionization process that generates positive or negative molecular ions even with thermally unstable compounds. With electrospray MS, the molecular ion is the base peak. But we found that ginkgolic acids did not ionize in the positive ESI mode; therefore, we employed the negative ESI mode at relatively high pH levels to improve ESI negative ion detection sensitivity according to the work done by He et al. [4]. Fig. 1 shows the selected ion recordings (SIRs) of ginkgo leaf extracts. The SIR of each individual ginkgolic acid appeared individually on the chromatograms. The mass spectra showed a deprotonated molecule $[M-H]^-$ at m/z 319, m/z 345, m/z 347 and m/z 373 for $GA_{13:0}$, $GA_{15:1}$, $GA_{15:0}$ and $GA_{17:1}$, respectively. In addition to three main ginkgolic acids published in the literature [4,5], we found, using

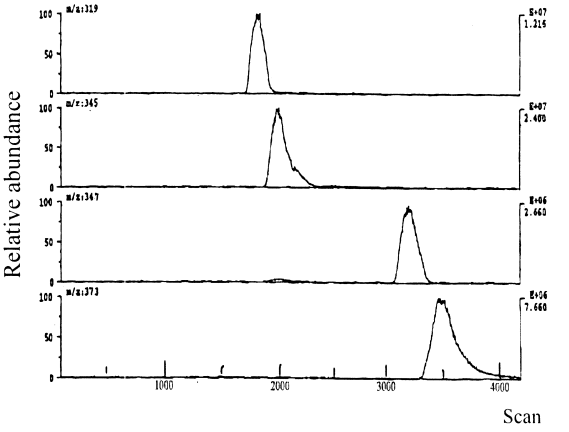

Fig. 1. Selected ion recordings (SIRs) of leaves extract from *Ginkgo biloba*. Chromatographic conditions were as described in the Experimental section.

LC–ESI-MS, trace amounts of $GA_{15:0}$ present in ginkgo leaves. Although $GA_{15:1}$–$GA_{13:0}$ and $GA_{17:1}$–$GA_{15:0}$ were not well separated on the common HPLC column when using UV detection, four ginkgolic acids eluting in the order: $GA_{13:0}$, $GA_{15:1}$, $GA_{15:0}$ and $GA_{17:1}$, were unambiguously identified based on their retention time and MS data, compared with those of standard compounds.

3.2. Development of the reversed-phase argentation HPLC method

The typical optimized reversed-phase chromatographic conditions were a C_{18} column with mixtures of methanol, acetonitrile or (and) tetrahydrofuran and water as mobile phase. In order to establish a HPLC–UV detection method for good separation of four ginkgolic acids, different compositions of solvent mixtures with equal total solvent strength or polarity but with different separation selectivity were systematically studied according to the optimization method of eluent composition in reversed-phase chromatography [10]. In the present work, three different mixtures consisting of three solvents and another two different mixtures consisting of four solvents were used. But all the attempts failed however the mobile phase was changed. Baseline separation by the common reversed-phase chromatographic technique with UV detection of the four

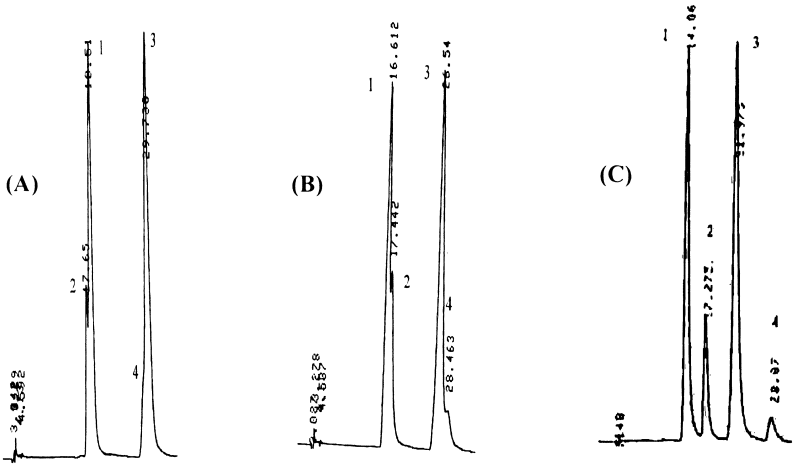

Fig. 2. HPLC separation of ginkgolic acids with and without addition of silver ion. Mobile phase: (A) methanol–5% aqueous acetic acid (90:10); (B) A+1·10^{-2} mol l^{-1} [Ag^{+}]; (C) A+3·10^{-2} mol l^{-1} [Ag^{+}]. Peaks: 1=$GA_{15:1}$; 2=$GA_{13:0}$, 3=$GA_{17:1}$, 4=$GA_{15:0}$.

