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Review

Chemical analysis of *Ginkgo biloba* leaves and extracts

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Abstract

The chemical analysis and quality control of Ginkgo leaves and extracts is reviewed. Important constituents present in the medicinally used leaves are the terpene trilactones, i.e., ginkgolides A, B, C, J and bilobalide, many flavonol glycosides, biflavones, proanthocyanidins, alkylphenols, simple phenolic acids, 6-hydroxykynurenic acid, 4-*O*-methylpyridoxine and polyprenols. In the commercially important Ginkgo extracts some of these compound classes are no longer present. Many publications deal with the analysis of the unique terpene trilactones. They can be extracted with aqueous acetone or aqueous methanol but also supercritical fluid extraction is possible. Still somewhat problematic is their sample clean-up. Various procedures, not all of them validated, employing partitioning or SPE have been proposed. Some further development in this area can be foreseen. Separation and detection can be routinely carried out by HPLC with RI, ELSD or MS, or with GC–FID after silylation. TLC is another possibility. No quantitative procedure for flavonol glycosides has been published so far due their difficult separation and commercial unavailability. Fingerprint analysis by gradient RP-HPLC is possible. After acidic hydrolysis to the aglycones quercetin, kaempferol and isorhamnetin and separation by HPLC, quantitation is straightforward and yields by recalculation an estimation of the original total flavonol glycoside content. For biflavones, simple phenols, 6-hydroxykynurenic acid, 4-*O*-methylpyridoxine and polyprenols analytical procedures have been published but not all assays are yet ideal. Lately a there is a lot of interest in the analysis of the undesired alkylphenols and a few validated procedures have been published. The analysis of Ginkgo proanthocyanidins is still in its infancy and no reliable assays exist.

Keywords: *Ginkgo biloba*; Reviews; Secondary metabolites; Phytochemical analysis; Terpene trilactones; Ginkgolides; Bilobalide; Flavonol glycosides; Quercetin; Biflavones; Proanthocyanides; Alkylphenols; Ginkgolic acid; Phenolic acids; 6-Hydroxykynurenic acid; 4-*O*-Methylpyridoxine; Polyprenols

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1. Introduction

Ginkgo biloba is among the most sold medicinal plants of this world with estimates of worldwide annual sales varying from a conservative US M$ 450 [1] to over 1 billion US $ in 1998 [2]. Most of the sales concern special extracts from the leaves which have been standardised for their content of terpene trilactones and flavonol glycosides. The extracts are mainly used for the improvement of the blood circulation, both peripherally and centrally [3]. The extracts are prepared in a multi-step process which may vary from manufacturer to manufacturer with the exact details remaining unknown. Most information can be found in a few patents [4,5]. During the process some compounds are enriched while others are removed. The final extracts contain a large number of constituents from various classes. Currently flavonol glycosides and terpene trilactones are considered the two pharmacologically most important groups present. A summary of the different classes of compounds present in the firstly developed and most sold special extract is given in Table 1.

All the larger manufacturers control their extracts for the presence of one or more of the groups given in Table 1 and a large number of other parameters. An example of a list of extract specifications is given in Table 2. Other manufacturers may or may not check for additional items such as proanthocyanidin content, organic acid content, limited ginkgolic acid content, individual content of bilobalide and ginkgolides A, B, C and J, solubility, qualitative fingerprints for terpene trilactones, flavonoid glycosides and organic acids, sulphated ash, total residual organic solvents, separate residual ethanol and chlorinated solvents, microbiological contamination, presence of phosphorous and chlorine containing pesticides, positive reaction in test tube assays for the presence of specific functional groups, pH-value and particle size. Many of those tests are well known, described in Pharmacopeias and not specific for Ginkgo. Therefore no attention will paid to them in this review. For more information on this topic see the recent overview by Camponovo and Soldati [7]. In recent years draft monographs on Ginkgo folium and extract for the United States Pharmacopeia (USP) [8,9] and Ginkgo folium and standardised Ginkgo extract for the European Pharmacopeia

Table 1
Different classes of compounds present in the standardised Ginkgo extract EGb 761 [6]

Compound class	%	Compound class	%
Flavonol glycosides	24.0	High molecular mass compounds	4.0
Terpene trilactones	6.0	Inorganic constituents	5.0
Proanthocyanidins	7.0	Water, solvent	3.0
Carboxylic acids	13.0	Various	3.0
Catechins	2.0	Unknown	13.0
Non-flavonol glycosides	20.0	Alkylphenols	$\leq$5 ppm

Table 2
Example of specifications for a standardised Ginkgo extract

Description	Brown powder with characteristic smell
Identity	Green-brown colour after adding $FeCl_3$ to a 0.1% solution (g/v) in alcohol–water (1:1)
Heavy metals	Not more than 20 ppm
Arsenic	Not more than 2 ppm
Ginkgolic acid	Not more than 10 ppm
Loss on drying	Not more than 5.0% (80 °C, vacuum)
Residue on ignition	Not more than 1.0%
Total flavonoid content	Not less than 24.0% (HPLC–UV)
Total terpene trilactone content	Not less than 6.0% (HPLC–RI)

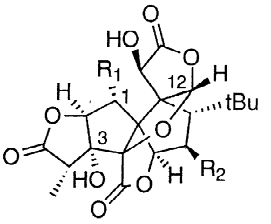
[10,11] have appeared for the quality control of these products and these publications will be briefly discussed.

This review will mainly focus on the quantitative chemical analysis of the secondary metabolites occurring in *Ginkgo biloba* leaves and extracts, i.e., terpene trilactones, flavonol glycosides, biflavones, proanthocyanidins, alkylphenols, simple phenolic acids, 6-hydroxykynurenic acid, 4-*O*-methylpyridoxine and polyprenols. No attention will be paid to preparative isolations [12–19] of particular constituents, qualitative tests in quality control like TLC [20], quantitative results without methodology [21] or extraneous constituents in phytopharmaceuticals [22] nor to investigations of finished drugs containing Ginkgo extracts [23]. Papers on constituents from other parts of the Ginkgo tree than leaves [24] and papers dealing with the biotransformation of flavonol glycosides [25–28] will not be reported on. Also analyses of Ginkgo leaf compounds which are neither secondary metabolites nor relevant for the medicinal activity like an antifungal protein [29], plant hormones [30] or chlorophyll [31] are not discussed. Earlier reviews of smaller scope on the analysis and quality control of Ginkgo leaves and extracts have been published by Sticher [32] and van Beek et al. [33,34].

2. Analyses of different classes of compounds occurring in *Ginkgo biloba* leaves and extracts

2.1. Terpene trilactones

Of all the compound classes present in *Ginkgo biloba*, the terpene trilactones have received by far the most attention. This is due to their chemical uniqueness, their importance in quality control and the analytical challenge. Their trivial names are ginkgolides A, B, C and J (further abbreviated as G-A, G-B, G-C and G-J) and bilobalide. The ginkgolides are diterpenes while bilobalide is a closely related C_{15} compound. The structures of these highly oxidised terpenes are given below. The structures of G-A, G-B and G-C were originally elucidated by two Japanese groups in the 1960s [2,35,36]. The structures of bilobalide [37] and G-J [38] were published a few years later. In the 1980s the interest in the ginkgolides suddenly soared when they were found to be potent and selective platelet-activating-factor antagonists and with the increase of sales of specialised Ginkgo extracts. A detailed review on their chemical analysis has appeared recently [39]. In the following only the more interesting papers and best approaches in addition to the most recent papers will be discussed in detail.

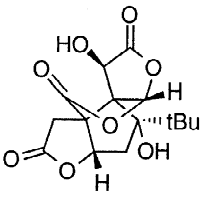


G-A: $R_1 = R_2 = H$

G-B: $R_1 = OH$, $R_2 = H$

G-C: $R_1 = R_2 = OH$

G-J: $R_1 = H$, $R_2 = OH$



Bilobalide

Technically speaking the chemical analysis of Ginkgo terpene trilactones can be divided in three

distinct parts: (1) extraction, (2) sample clean-up and (3) separation and detection. These steps should be described in detail and thoroughly validated but unfortunately this has not always been the case for Ginkgo publications. Several papers from industry and academia are lacking in sufficient experimental details and proper validation, respectively. In the following the extraction, clean-up and separation–detection will be discussed separately with some conclusions at the end of each section.

2.1.1. Extraction

To avoid too many apolar impurities, in almost all approaches water is an important constituent of the solvent initially used for the extraction of ginkgolides from Ginkgo leaves. Normally an organic solvent like methanol or acetone is added to improve the rate of extraction because G-A and especially G-B are poorly soluble in 100% water at room temperature. Examples are methanol–water (7:3) [40], refluxing or sonicating methanol–water (1:9) [41,42], acetone–water (4:1) after a prior defatting step with trichloroethylene [43,44], acetone–water (1:1) [45,46] and acetone–water (6:4) [10,47]. Pure methanol under ultrasonic agitation has also been used [48]. A few systematic investigations have been carried out to compare some of the above solvents. Mixtures of ethanol–water and acetone–water (ratios 5:5, 6:4, 7:3, 8:2 and 9:1) all gave satisfactory extractions of terpene trilactones but the amount of co-extracted components varied significantly [6,49]. The polar proanthocyanidins were not extracted at all with 90% organic solvent while the apolar ginkgolic acids were poorly extracted at less than 50% organic solvent content. Similar results were published by Aye and Müller [50]. Camponovo compared the efficiency of methanol, methanol–water (1:1) and refluxing water and reported that all three solvents extracted the terpene trilactones equally well. However methanol–water (1:1) was the preferred choice because it gave the most clean extract. The addition of a small percentage acid, e.g., 1% acetic acid, to the extraction solvent can be considered. It will reduce the amount of co-extracted chlorophyll and will diminish decomposition of the rather labile bilobalide during the extraction. Bilobalide is unstable above pH 7 [51,52]. Therefore the combination of extraction solvents recently proposed by Lang and Wai, should be considered a poor choice [53]. They first extracted leaves during 2 min with 100% water followed by a second extraction with refluxing 0.1% Na_2HPO_4 (pH 8) during 15 min. The degradation of bilobalide is obvious from the low values reported by them. This unusual extraction procedure as well as the ensuing sample clean-up invited a critical comment in the same journal [54].

Terpene trilactones can also be extracted supercritically (SFE). Carbon dioxide modified with 10% methanol at 335 atm and 45 °C can be used for a selective extraction of Ginkgo terpene trilactones from standardised extracts [55]. Advantages are reproducibility and automation. Unfortunately SFE was not successful for Ginkgo leaves due to the high amounts of co-extracted apolars like chlorophyll. The extraction of Ginkgo standardised extracts is less of a problem than Ginkgo leaves because matrix effects and diffusion do not play a role. Ginkgo extracts can be fully dissolved in 100% methanol for example.

Concluding one can state that there are several good solvents available for the extraction of terpene trilactones from leaves and the exact composition will more influence the concentration of other compounds than the terpene trilactone content. When selecting the extraction solvent it is good to keep already in mind the sample clean-up step.

2.1.2. Sample clean-up

The major problem in Ginkgo terpene trilactone analysis still lies in the sample clean-up of the crude initial leaf extracts or solutions of standardised extracts. Standardised extracts not only contain ~6% terpene trilactones but also ~24% flavonol glycosides and many other similarly polar constituents (Table 1) which can interfere with the ensuing separation and detection step if not removed. Additionally crude leaf extracts contain significant amounts of more apolar compounds like ginkgolic acids, biflavones and chlorophyll. Many procedures have been published during the last two decades. The first procedures were extremely time-consuming and error-prone comprising up to 35 partitioning steps or column chromatography steps and not validated leading to extracts which were either still not analysable [56] or gave wrong values [57,58]. Other early publications looked very promising but were lacking

Table 3
Quantitative SPE–HPLC–RI method for terpene trilactones in leaves by van Beek et al. [41]

(1) Reflux 600 mg Ginkgo leaves twice with 5 ml MeOH–H_2O (1:9) during 15 min
(2) Filter over Büchner, quantitatively collect aqueous extracts and apply to a 500-mg polyamide SC6 column connected in series with a 500-mg C_{18} SPE column
(3) Wash columns with 15 ml and 5 ml of 2 and 5% MeOH in H_2O, respectively, suck dry with air
(4) Disconnect polyamide column (discard) and wash C_{18} column with 6 ml hexane (discard)
(5) Elute C_{18} SPE column with 7 ml hexane–MeOAc (6:4) and evaporate solvent
(6) Dissolve in MeOH, add internal standard (benzyl alcohol) and inject into HPLC
(7) HPLC on a 5 μm 250×4.6-mm C_{18} column, 1 ml/min MeOH–H_2O (33:67), RI detection

in sufficient experimental detail for others to reproduce them [43,59]. The first validated method was published in 1991 [41]. An aqueous leaf extract was purified over a combination of a polyamide and C_{18} SPE column (see Table 3 for details and Fig. 3 lower trace for a chromatogram). Phenols (flavonoids) remained on the polyamide column while remaining impurities could be removed on C_{18}. Although the method worked it was complicated. An additional disadvantage was that it was not very robust. With different batches of C_{18} SPE columns breakthrough could occur leading to wrong results [60]. Instead of C_{18}, alumina has also been proposed as a stationary phase for the SPE clean-up of crude Ginkgo extracts [61,62]. Although this material gives a much better clean-up effect for ginkgolides than C_{18} or silica gel, others have reported that bilobalide decomposes on this material [55]. Perhaps acidic alumina might be useful. Recovery experiments are clearly indicated when this material is to be used.

Although perhaps not as reproducible and certainly more time-consuming than SPE, clean-up methods making use of partitioning steps by means of separatory funnels keep being published [10,45,46,53,63–67]. A problem with all of these procedures is the limited solubility of terpene trilactones in both water and more apolar organic solvents like diethyl ether and halogenated hydrocarbons. Additionally there are considerable differences in polarity between the individual terpene trilactones with G-B being the most apolar and G-C being the most polar. If one wants to extract 100% of G-C from the aqueous phase this necessitates a repeated extraction with a rather polar water-inmiscible solvent like ethyl acetate. This in turn implies the co-extraction of many impurities resulting in a poor clean-up effect. However, this is still to be preferred over a repeated extraction with more apolar solvents like diethyl ether [45] or worse dichloromethane [53]. On another occasion dichloromethane has even been used to remove (!) impurities from terpene trilactones [68]. The use of such solvents in combination with lack of recovery experiments will produce at least wrong results for G-C and G-J and possibly also bilobalide which remain partially in the aqueous phase [54]. The only usable exception is possibly the procedure published recently by Lang et al. [69]. They performed the partitioning in a 7-ml vial with the rather polar mixture of EtOAc and THF and an aqueous phase which was made more polar by the addition of salts. The full procedure is given in Table 4.

