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Supercritical fluid chromatography of ginkgolides A, B, C and J and bilobalide

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Abstract

Supercritical fluid chromatography (SFC) can be used to separate the five different ginkgo terpenetrilactones occurring in *Ginkgo biloba* leaf. The separation is carried out at 280 atm on a packed deactivated aminopropyl silica HPLC column with CO_2 modified with 12% methanol. The separation is better than what is currently achievable with RP-HPLC. Detection was done with evaporative light scattering (ELSD). The limit of detection was around 10 ng. The method can be used for the analysis of any ginkgo sample that has undergone an SPE clean-up and in some cases it is useful for samples without prior clean-up. This is due to the greater selectivity of the SFC separation mechanism relative to the RP-HPLC mechanism.

Keywords: Ginkgo biloba; Terpenetrilactones

1. Introduction

Ginkgolides A, B, C and J are chemically unique platelet activating factor (PAF) antagonists isolated from *Ginkgo biloba* leaves [1]. These diterpenetrilactones are largely held responsible for the beneficial effect of ginkgo phytopharmaceuticals which are currently among the most sold drugs in France and Germany. The closely related sesquiterpenetrilactone bilobalide lacks anti-PAF activity but has a neuroprotective effect [2]. (For structures see Scheme 1.)

In recent years, because of their interesting pharmacological properties and natural variations of leaf concentrations of more than a factor 150 [3], many papers have been published on the analysis of these substances in leaves, commercial standardised extracts and finished drugs. Initially HPLC with UV detection was suggested [4] but because these terpenes possess very poor UV characteristics [λ_{max}= 219 nm and $\varepsilon \approx 300$ l(cm·mol)$^{-1}$] [5] many trace impurities still interfere [6,7]. HPLC with RI detection is more suitable and has been used with considerable success [7–9] although sensitivity and baseline stability remain a problem. Other techniques which are used or have been suggested for quantitative purposes are: GC–FID after silylation [10], HPLC–ELSD [11], HPLC–MS [12] and NMR [13]. However, all of these methods have one or more disadvantages in terms of cost, time, low sensitivity, high R.S.D. values or poor separation and they all require some clean-up step (for reviews, see Refs. [14,15]).

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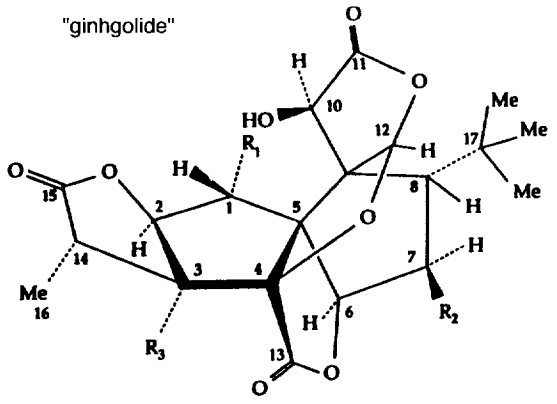
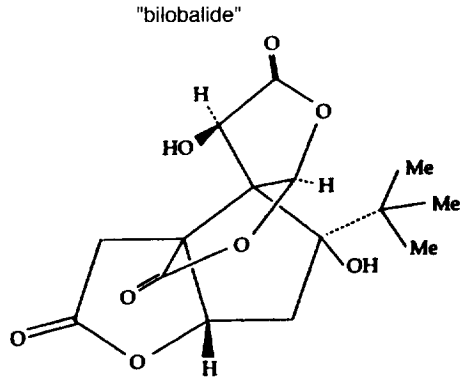


Ginkgolide A: $R_1=R_2=H$, $R_3=OH$
Ginkgolide B: $R_1=R_3=OH$, $R_2=H$
Ginkgolide C: $R_1=R_2=R_3=OH$
Ginkgolide J: $R_1=H$, $R_2=R_3=OH$



Bilobalide

Scheme 1. Structures of the compounds studied.