ginkgolic acids, especially $GA_{15:1}$–$GA_{13:0}$ and $GA_{17:1}$–$GA_{15:0}$, was difficult due to the opposite contribution of the number of the CH_2 group and the saturation degree in side chain moiety on the retention or partition equilibrium in reversed-phase HPLC. For instance, $GA_{15:1}$ has two more CH_2 groups than $GA_{13:0}$, which leads to an increase in retention (increased *k*), but introduction of unsaturation in the side chain leads to a decrease in retention (decreased *k*). Fig. 2A shows a typical HPLC–UV chromatogram with the common eluent. $GA_{15:1}$–$GA_{13:0}$ and $GA_{17:1}$–$GA_{15:0}$ were very poorly separated due to the above-mentioned reason.

In reversed-phase HPLC, control of the secondary chemical equilibrium in the mobile phase was an effective method leading to improved resolution [11]. In the present study we developed a reversed-phase argentation HPLC analytical method. Fig. 2C shows a HPLC chromatogram with addition of 0.03 mol l^{-1} silver ion to the eluent, which showed a sharp separation and a considerably increased selectivity compared with Fig. 2A under identical conditions except for no addition of silver ion to the eluent.

Addition of silver ion to the mobile phase selectively decreased the retention of ginkgolic acids with an unsaturation in the side chain moiety ($GA_{15:1}$, $GA_{17:1}$). Silver ions did not alter the retention of ginkgolic acids with a saturated side chain ($GA_{13:0}$, $GA_{15:0}$). Our data on the effect of silver ion concentration [Ag^{+}] on retention factor (*k*) are presented in Table 1 and visualized in Fig. 2. The decrease of *k* when silver ions are added to the eluent is due to complexation of isolated double bonds (containing π electrons) with silver ions (electronic acceptor), thus favoring partition toward the mobile phase. The retention of these two unsaturated ginkgolic acids can be easily regulated by adjusting the silver ion

Table 1
Effect of [Ag^{+}] on retention factor of ginkgolic acids on RP-HPLC

Solute	R*	Retention factor (*k*) (·10^{-2} mol l^{-1} [Ag^{+}])						
		0	0.5	1	2	3	4	5
$GA_{15:1}$	$C_{15:1}$	4.97	4.67	4.39	3.90	3.54	3.23	2.94
$GA_{13:0}$	$C_{13:0}$	4.69	4.67	4.66	4.66	4.57	4.61	4.64
$GA_{17:1}$	$C_{17:1}$	8.59	8.14	7.62	6.77	6.09	5.64	5.13
$GA_{15:0}$	$C_{15:0}$	8.20	8.14	8.24	8.27	8.05	8.31	8.11

R*: number of carbon atoms and unsaturation in the long side chain of ginkgolic acids.

concentration. In the common eluent without silver ion, four ginkgolic acids were eluted in the order: $GA_{13:0}$, $GA_{15:1}$, $GA_{15:0}$, $GA_{17:1}$, as confirmed and shown in Fig. 1 and Fig. 2A. With the increase of $[Ag^+]$, the order of appearance of the $GA_{15:1}$ and $GA_{17:1}$ was reversed and they emerged before the $GA_{13:0}$ and $GA_{15:0}$, respectively. As a practical consideration, we have chosen 0.03 mol l^{-1} silver ions in the eluent. Baseline separations of $GA_{15:1}$–$GA_{13:0}$ and $GA_{17:1}$–$GA_{15:0}$ were achieved and the separation selectivity (α) and resolution (R_s) between them were all above 1.2 and 1.5, respectively.

It might be expected that an Ag^+-containing mobile phase would cause increased corrosion, the deposition of metallic silver or changes of the properties of the support. No technical problems occurred during our work of the last 2 years when using the usual instrument arrangements. Columns were operated for several months with Ag^+-containing mobile phases without deterioration of the separation efficiency or peak shapes, mainly because of the low silver ion concentration involved.

3.3. Determination of ginkgolic acids from the sample matrix

Ginkgo biloba leaves are extracted with ethanol, but the resulting solution cannot be analyzed directly by HPLC before further purification because of the highly complex matrix. Interfering compounds present in ginkgo leaves, especially the weakly polar flavonoids, were coextracted and have to be removed due to their much stronger UV absorption near the detection wavelength and their much higher concentration than ginkgolic acids.