Table 4
Quantitative partitioning-GC–FID method for terpene trilactones in standardised extracts by Lang et al. [69]

(1) Sonicate 40 mg extract and 20 ml 10% NaH_2PO_4 (pH~4) in a 25 ml vial during 15 min at ~55 °C
(2) Shake the capped vials three to four times during the sonication, afterwards settling during 30 min
(3) Take 1.00 ml clear solution and transfer to a 7-ml sample vial, add 3 ml EtOAc–THF (7:3) and 25 μg squalane (I.S.), shake the flasks during 1 min and transfer 1–2 ml sample to a 4-ml vial
(4) Evaporate the solvent with N_2 and derivatise with 600 μl BSTFA–TMCS–DMF (99:1:100) during 45 min at 120 °C, after cooling down inject 1 μl into a GC
(5) GC analysis on a DB-5 column (15 m×0.32 mm×0.25 μm), oven temp. 200–280 °C, FID

Table 5
Quantitative partitioning-HPLC–RI method for terpene trilactones in leaves proposed for the European Pharmacopeia [10]

(1) Reflux 3 g powdered leaves with 100 ml Me_2CO–H_2O (6:4) for 30 min, filter and collect filtrate
(2) Repeat extraction with 80 ml solvent, filter, combine filtrates and evaporate Me_2CO
(3) Transfer to separatory funnel with 10 ml phosphate buffer, pH 5.8, extract 3× with 50 ml EtOAc
(4) Combine EtOAc layers, evaporate in vacuo, dissolve residue in 10 ml phosphate buffer, pH 5.8
(5) Transfer quantitatively to a column containing 20 g kieselguhr with an additional 5 ml of buffer
(6) Wait 15 min, elute column with 100 ml EtOAc, evaporate in vacuo
(7) Dissolve in 2.5 ml THF–MeOH–H_2O (10:20:75) and inject 100 μl into HPLC
(8) HPLC on a 5 μm 250×4 mm C_{18} column, 1.0 ml/min THF–MeOH–H_2O (10:20:75)
(9) RI detection, calculation by response factors against an external standard of benzyl alcohol

A better approach is to carry out such partitioning experiments in small SPE-like columns. This eliminates the problematic phase separation. Such procedures have been published [10,11,70–72] although not all of them have been properly validated. As an example the procedure proposed for the European Pharmacopeia is given in Table 5. The advantages of a sample clean-up by means of partitioning chromatography are that (1) the separation mechanism is different from the ensuing RP-HPLC or GC separation, (2) an aqueous solution can be applied on top of the column and (3) the ginkgolides are eluted in an easy to concentrate organic solvent. An almost identical procedure as the one in Table 5 was published in a draft United States Pharmacopeia monograph for standardised Ginkgo extract [8].

Still another useful stationary phase for sample clean-up is silica gel. A problem with silica gel is that the terpene trilactone extract to be purified needs to be applied in a very apolar organic solvent otherwise the ginkgolides are not retained. However, in such solvents the ginkgolides are poorly soluble. This problem was solved in an elegant way by Lolla et al. [47]. They applied their crude extract in methanol and subsequently removed the methanol at 60 °C in vacuo. Then the terpene trilactones could be eluted with a much more apolar solvent. The entire procedure is given in Table 6. An example chromatogram is given in Fig. 1. Validation experiments were carried out with respect to peak purity (GC–

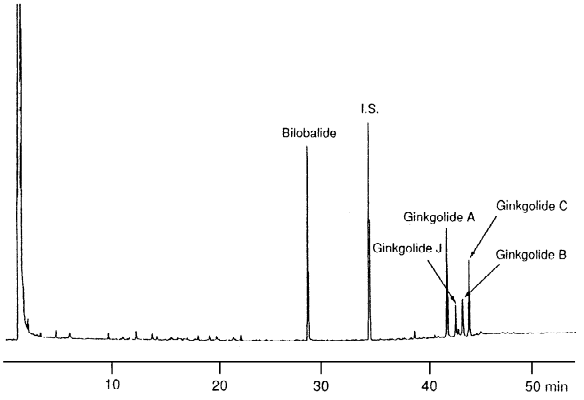


Fig. 1. GC–FID profile of an extract of Ginkgo leaves after sample clean-up on silica gel according to Lolla et al. [47]. Internal standard, squalane. Reproduced from Ref. [34] with permission of the editor.

Table 6
Quantitative SPE–GC–FID method for terpene trilactones in leaves by Lolla et al. [47]

(1) Extract 5 g powdered leaves in a Soxhlet with hexane for 4 h, discard and dry leaves in an oven
(2) Extract leaves with 120 ml Me_2CO–H_2O (6:4) overnight at room temperature with stirring
(3) Filter, evaporate in vacuo and dissolve in 5 ml MeOH under sonication
(4) Transfer 0.5 ml to an SPE column containing 1.2 g silica gel, dry column at 60 °C in vacuo for 4 h
(5) Elute column with 10 ml toluene–Me_2CO (7:3), add internal standard (squalane) to eluate
(6) Evaporate under N_2 at 60 °C, add pyridine and 1% TMCS in BSTFA and heat at 60 °C for 30 min
(7) GC analysis on a DB-1 column (15 m×0.25 mm×0.25 μm), oven temp. 120–300 °C, FID

Table 7
Quantitative SPE–GC–GC–MS method for terpene trilactones in tissue cultures by Balz et al. [44]

(1) Stir 1 g freeze-dried cells with Me_2CO–H_2O pH 2.5, (4:1) during 1 h, evaporate Me_2CO
(2) Push aqueous solution through a preactivated RP Seppak column, elute with MeOH
(3) Apply methanolic extract to silica gel column and dry overnight in a vacuum oven at 60 °C
(4) Elute with Me_2CO, derivatise overnight at 100° with BSTFA–pyridine (1:1)
(5) Inject 1 μl on-column into a two-dimensional GC (BP10 and 1) with MS detection
(6) Calibration with reference substances (G-A, G-B, G-C and G-J)

MS), linearity (over a factor 20), reproducibility (RSD = 2.5–3.5%) and recovery (99–99.5%). Although the entire procedure is rather lengthy, the sample clean-up is robust and has been applied successfully by others [55]. Similar clean-up procedures were used by Balz and co-workers and Peishan and co-workers for the analysis of Ginkgo tissue cultures and leaves, respectively [42,44,48,73]. Their procedures are given in Tables 7 and 10.

Silica gel has also been used for on-line sample clean-up of standardised Ginkgo extracts during SFE [55]. Some silica gel was placed in the extraction cell where it retained flavonoids. Silica gel was further used to trap the extracted terpene trilactones. After trapping they could be flushed from the trap with methyl acetate. This is the only procedure so far which combines extraction and sample clean-up. The entire procedure and an example chromatogram are given in Table 8 and Fig. 2, respectively. The final extract is also amenable to GC analysis after silylation, see Fig. 8 for an example chromatogram.

The most simple sample clean-up is no sample clean-up but just an extraction immediately followed by analysis. An early example of this approach can be seen in Fig. 10 where a liquid Ginkgo drug is directly analysed by SFC-ELSD. This method has not been validated for quantitative use. Very recently

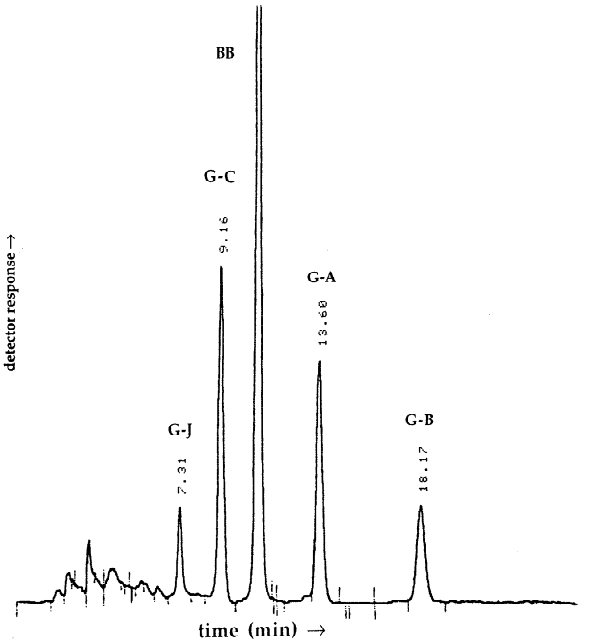


Fig. 2. HPLC–ELSD profile of a methanolic solution of a supercritical fluid extract of a standardised Ginkgo extract. Phenomenex column 250×4.6 mm filled with Spherisorb 5 ODS(2), solvent H_2O–THF–MeOH (68.5:10.5:21) 1.0 ml/min, Varex ELSD, N_2 as nebulizer gas at 2.06 l/min, drift tube 107°. From Ref. [55], reproduced with permission from John Wiley & Sons Limited.

Table 8
SFE–HPLC–ELSD method for standardised extracts by van Beek and Taylor [55]

(1) Apply ~18 mg standardised Ginkgo extract in MeOH on 2 g silica gel and 0.5 g sand in a thimble
(2) Extract supercritically at 335 atm and 45 °C with 10% MeOH in CO_2, first 5 min static, then 40 min dynamic at 1.5 ml/min, nozzle and trap both 80 °C, solid trap consists of 400 mg silica gel
(3) Stop flow, cool nozzle and trap to 50 and 30 °C, respectively, and wash trap with 2.5 ml MeOAc
(4) Collect first 1.7 ml, evaporate, dissolve in MeOH and inject 5 μl into the HPLC
(5) HPLC on a 5 μm 250×4.6 mm C_{18} column, 1.0 ml/min with H_2O–THF–MeOH (68.5:10.5:21) with ELS detection

Table 9
Quantitative HPLC–ELSD method with minimal sample clean-up by Ganzera et al. [75]

(1) Sonicate 500.0 mg sample three times during 10 min with each time 3 ml MeOH
(2) Centrifuge at 3000 rpm during 10 min, combine supernatants in a 10-ml volumetric flask
(3) Add MeOH to 10.0 ml, take sample and filter over a 0.45-μm membrane
(4) HPLC on a 4 μm 250×4.6 mm C_{18} column, 1.0 ml/min with a 10 m*M* NH_4OAc (pH 5) to MeOH–iBuOH (9:1) gradient with ELS detection, triplicate injections of 10 μl
(5) Calculation with external standardisation and log–log calibration curves

two papers with a validated quantitative procedure have appeared. Ganzera et al. [75] investigated a Ginkgo extract and several phytopharmaceuticals according to the procedure given in Table 9. The method is relatively fast and cheap but the required sample size is rather high. The method was validated in terms of recovery, peak purity, limit of detection, linearity, extraction efficiency and reproducibility. Some criticisms on the peak purity evaluation could be made. A chromatogram can be found in Fig. 4. The method of Jensen et al. is almost identical to the above method except for the use of a mass spectrometer as detector [74]. This leads to a much higher sensitivity and selectivity so the sample size could be reduced and peak purity should not pose a problem. The procedure was validated. RSDs were around 4%, the mean recovery 97% and the detection limits varied from 10 to 40 pg. There was a fair correlation between LC–MS and LC–RI results obtained for identical samples. A fast solvent gradient was used with a total run time of 14 min [74].

Concluding one can state that still significant developments in the sample clean-up of various Ginkgo samples for terpene trilactone analysis are taking place. In recent years there is a trend towards the application of partitioning materials like kieselguhr or adsorption materials like silica gel in small columns. Both methods give good results and deliver the purified terpene trilactones in an easy to remove organic solvent. Both techniques remove many polar impurities like flavonoids which could otherwise interfere with the separation and detection. Alumina appears to give more clean extracts but due to the possible degradation of bilobalide on this material its use is controversial. A new solvent–solvent partitioning system on a 4-ml scale also deserves attention. A very recent trend is the omission of any sample clean-up step. Problems related to such procedures are intrinsically more filthy extracts which will lead to a faster degradation of HPLC columns and possibly insufficient peak purity unless an expensive and more complex mass spectrometer is used as detector. Future improvements which can be expected are further simplifications, the introduction of internal standards for HPLC methods, a move towards a smaller scale resulting in a reduction of the required amounts of solvents which are still high and the development of one method which can be used for Ginkgo leaves, extracts and phytopharmaceuticals.

2.1.3. Separation and detection

2.1.3.1. HPLC

Initially RP-HPLC was applied for the analytical separation of terpene trilactones which is the most straightforward technique as they are high-melting non-volatile compounds. Already in the very first publication good solvent systems were published for standard C_{18} columns [56]. Two solvent systems, both isocratically, have remained in use until today: (1) H_2O–MeOH (~70:~30) and (2) H_2O–MeOH–THF (~70:~20:~10). The solvents show quite different selectivity (see Figs. 2 and 3, respectively). With solvent system (2) the peaks are somewhat better resolved and more evenly distributed over the chromatogram. However it contains THF and not all RI detectors can cope with THF. No further improvements in the RP-HPLC separation of terpene trilactones are to be expected. Normal-phase HPLC separations have not been published.

Unfortunately the first researchers selected UV for detection of terpene trilactones in HPLC eluates. In retrospect this was a poor choice as these compounds possess very low ε values around the non-selective wavelength of 219 nm. Therefore the peaks of

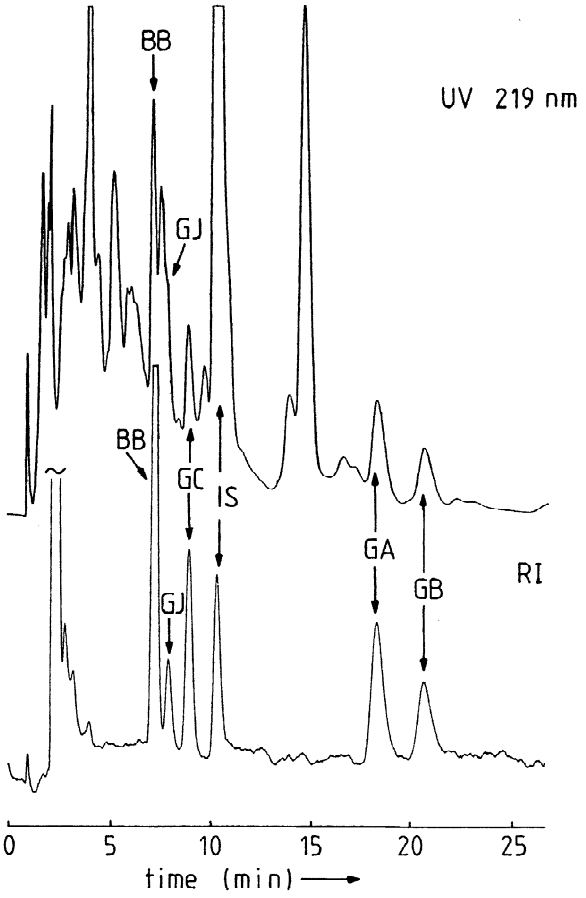


Fig. 3. HPLC profiles of 1.00 ml of purified Tanakan phytopharmaceutical with (upper trace) UV detection at 219 nm and (lower trace) RI detection. See Table 3 for chromatographic details. Internal standard (I.S.) is benzyl alcohol. Reprinted from Ref. [41], Copyright (1991), with permission from Elsevier Science.

interest were overshadowed by the absorbance of traces of other compounds remaining in the partially purified extracts [56]. This is clearly demonstrated in Figs. 3 and 4. A much better approach for ginkgolides is therefore a detector which shows less variation in response factors, e.g., refractive index detection (RI) [7,10,11,40,41,43,44,59,60,66,72,76–78] or evaporative light scattering detection (ELSD) [42,55,65,75]. Examples of UV/RI and UV/ELS detection are given in Figs. 3 and 4, respectively. Both methods are suitable for the routine analysis of all terpene trilactones after an RP-HPLC separation. Advantages of ELSD over RI are better baseline stability, compatibility with THF and gradients, small solvent peak and greater sensitivity. Advantages of RI over ELSD are larger linear range, lower costs and its broader availability.

The only other LC detection technique used is mass spectrometry [74,75,79–81]. With a thermospray interface and post-column addition of ammonium acetate strong quasi molecular ions $[M+NH_4]^+$ could be observed for all terpene trilactones and the method could be used for the selective detection and semi-quantitative analysis of the terpene trilactones after minimal sample clean-up [79]. Disadvantages were the large day to day variation necessitating recalibration every day and the much higher cost compared to other LC detectors.