A faster and more selective method for the separation of ginkgolides without derivatisation would be of considerable interest. Especially because the current RP-HPLC methods still have difficulty achieving an efficient separation of the ginkgolide pairs A/B and C/J. Additionally some impurities have retention times similar to the ginkgolides in reversed-phase systems. It has been suggested that normal-phase systems might be more selective [15] and indeed a normal-phase preparative medium pressure liquid chromatographic system was shown to be capable of separating the five individual terpenes [15]. Still another approach would be supercritical fluid chromatography. In this paper we report on the development of an SFC system for the separation of ginkgolides and bilobalide.

2. Experimental

2.1. Plant materials and phytopharmaceuticals

Ginkgo leaves were collected in 1993 from few-year-old *Ginkgo* trees growing on a plantation 45 km south of Reims, France. Standardised ginkgo extracts were kindly provided by various producers. Phytopharmaceuticals were provided by the producers or bought in a pharmacy.

2.2. Standards

Ginkgolide A, B and C and bilobalide were isolated by us from standardised ginkgo extracts by means of medium pressure liquid chromatography (MPLC). The purity was checked by TLC, HPLC and qualitative and quantitative NMR.

2.3. Solvents, chemicals and gases

Acetone, methanol and toluene (all HPLC grade) were from EM Science (Gibbstown, NJ, USA). Ethyl acetate was from Mallinckrodt (Paris, KY, USA). Tetrahydrofuran (HPLC grade) was from Aldrich (Milwaukee, WI, USA). Water was deionised. SFE-grade CO_2 was obtained from Air Products and Chemicals (Allentown, PA, USA). Nitrogen for removal of acetone from autosampler vials and helium 5.0 for gas chromatography were from Airco (Murray Hill, NJ, USA). Anhydrous sodium sulphate (certified A.C.S.) and silica 60–200 mesh (grade 950 for GC) were from Fisher (Fair Lawn, NJ, USA).

2.4. Instrumentation

2.4.1. SFC

A prototype of the Hewlett-Packard (HP) Model G1205 SFC system (Hewlett-Packard, Little Falls, DE, USA) was used for the optimization and separations. Overall system pressure was maintained electronically by a computer controlled back pressure regulator. The mobile phase flow-rate was measured as a liquid at the pump. This allowed the flow-rate and pressure to be controlled independently. Organic modifier was added via an auxiliary pump. Chromatographic conditions: oven temperature 40°C, pressure 280 atm, flow-rate 3.5 or 4.0 ml/min, 5 μl loop volume, mobile phase 12% methanol in CO_2. A post-column split was introduced to divert a percentage of column effluent (<90%) through a linear restrictor to the ELSD with the remaining flow (>10%) directed towards a standard HP 1050 multiwavelength detector which incorporated a 13 μl high-pressure flow cell. A commercially available Mark III evaporative light scattering detector (ELSD) (Alltech Associates, Deerfield, IL, USA) was used for detection. The nebulizer was removed and replaced with an integral restrictor (100 μm I.D.) which was made in-house to deliver 855 ml min^{-1} decompressed methanol-modified CO_2. To aid in the evaporation of the mobile phase, a nitrogen make-up gas flow-rate of 400 ml min^{-1} was used. The drift tube temperature was set at 65°C. In Fig. 1 a schematic drawing of the modified ELSD is given.

2.4.2. HPLC–ELSD

Waters 600-MS System Controller (Millipore), Waters (Milford, MA, USA) 600E multisolvent delivery system, automated VICI injector (Valco, Houston, TX, USA), column: Phenomenex (Torrance, CA, USA) filled with Spherisorb 5 ODS(2) 250×4.60 mm, mobile phase water–THF–MeOH (68.5:10.5:21) at 1.0 ml min^{-1}, loop volume 5 μl. Detector: Varex ELSD MK III (Deerfield, IL, USA), nitrogen nebulizer gas at 2.06 l min^{-1}, drift tube temperature 107°C, HP 3394 A integrator. The solvents were sparged with helium.