In this study several purification procedures were developed. Initially we chose different solid-phase purification columns including alumina, silica gel, magnesia, polyamide types eluted by different organic solvents in the polar order, but no single or combination of two or even three extraction columns gave an adequate recovery or improved the chromatograms. Then a multi-step liquid–liquid extraction combined with concentration procedure was used. This method gave an extract that could be analyzed by HPLC. However, large amounts of ginkgolic acids are lost in the time-consuming clean-up process and degrade because of their thermal instability in three times evaporation, which led to poor reproducibility and low recovery.

The best results were eventually obtained with a single extraction as described in Experimental. Much acidified solution and a little adsorbent were added to the matrix solution before the analytes were extracted with hexane and only one concentration was needed. Here, addition of sodium sulfate solution was very important because the salting-out effect changed the partition coefficient of ginkgolic acids between the organic phase and water phase, thus improving the extraction efficiency. Furthermore, the acidic conditions were also in favor of higher extractability of apolar solvent by restraining the ionization of ginkgolic acids. Fig. 3B shows a typical chromatogram after this purification procedure, which showed that four ginkgolic acids were present in ginkgo leaves and separated well from other impurities. They are identified by comparison with standards (Fig. 3A). Together with the retention time data, these peaks were also identified by comparing their UV spectra with those of the standards by means of HPLC–didoe array detection (PDA) in the wavelength range 200–400 nm. It was found that the UV spectra of peaks from samples and standards were essentially identical. There are two obvious absorption peaks near the wavelengths 310 and 250 nm as reported [3,4]. The differences in UV spectra between the four ginkgolic acids were very small because of the general similarity of functional groups in their chemical structures (salicylic acids derivatives).

In studying the method it was found that different extraction solvents had a great effect on analytical results. Comparison of extraction efficiency and characteristics showed that hexane, which has a selective extractability and high extraction efficiency up to 98.5% for ginkgolic acids, was superior to any other solvents such as light petroleum, cyclohexane. When the resulting solution was extracted with benzene or chloroform, which also has a high efficiency for both ginkgolic acids and other interfering compounds, only at the end of the chromatogram was the baseline back to zero (Fig. 3C), so ginkgolic acids could not be quantified at all due to the presence of impurities.

In order to further confirm that ginkgolic acids were not coeluted with other interfering compounds

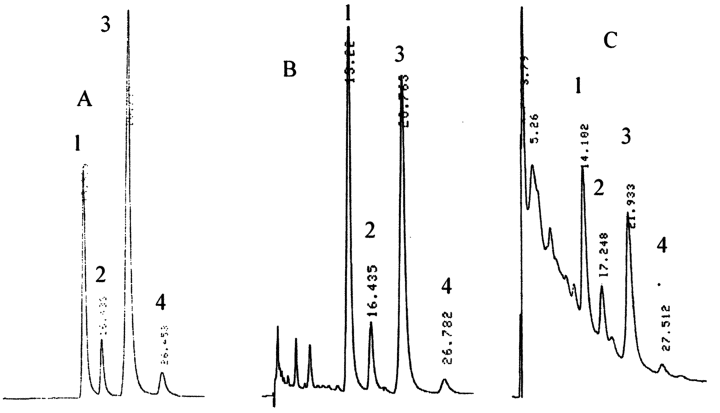

Fig. 3. Chromatograms of ginkgolic acids from standards (A); samples extracted by hexane (B) and benzene (C). 1=$GA_{15:1}$; 2=$GA_{13:0}$; 3=$GA_{17:1}$; 4=$GA_{15:0}$. Mobile phase: methanol–5% aqueous acetic acid (90:10) with 0.03 mol l^{-1} silver ions; the other conditions as in the Experimental section.

in Fig. 3B, peak purity was assessed on the basis of PDA and Millennium 2010 software. The acquisition of UV spectra was automatic at the apex, both inflection points and base of all peaks (200–400 nm, 2-nm step). The UV spectra of each peak, after subtraction of the corresponding UV base spectrum, were computer normalized and the plots were superimposed. The results proved there was exact correspondence among the corresponding spectra and purity match factors were all more than 990. So peaks are considered to be homogeneous and this method can be applied to quantitative analysis of ginkgolic acids by HPLC.