Electrospray ionisation MS (ESI-MS) was used by Mauri et al. and is more robust than the TSP interface [80]. Best ESI-MS results were obtained in the positive mode. All the terpene trilactones gave sodiated adducts $[M+Na]^+$ as the main ion. No additional sodium needed to be added to the sample or solvent for the sodiated adducts to be the main ions. Both direct infusion ESI-MS and on-line ESI-MS after an isocratic or gradient RP-HPLC run were possible. An example of the direct infusion technique showing also many flavonoids is given in Fig. 5. The detection limit for this technique was ~50 μg/ml for each terpene when present in standardised extracts. A much higher sensitivity could be achieved by selected ion monitoring in the LC ESI-MS mode: ~1 ng. Good linearity was obtained in the range 1–20 μg/ml for each terpene trilactone. Overall reproducibility was 3.4% (same day) and 5.8% (between days) [80]. Ganzera et al. also used ESI-MS but then in the negative mode with a 10 m*M* ammonium acetate buffer. $[M-H]^-$ could be observed for all five terpene trilactones [75]. A chromatogram is depicted in Fig. 6. In a more recent paper Mauri et al. used atmospheric pressure chemical ionization (LC–APCI-MS) in the negative mode. The high sensitivity and specificity of the method (~1 ng/ml) allowed the quantitation of terpene trilactones in plasma samples of volunteers. Due to a fast gradient the total separation time was 7 min [81]. A similar LC–MS procedure was used by Jensen et al. [74]. They used LC–MS as part of a quantitative method for Ginkgo extracts (vide supra).

Although the MS detector offers a high selectivity and sensitivity, due to its high price and more complicated operation and maintenance it remains to

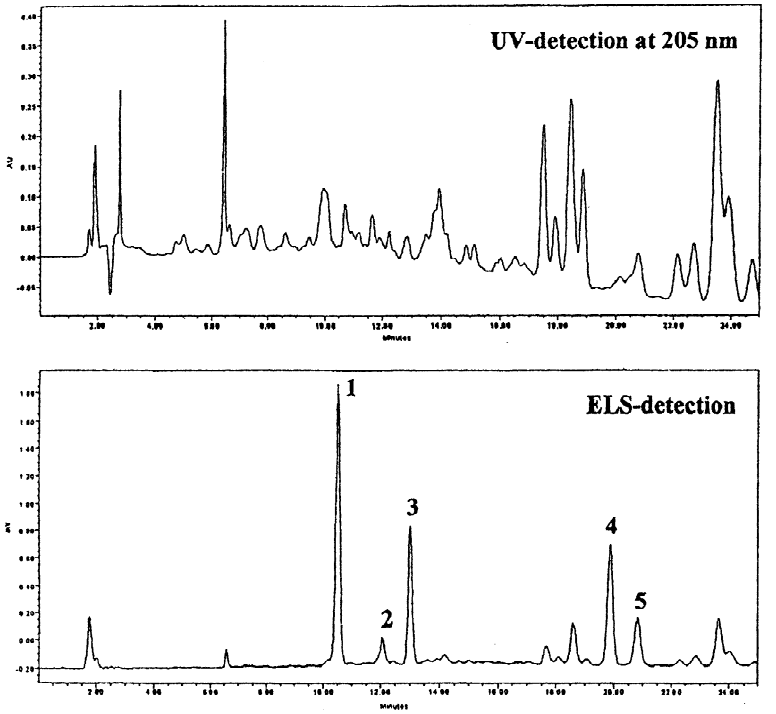


Fig. 4. HPLC–UV and HPLC–ELSD profiles of a filtered methanolic extract of a Ginkgo drug containing standardised extract. Phenomenex Synergi Max-RP 80 Å 4 μm column 150×4.6 mm, gradient from 10% B to 20% B in 10 min, then to 25% B in 15 min, 1.0 ml/min. (A) 10 m*M* NH_4OAc adjusted to pH 5; (B) MeOH–iBuOH (9:1). Sedex 55 ELSD, N_2 as nebulizer gas at 2.4 bar, drift tube 45°. Reproduced from [75] with permission of The Pharmaceutical Society of Japan.

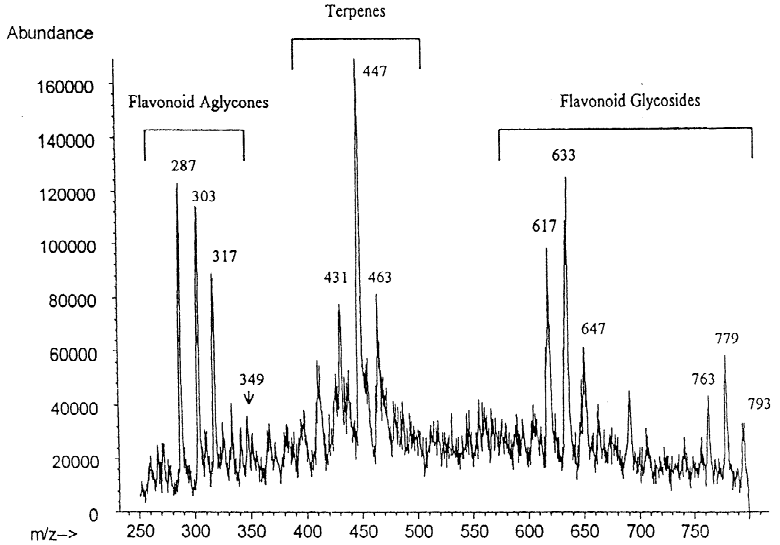


Fig. 5. Positive ion mass spectrum of Ginkgo standardised extract introduced by direct infusion into an ESI-MS. Peaks at m/z 349, 431, 447 and 463 correspond with $[M+Na]^+$ for bilobalide, G-A, G-B and G-J, and G-C, respectively. From Ref. [80], reproduced with permission from John Wiley & Sons Limited.

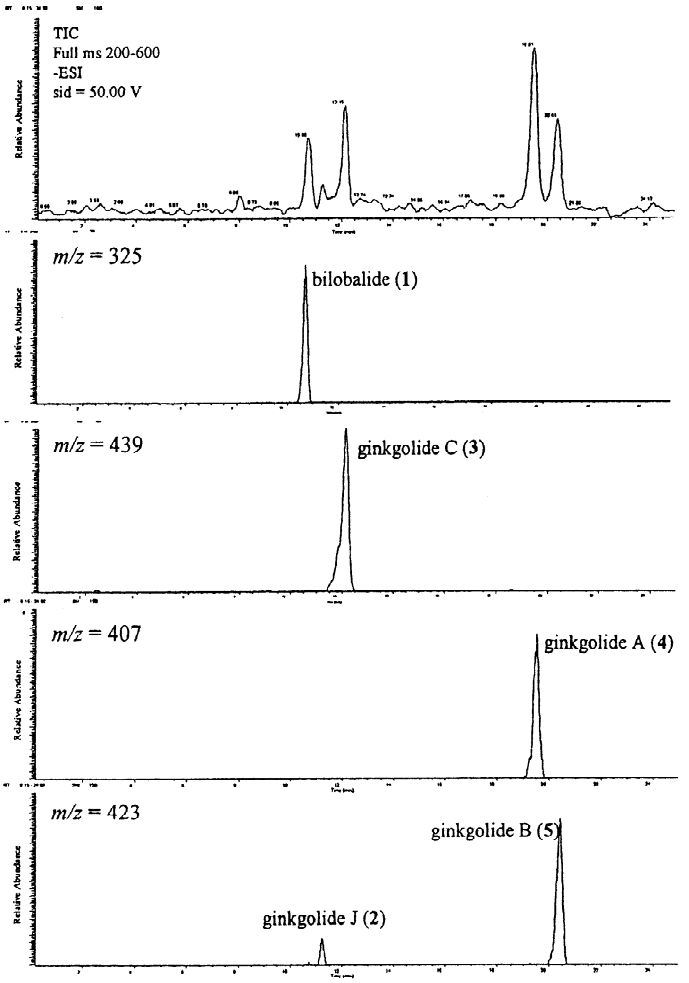


Fig. 6. LC–MS profiles of a filtered methanolic extract of a Ginkgo phytopharmaceutical. Upper trace: total ion current chromatogram. Lower four traces: selected ion monitoring, peaks at m/z 325, 439, 407 and 423 correspond with $[M-H]^-$ for bilobalide, G-C, G-A and G-J+G-B, respectively. ESI-mode, ionization voltage 50 V, source voltage 3.0 kV, probe 350 °C. Reproduced from Ref. [75] with permission of The Pharmaceutical Society of Japan.

be seen whether it will replace RI and ELSD in the near future for routine assays of terpene trilactones in Ginkgo leaves and standardised extracts.

2.1.3.2. Thin-layer chromatography

Ginkgolides and bilobalide can also be separated by TLC on normal-phase silica gel plates. Solvents that have been used include toluene–Me_2CO (7:3) [38,57,59], cyclohexane–EtOAc (1:1) [56], EtOAc–toluene–Me_2CO–hexane (4:3:2:1) [82] and $CHCl_3$–Me_2CO–HCO_2H (75:16.5:8.5) [83]. Prior impregnation of the plates with sodium acetate in combination with methyl acetate as solvent gives slightly sharper spots and a better resolution [84]. R_f values decrease in the order bilobalide, G-A, G-B, G-J, G-C. This system appeared in the draft monograph of the United States Pharmacopeia on Ginkgo leaf for the qualitative analysis of terpene trilactones [9,85] and was used in the only quantitative TLC procedure published so far [48,73]. In the latter procedure after a rather lengthy clean-up procedure, development and conversion to fluorescent derivatives by simple heating, the terpene trilactones were analysed by densitometry. The procedure is depicted

Table 10
Quantitative SPE–HPTLC-densitometric method for terpene trilactones in leaves by Peishan et al. [48,73]

(1) Extract 3 g Ginkgo leaf powder under sonication 20 + 15 min with 2× 80 ml MeOH–H_2O (1:9)
(2) Filter, combine filtrates and pass through a polyamide cartridge and elute with 100 ml water
(3) Evaporate eluate, dissolve in 5 ml MeOH, mix with 3 g silica gel, dry 4 h over P_2O_5 in vacuo
(4) Transfer silica gel to a 5-g activated silica gel SPE column, elute with 200 ml $CHCl_3$–MeOH (1:1)
(5) Evaporate eluate, dissolve in 1.0 ml MeOH
(6) Apply 6–10 μl and reference solutions on a NaOAc impregnated silica gel plate with autosampler
(7) Dry the plate >4 h over P_2O_5, condition the plate over aqueous H_2SO_4
(8) Develop HPTLC plate 9 cm with toluene–EtOAc–Me_2CO–MeOH (10:5:5:0.6)
(9) Evaporate solvent, heat plate 30 min at 160 °C, scan the chromatogram in a TLC scanner at $\lambda = 366$ nm, calibrate by second-order polynomial regresssion, calculate results

in Table 10. A chromatogram can be viewed in Fig. 7. The method was validated and gave accurate and reproducible results for well known standardised extracts. The results were comparable with those obtained by HPLC–ELSD or HPLC–RI [73].

2.1.3.3. Gas chromatography

Instead of HPLC, GC can also be used. However prior silylation is necessary because ginkgolides and bilobalide are non-volatile. This is the main disadvantage in comparison with HPLC. The separation is at least as good and detection by FID surpasses any available LC detector in reproducibility, baseline stability, ruggedness, costs and sensitivity. The best column is a 30-m capillary one coated with 100% dimethyl polysiloxane phase. One of the milestones in this area is the paper by Hasler and Meier [86]. They investigated among others the optimal silylation procedure. BSTFA with 1% TMCS at 120 °C was found to give the best results. Rather mystifying is the recent remark of Balz et al. that bilobalide cannot be derivatised [44]. This is in contradiction when many other publications (vide infra). After silylation the mixture should be injected directly into the GC to avoid desilylation problems. Detection limits varied from 50 to 100 ng and RSDs were low (1–2%). Various internal standards have been proposed: cholesterol [86], octacosane [55] and squalane

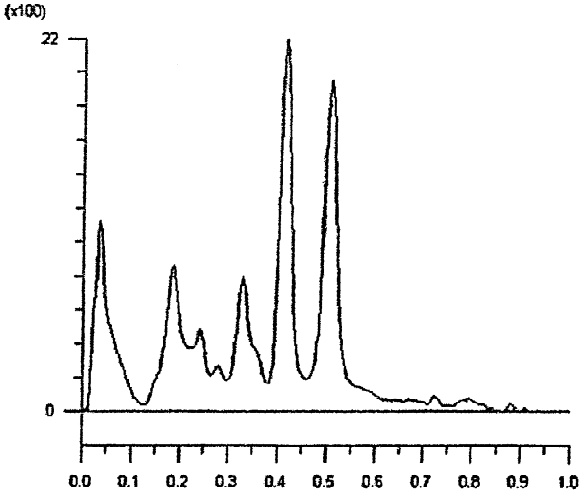


Fig. 7. TLC scanning profile of a Ginkgo leaf extract on a sodium acetate impregnated hand-made silica gel plate. Bilobalide, G-A, G-B and G-C can be observed at R_f values of 0.52, 0.42, 0.33 and 0.18, respectively. Reproduced in modified form from [73] with permission of the editor.

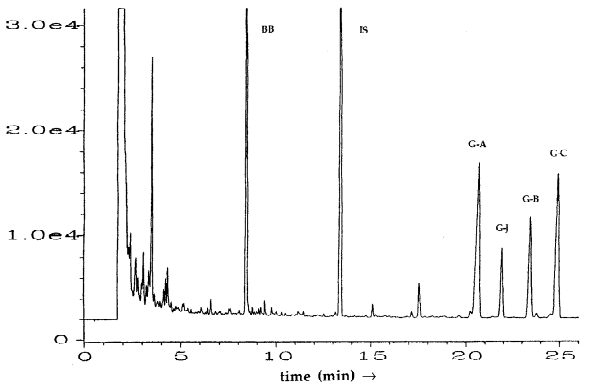


Fig. 8. GC–FID profile of a standardised Ginkgo leaf extract after supercritical fluid extraction and on-line sample clean-up after silylation [55]. See Table 8 for details on the sample preparation. GC analysis took place on an Ultra 1 column (25 m×0.2 mm× 0.33 μm), oven temp. 230–280 °C. Internal standard, octacosane. From Ref. [55], reproduced with permission from John Wiley & Sons Limited.

[47,69]. An example chromatogram can be seen in Fig. 8. Other publications describing silylation of terpene trilactones followed by GC are [45–47,53,55,63,64,69,83,87,88].

Instead of flame ionisation two other detectors can be used for the detection of silylated terpene trilactones: electron capture and mass spectrometry. ECD has been used only once [34]. The slight increase in selectivity and sensitivity of ECD compared with FID is offset by the problems associated with this detector: not generally available, limited linear range and its inherent radioactivity. GC–MS is only necessary when the concentrations are very low: in certain Ginkgo cell cultures [44,89] and for blood and urine samples in pharmacokinetic studies [51,90]. For the procedure used by Balz et al. see Table 7. ECD can also be used for a highly sensitive detection of terpene trilactones if the derivatisation takes place with halogen-containing reagents, e.g., heptafluorobutyric anhydride. Then detection limits of below 1 pg (Fig. 9) can be realised although the stability of the bilobalide derivative might be a problem [34]. This derivatisation is of little practical consequence for the routine analysis of leaves or extracts where such high sensitivity is not needed. For pharmacokinetic studies where sensitivity is an issue, this technique may be more valuable but still GC–MS with its inherent higher selectivity is probably more useful and practical.