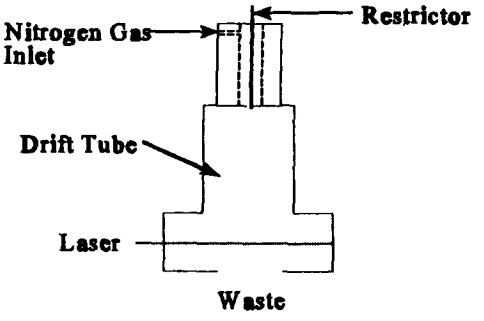


Fig. 1. Schematic drawing of the modified ELSD used with SFC.

2.5. SFC columns

The Deltabond® poly(ethylene glycol) (PEG) column, 15 cm×2.0 mm, 5 μm particle size and the Deltabond Amino 2 column, 15 cm×4.6 mm, 5 μm particle size were both obtained from Keystone Scientific (Bellefonte, PA, USA). The Lichrospher™ NH2 column, 25 cm×4.0 mm, 5 μm particle size was obtained from HP.

2.6. Solid-phase clean-up

A stock solution of ca. 35 mg ml^{-1} of standardised ginkgo extract in methanol was made. With a volumetric pipet 0.500 ml of this solution was applied to an SPE column containing 1.2 g silica. The methanol was removed by blowing nitrogen from top to bottom through the SPE column for 15 min while the column was inserted in a water bath of 70°C. Next the column was eluted with 10 ml of toluene–acetone (7:3). The eluate was collected, evaporated in vacuo and quantitatively transferred with 4×0.35 ml acetone to a 1.8-ml autosampler vial. After removing the acetone with a gentle stream of nitrogen and some heat, 0.500 ml methanol was added to the residue and the vial was closed with a cap and septum. This solution was investigated by means of SFC–ELSD or HPLC–ELSD.

2.7. Extraction of leaves

Approximately 500 mg of dried pulverised ginkgo leaves were twice extracted in a 50-ml Erlenmeyer with 8 and 5 ml of boiling 10% aqueous methanol, respectively. After filtration of the extracts over a cotton plug the combined filtrates were thrice extracted with 10, 7 and 4 ml ethyl acetate, respectively. The organic layers were pooled, dried for 5 min

over anhydrous sodium sulphate and evaporated in vacuo. The residue was quantitatively transferred with 3×0.4 ml acetone to a 1.8-ml autosampler vial. After removing the acetone with a stream of nitrogen and some heat, 0.500 ml methanol was added to the residue and the vial was closed with a cap and septum. This solution was investigated by means of SFC–ELSD.

3. Results and discussion

For our studies a supercritical fluid chromatograph equipped with a cooled CO_2 pump and a separate modifier pump in combination with a high pressure variable UV detector and an evaporative light scattering detector (ELSD) were available. From preliminary solubility studies, it was learned that ginkgolides are insoluble in pure CO_2 so it was decided to add 5–15% of methanol as modifier to achieve elution. Several packed columns such as cyanopropyl-, octyl- and octadecyl derivatised silica were initially tested. Although elution could be achieved there was no separation at all. Replacing methanol partially by water or THF gave no improvement. During these experiments it also became clear that UV detection was totally unsuitable and that ELSD was much more selective and sensitive for detecting ginkgolides.

As some polar stationary phases such as Sephadex LH-20 [8] and silica gel impregnated with sodium acetate [16] are capable of separating the ginkgo terpenes in order of increasing polarity, it was then decided to try some more polar, silica-based columns. The first column tried was a Deltabond PEG column. In combination with a 4–8% methanol gradient, this system was able to separate bilobalide from ginkgolide A/B and ginkgolides J/C, but unfortunately the A/B and J/C pairs were not baseline separated. Additionally some tailing was observed. In essence the separation that we observed was similar to that on unimpregnated silica which can also not resolve the A/B and J/C pairs. On the polar Lichrosphere aminopropyl silica column there was again separation but no complete resolution of the A/B and J/C pairs. To reduce the activity of the column, some water was added to the mobile phase. This gave some improvement in the separation of the A/B and J/C pairs but still no baseline separation. Instead of deactivation by adding water, a deactivated aminopropyl column (e.g., Deltabond) was then tried in combination with only methanol as modifier. This time a baseline separation was achieved with the terpenes eluting in the same order as on sodium acetate-impregnated silica: bilobalide, ginkgolide A, B, J and finally C. Further, the peak shape was excellent for all five terpenes with hardly any tailing.