The efficiency of the extraction of the leaves was determined by extracting the residues of leaves a second time. It was found that an additional amount of less than 0.5% of ginkgolic acids could be extracted. Thus in the normal procedure 99.5% of the ginkgolic acids were extracted. As there is always a possibility that extraction operation in the sample clean-up leads to evaporation of hexane, determination errors were assessed with pure reference compounds for varying the volume ratio of hexane to standards solution. It was found that accuracy was more than 98.5% when three or even more times volumes of hexane were used to extract ginkgolic acids solution and then concentrated by nitrogen gas according to the standard procedure.

Table 2
Reproducibility of extraction and purification process (n=5)

Compound	RSD (%)	Percentage composition (%)
$GA_{15:1}$ ($C_{15:1}$)	1.9	36
$GA_{13:0}$ ($C_{13:0}$)	1.7	13
$GA_{17:1}$ ($C_{17:1}$)	2.2	47
$GA_{15:0}$ ($C_{15:0}$)	5.1	3

3.4. Evaluation of the analytical method

Under the selected chromatographic conditions the chromatographic precision and linearity in the integration process were measured. Different volumes of ginkgolic acid standards solution were analyzed accurately three times, a good correlation was obtained between the integrated peak area and the total content over a range from at least 84 ng to 10 μg by linear regression, as confirmed by the correlation coefficient r (r=0.9998). The detection limit was 26 ng with a signal-to-noise ratio greater than 3. The relative standard deviation (RSD) of peak area was 1.0% for $GA_{15:1}$, 1.1% for $GA_{13:0}$, 0.4% for $GA_{17:1}$ and 1.6% for $GA_{15:0}$, which was evaluated in this work six times at the concentration level found in ginkgo leaves.

The reproducibility of the method when applied to leaves harvested in the autumn was determined. For this purpose five samples were extracted and purified according to the standard procedure. Each of the five extracts was analyzed three times by HPLC. The results are given in Table 2. $GA_{15:0}$ had the highest

Table 3
Results of recovery experiments for 1.0-g of ginkgo leaves ($n=6$)*

Added (μg)	Recovery (%)	RSD (%)
5280	95.8	2.1
2640	94.3	1.7
1060	101.8	5.9

*The initial amount of ginkgolic acids in leaves was 5230 μg.

RSD because of its low concentration in ginkgo leaves. Table 2 also gives the percentage composition (uncorrected) of the constituents from leaves, which was obtained by peak area percentage. The ginkgolic acids with unsaturated alkyl chain ($GA_{15:1}$ and $GA_{17:1}$) are the main constituents. Of the ginkgolic acids with saturated alkyl chain, we also found trace amount of $GA_{15:0}$ (which has GA_{15} side chain) by this HPLC method in addition to $GA_{13:0}$ as previously reported [2–5].

The recovery of the whole extraction and purification process was determined for various addition of standards solution into the sample just prior to extraction. The results are presented in Table 3. The average recovery of the method was 97.3% for the total amount of all ginkgolic acids and showed relatively small deviations. Obviously, the simple sample clean-up method described above offered a high extractability and recovery.

Finally, some different leaves collected from China were compared and large variations were found. The total concentrations varied from 5.4 to 17.8 mg g^{-1} dry leaves. Hubei leaves from late autumn (November 1996) contained the lowest amount of ginkgolic acids, which were more than three times lower than leaves from spring. The values for Jiangsu and Henan green leaves (September 1997) were nearly the same around 7 mg g^{-1} dry leaves. While the contents of ginkgolic acids for leaves collected in July from China (13 mg g^{-1} dry leaves) were less than leaves from Japan reported by Irie et al. (16 mg g^{-1} dry leaves) [3]. Therefore, one can presume that the sun and the time of harvest play an important role in the yield of these ginkgolic acids. But interestingly the percentage composition of the constituents from different leaves was found to vary by less than 5% in all instances. The approximate composition was generally similar to that in Table 2.

4. Conclusion

A new analytical method for determination of ginkgolic acids, allergenic components existing in *Ginkgo biloba* leaves, has been developed. Negative ionization mode was successful in obtaining a deprotonated molecule $[M-H]^-$ in the identification of ginkgolic acids from leaf extract by LC–ESI-MS. Extraction of ginkgo leaves with ethanol followed by addition of acidified saturated solution of sodium sulfate and adsorbent and then clean-up with hexane extraction gave an extract that can be readily analyzed by RP-HPLC containing silver ion in the mobile phase. Addition of silver ions as complexation agent improved the selectivity and resolution between ginkgolic acids. Four ginkgolic acids were well separated from each other and from other impurities within 30 min. All the leaves investigated were found to contain four ginkgolic acids and similar percentage compositions, although large differences between different leaf batches were observed.

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