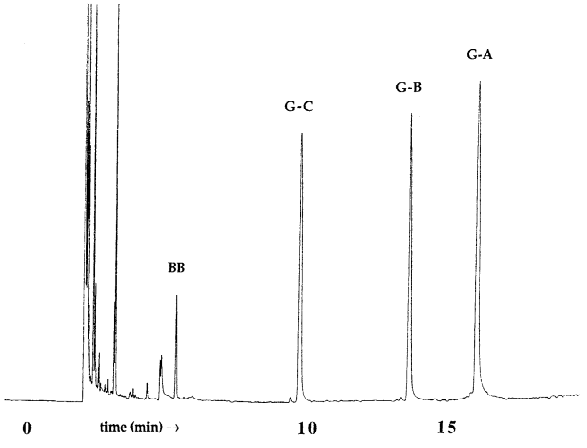


Fig. 9. GC–ECD profile of a toluene extract of bilobalide (BB), G-C, G-B and G-A reference substances after reaction with heptafluorobutyric anhydride. Each peak corresponds with approximately 10 pg of underivatised terpene trilactone. After 14.5 min attenuator value is halved. Reproduced from Ref. [34] with permission of the editor.

2.1.3.4. SFC

Supercritical fluid chromatography is also capable of separating Ginkgo terpene trilactones [91]. On a Deltabond deactivated aminopropyl HPLC column (150×4.6 mm, 5 μm) with 12% methanol in carbon dioxide as fluid (280 atm, 3.5 ml/min, 40 °C), a baseline separation of bilobalide and all four ginkgolides could be achieved within 9 min. Detection occurred through evaporative light scattering detection (ELSD). A detection limit of approximately 10 ng was reported. The selectivity of the system appears to be higher than that of RP-HPLC. The explanation given was that the separation mechanism is essentially a normal-phase one. As most impurities present in Ginkgo extracts are more polar than terpene trilactones, the latter elute first and the impurities remain on the column. Although a sample clean-up over silica did give cleaner chromatograms, some standardised extracts and phytopharmaceuticals could be analysed without any clean-up (Fig. 10). Thus SFC is an alternative for the analysis of Ginkgo extracts: quick, low consumption of organic solvent and a more simple sample clean-up. However in the near future it is unlikely to replace either HPLC or GC in the average quality control laboratory.

2.1.3.5. Capillary electrophoresis

Oerhle has demonstrated that G-A, G-B and bilobalide can be separated by micellar electrokinetic capillary electrophoresis (MECC) [92]. The three compounds were separated in a capillary of 60 cm×75 μm at 30 °C with a buffer consisting of 25 m*M* phosphate and 90 m*M* SDS (Fig. 11). The voltage was not given. Detection took place by UV at 185 nm. There was a good separation between bilobalide (10 min) and the two ginkgolides but G-A and G-B were not baseline separated (16.5 and 16.7 min, respectively). No quantitative data or chromatograms of leaf extracts were included in this paper. Unless the separation considerably improves, it is clear that capillary electrophoresis cannot yet compete with HPLC, GC or SFC for the separation of Ginkgo terpene trilactones.

Two less straightforward techniques for the quantitative analysis of terpene trilactones are quantitative NMR [71] and biological standardisation [83,93]. Neither of these techniques are likely to be routinely used for quality control of Ginkgo leaves or extracts because of one or more disadvantages. NMR is too

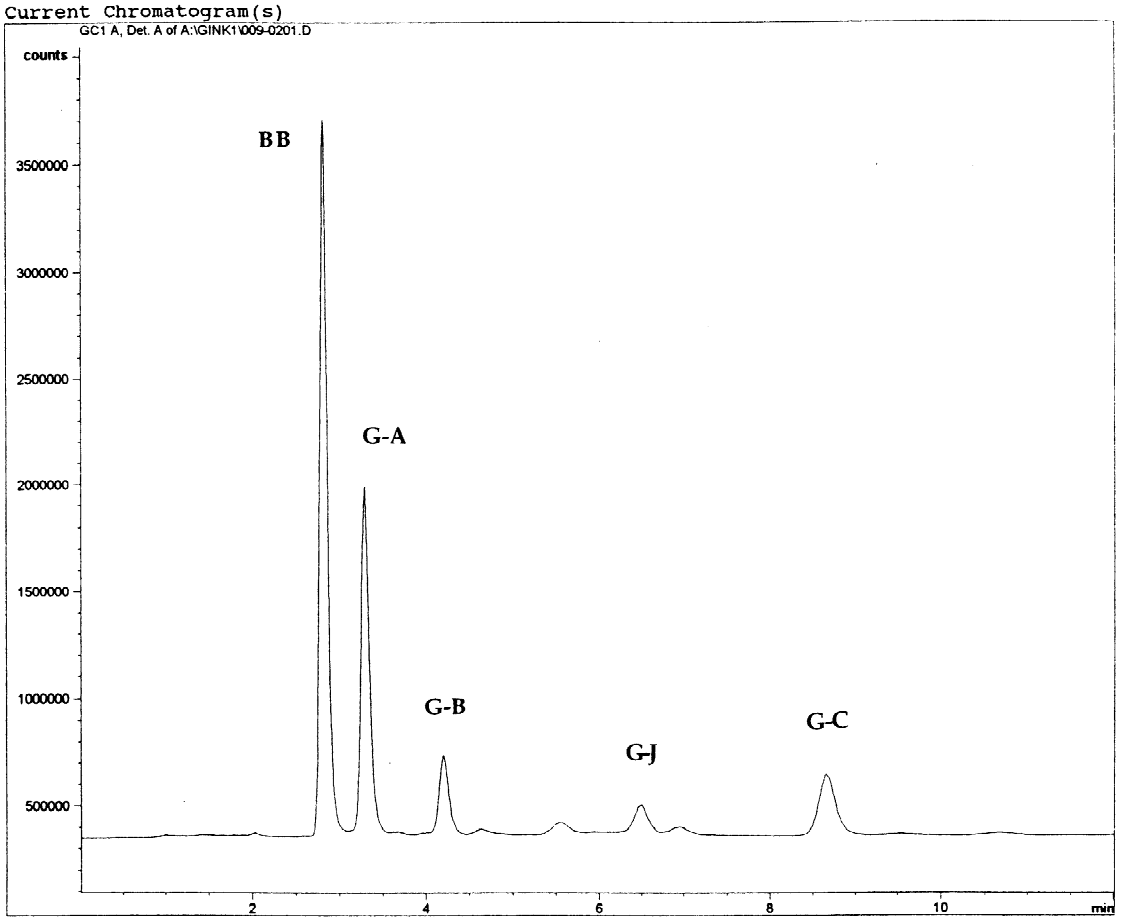


Fig. 10. SFC–ELSD profile of a methanol solution of Indena standardised Ginkgo extract without any prior sample clean-up. Deltabond Amino 2 column, 5 μm 15 cm×4.6 mm. Pressure 280 atm, flow 3.5 ml/min, fluid 12% MeOH in CO_2, 40 °C. Reprinted from Ref. [91], Copyright (1996), with permission from Elsevier Science.

expensive and not widely available in quality control departments and biological assays based on PAF–antagonistic activity need blood or blood-derived products and fail to assay bilobalide. The only unique advantage of quantitative NMR is that with this technique it is possible to determine the absolute purity of every reference terpene trilactone which is difficult to determine in another way.

Separation and detection of terpene trilactones is non-problematic. Both GC on an apolar column and RP-HPLC and TLC give acceptable resolution within 20 min. SFC is a little faster and a possible alternative with a different separation mechanism. Detection is not an issue. For LC, both RI and ELSD are low budget detectors with sufficient sensitivity. UV should not be used. For GC, FID is the technique of choice. If a much higher selectivity and sensitivity are necessary, for both LC and GC mass spectral detection is available.

2.2. Flavonoids and proanthocyanidins

Ginkgo leaves contain large amounts of flavonol glycosides, biflavones and proanthocyanidins. Flavonol glycosides and proanthocyanidins both occur in standardised extracts (24 and 7%, respectively) [6] and both are considered to be of importance for the beneficial effects of Ginkgo extracts. While biflavones do not occur in standardised extracts, they are not devoid of pharmacological activi-

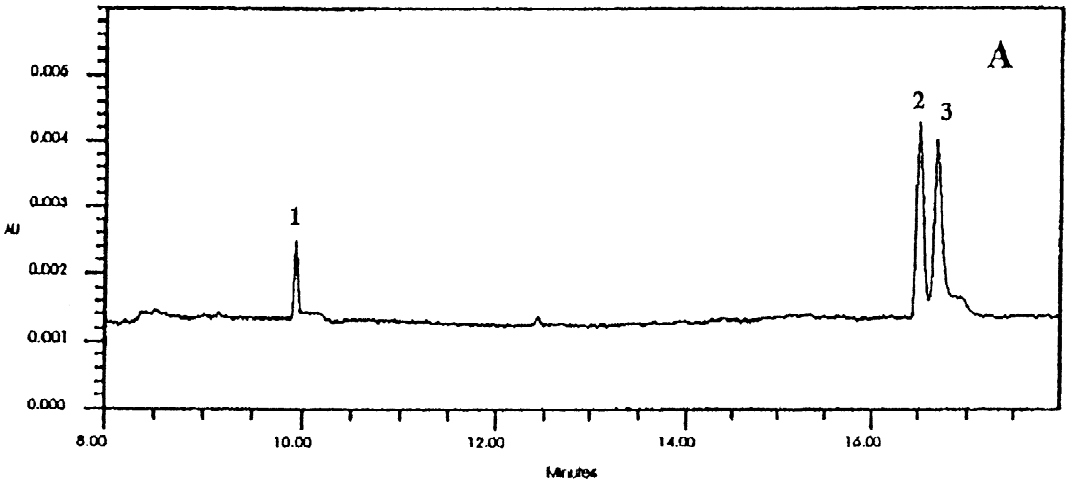


Fig. 11. MECC profile of standards of G-A, G-B and bilobalide. Capillary 60 cm×75 μm, buffer 25 m*M* phosphate and 90 m*M* SDS, 30 °C. Detection UV at 185 nm. Reprinted from Ref. [92] by courtesy of Marcel Dekker Inc., 1995.

ty. Lately they have been applied in cosmetics [94]. In the following these three different groups of polyphenolics are discussed. An earlier review on the chemical analysis of Ginkgo flavonoids was published by Sticher et al. [95].

2.2.1. Flavonol glycosides

In Ginkgo leaves and extracts many different flavonol glycosides occur most of them being derivatives of quercetin, kaempferol and isorhamnetin. The aglycones themselves occur only in relatively low

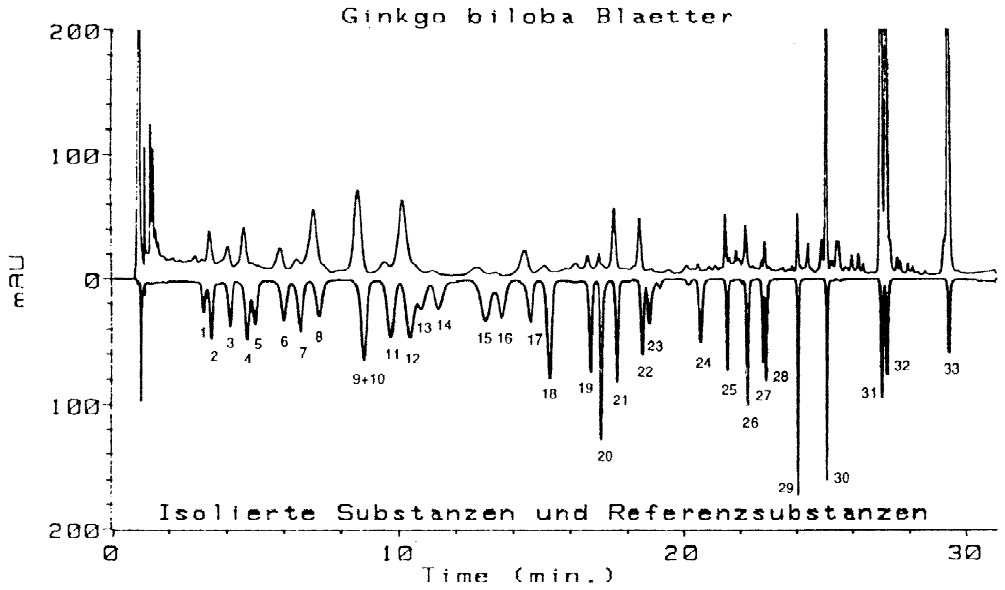
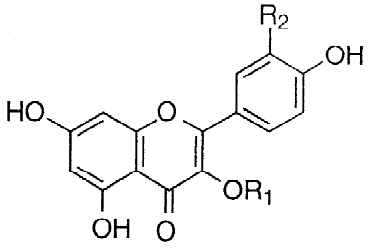


Fig. 12. Upper: HPLC profile of 33 different flavonol glycosides (1–22), flavonol aglycones (23–28) and biflavones (29–33) occurring in an alcoholic Ginkgo leaf extract. Lower: HPLC profile of a mixture of 33 reference compounds. For chromatographic details see Table 11, line 8. Reproduced with permission from Ref. [96].

concentrations. The structures of the three aglycones and the major glycosides are given below.



kaempferol $R_1 = H, R_2 = H$

quercetin $R_1 = H, R_2 = OH$

isorhamnetin $R_1 = H, R_2 = OMe$

3-O-(6''-O-(α-L-rhamnosyl)-β-D-glucosyl)kaempferol R_1 = glc-rha, $R_2 = H$

3-O-(6''-O-(α-L-rhamnosyl)-β-D-glucosyl)quercetin R_1 = glc-rha, $R_2 = OH$

3-O-(2''-O,6''-O-bis(α-L-rhamnosyl)-β-D-glucosyl)kaempferol $R_1 = glc\text{-}(rha)_2$, $R_2 = H$

3-O-(2''-O,6''-O-bis(α-L-rhamnosyl)-β-D-glucosyl)quercetin $R_1 = glc\text{-}(rha)_2$, $R_2 = OH$

3-O-(2''-O-(6'''-O-(*p*-coumaroyl)-β-D-glucosyl)-α-L-rhamnosyl)kaempferol R_1 = glc-rha-coumaroyl, $R_2 = H$

3-O-(2''-O-(6'''-O-(*p*-coumaroyl)-β-D-glucosyl)-α-L-rhamnosyl)quercetin R_1 = glc-rha-coumaroyl, $R_2 = OH$

Most of the work on the separation of the flavonol glycosides has been carried out by Hasler and co-

Table 11
Qualitative SPE–HPLC–UV fingerprint method for flavonol glycosides and biflavones by Hasler and co-workers [96,97]

(1) Extract 4 g leaves or 2 g extract with 50 ml 80% EtOH during 2 min with mixer
(2) Remove insolubles with a glass filter (G3) covered with filter paper
(3) Re-extract the residue with 30 ml 80% EtOH for 1 min in mixer, wash with 20 ml 80% EtOH
(4) Pool the three extracts and remove the solvent in vacuo until 40 ml remains
(5) Dilute with 80% EtOH to 50 ml in a volumetric flask
(6) Filter 5 ml through a Bond Elut C_{18} SPE cartridge equilibrated with 80% EtOH
(7) Elute with 4 ml MeOH and dilute with 80% EtOH in a 10 ml volumetric flask
(8) Analyse 10 μl with HPLC (Nucleosil 100-C_{18}, 3 μm, 100×4 mm), 30 min ternary gradient with A=iPrOH–THF (25:65), B=MeCN and C=0.5% H_3PO_4 in H_2O, 1 ml/min, 30 °C, UV detection at 350 nm

workers [96,97]. They published an excellent reversed-phase separation of 33 flavonol glycosides, flavonols and biflavones using a complex ternary gradient (Fig. 12). Unfortunately due to a lack of commercially available reference compounds no quantitation of individual glycosides is possible. The procedure (see Table 11) can be used for fingerprint analysis to check the genuine origin and proper storage conditions of Ginkgo extracts. A similar but binary gradient HPLC separation was published by Pietta and co-workers [98–100]. They used a C_8 Aquapore RP-300 (220×4.6 mm, 7 μm) column in combination with a linear gradient from 20–60% B in 40 min (A: iPrOH–H_2O (5:95); B: iPrOH–THF–H_2O (4:1:5)) at 1 ml/min with UV detection at 260 or 360 nm. A more simple isocratic reversed-phase system for flavonol glycosides was published by Chen et al. [101]. The solvent was MeOH–MeCN–0.5% H_3PO_4 in H_2O (35:5:60) at 1 ml/min in combination with a 250×4.6 mm column. Detection took place by UV at 330 nm. The peaks were less well resolved compared to the ternary gradient system of Hasler. Calculated as rutin the combined content based on 11 major peaks was approximately 1% in five different leaf batches [101].