Next various parameters, like percentage modifier, pressure and flow-rate were altered to find the optimum conditions for this separation. It was found that a good baseline separation could be achieved within 10 min with 12% modifier at 280 atm, 40°C and a constant flow of 3.5 or 4.0 ml min^{-1}. In Fig. 2a, a supercritical fluid chromatogram of a test mixture containing all five terpenes is shown. The quality of the separation is comparable to that obtained with a 25 cm HPLC column but it takes 50% less time. In Fig. 2b the same sample but now recorded on a RP-HPLC with ELSD is presented for comparison. Relative standard deviations (R.S.D. values) for the SFC/ELSD integration varied from 1–7%, depending on the concentration, which is better than what we could achieve with HPLC–ELSD and about similar to HPLC–RI but significantly higher than the 0.5% R.S.D. of GC–FID. Therefore, GC–FID remains the method of choice for very accurate quantitations of ginkgo terpenes. Limits of detection for SFC–ELSD were around 20 ng for bilobalide and ginkgolide A and B, and around 40 ng for ginkgolide C. This is comparable to GC–FID [10,11] and much better than HPLC–RI [7].

The aim of this study was to see whether ginkgolides could be successfully separated by means of SFC. This being achieved, a few real life ginkgo samples were run with the method to explore the possibilities and limits of the technique. In Fig. 3a a standardised ginkgo extract containing ca. 7.5% terpenes after SPE clean-up is shown. Essentially only peaks of the compounds of interest are visible. A difference with an HPLC chromatogram (not shown) of the same sample is the lower abundance of peaks in the initial part of the chromatogram. This is due to the fact that most of the remaining impurities after SPE are of a polar nature and, therefore, elute