An entirely different and so far only qualitative approach is the direct infusion at 10 μl/min of Ginkgo standardised extracts into a mass spectrometer equipped with an electrospray interface [100,102]. No sample clean-up or separation was used. In the positive ion mode clear peaks at *m/z* 617, 633 and 647 corresponding with the sodium adducts of kaempferol-, quercetin- and isorhamnetin-3-*O*-rutinosides, respectively, and at *m/z* 763, 779 and 793 corresponding with the sodium adducts of 3-*O*-[rhamnosyl-(1→2)-rhamnosyl-(1→6)-glucoside] derivatives of kaempferol, quercetin and isorhamnetin, respectively, can be observed (Fig. 5). With this technique sodium adducts of terpene trilactones can be observed simultaneously. Further development is necessary to convert it to an — in principle — very fast quantitative procedure for the main Ginkgo flavonol glycosides.

Due to lack of commercially available reference substances and the complex separation conditions, the normal procedure for the quantitative analysis of flavonol glycosides and flavonols in Ginkgo leaves and extracts is an acidic hydrolysis followed by HPLC of the resulting aglycones. Because only three flavonol aglycones (quercetin, kaempferol and isorhamnetin) occur in significant concentrations, this greatly facilitates the analysis. Wagner et al. have published a method for Ginkgo extracts where 10 ml of a filtered extract solution in 70% MeOH is heated in a closed flask at 100 °C during 30 min after addition of 10 ml 5.5% HCl in MeOH [57]. After cooling, this solution could be directly investigated by RP-HPLC with UV detection at 370 nm. In spite of the presence of only three similar compounds a gradient was needed. The chromatogram looks relatively clean so indeed no sample clean-up appears to be necessary. The content of the three aglycones was recalculated to an acylated flavonol diglycoside (MW=755.6) or rutin (MW=665) content. No validation was carried out [57].

A similar procedure was proposed by Hasler et al. [96,97]. It is summarised in Table 12. In contrast to the procedure of Wagner et al., in this case a sample

Table 12
Indirect quantitative SPE–HPLC–UV method for flavonol glycosides by Hasler et al. [96,97]

(1) Reflux 4 g leaves or 2 g extract with 70 ml MeOH and 10 ml 25% HCl during 1 h
(2) After cooling remove insolubles with a glass filter (G3) covered with filter paper
(3) Wash residue with 100 ml MeOH and pool both filtrates
(4) Evaporate solvent in vacuo until 80 ml and add MeOH until 100.0 ml of volume
(5) Filter 5 ml through a Bond Elut C_{18} SPE cartridge equilibrated with MeOH
(6) Elute with 4 ml MeOH and dilute with MeOH in a 10-ml volumetric flask
(7) Analyse 10 μl with HPLC (Hypersil 100-C_{18}, 5 μm, 100×4 mm), 12 min gradient from 38 to 48.2% A, A=MeOH, B=0.5% H_3PO_4 in H_2O, 2 ml/min, 25 °C, detection UV 370 nm
(8) Calculate quercetin, kaempferol and isorhamnetin content by external standardisation; recalculate to original acylated flavonol diglycoside content (MW=756.7) by multiplication of the total aglycone content with a factor 2.51

clean-up step was part of the procedure. Perhaps this is caused by the fact that the procedure is suitable for both Ginkgo leaves and extracts. On the other hand, few leaf constituents eluting around the same time as flavonol aglycones and which absorb light at the selective wavelength of 370 nm occur in Ginkgo leaves. The chromatogram (Fig. 13) is of high quality and the separation time is 12 min excluding 10 min washing and re-equilibration. Also in this case a mild gradient was used. Kim et al. and Chen et al. have both experimented with isocratic systems but the resulting chromatograms are not as good as those of Hasler [101,103].

The draft monographs on Ginkgo leaf for the United States [9,85] and European [10] Pharmacopeias are identical and use a variation of the procedures described above. As initial extraction solvent they use acetone–water (6:4). After removal of the acetone and addition of methanol and HCl the glycosides are hydrolysed. Separation takes place isocratically on a 125×4.6-mm C_{18} column with iPrOH–MeCN–0.6% citric acid in H_2O (5:47:100) as solvent at 1.0 ml/min. UV detection occurs at 370 nm. This solvent system resolves isorhamnetin and kaempferol rather poorly but on the other hand is very simple and fast (7 min). All peaks are first calculated as quercetin by external standardisation and then recalculated to a flavonol glycoside of mass 756.7 by multiplication with 2.514. Why the kaempferol–isorhamnetin peak is not calculated as kaempferol by external standardisation is unclear.

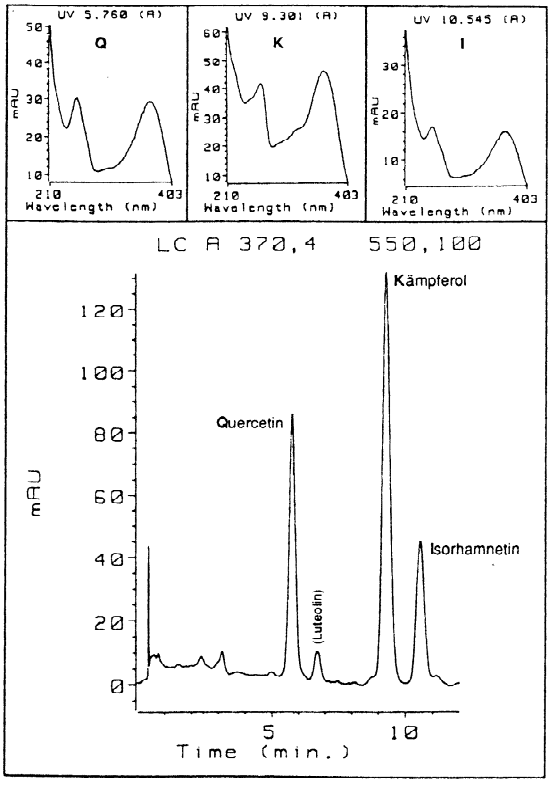


Fig. 13. HPLC profile of hydrolysed Ginkgo leaf sample. For chromatographic details see Table 12, line 7. Reproduced with permission from Ref. [96].

Recalculation of the aglycones to flavonol glycosides with an average weight of 756 is slightly incorrect as many flavonol glycosides possess a lower molecular mass and free aglycones are also always present to some extent. Thus the true flavonol glycoside content of standardised extracts is somewhat lower than 24% but this does not lead to problems as long as everyone uses the same procedure and method of calculation. Additionally extracts should be stored properly to avoid hydrolysis.

Two quantitative indirect methods for kaempferol and quercetin employing TLC or HPTLC and densitometry have been published [104,105]. The procedure used by Jamshidi et al. [105] is given in Table 13. A baseline separation of the two analytes of interest but not of quercetin and an unknown matrix component was obtained. Calibration curves were linear from 0.5 to 2.0 μg for both kaempferol and quercetin. Repeatability was good with RSDs of 1.4%. The recovery varied from 94 to 97%. The extraction efficiency was not checked.

There is one paper where micellar electrokinetic capillary chromatography (MECC) has been used for the separation of Ginkgo flavonol glycosides [99]. The separations were performed with a 72-cm×50-μm fused-silica capillary column in combination with a 20 m*M* sodium borate buffer (pH 8.3) and 50 m*M* sodium dodecyl sulphate (SDS) at 20 kV and 27 °C. The injection volume was 4 nl and UV detection took place at 260 nm. Compared to HPLC the separation is faster with about the same resolution. Disadvantages of MECC mentioned by the authors themselves relative to HPLC are poorer reproducibility of retention times and poor compatibility with mass spectrometric detection [100].

Table 13
Indirect quantitative HPTLC–densitometric method for flavonol glycosides in leaves by Jamshidi et al. [105]

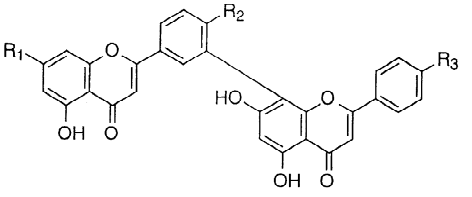
(1) Reflux 2 g leaves with MeOH for 30 min, filter, add 2 ml 25% HCl solution
(2) Reflux acidic extract for 60 min, cool and neutralize with 25% ammonia solution
(3) Reduce volume on a water bath with N_2 and dilute with MeOH in a 10-ml volumetric flask
(4) Leave overnight and apply supernatant by means of a Linomat IV on a 10×20-cm silica gel HPTLC plate prewashed with $CHCl_3$–MeOH, band length 6 mm
(5) Develop plates by the incremental multiple development technique with toluene–Me_2CO–MeOH–HCO_2H (46:8:5:1) in an unsaturated chamber, dry during 5 min with N_2 at 40 °C
(6) Evaluate plates with a TLC scanner (densitometry) in the reflectance mode at 254 nm
(7) Calculate content of kaempferol and quercetin by external standardisation

Thus HPLC is likely to remain the technique of choice for the separation of Ginkgo flavonoids.

A good and simple method for the analysis of all Ginkgo flavonol glycosides does not exist and is unlikely to appear in the near future mainly due to a lack of reference compounds. The best approach might be the use of an internal standard which is calibrated against some standard mixture of known composition. As at this moment it is not known which of the many genuine glycosides are important for in vivo or clinical activity, there is not much interest in the development of such a procedure. Indeed—as suggested by Sticher et al. [95]—the analysis of the glycosides is most useful for a qualitative fingerprint check of Ginkgo extracts. The pattern is unique and the presence of more aglycones than normal could indicate improper storage. Additionally a high aglycone content could lead to a flattered flavonol glycoside content after acidic hydrolysis and recalculation. Acidic hydrolysis of flavonol glycosides to the corresponding aglycones is a well-developed simple procedure and the three main aglycones are all commercially available and easy to analyse by RP-HPLC. Little further development of this robust and widely accepted method is expected.

2.2.2. Biflavones

A number of biflavones occur in Ginkgo leaves and full extracts (e.g., homeopathic mother tinctures). The four major ones are bilobetin, ginkgetin, isoginkgetin and sciadopitysin. The first analytical papers on Ginkgo biflavones appeared in the early 1980s. Briançon-Scheid et al. published an HPLC separation on a Lichrosorb DIOL column in combination with a hexane–$CHCl_3$ (1:3)–THF gradient [106,107]. A disadvantage of this normal-phase system is the early elution of sciadopitysin. The system was not suitable for a quantitative determination of biflavones in a crude alcoholic extract [107]. Later the same group published a reversed-phase system which showed a much improved separation of the biflavones from other constituents but a poor resolution of the pair ginkgetin–isoginkgetin. This method has not been validated [108].



Amentoflavone	$R_1 = R_2 = R_3 =$ OII
Bilobetin	$R_1 = R_3 =$ OH, $R_2 =$ OMe
Sequojaflavone	$R_1 =$ OMe, $R_2 = R_3 =$ OH
Ginkgetin	$R_1 = R_2 =$ OMe, $R_3 =$ OH
Isoginkgetin	$R_1 =$ OH, $R_2 = R_3 =$ OMe
Sciadopitysin	$R_1 = R_2 = R_3 =$ OMe

Pietta et al. described an isocratic reversed-phase system which was applied to unspecified Ginkgo extracts. The sample clean-up consisted only of dissolving the extracts in alcohol and membrane filtration. The solvent system was THF–PrOH–H_2O (21:10:69). The resolution and total analysis time (<15 min) are good. The biflavone content in the

extracts was calculated by external standardisation. Total concentrations varied from a high 1.68% to a low 0.047% in three different extracts. Hasler et al. have described a special RP ternary gradient system capable of resolving most flavonol glycosides as well as all biflavones (Table 11) [97]. Because of the complexity of the gradient, the long retention times for the biflavones (~30 min) and lack of validation experiments, this method is less suitable for quantitative analysis. On the other hand it is eminently suitable for fingerprint analysis. In the same article a second system employing a different ternary gradient was described yielding a retention time of 20 min for the last eluting biflavone [97]. Zhong and Xu have published a quantitative procedure for Ginkgo biflavones on an RP column with MeOH–H_2O–HCO_2H (85:15:0.8) as eluent and UV detection at 330 nm. Anthracene was used as internal standard. A potential problem with this system is the severe tailing of the later eluting peaks and the very long retention time of sciadopitysin (not visible in the chromatogram). Recoveries and RSDs were presented. Gobbato and Lolla [109] have described a straightforward H_2O–MeCN gradient system giving a good separation of all six biflavones (Fig. 14) within 25 min in combination with a SupelcoSil C_{18} column [109]. Detection occurred with UV at 330 nm. This method was partially validated (recovery experiments for ginkgetin only). Peak purity was confirmed by LC–MS experiments. Regrettably no details were given about the sample preparation and no leaves were analysed. The total amount of biflavones calculated by external standardisation was expressed as ginkgetin although reference substances of the other biflavones were available [109]. Why they were expressed as ginkgetin is unclear.

A simple, accurate, reproducible and fully validated quantitative method for the analysis of biflavones in full Ginkgo leaf extracts is not yet available. Several chromatographic separation systems have been published though. The simple RP system proposed by Gobbato et al. appears promising al-

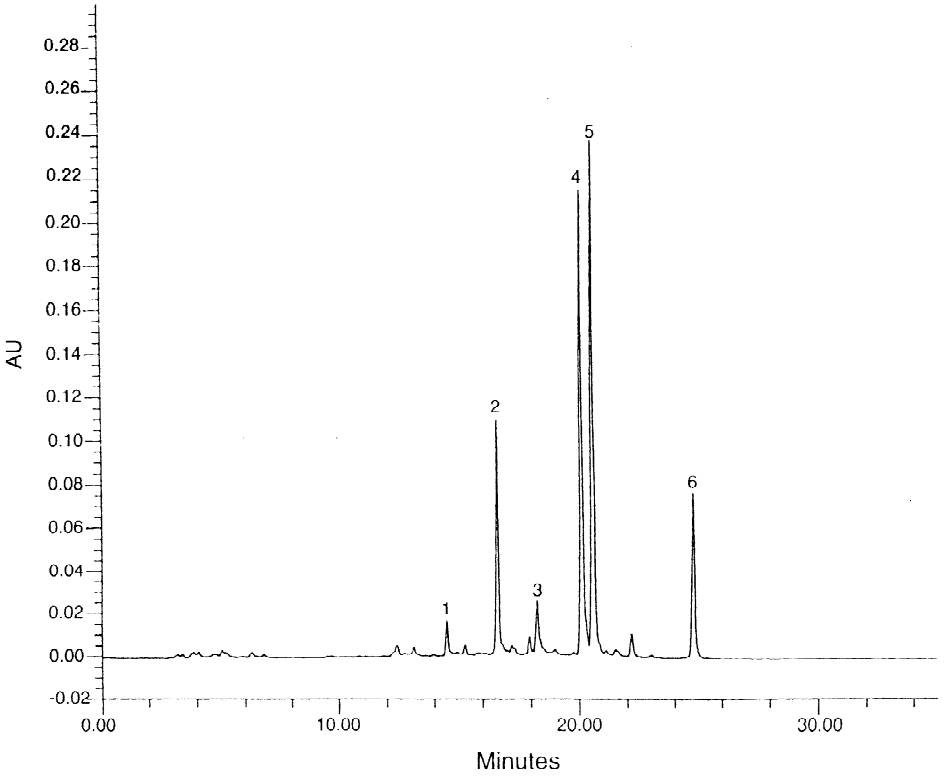
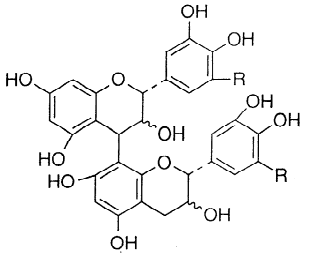


Fig. 14. HPLC profile of biflavones in a purified extract of Ginkgo leaves. Reproduced from Ref. [109] with permission of the editor.

though it is unknown how much overlap this system will give with other components present in Ginkgo extracts [109]. UV detection at 330 nm is the most selective and currently the best detection method available for Ginkgo biflavones.