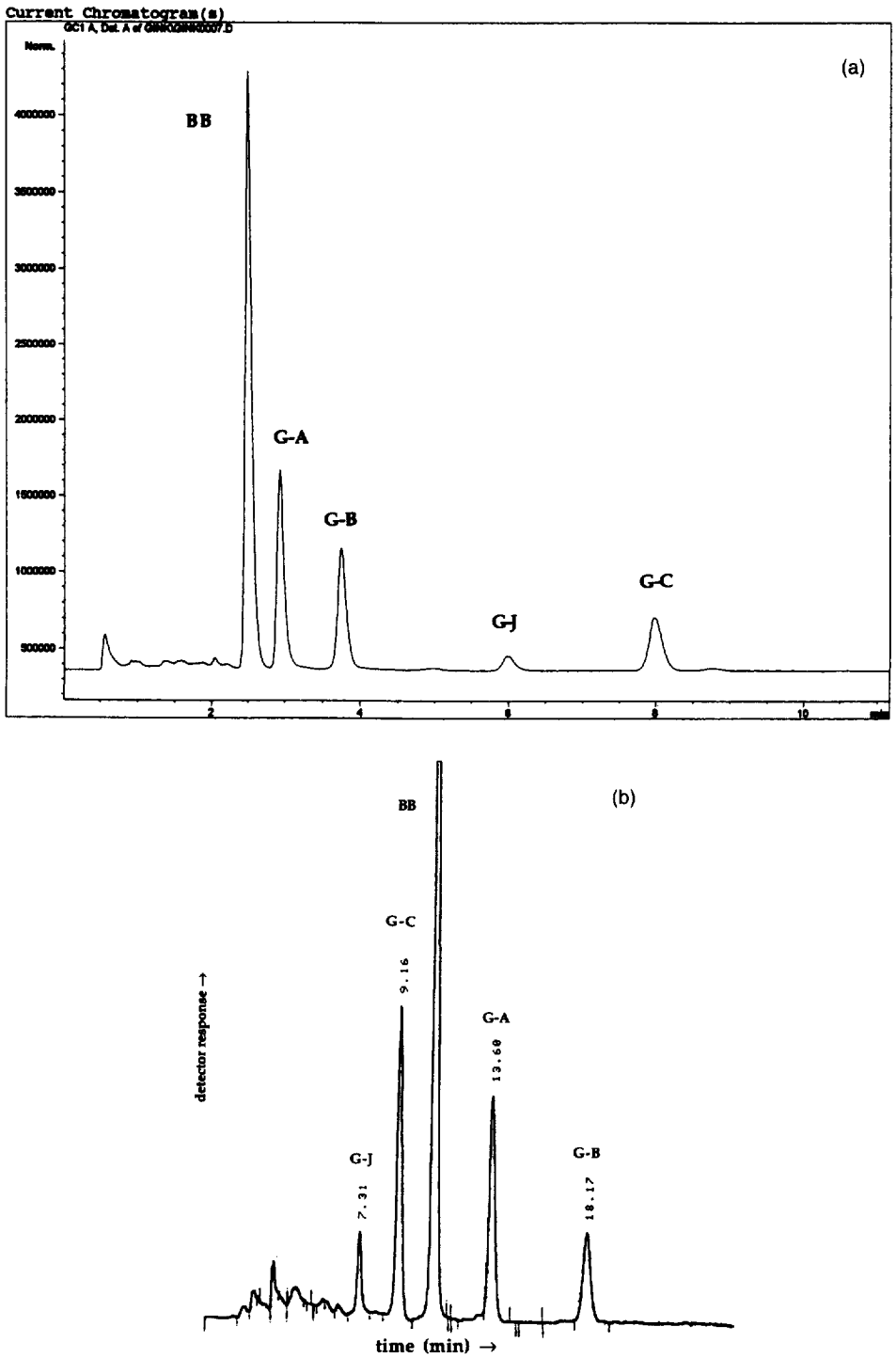


Fig. 2. SFC–ELSD and HPLC–ELSD traces of a test mixture of the five ginkgo terpenes. (a) SFC–ELSD, flow-rate 4.0 ml min^{-1}, for other conditions see Section 2. (b) HPLC–ELSD, for conditions see Section 2. BB, bilobalide; G-A, G-B, G-J and G-C, ginkgolide A, B, J and C, respectively.