2.2.3. Proanthocyanidins

Large amounts of proanthocyanidins occur in both Ginkgo leaves (4–12% [110]) and standardised extracts (7.0% [6]). They are considered to be of importance for the beneficial properties of Ginkgo leaf extracts. In spite of this, relatively little attention has been paid to them in terms of phytochemical publications. Stafford et al. have identified four dimers with the general structure given below [111].



Procyanidin R = H

Prodelphinidin R = OH

Two publications have appeared with deal with the quantitative analysis of Ginkgo proanthocyanidins [110,112]. Due to a lack of knowledge on the precise structures of these compounds and the absence of commercially available pure reference compounds, specific chromatographic separation and quantitation is not (yet) possible. Instead four group reactions are used:

(1) acid hydrolysis in the presence of iron(III) and measurement of the formed anthocyanidins at 563 nm [113];

(2) reaction with Folin Ciocalteus phenol reagent (disadvantage: interference by other phenols);

(3) reaction with vanillin (disadvantage: also monomeric flavanols react); and

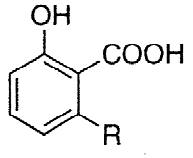
(4) reaction with proteins (hide powder) and gravimetric determination.

Lang and Wilhelm found for one standardised Ginkgo extract values of 2.3, 15.7, 0.9 and 22.2% for methods (1)–(4), respectively [112]. According to them, methods (2) and (4) gave too high values, while method (3) gave a too low value. Best results were obtained with method (1) which was also used by Schennen [110]. However, even this best method has a high degree of uncertainty which is perhaps best illustrated by the value of 7.0% given by Stumpf [6] for the proanthocyanidin content of the same extract as investigated by Lang and Wilhelm. Clearly there is significant scope for improving existing or developing new phytochemical methods for this analytically difficult class of compounds.

2.3. Alkylphenols

Three different classes of alkylphenols (ginkgolic acids, ginkgols and bilobols) occur in various parts of *Ginkgo biloba*. Only the two first mentioned classes had been detected in Ginkgo leaves [114] until recently Zarnowska et al. identified pentadecylresorcinol (dihydrobilobol) in Ginkgo leaves [115]. In the latter study the main alkylresorcinol (syn. cardol) occurring in Ginkgo fruits, bilobol (syn. 5-pentadec-8[*Z*]-enylresorcinol), could not be identified in leaves [115]. Synonyms for ginkgolic acids are 2-hydroxy-6-alkylbenzoic acids, 6-alkylsalicylic acids or anacardic acids. Synonyms for ginkgols are 3-alkylphenols or cardanols. The alkyl sidechain varies from 13 to 17 carbons in length with zero to two double bonds. The double bonds possess the *Z*-configuration. When only one double bond is present, the position is most frequently 8 [116]. The most important structures of alkylphenols occurring in Ginkgo leaves [24,114–120] are given below.

These compounds possess contact allergenic [121], cytotoxic [122], mutagenic [123] and slight neurotoxic properties [124] and their presence is considered undesirable in Ginkgo special extracts [7,125–127]. On the other hand also antitumor activities have been reported for these compounds [24]. Furthermore it should be remarked that there is no solid proof of a strong allergic reaction to these alkylphenols when taken orally. For instance, no reports have been filed on adverse effects of Ginkgo homeopathic mother tinctures [128,129]. This in spite of the fact that such extracts contain 2.2% of ginkgolic acids (22 000 ppm !) [122] and that they have been taken by many people during several



Ginkgolic acids

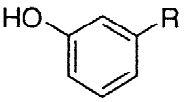
R = $C_{13}H_{27}$ (C13:0)

R = $C_{15}H_{31}$ (C15:0)

R = $C_{15}H_{29}$ (C15:1)

R = $C_{17}H_{33}$ (C17:1)

R = $C_{17}H_{31}$ (C17:2)



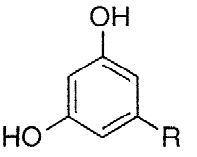
Ginkgols

R = $C_{13}H_{27}$ (C13:0)

R = $C_{15}H_{31}$ (C15:0)

R = $C_{15}H_{29}$ (C15:1)

R = $C_{17}H_{33}$ (C17:1)



Cardols

R = $C_{15}H_{31}$ (C15:0)

years. Nevertheless these compounds are suspect and the larger manufacturers limit the total alkylphenol concentration in the final standardised extract to 5 or 10 ppm. Technically this poses no problems. Unfortunately it is often not totally clear whether the maximum limit relates only to all ginkgolic acids combined or also includes ginkgols. The concentration of ginkgols is approximately a factor 10 lower than that of the ginkgolic acids and in an animal model for allergy the ginkgolic acids appear to be more important than the ginkgols [127]. However in another publication it was claimed that the concentration of ginkgols was equal to that of the gingkolic acids [130]. During the manufacturing process the alkylphenols can be removed together with other fat-soluble constituents by a partitioning step with an apolar solvent like heptane. In ''full'' extracts (e.g., alcoholic or acetone–water extracts without multiple purification steps) these compounds are still present in significant percentages [49,50,122,127].

Most phytochemical analyses on Ginkgo alkylphenols have been qualitative in nature and only during the last two years three validated quantitative procedures for leaves or extracts have been published [131–134]. The alkylphenols are very apolar constituents which can be readily extracted with hexane but also with more polar less selective solvents like methanol. Water has—not unexpected—a negative influence on the extraction. Aye and Müller (1991) have shown that the concentration of ginkgolic acids ($C_{15:1}$ and $C_{17:0}$) in the initial crude extract is 0.13 and 10.4% for acetone–H_2O (7:3) and pure acetone, respectively [50]. For the same two solvents Lang and Stumpf gave values of 7.24 and 20%, respectively [49]. Supercritical carbon dioxide at 300 atm and 55 °C can be used for a more selective extraction from leaves. Fatty acids are already extracted at lower pressures while chlorophyll remains unextracted [118]. Purification is possible by partitioning, silica gel column chromatography, ion-exchange chromatography, Sephadex LH-20 and RP-HPLC.

Irie et al. have published concentrations for four ginkgolic acids in Ginkgo leaves using the quantitative procedure below (see Table 14) [119]. They reported 0.04% $C_{13:0}$, 1.20% $C_{15:1}$ (8Z), 0.44% $C_{17:1}$ (8Z) and 0.05% $C_{17:2}$ (9Z, 12Z). A similar procedure is used by the Pharmaton company (Switzerland) except that they used an RP-8 column and detect at 210 nm. The total content is expressed as the combined amount of ginkgol and ginkgolic acid [7].

More recently a limiting test ($\leq$5 ppm) for ginkgolic acids was published in draft Pharmacopeia monographs on standardised dry Ginkgo extract

Table 14
Quantitative HPLC–UV method for alkylphenols by Irie et al. [119]

(1) Collect leaves and freeze dry them
(2) Extract 1-g sample 3× with 20 ml hexane each
(3) Pool the extracts and remove the solvent
(4) Analyse with HPLC (YMC pak R-ODS-10, 4.6×250 mm), MeCN–5% HOAc in H_2O (8:2) for 60 min, then (9:1) for 10 min, 1 ml/min, detection UV 280 nm

[8,11]. The procedure is given in Table 15 and an example chromatogram can be seen in Fig. 15. From this figure it is obvious that this is not an easy assay and that there are still many impurities remaining after the partitioning step. The baseline returns only to zero after 30 min. Why such a relatively polar solvent as ethyl acetate was used for the sample clean-up does not become clear. The peaks marked

Table 15
Quantitative partitioning-HPLC–UV method for ginkgolic acids in standardised extract as occurring in draft monographs of the European and United States Pharmacopeias [8,11]

(1) Stir 1.00 g of extract 5 min in 10 ml H_2O
(2) Transfer suspension quantitatively with an additional 10 ml of H_2O to a separatory funnel
(3) Extract 4× with 10 ml EtOAc each, combine the EtOAc layers and wash twice with 5 ml H_2O
(4) Filter the EtOAc layer, evaporate to dryness in vacuo at 40 °C, dissolve residue in 2 ml MeOH
(5) HPLC on a 5 μm 125×4-mm C_{18} column, 1.2 ml/min H_2O–MeCN–H_3PO_4 (70:30:0.3) to MeCN–H_3PO_4 (100:0.3) gradient, UV detection at 310 nm, 200 μl injection
(6) Calculate by external standardisation against a mixture of C_{13}, C_{15}, C_{17} alkylsalicylic acids

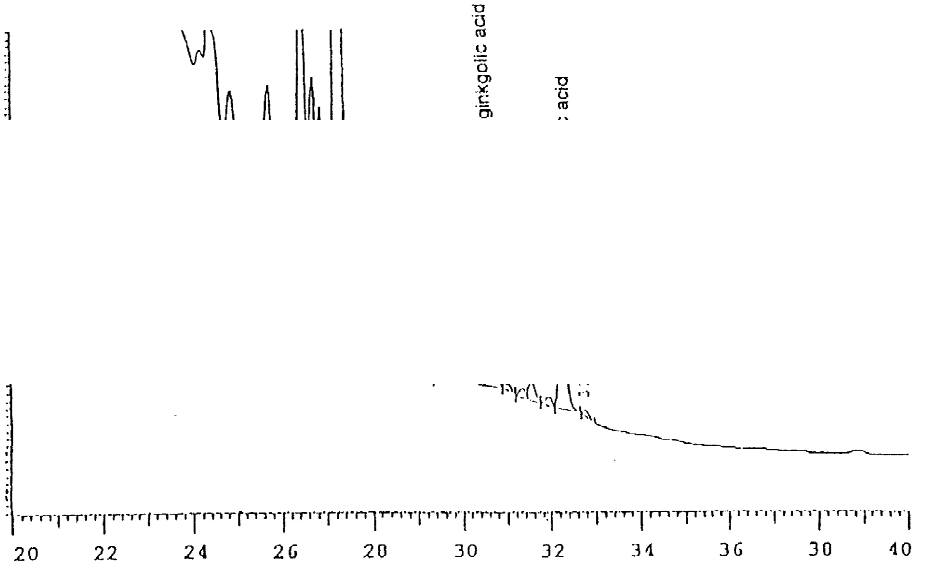


Fig. 15. HPLC–UV profile of a purified ginkgolic acid sample from a standardised *Ginkgo biloba* extract on a C_{18} column with a H_2O–MeCN–H_3PO_4 (70:30:0.3) to MeCN–H_3PO_4 (100:0.3) gradient at 1.2 ml/min. Detection UV 310 nm. From Ref. [11], Copyright (1999), with the kind permission of the Council of Europe.

as ginkgolic acid are not clearly assigned and the exact composition of the reference mixture is not given. Most likely the first peak consists of a mixture of $C_{13:0}$ and $C_{15:1}$ while the second peak represents $C_{17:1}$ [119,135].

One validated method for Ginkgo extracts uses supercritical extraction with carbon dioxide modified with 8% methanol at 55 °C and 30 MPa [131]. The sample had to be mixed with silica gel for obtaining clean extracts. The extracts were subsequently analysed by RP-HPLC with UV detection at 254 nm. The three main peaks were identified as $C_{15:1}$ Δ8, $C_{15:1}$ Δ10 and $C_{17:1}$ Δ8. The correct identification of the two $C_{15:1}$ double bond isomers needs to be reconfirmed as one of them might have been confused with $C_{13:0}$ [136]. The recovery was 95.3%, the RSD 4.2% and the limit of detection 4.2 ng with a 10-μl loop [131]. Due to the combined extraction–sample clean-up step the method is simple. A disadvantage is that an SFE apparatus is required. Another validated procedure for the quantitative analysis of the two main ginkgolic acids ($C_{15:1}$ and $C_{17:1}$) in Ginkgo extracts is given in Table 16 [132]. In this case detection took place by ESI-MS in the negative mode. A corresponding chromatogram can be seen in Fig. 16. Calibration curves were linear between 0.5 and 10 μg/ml and 0.1–7.5 μg/ml for $C_{15:1}$ and $C_{17:1}$, respectively. Detection limits were 0.25 and 0.1 μg/g. The sensitivity was superior to UV detection. The recovery of the method was around 100% for both compounds and the RSDs were around 3.5%. Disadvantages of the method are the triple extraction with a halogenated solvent and the absence of data on $C_{13:0}$, $C_{15:0}$ and $C_{17:2}$. A very similar separation and detection system using a C_{18} column in combination with isocratic elution with MeCN–H_2O–HOAc (92:7:1) and quadrupole MS with negative ion ES operation was published independently [137]. The latter study was only qualitative in nature. Tentatively two 2,4-dihydroxy-6-alkylbenzoic acids were identified in leaf extracts.

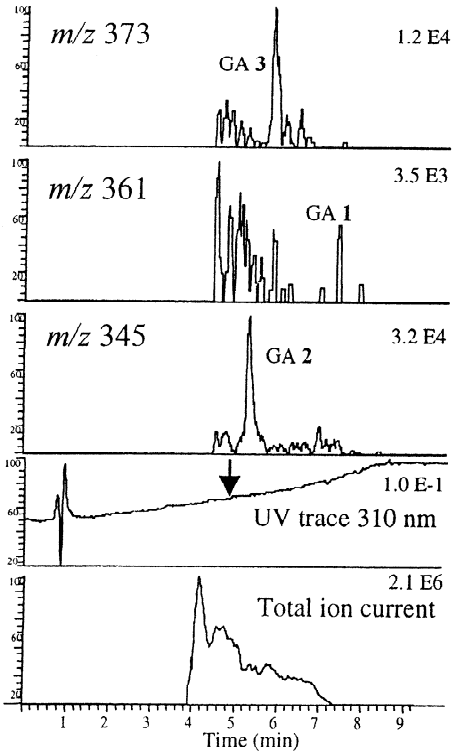


Fig. 16. HPLC–MS profile (ESI, SIM mode and negative ion mode) of $C_{15:1}$ and $C_{17:1}$ ginkgolic acids (GA2 and GA3, respectively) occurring in GK 501 *Ginkgo biloba* standardised extract. $C_{15:1}$ and $C_{17:1}$ ginkgolic acids occurred at 0.50 and 0.17 μg/g extract, respectively. For experimental details see Table 16. Reprinted from Ref. [132], copyright (2000), with permission from Elsevier Science.