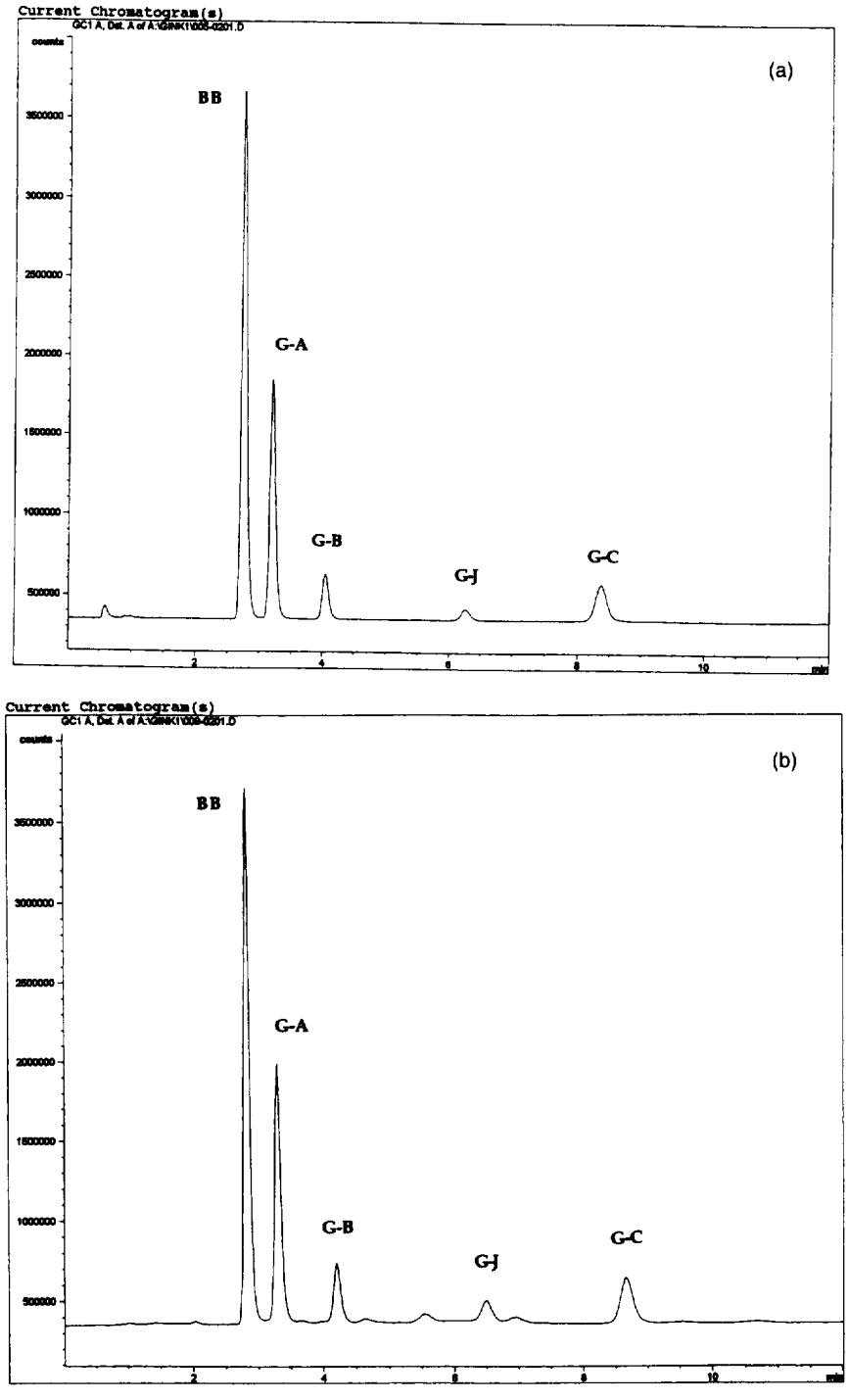


Fig. 3. SFC–ELSD traces of Indena standardised ginkgo extract. (a) Sample after SPE clean-up. (b) Sample dissolved in methanol without any further clean-up. For conditions see Section 2. BB, bilobalide; G-A, G-B, G-J and G-C, ginkgolide A, B, J and C, respectively.

early in the RP-HPLC system and are retained by the SFC column which is essentially a normal-phase system. As these impurities remain on the column, a wash with pure methanol or replacement of a guard column may be necessary after a large number of SFC runs. The selectivity of the SFC system was further assessed by injecting the same sample without prior SPE clean-up (Fig. 3b). Although the chromatogram shows naturally more impurities than the one in Fig. 3a an accurate integration of bilobalide, ginkgolide A, B and C is still possible. Only ginkgolide J which is the least important ginkgolide both in terms of biological activity and total amount present co-eluted with an impurity. If the labour intensive clean-up step could be omitted in the routine analysis of standardised ginkgo extracts this would be of considerable interest.

A chromatogram (not shown) of ginkgo leaves after a one partition clean-up showed essentially only the five peaks of interest. Finally, in Fig. 4 a chromatogram of a direct injection, without any purification, of 5 μl Tanakan, a liquid ginkgo phytopharmaceutical and the most sold ginkgo drug in France, is presented. Again a stable baseline is observed and the peaks of bilobalide and ginkgolide A, B and C are well separated. Near ginkgolide J a big impurity elutes which makes an accurate integration impossible. When Tanakan is purified by SPE ginkgolide J could also be analysed. This shows that the method could in principle be applied to all the three different types of economically important ginkgo samples: leaves, standardised extracts and finished phytopharmaceutical drugs. Quantitation could be carried out by means of external standardisation with reference solutions.