An isocratic dual column system was published which is capable of a near baseline separation of $C_{13:0}$, $C_{15:1}$, $C_{17:2}$, $C_{15:0}$ and $C_{17:1}$ separation in 20 min [136]. The two HPLC columns were a 150×4.6-

Table 16
Quantitative partitioning-HPLC–ESI–MS method for $C_{15:1}$ and $C_{17:1}$ ginkgolic acids in standardised extracts by Ndjoko et al. [132]

(1) Dissolve 1.00 g of extract in 50 ml MeOH–H_2O (1:1), extract 3× with 20 ml of $CHCl_3$ each
(2) Combine $CHCl_3$ phases, filter through Na_2SO_4, evaporate to dryness, redissolve in 1 ml MeOH
(3) HPLC on a 4-μm 125×4-mm C_4 column with a 8×4 mm precolumn, 1 ml/min H_2O–MeCN–HOAc (49.75:49.75:0.5) to MeCN–HOAc (99.5:0.5) gradient, 10% was split to an ion trap MS equipped with an electrospray (ES) interface in the negative ion mode using SIM, 5 μl injection
(4) Calculate by external standardisation against pure $C_{15:1}$ and $C_{17:1}$ ginkgolic acids

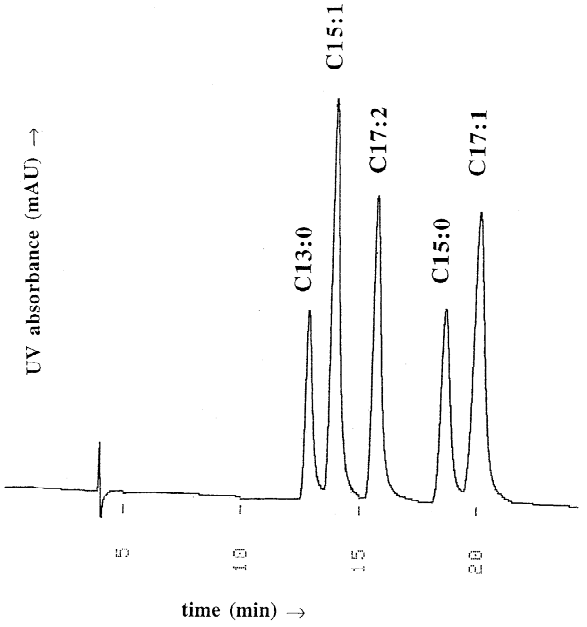


Fig. 17. HPLC–UV profile of a reconstituted mixture of pure $C_{13:0}$, $C_{15:0}$, $C_{15:1}$, $C_{17:1}$ and $C_{17:2}$ ginkgolic acids separated on a combination of a C_{18} column and a Ag(I) coated cation-exchange column. For experimental details see text. Reprinted from Ref. [136], copyright (2001), with permission from Elsevier Science.

mm end-capped 5 μm C_{18} column connected in series with a 200×4-mm Ag(I)-coated 5-μm cation exchanger. The eluent was methanol–water–formic acid (929:70:1) at 1.0 ml/min and the column temperature 35 °C while detection took place by UV at 311 nm. A chromatogram can be seen in Fig. 17. This dual column system gave a better resolution than either of the two columns alone. Reversed-phase columns have difficulties in separating the pairs $C_{13:0}$–$C_{15:1}$ and $C_{15:0}$–$C_{17:1}$ while Ag(I)-coated columns give a baseline separation of these pairs due to their complexation of compounds with olefinic double bonds. In the same study a trace of $C_{17:3}$ was tentatively detected in Ginkgo leaves [136]. Independently a similar separation based on argentation chromatography was published by He and Xie [133,134,136]. This second method can also be used for a quantitative determination of the four major ginkgolic acids in Ginkgo leaves. The exact procedure is given in Table 17. The identification was confirmed by LC–ESI-MS in the negative ion mode. The method was validated with respect to extraction efficiency, recovery, peak purity, linearity, reproducibility and sensitivity. The method was not evaluated in combination with standardised Ginkgo extracts which is the normal sample type encountered in quality control. The method may lack in sensitivity for such samples.

A procedure for the quantitative analysis of 5-pentadecylresorcinol in Ginkgo leaves has been published [115]. The procedure is given in Table 18. The standard error for triplicate analyses did not exceed 5%. Seven leaf samples were analysed corresponding to harvest times from the end of May until early October. The content of 5-pentadecylresorcinol was found to vary from 26.5 to 87 μg/g. The highest content was found early July [115].

Currently there is a lot of interest in the potentially allergenic alkylphenols but this is not yet reflected in the publication of a large number of simple, sensitive and validated analytical procedures. For a selective extraction, hexane or supercritical carbon dioxide

Table 17
Quantitative partitioning-HPLC–UV method for $C_{13:0}$, $C_{15:0}$, $C_{15:1}$ and $C_{17:1}$ ginkgolic acids in leaves by He and Xie [134]

(1) Dry leaves at 70 °C during 6 h under forced ventilation and pulverise mechanically
(2) Extract 1.0 g of leaves with 40 ml EtOH under reflux during 90 min, filter after cooling
(3) Wash flask and leaves 3× with 3 ml EtOH, combine all EtOH and dilute to 50.0 ml with EtOH
(4) Take 1.0 ml, add 3.0 ml hexane and 10 ml of satd. Na_2SO_4–H_2SO_4 (pH 3) and siliceous earth
(5) Shake the solution in a stoppered tube and take 1.5 ml of the upper layer, evaporate with N_2
(6) Dissolve in 0.5 ml $CHCl_3$ and inject 20 μl into the HPLC
(7) Separate on a 7 μm 250×4.6-mm C_{18} column with MeOH–5% HOAc in H_2O (9:1) with 0.03 mol/l $AgNO_3$ at 1.0 ml/min and 30 °C, detection UV at 310 nm
(8) Calculate content of $C_{13:0}$, $C_{15:0}$, $C_{15:1}$ and $C_{17:1}$ ginkgolic acids by external standardisation against $C_{17:1}$ ginkgolic acid

Table 18
Quantitative colorimetric method for 5-pentadecylresorcinol in leaves by Zarnowska et al. [115]

(1) Collect leaves, cut, freeze in liquid N_2, store at -70 °C until analysis, grind in a mortar
(2) Extract with Me_2CO for 24 h, filter and reextract with Me_2CO, combine and evaporate in vacuo
(3) Redissolve in 1 ml $CHCl_3$ and apply to silica gel column, flash chromatography with a $CHCl_3$–EtOAc gradient, pool fractions with 5-pentadecylresorcinol, concentrate under N_2
(4) Spot on a silica gel prepTLC plate, develop with hexane–Et_2O–HCO_2H (70:30:1), scrape off 5-pentadecylresorcinol band and extract with Me_2CO during 2 h, centrifuge, remove solvent
(5) Dissolve in 0.5 ml $CHCl_3$, put in glass tube, evaporate under N_2, add 4 ml of 0.05% (w/v) Fast Blue B×BF_4 in 5% HOAc diluted 5× with PrOH, leave in the dark for 1 h, read absorbance at 520 nm against a reagent blank
(6) Estimate content of 5-pentadecylresorcinol using a calibration curve (1–10 μg) of the reference

seem the best choices. Purification is possible over an anion-exchange column in the OH^- form although this may not be necessary in all cases. Separation by RP-HPLC is possible but the resolution of the pairs $C_{13:0}$–$C_{15:1}$ and $C_{15:0}$–$C_{17:1}$ is poor. Two papers have come up with a mixed argentation-reversed-phase separation mechanism which can resolve these two pairs. Resolution of positional double bond isomers (Δ8 or Δ10) of $C_{15:1}$ and $C_{17:1}$ ginkgolic acids is not possible with LC but can be achieved with capillary GC. Prior derivatisation is then necessary. For GC both FI and MS detection are possible and satisfactory. After the HPLC separation, detection can be carried out with UV at 210, 243 or 311 nm or by mass spectrometry in the negative ion mode with an electrospray interface. The latter technique is more selective and sensitive, especially in the selected ion monitoring (SIM) mode. For routine quality control of Ginkgo extracts this detector is expensive; however, for these trace constituents it is a good choice. An additional advantage is that deconvolution of co-eluting peaks is possible. Thus simple RP-HPLC may be sufficient in combination with MS detection. The only alternative is UV detection with an optimised sample clean-up and a more complex HPLC system.

2.4. Carboxylic acids

Significant amounts of various carboxylic acids occur in *Ginkgo biloba* leaves and extracts. Stumpf mentions 13% as the carboxylic acid content of the standardised Ginkgo extract EGb 761 [6]. This group can be subdivided in non-phenolic and phenolic acids. The former group comprises compounds like ascorbic acid, D-glucaric acid, quinic acid and shikimic acid [114]. No publications have been devoted to the specific quantitative analysis of these compounds in Ginkgo leaves or extracts. Schennen has reported a shikimic acid content of 1.9% based on dried Ginkgo leaves [110]. There is one publication on the analysis of free and bound phenolic acids [138]. The analytical procedure is given in Table 19 and an example chromatogram in Fig. 18.

Table 19
Quantitative partitioning-HPLC–UV method for simple phenolic acids in leaves by Ellnain-Wojtaszek and Zgórka [138]

(1) Extract 25 g powdered leaves twice with boiling 85% aqueous MeOH during 1 h
(2) Concentrate in vacuo, dilute with 100 ml distilled water, filter
(3) Extract with 2× 30 ml petrol ether, followed by 15× 50 ml Et_2O
(4) Extract combined Et_2O layers with 10× 10 ml 5% $NaHCO_3$, acidify with HCl until pH 3
(5) Extract combined aqueous layers with 10× 25 ml Et_2O
(6) Evaporate Et_2O layers until dryness, dissolve in 25 ml 50% aqueous MeOH
(7) Inject 20 μl on a 200×4.6 mm 5 μm Hypersil C_{18} column, eluent MeOH–H_2O (25:75) with 1% HOAc at 1 ml/min, UV detection at 254 nm
(8) Compare with calibration curves (external standardisation)

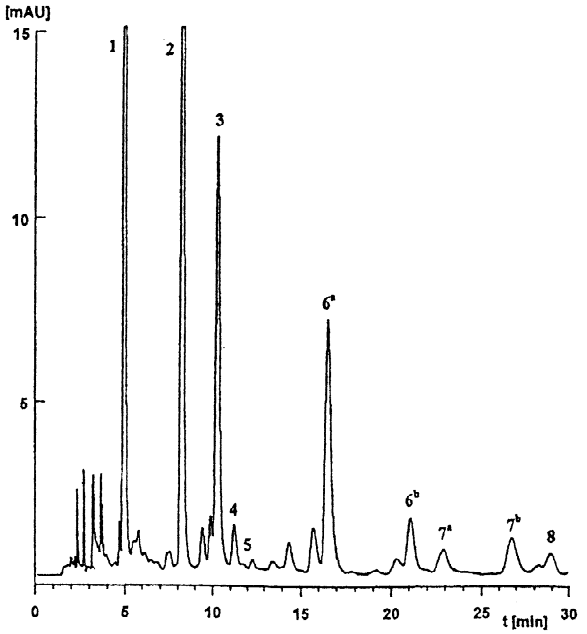
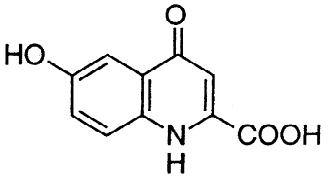


Fig. 18. HPLC–UV profile of phenolic acids in a purified extract of *Ginkgo biloba* leaves on a C_{18} column with MeOH–H_2O (25:75) with 1% HOAc at 1 ml/min: (1) protocatechuic acid; (2) *p*-hydroxybenzoic acid; (3) vanillic acid; (4) caffeic acid; (5) isovanillic acid; (6) *p*-coumaric acid; (7a) *E*-ferulic acid; (7b) *Z*-ferulic acid; (8) sinapic acid. Reprinted from Ref. [138] by courtesy of Marcel Dekker Inc., 1999.

The sample clean-up appears time-consuming with 37 partitioning steps and has not been validated. The authors followed the concentration of the phenolic acids in leaves from June 28 until October 28. The concentration of the free acids increased until a maximum at the end of October. The concentration of bound phenolic acids (measured after acidic or alkaline hydrolysis) showed a less clear pattern. The major phenolic acid was *p*-coumaric acid with a concentration of 0.25 mg/g leaves at the end of October [138].

Apart from these common phenolic acids, one rare nitrogen-containing phenolic acid named 6-hydroxykynurenic acid (6-HKA) occurs in Ginkgo leaves. Together with some other organic acids it is also present in standardised extracts. Little is known about the importance of his compound for the clinical activity of Ginkgo. 6-HKA may be an antagonist of *N*-methyl-D-aspartate (NMDA) [139]. 6-HKA is probably a degradation product arising from tryptophan [110]. Schennen was the first to analyse 6-HKA in Ginkgo leaves. His procedure is given in Table 20. It was discovered that the 6-HKA content increases dramatically throughout the year to a maximum of 1–2 mg/g in November. A similar procedure was used by Chen et al. except that they removed the spot of 6-HKA from a TLC plate and then used off-line UV spectroscopy after elution to determine the content [52].



6-Hydroxykynurenic acid

Gräsel and Reuter have used a more modern approach by means of HPLC or HPTLC [139]. Their HPLC procedure is given in Table 21. An example chromatogram can be found in Fig. 19. The method was validated with respect to extraction efficiency, recovery, reproducibility and linearity. The detection

Table 20
Quantitative partitioning-paper chromatography–fluorescence method for 6-HKA in leaves by Schennen [110]

(1) Extract 40–100 mg powdered leaves twice with 10 ml 70% MeOH under reflux
(2) Extract the combined filtrates with 20 ml petrol ether
(3) Evaporate the aqueous phase to dryness, dissolve residue in 2 ml 60% Me_2CO
(4) Separate 20 μl solution by two-dimensional paper chromatography
(5) Extract 6-HKA and blank spot with 0.05 *M* H_2SO_4 at 50 °C
(6) Measure the fluorescence (ex. 350 nm, em. 540 nm) of both extracts
(7) Compare with calibration curve (also after two-dimensional paper chromatography)

Table 21
Quantitative SPE–HPLC–UV method for 6-HKA in leaves by Gräsel and Reuter [139]

(1) Extract 0.5–2 g powdered leaves in a Soxhlet with 80 ml petrol ether during 90 min
(2) Re-extract leaves in a Soxhlet with 80 ml 70% aqueous MeOH during 150 min
(3) Evaporate the aqueous extract to dryness, dissolve residue in 5 ml 70% MeOH
(4) Filter and perform sample clean-up by SPE with an aromatic cation exchanger, wash solvents: H_2O and 1 *M* HCl, elution solvent: 2 *M* HCl
(5) Collect 6-HKA fraction, evaporate and dissolve in 2 ml 70% MeOH
(6) Inject into HPLC, LiChrosorb RP 8 column, 250×4 mm, MeCN–MeOH–H_2O (5:1:15) with H_3PO_4 at pH 3.2, 1 ml/min, UV 254 nm detection, calculate with calibration curve

limit was calculated as 12 ng. In the HPTLC method (silica gel, $CHCl_3$–HOAc–H_2O (4:4:1)) the detection took place by fluorescence. The detection limit was 7 ng. Both methods gave similar results. The 6-HKA content for three different commercial batches of Ginkgo leaves varied from 0.121 to 0.269 mg/g dry plant material.