4. Conclusion

SFC with 12% methanol-modified CO_2 in combination with a packed deactivated aminopropyl silica column is capable of achieving a baseline separation

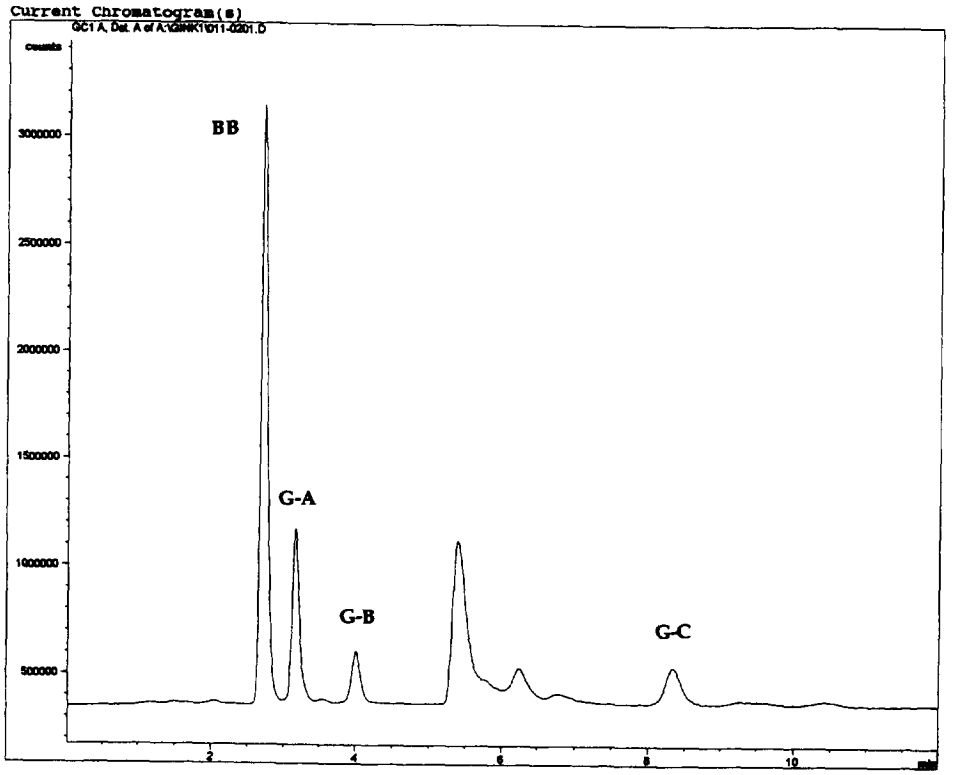


Fig. 4. SFC–ELSD trace of 5 μl Tanakan liquid phytopharmaceutical without any prior clean-up. For conditions see Section 2. BB, bilobalide; G-A, G-B, G-J and G-C, ginkgolide A, B, J and C, respectively.

of, in order of elution, bilobalide and ginkgolides A, B, J and C within 10 min. After one run the apparatus is immediately available for the next separation because the SFC conditions are simple: isothermal, isobaric and isocratic. Of the two available detectors, UV and ELSD, only the last one was both compatible with the mobile phase and capable of giving a sensitive and selective detection of ginkgo terpenes. The separation, baseline stability, as well as the detection are better than that of current RP-HPLC with RI detection and it is much faster than GC–FID because no derivatisation is needed. A disadvantage for quantitative purposes is that the R.S.D. values are higher than for GC–FID.

Although no in-depth studies have been carried out with regard to the long term practical usefulness of SFC–ELSD for the quantitative analysis of ginkgo leaves, extracts and phytopharmaceuticals, the first results seem to indicate that it is a selective, sensitive and promising technique for the analysis of ginkgo terpenes in different matrices especially if it could be combined with some on-line sample preparation step like for instance SFE. For some samples no clean-up at all might be needed.

Acknowledgments

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