2.5. 4-O-Methylpyridoxine

In Ginkgo seeds significant quantities of the antivitamin B6 4-*O*-methylpyridoxine occur. An overconsumption of seeds has led to toxicity and even death in the past [140]. Especially children are vulnerable. A review on 4-*O*-methylpyridoxine is available [141]. Until recently this compound could not be detected in Ginkgo leaves and was thus assumed not to occur in Ginkgo phytopharmaceuticals [68]. This was supported by the absence of any reports on the typical 4-*O*-methylpyridoxine intoxication signs (convulsions) in the millions of people taking standardised Ginkgo extracts. However, recently this compound was reported to occur in leaves and could also be detected in Ginkgo drugs [142], but the concentrations are so low that no toxic effects can be observed. The compound is extracted with boiling water and separated on a strong cation exchanger. Due to the selectivity of the extraction solvent, the separation mechanism and the fluorescence detection, no sample clean-up was necessary. The entire procedure is given in Table 22 while a chromatogram is depicted in Fig. 20. The sensitivity

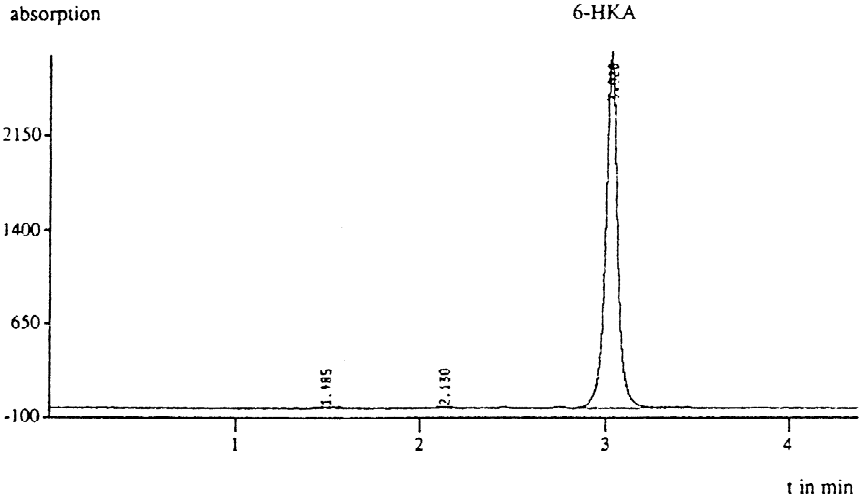


Fig. 19. HPLC–UV profile of an infusion of Ginkgo leaves. 6-HKA = 6-hydroxykynurenic acid. Analysed according to the method of Gräsel and Reuter [139]. Reprinted from Ref. [139], with permission from Georg Thieme Verlag.

Table 22
Quantitative HPLC–fluorescence method for 4-*O*-methylpyridoxine in leaves by Arenz et al. [142]

(1) Freeze leaves in liquid nitrogen and subsequently powder them in a mortar
(2) Suspend 0.4 g leaves in 2 ml H_2O, heat in an almost closed test tube at 100 °C during 30 min
(3) Centrifuge the suspension after cooling at 10 000 rpm
(4) Inject the supernatant into an HPLC, Nucleosil 5SA column, 250×4 mm, gradient from 100% 0.01 *M* HCl to 100% 0.5 *M* KH_2PO_4 (pH 5) in 15 min, 1 ml/min, fluorescence detection 330 nm excitation, 400 nm detection

was reported as <0.5 μg. The method was validated. Concentrations in leaves varied from 2 to 5 μg/fresh leaf. One fresh leaf equals approximately 0.25 g dry weight. In standardised liquid Ginkgo drugs the concentrations varied from 3.8 to 9.8 μg/ml. In contrast in the albumen of seeds a concentration of 105 μg/g was measured [142]. This latter value corresponds well with values of 0.01–0.02% in raw Ginkgo seeds reported by others [141,143]. In seeds also the 5-glucoside occurs [143].

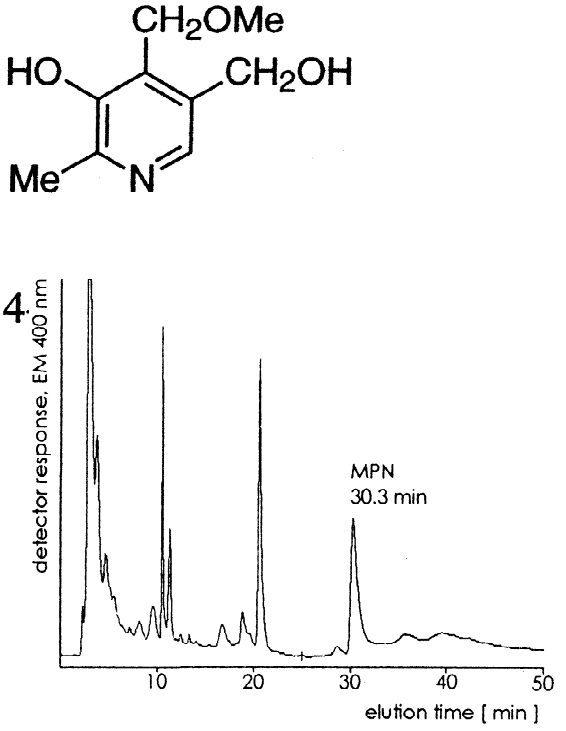


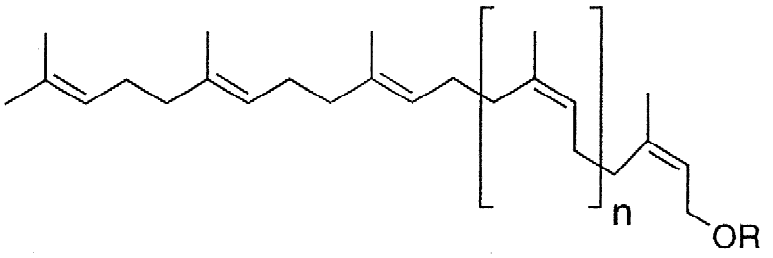
Fig. 20. HPLC profile of a German homeopathic Ginkgo drug (Ginkgo loges Tropfen). MPN = 4-*O*-methylpyridoxine. Analysed according to the method of Arenz et al. [142]. For chromatographic details see Table 22, line 4. Reprinted from Ref. [142], with permission from Georg Thieme Verlag.

2.6. Polyprenols

Polyprenols consist of a large number (10–30) of isoprene units linked in a linear fashion. They occur in many plants besides Ginkgo leaves. They could be involved as sugar carriers in protein glycosylation. In Ginkgo the polyprenols are mostly present as acetates. The chain consists of an ω-unit, two *trans* isoprene units, a large number (10–20) of *cis*-isoprene units and a terminal (α) *cis*-isoprene unit with the ester group. They are removed during the manufacturing process of standardised Ginkgo extracts and therefore do not occur in such extracts. Thus the two most relevant publications on Ginkgo polyprenols deal with the analysis of leaves only. Polyprenols are very apolar compounds which can be extracted with solvents like hexane. Reviews on Ginkgo polyprenols [144] and chromatography of polyprenols in general [145] have been published before.

The initial work on Ginkgo polyprenols was carried out by Ibata et al. [146]. Their method of analysis is given in Table 23. The procedure is preparative in nature and not very suitable for routine quantitative analysis. They characterised the polyprenols by a combination of IR, FD-MS, ^{1}H NMR and ^{13}C NMR, and saponification. The percentages by weight of polyprenyl-14–23 acetates were given as 0.9, 1.6, 6.5, 24.9, 36.5, 17.7, 6.7, 2.9, 1.6 and 0.8%. During the growing season the total concentration of polyprenol acetates steadily increased from an initial 0.05 to 2.0% in late autumn. The free polyprenol concentration never increased above 0.05% [146].

A more simple assay has been described by Huh et al. and is summarised in Table 24 [147]. They extracted the leaves with the more selective hexane and removed many slightly more polar constituents with 90% methanol in water. The extract was then



Ginkgo polyprenols: R = H, n = 10 - 20

Ginkgo polyprenol acetates: R = Ac, n = 10 - 20 (n = 14 is most abundant)

saponified with base converting the acetates to the alcohols (free polyprenols). By means of supercritical fluid chromatography (SFC) with pure carbon dioxide on an apolar capillary column, they were able to obtain a good separation of the individual C_{70} to C_{120} polyprenols present. Similarly to Ibata et al. they found C_{90} polyprenol to be the most abundant. Quantities of C_{85}, C_{90} and C_{95} polyprenols (commercially available) were calculated via internal standardisation (I.S. = dodecaprenol (C_{60}) from *Rhus typhina*). The method was partially validated. RSDs were calculated (6–8%) and the calibration curves

Table 23
Quantitative gravimetric method for polyprenols in leaves by Ibata et al. [146]

(1) Dry leaves at 50–60 °C during 1–2 days in an oven with forced ventilation, afterwards crush them
(2) Extract 200 g with 600 ml hexane–Me_2CO (1:1) 3×, each time for 3–9 days at 20 °C
(3) Purify over silica gel with hexane–Et_2O (19:1), collect 72% pure polyprenol acetates
(4) Purify by GPC on styrene–divinylbenzene gel with $CHCl_3$, collect 96.4% pure fraction
(5) Purify by dissolving in a 20-fold volume of Me_2CO, discard precipitate
(6) Separate into individual polyprenols by HPLC, Nucleosil $5C_{18}$ column, 300×10 mm, Me_2CO–MeOH (9:1), 3 ml/min, RI detection
(7) Determine the weight of each fraction (weight is proportional to peak area in HPLC profile)

Table 24
Quantitative partitioning-SFC–FID method for polyprenols in leaves by Huh et al. [147]

(1) Dry leaves at 60 °C, pulverise them afterwards
(2) Extract 200 mg with hexane during 1 h after addition of 500 μg dodecaprenol (I.S.)
(3) Wash the hexane 3× with 90% aqueous MeOH, discard aqueous MeOH
(4) Stir hexane vigorously with K_2CO_3 and MeOH during 12 h at room temperature
(5) Wash once with H_2O and twice with saturated NaCl solution, dry over Na_2SO_4
(6) Evaporate hexane, reconstitute in 1 ml hexane
(7) Inject 0.2 μl (time split 0.2 s) into an SFC apparatus equipped with a chemically bonded phenyl capillary column (10 m×50 μm I.D., 0.25 μm film) at 100 °C; fluid 100% CO_2; pressure gradient from 200 to 400 atm with 20 atm/min, FI detector at 325 °C

were linear from 0.5 to 2.0 mg/ml. Recovery of the internal standard was 85.5%. The assumption was made that the recoveries of the C_{85}, C_{90} and C_{95} polyprenols were similar to that of dodecaprenol. The extraction efficiency was not determined. No distinction was made between polyprenol acetates and free polyprenols. The polyprenol concentrations that were determined by Huh et al. in Ginkgo leaves throughout the growing season were close to those published earlier by Ibata et al. An ontogenetic study of Ginkgo polyprenols was published separately by the same group [148].

Shen et al. have compared 19 different solvents for the extraction of polyprenols from Ginkgo leaves [149]. Petroleum ether gave the most clean extract (yield 0.92 g, purity 19.8%) while hexane–acetone (8:2) gave the highest yield (yield 1.16 g, purity 18.3%). Prior to quantitative HPLC analysis, a sample clean-up on silica gel was necessary (no details given). Polyprenols were determined in the purified extract on a Spherisorb C_{18} column with PrOH–MeOH (9:1) as solvent at 2.0 ml/min. The system was capable of resolving both the alcohols and acetates of various chain length. The detection method was not given. The total content varied but an average value of 1.5% was reported.

3. Conclusion

Although a lot of different analytical methods have been proposed by academia and industry for various constituents of Ginkgo leaves during the last 20 years, in most cases no consensus has yet been reached about the use of one best method for each class of compounds (terpene trilactones, flavonol glycosides, etc.). In fact for the terpene trilactones, one simple, reproducible method suitable for both leaves and extracts has not yet been published. The extraction of terpene trilactones from leaves can be carried out with mixtures of acetone, methanol and water, at room temperature or under reflux conditions and with or without a pre-extraction with a highly non-polar solvent. In principle all give a quantitative extraction but not all methods are equally fast, environmentally friendly and yield equally clean extracts. Comparative assays are unfortunately scarce. This is even more true for the main bottleneck in terpene trilactone analysis: the sample clean-up step. Nevertheless two methods appear to have a slight edge over other published methods. One starts with an aqueous solution which is purified over diatomaceous earth (partitioning), the other starts with a methanolic solution which is purified over silica gel (adsorption). Both methods use for this step a small SPE-type column with 0.5 g stationary phase. Good separation and detection is not an issue and can take place equally well by RP-HPLC with refractive index or evaporative light scattering detection or by GC–FID. The last technique has as disadvantage that a prior derivatisation step is necessary. Recently several LC–MS studies of terpene trilactones appeared. This detector is so selective that it is possible to forgo any sample clean-up.

The analysis of the flavonol glycosides is straightforward. First they are hydrolysed after which the resulting three aglycones are quantified by RP-HPLC. A simple recalculation provides the original yield of the glycosides. The European and United States Pharmacopeia have adopted the same procedure. The direct analysis of the complex mixture of genuine flavonol glycosides is still in its infancy. Only fingerprint analysis is currently feasible. Although biflavones do not occur in significant concentrations in Ginkgo standardised extracts, interest in them is rising because they possess interesting pharmacological properties as well. They are present in full extracts and certain cosmetics. A good assay has not yet been published. The analysis of Ginkgo proanthocyanidins which do occur in standardised extracts is still in its infancy. Different assays give widely different values.

Lately there is a lot of interest in Ginkgo alkylphenols (ginkgolic acids) because of their potentially harmful effects. Most manufacturers limit the concentration of alkylphenols to 5 ppm. For the quantitation of these trace constituents in extracts LC–MS is probably the method of choice. Due to the high selectivity of LC–MS a complete chromatographic separation of all ginkgolic acids is not necessary. Such a separation is difficult to achieve by RP-HPLC. Further developments in the analysis of this group are foreseen.

From a phytopharmaceutical point of view the interest in the remaining groups discussed in this

review is less. Polyprenols and 4-*O*-methylpyridoxine do not occur in significant amounts in standardised Ginkgo extracts. Various simple phenolic carboxylic acids do occur in Ginkgo leaves and standardised extracts and some methods for their analysis in leaves have been published.

4. Abbreviations

APCI	atmospheric pressure chemical ionisation
BP1	type of GC stationary phase
BP10	type of GC stationary phase
BSTFA	*N,O*-bis(trimethylsilyl)trifluoroacetamide
C_{18}	type of HPLC stationary phase
C_4	type of HPLC stationary phase
DB-1	GC stationary phase
DMF	dimethylformamide
ECD	electron capture detector
ELSD	evaporative light scattering detector
ES	electrospray
ESI	electrospray ionisation
Et_2O	diethylether
EtOAc	ethyl acetate
EtOH	ethanol
FD	field desorption
FI	flame ionisation
FID	flame ionisation detector
G-A	ginkgolide A
G-B	ginkgolide B
G-C	ginkgolide C
G-J	ginkgolide J
GC	gas chromatography
GPC	gel permeation chromatography
6-HKA	6-hydroxykynurenic acid
HCO_2H	formic acid
HOAc	acetic acid
HPLC	high-pressure liquid chromatography
HPTLC	high-performance thin layer chromatography
iPrOH	isopropanol
I.S.	internal standard
IR	infrared
LC	liquid chromatography
Me_2CO	acetone
MECC	micellar electrokinetic capillary electrophoresis
MeCN	acetonitrile
MeOAc	methyl acetate
MeOH	methanol
MS	mass spectrometry
MW	molecular mass
NaOAc	sodium acetate
NMR	nuclear magnetic resonance
PrOH	propanol
RI	refractive index
RP	reversed-phase
RSD	relative standard deviation
SDS	sodium dodecyl sulphate
SFC	supercritical fluid chromatography
SFE	supercritical fluid extraction
SIM	selected ion monitoring
SPE	solid-phase extraction
THF	tetrahydrofuran
TLC	thin-layer chromatography
TMCS	trimethylchlorosilane
TSP	thermospray
UV	ultraviolet

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